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Genetic Techniques

for biological research

A CASE STUDY APPROACH

Corinne A. Michels

Genetic Techniques for Biological Research
A case study approach

For Harold
and for our F1 generation Catherine and Bill

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A case study approach

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Introduction

Molecular genetics is a tool used by today's biologist interested in understanding—not simply describing—the underlying mechanisms of processes observed in cellular and developmental biology. It is a fusion of the biochemical and genetic approaches to problem solving developed over the past decades and the resulting synergy of these approaches has produced an extremely powerful tool for the investigation of living systems.

The biochemical approach has been very productive in identifying the major macromolecular components of cells and the pathways of metabolism. Nevertheless, used exclusively, it is not an adequate tool for elucidating the details of the regulation of these pathways and their physiological coordination. The biochemist's tools, although powerful, are limited. The biochemist identifies and characterizes a component of interest (such as a protein) by purifying it or by monitoring its presence based on an assay of the reaction or cellular process it catalyzes. It is hoped that investigations of characteristics such as subcellular localization, structure, and identification of interacting proteins will provide clues to its cellular function. But, if these studies are uninformative, if the component is present at a very low level or is unstable, or an assay method cannot be developed, the biochemical approach will fall short.

The genetic approach does not have these limitations but does have others. No information regarding the number, function, location, or structure of the gene functions involved is required. One only needs to be able to observe the process of interest (the wild-type phenotype) and identify individuals exhibiting alterations or aberrations in this process (the mutant phenotype). The genetic approach assumes that few, if any, cellular processes occur spontaneously *in vivo,* and that there is a gene(s) encoding a protein(s) or RNA(s) that is responsible for catalyzing the process and allowing it to occur at a rate that is adequate for sustaining growth and development. The geneticist isolates mutant individuals exhibiting alterations in the process, uses genetic analysis to identify the full battery of genes encoding the products involved in regulating the process of interest, and explores the genetic interactions among these genes. To carry these studies further, the geneticist needs to isolate and functionally characterize the gene products and this requires the tools of biochemical analysis. Moreover, major limitations for the geneticist come from the availability of specific genetic techniques for the particular organism under study.

Thus, through the skilled use of the techniques of genetic analysis and biochemical methods, molecular genetic analysis allows the researcher to identify all the genes controlling a process, isolate the protein(s) or RNA(s) involved, and reveal their molecular mechanism of action. Numerous reference books, review articles, and journal articles are available to the laboratory researcher to learn the theory and practice of the vast array of biochemical methods available. Only a very few review articles on some methods of genetic analysis have been published. Thus,

learning the tools of the trade for geneticists has been largely a hands-on experience and only those fortunate enough to be trained in genetic model systems like *Escherichia coli*, bacteriophage, *Saccharomyces*, *Drosophila*, and more recently *Caenorhabditis elegans* and *Arabidopsis thaliana* completely integrate these methods into their research.

The genetic approach is straightforward but not easy. One needs to be a creative and shrewd observer with a critical, clear-thinking mind. The geneticist's tools include mutant selections/screens, complementation analysis, fine structure mutation analysis, suppressor and enhancer analysis, and more recently gene cloning, sequence analysis, and genomics. This book outlines the tools of molecular genetic analysis and presents examples of their use through case studies. The goal is to provide the novice geneticist with the skill to use these tools for his/her own research. The case studies use *Saccharomyces* because the tools of molecular genetic analysis available for *Saccharomyces* are the most straightforward and highly developed of all of the eukaryotic research organisms. As similar tools develop for genetic analysis of other systems, particularly the mammalian systems, the ability to carry out sophisticated genetic analysis to the level seen in *Saccharomyces* will also develop. Nevertheless, the theoretical basis of the methods will remain the same. To quote David Botstein (1993), a renowned geneticist who has contributed greatly to the theoretical development of molecular genetics, 'The many different organisms upon which we practice genetics present diverse difficulties and opportunities in execution, but underneath the fundamentals remain always the same.' The methods of molecular genetic analysis learned using *Saccharomyces* are directly applicable to other organisms.

Section I of this book describes *Saccharomyces cerevisiae* as a genetic model organism. The genome, life cycle, sexual cycle, basic genetic methods, plasmids, and tools for molecular genetic manipulation are described. An overview of important standard techniques in cell and molecular biology is presented along with *Saccharomyces* cell structure. This summary is presented largely to facilitate reading of the research literature articles included in the case studies. Section II presents the various methods and tools of molecular genetic analysis and takes a theoretical approach. Specific protocols for procedures are not presented. These are available from the literature and differ from organism to organism. The methods described in Section II are intended to be general in nature and adaptable to any organism. Section III consists of the *Saccharomyces* case studies. With each case study one is expected to read, interpret, and critique a series of original research articles by responding to a series of homework questions based on each article. These articles were published over the past several decades and illustrate, step by step, the molecular genetic analysis of important cellular processes in the budding yeast *S. cerevisiae*. Along the way, the reader will develop an appreciation for the molecular genetic method of analysis and the synergy between the genetic, biochemical, and cytological approaches to problem-solving in biological systems. More important, the critical thinking skills illustrated by the case studies presented here should translate quite readily to the reader's own research projects and scientific decision-making.

The following fable, 'A Tale of Two Retired Scientists and Some Rope', by William T. Sullivan (1993), describes in anecdotal fashion the differences between

the biochemical approach and the genetic approach to problem-solving. The real take-home message of this story, and also of this book, is that while both the biochemical and genetic approaches are very valuable, the synthesis of the two, that is the molecular genetic approach, is far more powerful than either method used exclusively.

'The Salvation of Doug— A Tale of Two Retired Scientists and Some Rope', by William T. Sullivan

On a hill overlooking an automobile factory, lived Doug, a retired biochemist, and a retired geneticist (nobody knew his name). Every morning, over a cup of coffee, and every afternoon, over a beer, they would discuss and argue over many issues and philosophical points. During their morning conversations, they would watch the employees entering the factory below to begin their workday. Some would be dressed in work clothes carrying a lunch pail, others, dressed in suits, would be carrying briefcases. Every afternoon, as they waited for the head on their beers to settle, they would see fully built automobiles being driven out of the other side of the factory.

Having spent a life in pursuit of higher learning, both were wholly unfamiliar with how cars worked. They decided that they would like to learn about the functioning of cars and having different scientific backgrounds they each took a very different approach. Doug immediately obtained 100 cars (he is a rich man, typical of most biochemists) and ground them up. He found that cars consist of the following: 10% glass, 25% plastic, 60% steel, and 5% other materials that he could not easily identify. He felt satisfied that he had learned of the types and proportions of material that made up each car.

His next task was to mix these fractions to see if he could reproduce some aspects of the automobile's function. As you can imagine, this proved daunting. Doug put in long hard hours between his morning coffee and afternoon beer.

The geneticist, not being inclined toward hard work (as is true for most geneticists) pursued a less strenuous (and less expensive) approach. One day, before his morning coffee, he hiked down the hill, selected a worker at random, and tied his hands. After coffee, while the biochemist zipped up his blue jump suit, adjusted his welder's goggles, and lit his blowtorch to begin another day of grinding, the geneticist puttered around the house, made himself another pot of coffee, and browsed through the latest issue of *Genetics*.

That afternoon, while the automobiles were rolling off the assembly line, Doug, wet with the sweat of his day's exertions, took a sip of beer and as soon as he caught his breath began discussing his progress.

'I have been focusing my efforts on a component I consistently find in the plastic fraction. It looks like this (he draws the shape of a steering wheel on the edge of a napkin). Presently I have been mixing it with the glass fraction to see if it has any activity. I am hoping that with the right mixture I may get motion, although I have not had any success so far. I believe with a bigger blow torch, perhaps even a flame thrower, I will get better results.'

The geneticist was only half listening because his attention was drawn to the cars rolling off the assembly line. He noticed that they were missing the front and rear windows, but not the side windows. As soon as the biochemist finished speaking (geneticists are very polite conversationalists), the geneticist proclaimed, 'I have learned two facts today. The worker whose hands I tied this morning is responsible for installing car windows and the installation of the front and back windows.'

The following day the geneticist tied the hands of another worker. That afternoon he noticed that the cars were being produced without the plastic devices the biochemist was working on (steering wheels). In addition, he noticed that as the cars were being

driven off to the parking lot, none of them make the first turn in the road and they begin piling up on the lawn.

That evening, to Doug's dismay, the geneticist concluded that steering wheels were responsible for turning the car and, in addition, that he had identified the worker responsible for installing the steering wheels.

Emboldened by his successes, the next morning the geneticist tied the hands of an individual dressed in a suit and carrying a briefcase in one hand and a laser pointer in the other (he was a vice president). That evening the geneticist, and Doug (although he would not openly admit it), anxiously awaited to see the effect on the cars. They speculated that the effect might be so great as to prevent the production of the cars entirely. To their surprise, however, that afternoon the cars rolled off the assembly line with no discernible effect.

The two scientists conversed late into the evening about the implications of this result. The geneticist, always having had a dislike for men in suits, concluded that the vice-president sat around drinking coffee all day (much like geneticists) and had no role in the production of the automobiles. Doug, however, held the view that there was more than one vice president so that if one was unable to perform, others could take over his duties.

The next morning Doug watched as the geneticist, in an attempt to resolve this issue, headed off towards the factory carrying a large rope to tie the hands of all the men in suits. Doug, after a slight hesitation, abandoned his goggles and blowtorch, and stumbled down the hill to join him. (Reproduced by permission of the Genetics Society of America.)

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I *Saccharomyces cerevisiae* as a Genetic Research Organism

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1 *Saccharomyces cerevisiae* as a Genetic Model Organism

OVERVIEW

Baker's/brewer's yeast, *Saccharomyces cerevisiae*, is a molecular genetic model organism. It is a eukaryote with a nucleus and membrane-bound organelles like mitochondria, peroxisomes, endoplasmic reticulum, and a Golgi complex. As such, complex processes like chromosome replication, transcription and translation, cell division, secretion, membrane trafficking, subcellular compartment structure and function, energy metabolism, cytoskeletal structure and mechanics, and intracellular signaling that are carried out by all eukaryotes can be explored in detail in an organism with a well-developed and simple-to-use genetic system. *Saccharomyces* is easy to culture and obtain in quantity, thus making it amenable to biochemical analysis. Gene manipulation techniques for *Saccharomyces* are extremely powerful. The major disadvantage of working with *Saccharomyces* is cell size, which makes cytological analysis difficult. Nevertheless, continued development of new microscopic techniques and analytical tools has improved the situation greatly. It is likely that the function of each of *Saccharomyces*' 6000+ genes will soon be known making *Saccharomyces* a tool for *in vivo* testing of the function of genes derived from other organisms with less-well-developed genetic systems. Detailed protocols for many of the techniques described in Chapter 1 can be found in Section 13 of *Current Protocols in Molecular Biology* (Ausubel *et al.*, 2001). Other excellent guides to yeast genetic methods are *The Guide to Yeast Genetics and Molecular Biology* (Guthrie & Fink, 1991), *Methods in Microbiology*. Vol. 26: *Yeast Gene Analysis* (Brown & Tuite, 1998), and *Methods in Yeast Genetics* (Burke *et al.*, 2000).

CULTURE CONDITIONS

Saccharomyces can be grown in defined media, either liquid or solid, that provide the energy and nutrients required for growth and proliferation. In a liquid medium, in which the components are dissolved in water, the individual cells are in suspension. Agar is added to a liquid medium to make solid media. Individual cells placed on the surface of a solid medium grow and divide many times using the nutrients that diffuse to them from the surrounding medium. They form well-defined colonies that are clones containing billions of genetically identical individual cells. Dividing cells are said to be in the **logarithmic phase** of growth because the number of cells is doubling at a rate that is dependent on the nutritional quality of the medium. When one or more essential nutrients become limiting, growth and division will slow or even stop and the cells are said to be in **stationary phase** and the culture is referred to as a saturated culture. This

terminology is most often used to describe a liquid culture, but cells in colonies also go through similar phases.

Both rich and synthetic minimal media are used to culture *Saccharomyces*. **Rich medium**, called YEP or YP, is made from commercially available yeast extract and peptone (a complex protein digestion product). It contains all essential nutrients including ammonia (a rich nitrogen source), phosphate, sulfate, sodium, magnesium, calcium, copper, iron, etc. and certain other compounds that all *Saccharomyces* strains are unable to synthesize. In addition, rich medium provides many macromolecular precursors such as amino acids and nucleotides that wild-type *Saccharomyces* strains are able to synthesize if necessary. A sugar or other carbon energy source must be added, such as glucose (dextrose), sucrose, lactic acid, or others depending on the genotype of the strain and its ability to utilize various carbon sources. Glucose is the richest and most readily available carbon source and a rich medium containing glucose is referred to as YEPD or YPD. Because of the abundant nutrient supply, cells divide rapidly on a rich medium with a division time of about 90 minutes and easily visible colonies are formed in about 2 days.

Synthetic minimal medium, referred to as SM, is made from commercially available yeast nitrogen base plus a carbon source, usually glucose unless specified. It provides the essential nutrients listed above but lacks the amino acids, nucleotides, and other precursors that are in a rich medium. Thus, a strain must be able to synthesize these in order to grow and divide on SM medium. Growth is significantly slower on SM medium, with a doubling time of about 4 hours. *Saccharomyces* can be grown on a completely chemically defined medium made from about two dozen organic and inorganic compounds, but for most research this is not necessary. A strain capable of growing in a defined minimal medium is called a **prototrope**. Ideally this minimal medium contains only a carbon source plus inorganic salts, but it is usual for wild-type microorganisms to require supplements, such as a vitamin, to this ideal minimal medium. Despite this, the wild-type genotype is generally considered to be a prototrope. Mutant strains unable to synthesize an essential nutrient are an **auxotrope** for that particular nutrient.

The following points are very important for the geneticist to note and understand. If a strain is unable to synthesize a particular essential nutrient, then that nutrient will have to be added to the synthetic minimal media to allow the strain to grow on an SM medium. For example, a strain containing a mutation in an *ADE* gene encoding an enzyme for the biosynthesis of adenine is unable to synthesize adenine and must have adenine added to the synthetic minimal medium to allow it to grow. This mutant strain is an adenine auxotrope. Thus, an *ade2* mutant strain requires adenine in the growth medium. In contrast, if a strain is unable to utilize a particular carbon source, for example sucrose, then the strain will not be able to grow on media that provide that carbon source as the sole carbon source. A strain that contains a mutation in a *SUC* gene is unable to utilize sucrose because it does not synthesize functional invertase, the enzyme required to hydrolyze sucrose to glucose and fructose. Thus, a *suc2* mutant strain will not grow if sucrose is the only carbon source provided and some other carbon source, such as glucose, must be available. In summary, strains carrying mutations in anabolic pathways require the product of the pathway for growth while strains carrying mutations in catabolic pathways cannot grow if the substrate of the pathway is provided.

THE MITOTIC LIFE CYCLE

Saccharomyces is a budding yeast, that is, the ovoid (or egg-shaped) mother cell produces a small protrusion or bud on its surface that grows in size during the course of interphase of the cell cycle into what will become the daughter cell. After the S phase is complete and the DNA has been replicated, the nucleus localizes to the neck region between the mother and the bud, divides into two nuclei, and one nucleus enters the bud while the other remains in the mother (karyokinesis). Following karyokinesis the cytoplasms of the mother and daughter cells divide with the formation of separate plasma membranes and cell walls (cytokinesis), and eventually the daughter cell grows to the size of the mother. Both cells are then capable of dividing again. This is outlined in Figure 1.1. A more in-depth description of the cytological changes that occur during mitosis is presented in Chapter 3.

Both haploid and diploid *Saccharomyces* cell types can divide by mitotic division. Many eukaryotic organisms favor either the haploid (lower plants, slime molds, many fungi) or diploid (animals, higher plants) portion of the life cycle and proceed through the alternate stage very rapidly. For *Saccharomyces* the existence of stable haploid and diploid cell types means that the researcher can culture large numbers of genetically identical individuals (**clones**) and use them for analysis of the phenotype via cytological or biochemical analysis. Other than dealing with different numbers of chromosomes, mitosis of diploid and haploid strains is essentially the same at the level of the chromosome. There are some cytological differences between haploid and diploid cells during mitosis, particularly in bud-site selection, that are discussed in Chapter 3. These do not affect the genetic analysis of other traits.

MATING TYPE, MATING, AND THE SEXUAL LIFE CYCLE

In nature most strains of *Saccharomyces* are diploid and carry the functional allele of the *HO* gene, homothalic diploids. Laboratory research strains carry mutant *ho* and can be grown as stable haploid cells. Haploids occur in two mating types, the **a** mating type and the α mating type, and these differ from one another at a single locus called the *MAT* locus. The two alleles of this locus are referred to as *MAT***a** and *MAT*α. Stable **a** or α strains divide mitotically to produce genetically identical clones of cells. The existence of a stable haploid stage in the life cycle of *Saccharomyces* is attractive to the geneticist because strains carrying recessive mutations can be isolated and identified in the haploid cell type and it is not necessary to inbreed mutagenized cells to obtain a homozygous mutant diploid.

*MAT***a** strains mate with *MAT*α strains by a complex process of cytoplasmic and nuclear fusion that results in a diploid cell (described in Chapter 3). This diploid cell is also stable and divides by mitosis to produce a genetically identical diploid clone. The existence of the stable diploid cell type is also extremely useful for the geneticist. It allows one to determine if a mutant allele is dominant or recessive and it provides a simple means for carrying out a complementation test. Complementation analysis is described in Chapter 5 and is used to determine if different mutations map to the same or different genes.

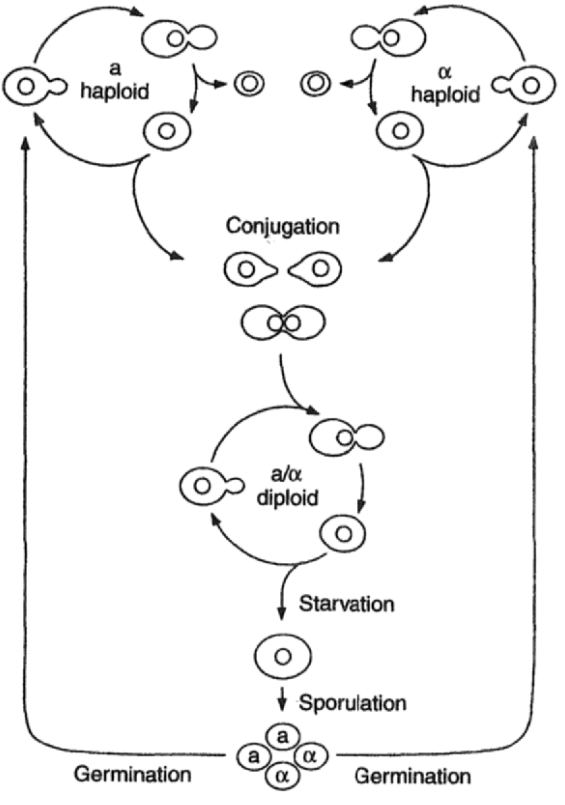

Figure 1.1 Life cycle of *Saccharomyces cerevisiae*. Diploid (**a**/α) and haploid, **a**- or α-mating type, *Saccharomyces cerevisiae* can reproduce by mitosis to form clones of genetically identical cells. Haploid cells of opposite mating type can fuse with one another to form an **a**/α diploid. When subjected to nutrient starvation conditions, diploid cells undergo meiosis producing four haploid meiotic products called ascospores all contained in a single structure called an ascus. If restored to nutrient sufficient conditions, each of these four ascospores will germinate and reproduce as haploid cells as follows: two α-mating type cells and two **a**-mating type cells. From *The Cell Cycle: An Introduction* by Andrew Murray and Tim Hunt, copyright © 1993 by Oxford University Press, Inc. Used by permission of Oxford University Press, Inc.

In starvation conditions, the **a**/α diploid undergoes meiotic division and produces four haploid cells that mature into **ascospores**. The four haploid products of a single diploid cell are contained in a sack called an **ascus** that is designed for survival under difficult conditions. Using a microdissection device mounted on a microscope stage, one can separate the individual haploid ascospores and germinate them in nonstarvation media. These will divide to produce genetically identical haploid clones. This process is shown in Figure 1.1. The simplicity by which the reseacher can manipulate the sexual life cycle of *Saccharomyces* is a tremendous advantage for genetic analysis.

SACCHAROMYCES GENOME AND NOMENCLATURE

GENOME SEQUENCE

Saccharomyces cerevisiae has a haploid chromosome number of 16. The entire *Saccharomyces* genome of strain S288C is sequenced and available on the *Saccharomyces* Genome Database (called SGD) at http://genome-www.stanford.edu/Saccharomyces. The site has a variety of tools for sequence analysis that are particularly useful for the *Saccharomyces* researcher, including gene and restriction maps of the chromosomes. The site is interconnected with genome databases for other genetic model organisms and sites for protein analysis. There are literature guides for the known *Saccharomyces* genes, announcements of interest to the yeast research community, and contact information for yeast researchers. SGD is well worth a visit.

The *Saccharomyces* genome contains more than 13 million basepairs (13 Mbp) including the rDNA and more than 6000 open reading frames (ORFs). Each ORF is named to indicate the chromosome number (A for chromosome *I* to P for chromosome *XVI*), whether the gene is found on the right (R) or left (L) arm of the chromosome (that is, to the right or left of the centromere), and the ORF number. All ORFs are numbered on each chromosome arm starting at the centromere and going in the direction of the telomere regardless of which strand is the coding strand. Finally, the direction of transcription is indicated by a W (for Watson, the upper strand) or C (for Crick, the lower strand) depending on which strand is the coding strand. Thus, ORF YBR288C is found on the right arm of chromosome *II*. It is the 288th ORF from the centromere, and the lower strand is the coding strand; that is, it is transcribed from right to left which for the right arm means towards the centromere.

GENETIC NOMENCLATURE

Saccharomyces gene names consist of three letters and a number (usually 1–3 digits). The letters chosen are most often based on the phenotype or function of the gene. Note that the number follows immediately after the letters with no space. For example, a gene encoding one of the enzymes of histidine biosynthesis is referred to as *HIS3*. As in all organisms, gene names are italicized. Often, several mutant alleles of a particular gene have been identified. These can be distinguished by placing a suffix after the gene name; frequently a hyphen followed by the allele number is used, all with no spaces.

In *Saccharomyces*, dominant alleles of a gene are capitalized and recessive alleles are in lower case. For example, mutant allele #52 is a recessive mutation of *URA3* and is written *ura3-52*. Mutant strains resistant to the toxic effects of the arginine analogue canavanine carry dominant alterations in *CAN1* encoding the arginine permease and are written *CAN1-R*. It is important that you do not confuse the concept of a wild-type allele versus a mutant allele with capital letters (for dominant alleles) versus lower case letters (for recessive alleles). There are many examples of mutant alleles that are dominant. The interpretation of dominant versus recessive will be discussed in Chapter 4 in more detail.

PHENOTYPE NOMENCLATURE

Descriptive words or abbreviations derived from the gene name can be used when discussing the phenotype of a strain. For example, a strain carrying a *lys2* mutant allele (genotype *lys2*) will not grow in the absence of added lysine. This phenotype can be referred to as lysine minus, lysine$^-$, or lys$^-$. Note that the letters are not italicized and that no gene number is given. Lysine synthesis requires several enzymatic steps and therefore mutations in any of several genes encoding these enzymes can cause a lysine minus phenotype. When observing the phenotype of a strain one has no information as to genotype. Therefore it is inappropriate to use the gene number. Genotype can only be determined by doing appropriate crosses to known genetic tester strains.

STRAIN NOMENCLATURE

In journal articles on *Saccharomyces* it will be noted that researchers name their strains in a wide variety of ways. There are certain standard strains, like S288C, W303, or YPH500, that are commonly used in research laboratories and these will be referenced in the Materials and Methods section of an article. If the authors have done some genetic manipulations with these strains, then they will rename the strain often using their initials. For example, strain YPH500 was constructed by Phil Hieter and coworkers, and the letters stand for Yeast Phil Hieter. The article will state that the new strain is a derivative of the original strain and a literature reference to the original strain will be given. Often a strain list is presented with the relevant genotype of the strains used in the study along with information on the derivation of the strain. The genotype will indicate all of the genes that are mutant. If a gene is not listed it is assumed to be the wild-type allele found in the strain from which the mutant was derived, such as S288C. While all of these strains are highly similar at the sequence level they are not identical. Strain differences may be very few but could potentially be significant for the particular research project being described. Geneticists pay very careful attention to strain backgrounds and do their best to keep them constant.

PROTEIN NOMENCLATURE

The protein product of a *Saccharomyces* gene can be named based on the gene name or the function, if it is known. For example, *GAL1* encodes galactokinase, the first enzyme in the catabolism of the sugar galactose. The product of the *GAL1* gene is referred to as galactokinase, Gal1 protein, or Gal1p. Note that only the first letter is capitalized and that the protein name is not italicized.

GENETIC CROSSES AND LINKAGE ANALYSIS

Saccharomyces diploids undergo meiosis when placed in starvation conditions and form four haploid ascospores, or just spores for short, all contained in a single sack called an ascus. These four spores are referred to as a **tetrad** since each spore

contains one chromatid from each of the 16 tetrads of chromatids found in prophase I of meiosis.

To fully understand the crosses outlined below, it would be helpful to first review the process of meiosis including chromatid segregation patterns and independent assortment, i.e. basic Mendelian genetics. The genetic cross is a powerful tool. In the initial stages of a genetic analysis the researcher must know whether a single mutation is producing the mutant phenotype under investigation. A simple genetic cross can demonstrate this. Crosses are used to construct heterozygous diploids to determine whether a mutation is dominant or recessive and can be used to demonstrate linkage. As can be seen from the literature, linkage analysis is used in a variety of ways and not simply to map genes on a chromosome.

SINGLE GENE CROSS

If two haploid strains carrying different alleles of the same gene are mated and the resulting diploid sporulated, the two alleles will segregate to different spores. The resulting four-spored tetrad will consist of two spores containing one allele of the gene and two spores containing the second allele of the gene. This is shown below in Cross 1 in which a strain containing a recessive mutation, *gen1-62*, is crossed to a strain carrying the wild-type dominant allele *GEN1*.

Cross 1: *GEN1* × *gen1-62* (genotypes of parental strains)
(wild-type) (mutant) (phenotype of parental strains)

Diploid: *GEN1* (genotype of diploid)
gen1-62
(wild-type) (phenotype of diploid)

2:2 Single gene segregation:

Spore	Genotype	Phenotype
A	*GEN1*	wild-type
B	*GEN1*	wild-type
C	*gen1-62*	mutant
D	*gen1-62*	mutant

Since *gen1-62* is a single alteration in the *GEN1* gene, the only possible tetrad that can result from this cross is one containing two *GEN1* spores and two *gen1-62* spores. If a thousand tetrads were dissected all would be 2 wild-type : 2 mutants because only a single mutant gene is segregating in this cross. Thus, the 2 : 2 segregation pattern is consistent with the fact that a single genetic difference exists between the parent strains in the cross.

But what if one does not know whether a mutant strain contains a single mutant alteration? Perhaps two or more mutations in different genes are needed to produce the mutant phenotype observed. Perhaps the mutant strain has a complex phenotype and exhibits several abnormalities. Are all these abnormal phenotypes associated with a single gene mutation or are there several mutations in the strain

each causing a specific phenotype? These questions can be answered by crossing the mutant strain to a wild-type strain (one that has not been exposed to mutagenesis and selection). If tetrad analysis of many tetrads derived from this heterozygous diploid gives only two mutant spores and two wild-type spores, then this is strong evidence that one is working with a mutant strain carrying a mutation in a single gene. If all the mutant phenotypes are exhibited by all the mutant spores, then one can conclude that the single gene mutation has several phenotypic effects, i.e. it is **pleiotropic**. If one finds some other segregation pattern, such as one mutant spore to three wild-type spores, or if the phenotypes segregate from one another, then one must consider the possibility that the mutant strain contains mutations in two or more genes (see Cross 3 below). There are other interpretations of a 1 : 3 ratio, such as a high rate of gene conversion or aneuploidy, but these are less likely particularly if the mutations were induced by a mutagen and are not spontaneous.

The 'single gene cross' has other uses. If one crosses two mutant strains believed to contain different mutant alleles of the same gene, such as a deletion and a single base change, then these alleles should always segregate to different spores. All of the haploid spores resulting from the heterozygous diploid should contain either one or the other mutant allele. Given this segregation pattern, only tetrads containing four mutant and no wild-type will result, except for rare recombinants between the alleles if recombination is possible. Thus, a 4 : 0 result in all tetrads is strong evidence that the mutations are in the same gene, or extremely closely linked genes. This is shown in Cross 2 below.

Cross 2: *gen1-33* (mutant) × *gen1-62* (mutant) (genotypes of parental strains) (phenotype of parental strains)

Diploid: *gen1-33* / *gen1-62* (genotype of diploid)
(mutant) (phenotype of diploid)

2:2 Single gene segregation:

Spore	Genotype	Phenotype
A	*gen1-33*	mutant
B	*gen1-62*	mutant
C	*gen1-33*	mutant
D	*gen1-62*	mutant

TWO GENE CROSS

If strains carrying mutations in two different genes are crossed, then the genes will recombine producing recombinant meiotic products with a wild-type and double mutant genotype. The frequency of recombination will depend on whether the genes are linked (map close to one another on the same chromosome) and, if linked, how tightly they are linked. When the genotypes of the spores in different tetrads from such a diploid are determined, three classes of tetrads are obtained as follows.

Parental ditype (PD) tetrads result when no recombination occurs between the mutant genes during meiosis of the diploid cells. These will contain four spores, two of each parental genotype. When recombination occurs during meiosis of the diploid cell, either **tetratype** (TT) or **nonparental ditype** (NPD) tetrads are obtained. A tetratype tetrad contains four spores each with a different genotype, including the two parental genotypes and the two recombinant genotypes, which are wild-type and the double mutant. A nonparental ditype tetrad contains two types of spores neither of which is the parental genotype, i.e. both are recombinant types, including two wild-type spores and two double mutant spores. This is shown below in Cross 3.

Cross 3: *gen1 GEN2* × *GEN1 gen2* (genotypes of parental strains)
(mutant) (mutant) (phenotype of parental strains)

Diploid: $\frac{gen1}{GEN1}\ \frac{gen2}{GEN2}$ (genotype)
(wild-type) (phenotype)

Parental ditype:

Spore	Genotype	Phenotype
A	*gen1 GEN2*	mutant
B	*gen1 GEN2*	mutant
C	*GEN1 gen2*	mutant
D	*GEN1 gen2*	mutant

Tetratype:

Spore	Genotype	Phenotype
A	*gen1 GEN2*	mutant
B	*gen1 gen2*	mutant
C	*GEN1 gen2*	mutant
D	*GEN1 GEN2*	wild-type

Nonparental ditype:

Spore	Genotype	Phenotype
A	*gen1 gen2*	mutant
B	*gen1 gen2*	mutant
C	*GEN1 GEN2*	wild-type
D	*GEN1 GEN2*	wild-type

The frequency of each type of tetrad will depend on the frequency of recombination. If the two mutant genes are completely unlinked, that is 50% recombination, then the frequency of PD : TT : NPD tetrads will be 1 : 4 : 1. If there is any linkage, that is the frequency of recombination is less than 50%, then the relative number of PD tetrads will increase to greater than the expected 1/6 of the total number of tetrads analyzed and the number of PD tetrads will exceed the number of NPD

tetrads. Ultimately, for crosses between two alleles, 100% of the tetrads will be PD, as is shown in Cross 2.

It is important to note that the phenotype of the double mutant may be unique. This occurs most often when the two genes encode functions involved in the same process. If mutant strains containing alterations in unrelated gene functions, such as *ade2* and *suc2*, are crossed, then the double mutant is expected to exhibit both phenotypes, adenine requiring and unable to utilize sucrose. If mutant strains containing alterations in two related gene functions are constructed, such as *MCM2* and *MCM7* encoding different components of the origin recognition complex (ORC), then the double mutant could exhibit an unexpected phenotype. For example, the double mutant combination could be lethal even though each single mutant strain is viable. Often mutant genes are crossed for the purpose of determining the double mutant phenotype. As will be discussed in detail in the chapters on epistasis, suppression, and enhancement, a great deal of insight into the function and relationship between gene products can be obtained from observing the phenotype of the double mutant.

CLASSES OF *SACCHAROMYCES* CLONING PLASMID VECTORS

Saccharomyces plasmids were developed from *Escherichia coli* plasmid vectors. The basic *E. coli* vector is small [2–4 kilobasepairs (kbp) of DNA] and includes genes needed for plasmid replication, an origin of replication (ORI) derived from an *E. coli* plasmid, and a selectable marker gene such as AMP^r (for ampicillin resistance) to be used to identify *E. coli* transformants containing the plasmid. The *E. coli* ORI allows the plasmid to replicate independent of the *E. coli* chromosome as an extra-chromosomal element or plasmid. As such it is easy to purify in large amounts. Additionally, one or more restriction sites will be present for cloning foreign DNA sequences.

E. coli plasmids are the foundation for the construction of the *Saccharomyces* yeast cloning vectors. *Saccharomyces* sequences were added to the *E. coli* vectors to create what are referred to as *E. coli*/yeast shuttle vectors, meaning that these plasmid vectors are able to establish themselves in either organism. First, a **marker gene** capable of being selected in a yeast host strain was included in order to be able to select yeast transformants. Good antifungal agents, comparable to the ampicillin and tetracycline used in *E. coli*, were not initially available. Therefore, nutritional genes encoding enzymes in biosynthetic pathways were the first to be used as **selectable marker genes** in *Saccharomyces*. More recently antifungal agents like kanamycin (also called G418 or neomycin) and hygromycin have come into use.

URA3, *LEU2*, *TRP1*, and *HIS3* are the genes most commonly used as selectable marker genes for *Saccharomyces* transformation. The *Saccharomyces* strains used as hosts for plasmid vectors carrying these nutritional marker genes must contain recessive mutant alleles of these genes in order to be an appropriate host. Suitable mutant alleles of *URA3*, *LEU2*, *TRP1*, *HIS3* and other genes are available. Strains like YPH500 have been specially constructed to carry several of these mutant genes. It is important to keep in mind that transformation is rare, about one in 1000 cells

or less, and not so different from the rate of mutation. To facilitate selection of transformants as opposed to the back mutations to wild-type, the mutant alleles of these genes do not revert at any appreciable rate because they are deletions, multiple point mutations, or transposon insertion mutations.

A typical *Saccharomyces* transformation is carried out as follows. An appropriate host/vector pair is selected. For example, a host strain carrying the *ura3-52* allele is unable to grow on a minimal medium that lacks uracil because it is unable to synthesize uracil, which is essential for various cellular processes including RNA synthesis. If a plasmid carrying the wild-type dominant *URA3* gene is introduced into this host strain by transformation, then the transformant will be able to grow on a minimal medium lacking uracil. The plasmid DNA is transformed into the host cells by any one of a number of methods including chemical treatments, electroporation, or pellet guns. The DNA treated cells are plated on a solid synthetic medium lacking uracil. Only those individuals that have acquired a stable copy of *URA3* by transformation with the plasmid vector will be able to form colonies. Of course this must be confirmed by appropriate tests.

The fate of the plasmid after entering a *Saccharomyces* cell depends on the particular *Saccharomyces* sequences it contains. If a *Saccharomyces* origin of replication is included, then the plasmid will replicate as an extrachromosomal element. Its copy number, the average number of plasmids per cell, is determined in part by the class of *Saccharomyces* ORI and whether or not a *Saccharomyces* centromere is also included in the plasmid. If the plasmid vector lacks a *Saccharomyces* replication origin, then the plasmid must integrate at a chromosomal site (usually by homologous recombination between vector sequences and the chromosome) to produce a stable transformant. If the plasmid vector integrates, then it will replicate as part of the chromosome.

YIp PLASMID

A YIp plasmid consists of the basic *E. coli* vector described above plus a *Saccharomyces* selectable marker gene, but does not contain a *Saccharomyces* origin of replication. Therefore, YIp plasmids must integrate into a chromosome in order to be replicated at each cell division. If integration does not occur, the transforming DNA will be lost due to degradation or dilution by cell division.

Integration occurs by means of a single crossover (recombination) event between the plasmid DNA and the chromosome. This is illustrated below in Figure 1.2. The crossover occurs *only* between homologous DNA sequences and is carried out by the generalized recombination enzymes. After the integration event, the plasmid sequences are part of the chromosome, are replicated when the chromosome is replicated, and are passed into both the mother and daughter cells during cell division, as are all the other sequences of the chromosome. Figure 1.2 shows a recombination event occurring between a yeast sequence carried by this vector and a homologous chromosomal sequence. This recombination event might also have occurred between the *URA3* sequence on the plasmid and the mutant *ura3-52* gene in the host since sequences are still present at this site. To prevent this, one could use *ura3* deletion mutation.

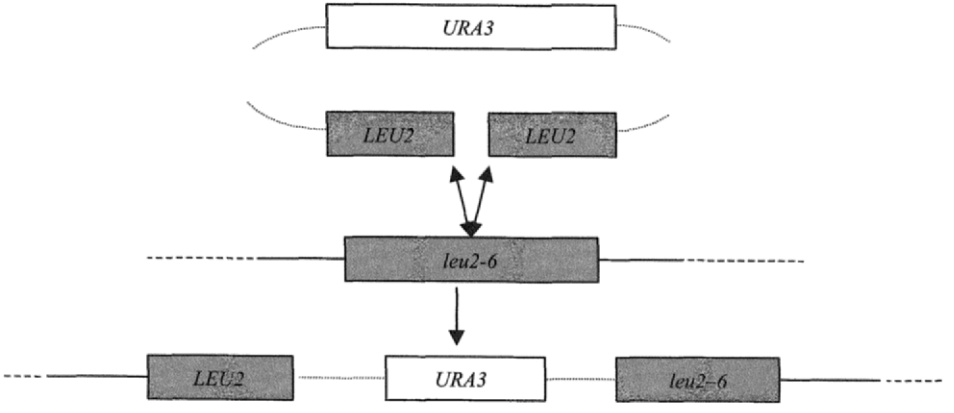

Figure 1.2 Targeted integration

The integration of a YIp plasmid can be targeted to a specific chromosomal site (shown in Figure 1.2). Integration requires recombination between homologous sequences on the plasmid and chromosome. For supercoiled plasmid DNA, this recombination occurs in about one in 10 000–100 000 transformed cells. But, if prior to transformation the plasmid is digested with a restriction enzyme that cuts at a site within the homologous sequence creating a highly recombinogenic double-strand break, then the frequency of recombination will increase about 1000-fold. Therefore, as is shown in Figure 1.2, if a particular YIp plasmid carrying two *Saccharomyces* sequences, for example *URA3* and *LEU2*, is digested at a site in the *LEU2* gene and transformed into a *ura3 leu2* host strain, then it will integrate 1000 times more often at the *leu2* locus than at the *ura3* locus. If transformant strains are crossed to another strain of opposite mating type with the *ura3 leu2* genotype and sporulated, then all of the tetrads will be PD with two uracil$^-$ leucine$^-$ spores and two uracil$^+$ leucine$^+$ spores. That is, the *URA3 LEU2* alleles will segregate together because they are both linked to the site of plasmid integration.

YRp PLASMID

YRp plasmids are constructed from the basic YIp vector by the addition of a *Saccharomyces* origin of replication derived from a chromosomal sequences. These yeast ORI sequences are commonly called ARS sequences for autonomously replicating sequence. Chromosomal replication initiates at these sites and, on average, they are found every 40 kilobasepairs of DNA in *Saccharomyces*. YRp plasmids can be integrated but normally they are not and are able to replicate as independent extrachromosomal plasmids. Depending on the particular ARS element, they are present in 5–10 copies per cell on average.

YRp plasmids are unstable because there is no mechanism to move the plasmid copies into the bud (like a spindle) and they often get left behind in the mother nucleus. Because of this, the growth of cells transformed with YRp plasmids in nonselective media (that is, media containing the nutrient synthesized by the

selection marker carried on the plasmid) leads to the spontaneous loss of the plasmid. To ensure that the plasmid is maintained by most of the cells in a culture, transformants must be grown in a selection medium lacking the nutrient at all times so that only cells inheriting the plasmid will be capable of growing and dividing.

YEp PLASMID

A YEp plasmid contains an origin of replication derived from the naturally occurring *Saccharomyces* plasmid called the 2μ circle in the basic YIp vector. The *Saccharomyces* 2μ circle is a very abundant plasmid found in many natural strains and most laboratory strains. The 2μ circle ORI is a very active origin and YEp plasmids normally replicate as high-copy independent extrachromosomal plasmids present in 25–50 copies per cell on average. YEp plasmids are also unstable and transformants must be grown under selection to maintain the plasmid.

YCp PLASMID

A YCp plasmid contains a centromere sequence, CEN, derived from one of the 16 *Saccharomyces* chromosomes added to a YRp vector. These plasmids are treated like mini chromosomes by the dividing *Saccharomyces* cell. YCp plasmids attach to spindle fibers during division and are very efficiently transmitted to both mother and daughter cells in mitosis and meiosis, although not as efficiently as the normal chromosomes. Therefore, YCp plasmids are very stable plasmids that replicate and segregate along with the remainder of the chromosomes. As a result, YCp plasmids are low-copy independent extrachromosomal plasmids present in 1–2 copies per cell on average and are lost from transformant cells at a very low rate even in the absence of nutritional selection.

YAC PLASMID

YAC vectors are designed to carry large chromosomal fragments of DNA and have been very useful in cloning fragments for various genome sequencing projects and for positional cloning studies. YAC stands for yeast artificial chromosome. The major difference between YAC vectors and YCp vectors is the inclusion of two copies of a sequence derived from *Saccharomyces* telomere DNA consisting of many repeats of the short nucleotide sequence $5'C_{2-3}A(CA)_{1-3}$ on one strand with the complementary GT-rich sequence repeated on the other strand. In the circular YAC vector plasmid, the two copies are separated by a stuffer fragment that is cut out using restriction enzymes prior to transformation into the host *Saccharomyces* strain. Once in the host cell, endogenous telomerase enzyme will elaborate a full telomere at each end of the linearized YAC vector DNA. Natural *Saccharomyces* chromosomes range from 230 to 1700 kbp but these have several origins of replications each. Inserts up to 1400 kbp can be accommodated in certain YAC vectors.

LIBRARIES

Saccharomyces plasmid libraries can be constructed from any of these types of vector. The choice depends on whether a stable or unstable *Saccharomyces* transformant is desired and whether one or many copies per cell are needed.

GENE DISRUPTION/DELETION IN *SACCHAROMYCES* (ONE-STEP GENE REPLACEMENT)

Gene disruption is a method by which a DNA fragment is used to replace a genome sequence with a selectable marker gene, such as *HIS3* or kanavanine resistance. In so doing, a deletion is created. The process occurs by homologous recombination and uses the enzymes of the homologous recombination pathway, such as Rad52p. The ends of the exchange fragment must be long enough and have sufficient homology to the chromosomal site so that homologous recombination can occur. Moreover, the size of the region to be deleted can be quite large but must be contained in a single chromosome.

One-step gene replacement is a relatively efficient process. Free DNA ends are very 'recombinogenic' in yeast. This means that free 3' and 5' ends of double-stranded DNA fragments *in vivo* search out homologous sequences in the chromosomes with very high efficiency. When a homologous sequence is found, the free ends invade the chromosomal sequence and this leads to a crossover event at a site near the free end. If this happens at both ends of a DNA fragment, then the fragment replaces the genomic copy. This is shown in Figure 1.3 for a fictitious gene, *YGI1* (Your Gene of Interest).

Recombinant DNA methods can used to construct the disruption fragment. This method was used prior to the development of polymerase chain reaction (PCR)-based methods (see below) and is often seen in the literature. Disruption constructs for many genes are available from researchers in the yeast community and are provided upon request. To make a disruption construct, one starts with the cloned genomic fragment (contained in an *E. coli* plasmid vector). Restriction digestion or other related methods can be used to cut out the internal sequences and replace them with the selectable marker gene. This is shown in Figure 1.4.

More recently, PCR-based methods have been used for the construction of the disruption fragment. In yeast, only about 40 bp of sequence are needed at each end of the disruption fragment in order for the crossover events to occur properly, but the sequence must be identical to the genomic target sequence. The PCR primers are used to amplify the selection gene and place a target site sequence at either end. Each primer consists of 40 bp of target site sequence at the 5' end followed by a short sequence homologous to the selection gene. The complete selection marker gene must be amplified, including the promoter and ORF. Longtine *et al.* (1998) describe plasmid constructs designed specifically to provide selection marker templates for PCR amplification. The *kanMX6* resistance gene and the *Saccharomyces pombe his5*$^{+}$ gene fused to appropriate *S. cerevisiae* promoter and terminator sequences as well as the *S. cerevisiae TRP1* gene are available in this series. The *kanMX6* and *his5*$^{+}$ genes are particularly useful because they lack homology to *S.*

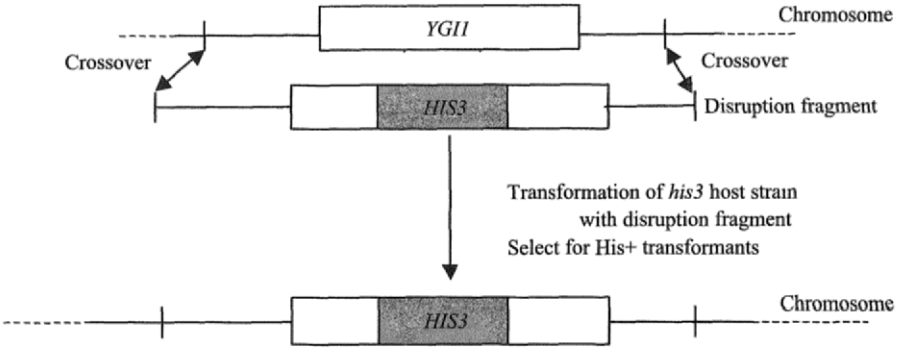

Figure 1.3 One-step gene disruption of *YGI1*

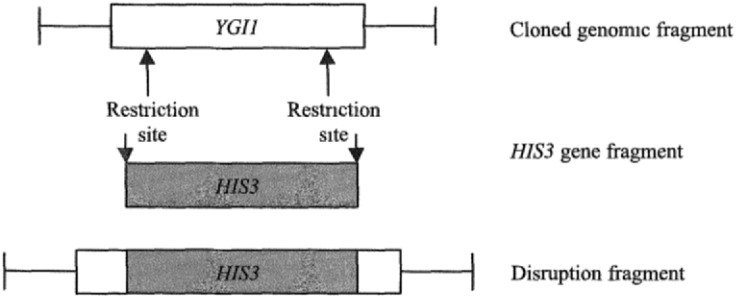

Figure 1.4 Construction of a disruption fragment using available restriction sites

cerevisiae genomic sequences and thus preclude the possibility of recombination at sites in the genome other than at the intended disruption site. When deleting a gene, it is best to remove sequences starting in the promoter and extending into the ORF or past the stop codon. This ensures that the gene has been functionally knocked out. If the transcription and translation start sites are not removed and the deletion is internal to the ORF, it is conceivable that some gene function could be retained.

Whether the traditional or the PCR-based method is used for one-step gene disruption, it is important to confirm that the event has occurred correctly. This can be done by Southern analysis or by PCR of genomic DNA using one primer that anneals to sequences outside the deleted region and one primer that anneals to internal sequences in the selection marker gene.

GAP REPAIR

This is a method frequently used to recover a specific sequence from the chromosome onto an episomal plasmid. Gap repair utilizes the host cell's recombination/repair and DNA replication machinery to fill an artificially created deletion in a

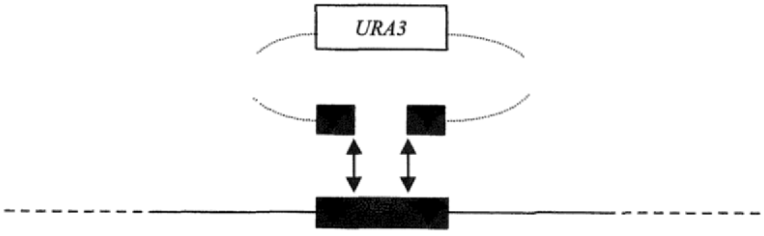

Figure 1.5 Gap repair

homologous sequence carried on the plasmid. Its most common use is for cloning different alleles of a cloned gene.

One starts with the DNA fragment of interest cloned into a plasmid vector that is maintained as an extrachromosomal element, such as YRp or YEp. Using restriction endonucleases that cut sites in the insert fragment but not in vector sequences, one creates a deletion internal to the yeast DNA fragment. It is essential to leave at least 50 bp of insert fragment at either end to provide homology to the chromosomal site as a substrate for recombination. This linearized and gapped fragment is then transformed into the host cell and transformants are selected using the marker gene carried by the plasmid vector. For the example shown in Figure 1.5, the host strain is *ura3* and repair of the gap is essential if the cell is to maintain the plasmid and to be able to grow on a selection medium lacking uracil. The gapped region is filled by a gene-conversion-like event between the gapped plasmid and the homologous chromosomal site. The arrows in Figure 1.5 indicate the end-points of the gap and the positions where the exchange events will initiate. The free ends of the gapped fragment invade the homologous chromosomal sequence, DNA replication of the gapped region occurs from these ends using the chromosomal sequence as template, and the gap is filled.

Gap repair is used to recover different alleles of the cloned sequence from the chromosome. For example, one has cloned the wild-type allele of a gene and wants to clone the available mutant alleles. Another use of gap repair is in fine structure mapping of recessive mutant alleles. If a mutation maps outside the gapped region, then filling in the gapped region of the wild-type allele carried on the plasmid with the chromosomal sequence will result in the restoration of the wild-type allele on the plasmid copy of the gene and stable transformants with the wild-type phenotype of the gene of interest will result. If the mutation maps within the gap, then only stable transformants with the mutant phenotype will be obtained.

REPORTER AND OTHER TYPES OF FUSION GENE

A reporter gene is used to follow gene expression *in vivo*. It is a fusion between all or part of a gene of interest with another gene whose product is easy to detect or measure qualitatively and/or quantitatively. Most often, the researcher will choose to use a reporter gene if the product of the gene of interest is difficult to assay or detect. Thus, the reporter gene product acts as a surrogate.

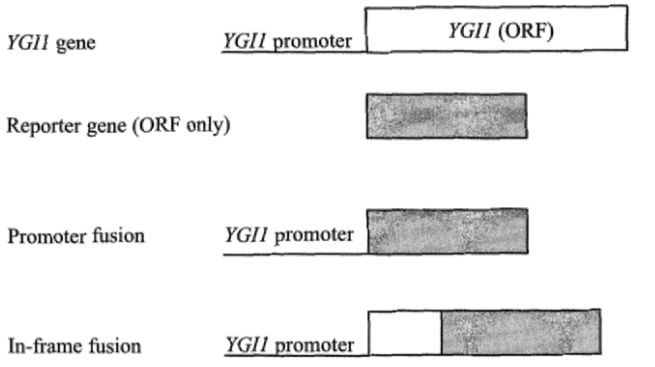

Figure 1.6 Reporter gene fusion constructions

A fusion gene between the gene of interest and the reporter gene can include only the upstream promoter of the gene of interest or part or all of its ORF. If a coding region is included, then the sequence at the fusion junction must maintain the correct reading frame so that a single ORF is produced that encodes a fusion of the two proteins. This is shown in Figure 1.6. Fusion gene constructions are often carried on plasmid vectors but they can also be integrated into chromosomes, depending on the needs of the experiment.

There are several commonly used reporter genes including *lacZ* (encoding β-galactosidase from *E. coli*), *CAT* (encoding chloramphenicol acetyltransferase, the bacterial protein providing chloramphenicol resistance), luciferase gene (encoding the phosphorescent protein from firefly), and *GFP* [encoding green fluorescent protein (GFP) from a jellyfish]. To be useful, the host organism must not encode a protein with the same activity, otherwise one could not be sure whether one was observing the activity of the endogenous protein or the reporter protein. Using a variety of techniques, these proteins can be measured either *in vivo* or *in vitro*. β-Galactosidase can be assayed *in vitro* using cell extracts by measuring the rate of hydrolysis of an uncolored compound called ONPG to a yellow dye or another uncolored compound called X-gal to a blue dye. The X-gal reaction is particularly useful because is can be done on whole cells in tissues or colonies growing in a petri dish. Cells expressing the β-galactosidase reporter will be bright blue. GFP is very useful for determining the subcellular location of a protein and the type of fusion used for this analysis is an in-frame fusion between the full-length gene of interest and the *GFP* gene (discussed in detail in Chapter 2). Alternately, portions of the gene of interest can be fused to GFP to localize the portion of the protein of interest responsible for targeting the protein to a particular subcellular compartment. There are many other uses of these types of construction.

A variety of *E. coli*/yeast shuttle vectors are available for the construction of fusion genes. These contain a multiple cloning sequence at the junction site of the fusion. The DNA sequence to be fused to the vector gene, whether it is a promoter or an ORF, is typically amplified by PCR using primers that place appropriate restriction sites at the ends of the fragment. The fragment is then cloned into the multiple cloning site to create the fusion. PCR-based methods are also available for

creating fusions at sites in the genome and these are described in Chapter 2 (Longtine *et al.*, 1998).

EXPRESSION VECTORS

Expression vectors are vectors that allow one to construct gene fusions that replace the native promoter of a gene with another promoter for any of a variety of reasons. For example, the native promoter might initiate transcription at a very low rate, too low to allow for purification or detection of the protein product of the gene, or only under very special conditions. Placing the ORF of the gene of interest under the control of a high-level constitutive promoter in a YEp vector would increase expression of the protein hopefully to levels that would allow the researcher to purify and characterize the product.

Several expression vectors are available to the *Saccharomyces* researcher and can be obtained from colleagues or from commercial sources. The *ADH1* promoter is commonly used for high-level constitutive expression in glucose-grown cells. *GAL1* and *GAL10* are frequently used when regulated expression is desired. The *GAL1* and *GAL10* are induced to very high levels in galactose grown cells but expression is dramatically repressed by growth on glucose.

An expression system developed by Mumberg *et al.* (1995) allows for the constitutive production of a gene product over a 1000-fold range. One can choose from the promoters of either *CYC1* encoding cytochrome-c oxidase isoform 1, *ADH1* encoding alcohol dehydrogenase 1, *TEF2* encoding translation elongation factor 1α, or *GPD1* encoding glyceraldehyde-3-phosphate dehydrogenase. These are available in either YEp or YRp vectors, which provides another mechanism for varying the expression level. Additionally, one can choose from either *HIS3*, *LEU2*, *URA3*, or *TRP1* as the selectable marker. If one prefers to be able to regulate the expression of the gene of interest, Labbé & Thiele (1999) developed a similar vector series but use the *CUP1*, *CTR1*, and *CTR3* copper-regulated promoters.

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2 Techniques in Yeast Cell and Molecular Biology

The techniques used to study *Saccharomyces* are not unique to *Saccharomyces* but have been adapted where possible to the special needs of this small eukaryote. Those methods that are most commonly used by researchers are described in this chapter and are presented in enough detail to allow one to understand the basics and read the literature. Additional reading material and techniques manuals are listed at the end of the chapter for those needing more information.

CELL FRACTIONATION

The study of subcellular components often requires purifying a large amount of a particular component. One might be interested in isolating and purifying for further analysis the components of a complex unit like a ribosome, proteasome, or spliceosome. Or one might want to measure the metabolic activity of organelles such as the mitochondrion or peroxisome. Cell fractionation methods have been developed that allow the researcher to separate relatively pure samples of subcellular components in reasonably active states. Lloyd & Cartledge (1991) and Zinser & Daum (1995) review methods of isolation of yeast subcellular organelles. Additional methods for specific organelles are listed in Walker (1998).

PREPARATION OF THE CELL EXTRACT

The first step is to rupture the cells and release the contents. A variety of methods are available for *Saccharomyces* that are similar to those used for other types of cell with variations to accommodate the rigid cell wall and the high level of protease activity found in *Saccharomyces*. Cells are grown under the appropriate culture conditions, harvested by centrifugation or filtration, and resuspended in a buffered salt solution containing protease inhibitors. A number of mechanical, chemical, or enzymatic methods are available for breaking open the cells and releasing the contents. A French press is used for large-sized samples (several grams of cells). This device forces cells through a small hole under pressure. Cells can also be ruptured by aggressive agitation of a cell suspension with glass beads. This can be done on small samples simply by vortexing the cell–glass bead suspension or by shaking the suspension in any of a variety of devices designed specifically for this purpose. Alternately, the *Saccharomyces* cell wall may be stripped using glusulase or zymolyase, enzymes that attack the structural components of the cell wall, after which the cells are burst by altering the osmolarity of the cell suspension.

DIFFERENTIAL-VELOCITY CENTRIFUGATION

Differential-velocity centrifugation separates subcellular components based on size/shape and density. The theory is that the larger more dense components will pack at the bottom of a centrifuge tube faster and at lower speeds then smaller less dense ones. Initially, the total cell extract is centrifuged at a low speed for a short time to remove unbroken cells. In the next steps, the speed and time of centrifugation are progressively increased removing some components (packing them at the bottom of the tube as a precipitate) and leaving others in the supernatant at each step. The final supernatant, after the step-wise removal of nuclei, mitochondria, vacuoles, peroxisomes, plasma membrane and vesicles, endoplasmic reticulum, and ribosomes, is called the soluble fraction and contains soluble proteins and other small molecule components of the cytosol such as tRNAs.

EQUILIBRIUM DENSITY GRADIENT CENTRIFUGATION

Equilibrium density gradient centrifugation separates subcellular components based only on their density. For this method, one must first prepare a density gradient in a centrifuge tube. A nonionic molecule like sucrose, glycerol, or Ludox is used to vary the density of the buffer solution. The concentration of the molecule is varied, and therefore the density of the solution, and the concentration, is greatest at the bottom of the centrifuge tube and decreases slowly towards the top of the tube. Special devices are available for making these gradients. A step gradient can also be prepared. Here a series of solutions of different concentration (30%, 25%, 20%, etc.) are layered on top of one another with the step with the highest concentration at the bottom.

The cell extract is layered at the top of the gradient and the tube is subjected to centrifugation at high speed for several hours. During this time the different subcellular components move down the tube until they reach the position in the density gradient that corresponds to the density of the component and will remain in this position indefinitely. In a step gradient, the organelle will position itself between two steps. Using a fraction collector, individual small samples are gently removed from the tube starting at the top or bottom in a manner that does not disturb the gradient. The samples are then analyzed by Western analysis, electron microscopy (EM), or biochemical assay to identify the subcellular location of a particular protein. The purified fractions also can be used for other biochemical studies.

Figure 2.1 illustrates the results from a typical equilibrium density gradient separation experiment. Western analysis (see below) was used to identify the fractions containing the protein of interest, Gap1p (the general amino acid permease) in this experiment, and biochemical assays of marker enzymes from the different subcellular compartments were carried out to identify the location of the compartment in the gradient.

The purity of the subcellular fractions is often at issue. Samples obtained by differential-velocity centrifugation are generally not considered to be a highly homogeneous purified product. No matter which method is used it is essential to test the purity. The samples can be observed by EM to determine the presence of contaminating components. Marker enzymes or proteins characteristic of a particular

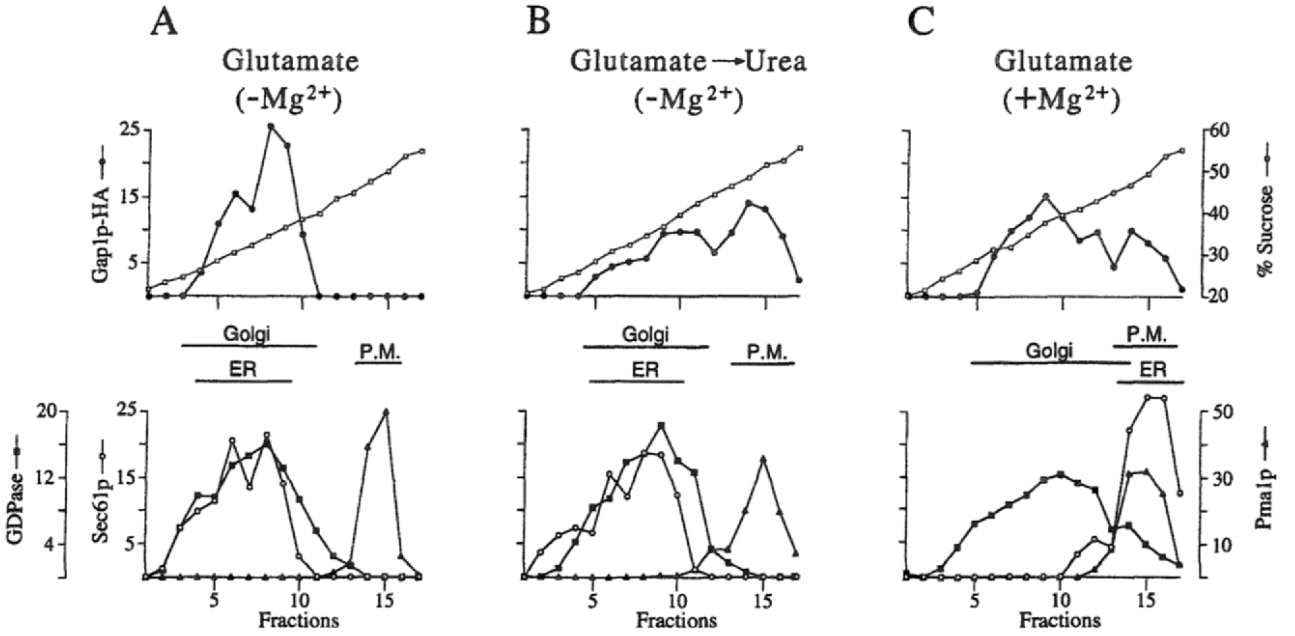

Figure 2.1 Equilibrium density gradient analysis. Shown are the results of an equilibrium density gradient analysis carried out to determine the subcellular localization of Gap1p, the general amino acid permease, under different culture conditions (glutamate or glutamate transferred to urea). (Taken from Roberg *et al.*, 1997.) The density gradient is 20%–60% sucrose with and without Mg^{2+}. The upper row of panels shows, for each fraction, the sucrose concentration (open circles) and the relative levels of Gap1p-HA (hemagglutinin-tagged Gap1 protein) as determined by Western analysis. The lower row of panels shows the relative levels of Pma1p (plasma membrane marker) and Sec61p (endoplasmic reticulum marker) as determined by Western analysis and the activity of GDPase (Golgi marker) as determined by enzymatic assay. The horizontal bars above the lower set of panels indicates the position in the gradient of each membrane type. Reproduced from *The Journal of Cell Biology*, 1997, by copyright permission of The Rockefeller University Press

subcellular component can be assayed (either biochemically or by Western analysis) to assess the purity of a sample. Examples of such marker enzymes can be found in Kreutzfeldt & Witt (1991), Griffin (1994), and are listed in Walker (1998).

MICROSCOPY TECHNIQUES

In standard light (bright-field) microscropy, a beam of light from a source (usually placed below the specimen) is focused onto a specimen, passes through the specimen, is focused by a second series of lenses, and is then observed by eye or photographed. Samples are usually fixed to denature the proteins in the specimen, sectioned into thin slices (if needed), attached to a solid substrate (the slide), and stained using any of a series of chemicals that specifically react with cellular components such as DNA or protein. It is hoped that these treatments do not significantly alter the subcellular structures or their organization. Because of the small cell size of *Saccharomyces*, bright-field microscropy is very uninformative and researchers have developed other methods for visualizing subcellular structures.

FLUORESCENCE MICROSCROPY, IMMUNOFLUORESCENCE, AND GFP

Fluorescence microscropy allows the researcher to localize a specific protein to its subcellular site by providing a mechanism for a fluorescent dye to specifically bind to a particular protein or subcellular component. A fluorescent molecule is one that becomes activated by absorbing light of one wavelength (the excitation wavelength) and then returns to the resting state by emitting light at a longer wavelength (fluorescence wavelength) still in the visual range. The fluorescent molecule is visualized in the specimen using a fluorescence microscope that is designed to shine light of the excitation wavelength on the specimen (usually from above) and to allow one to observe the emitted light (again from above). The emitted light is passed through filters that block all except light of the fluorescent wavelength before it is viewed or photographed.

The most commonly used fluorescent dyes are rhodamine (which emits light in the red range) and fluorescein (which emits light in the green range). In immunofluorescence the dye is covalently conjugated to an antibody specific to the protein of interest. Cells are fixed and made permeable to the antibody. The sample is then treated with the fluorescent dye-conjugated antibody and the antibody then binds to its target antigen/protein. The position of the antibody is visualized using a fluorescence microscope. Microscopes can be fitted with several different sets of filter pairs thereby allowing one to observe, in a single cell, the location of two or even three antibodies each conjugated to a different dye and emitting light of a different fluorescent wavelength. In this way one can compare the localization of two or more different proteins within a single cell. For further information, the *Handbook of Fluorescent Probes and Research Chemicals* from Molecular Probes, a supplier of such reagents, is an excellent resource (http://www.probes.com).

A number of variations on the immunofluorescence theme have been developed for use in *Saccharomyces* and other cells. The fluorescent dye can be conjugated to a

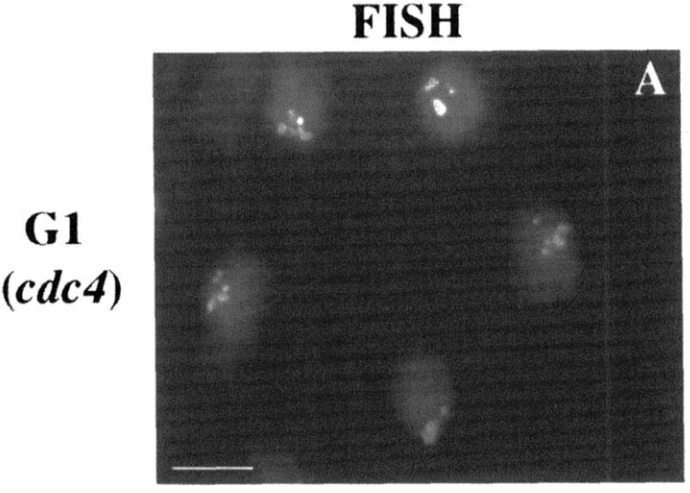

Figure 2.2 Fluorescence *in situ* hybridization—FISH. Shown is the result of FISH analysis of centromere localization in the nuclei of diploid cells arrested in G1 of the cell cycle (using a temperature sensitive mutation of *cdc4*). Chromosomal DNA was stained with propidium iodide (grey) to show the outlines of the nucleus. Oligonucleotide probes were made from sequences tightly linked to *CEN1*, *CEN4*, and *CEN16* and thus should hybridize to six chromosomal sites in these diploid cells. Each probe is tagged with an antigenic compound called digoxigenin and the location of the tagged oligonucleotide is visualized by immunofluorescence using dye-conjugated antibodies. The photograph shows that the centromeres are clustered and, using immunofluorescence to localize the position of the microtubule attachment to the nuclear envelope (data not shown), the authors demonstrate that the clustering is in the region closest to the spindle pole body. Taken from Guacci *et al.* (1997). Reproduced by permission of the American Society for Cell Biology

molecule other than an antibody that specifically interacts with a particular cellular component. For example, phalloidin is a small cyclic peptide derived from the death cap fungus *Amanita phalloides*. It specifically binds to polymerized actin microfilaments. Rhodamine-conjugated phalloidin will enter permeablized cells and bind to the actin cytoskeleton thereby allowing this complex meshwork to be visualized by fluorescence microscropy (see Chapter 3, Figure 3.9). Fluorescence *in situ* hybridization, or FISH, uses nucleic acid hybridization to bind the fluorescent dye to specific DNA sequences in chromosomes. The fluorescent dye is conjugated to a DNA oligonucleotide probe, introduced into cells, and allowed to hybridize to the complementary site(s) on the chromosomes. Examination by fluorescence microscropy allows these positions to be visualized. FISH has been used to demonstrate the location of chromosomal telomeres in interphase *Saccharomyces* nuclei and to study chromosome separation during cell division (Figure 2.2). Finally, Walker (1998) lists several cytofluorescent dyes for yeast microscropy that interact with specific cellular molecules. One example is calcofluor white that binds to the chitin found at the site of bud scars. Another is DAPI (4,6-diamidino-2-phenylindole) that binds specifically to DNA and can be used to visualize the nucleus and even mitochondrial DNA (Figure 2.3).

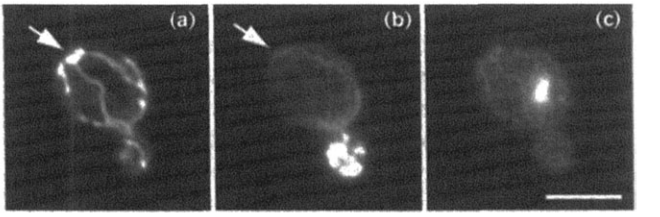

Figure 2.3 Visualization of mitochondria by different methods. Cells were grown to the midlog phase on a rich medium with galactose as the carbon source. Panel (a) shows the mitochondria stained by immunofluorescence. In panel (b) Alexa-phalloidin was used to detect polymerized actin. Panel (c) shows cells stained with the DNA binding dye DAPI. The large bright spot in the mother cell in panel (c) is the nucleus and the less brightly staining spots are the mitochondria, indicating that several copies of mtDNA can be found. Note that the mitochondria accumulate in the mother cell distal to the site of bud emergence and appear to lie along the length of the actin cables. Taken from Yang *et al.*, 1999. Reproduced with permission from Elsevier Science

Perhaps the most powerful advance in fluorescence microscropy came with the development of GFP, by Martin Chalfie and coworkers (Chalfie *et al.*, 1994). GFP is responsible for the bioluminescence exhibited by the jellyfish *Aequorea victoria*. What makes GFP such a valuable tool for the study of cell biology in the age of recombinant DNA technology? GFP fluorescence occurs *in vivo* in the living cell simply by shining light of the correct excitation wavelength on the cells. Since there is no fixation or staining necessary, it is believed that a more accurate view of the *in vivo* situation is obtained. The amino acid sequence and structure of GFP is solely responsible for its fluorescent activity, and no exogenously added cofactors or exogenously produced modifications are required. In fact, the GFP chromophore is synthesized autocatalytically by a series of intramolecular reactions involving the side-chains of several amino acid residues in the GFP sequence. Therefore, whether expressed in the native organism *Aequorea victoria* or heterologously in the cells of any other species, GFP undergoes this autocatalytic reaction to produce the chromophore and emits light at its characteristic fluorescent wavelength. GFP is nontoxic and thus can be expressed in all cell types. Most importantly, GFP fusions are usually functional and results obtained with these fusions are therefore biologically relevant.

To use GFP to study the localization of any protein, one constructs an in-frame fusion of the GFP ORF to that encoding the protein of interest. Plasmid vectors containing the full ORF of the GFP gene are available (Niedenthal *et al.*, 1996). These contain multiple cloning sites positioned so as to allow the gene of interest to be inserted either upstream or downstream of the GFP. For use in *Saccharomyces*, a high-level constitutive promoter, such as the *ADH1* promoter, is usually included. Using PCR-based methods, the sequence of the GFP ORF can be inserted at any position in any gene of interest. The GFP-fusion construction is then introduced into cells and transformants will produce a GFP-fusion protein whose subcellular localization can be visualized by fluorescence microscropy. In *Saccharomyces* time-lapse photography of cells expressing a GFP-fusion protein has been used to

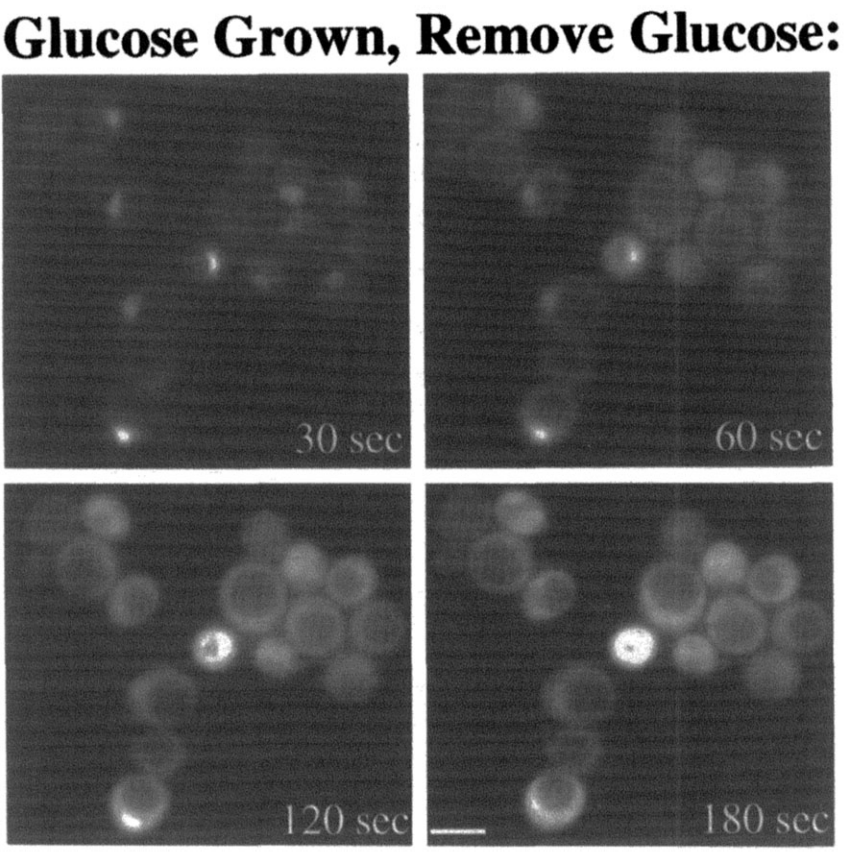

Figure 2.4 Green fluorescent protein fusions for visualization of living cells. The photo shows a time-course of Mig1 repressor exit from the nucleus. These cells are expressing a fully functional Mig1p–GFP fusion protein. Following growth on glucose, the cells are harvested and placed in medium lacking glucose. The first panel shows the cells 30 seconds after glucose removal. Mig1p–GFP is seen localized to a discrete subcellular site that, by DAPI staining, is shown to be the nucleus (data not shown). Very little fluorescence is observed in the cytoplasm. The same cells are photographed every 30 seconds for 1.5 minutes during which time the fluorescence can be seen to leave the nucleus and accumulate in the cytoplasm. The large poorly fluorescent region seen in the cytoplasm is the vacuole (based on Nomarski optics). Taken from De Vit *et al.* (1997). Reproduced by permission of John Wiley & Sons Publishers

demonstrate changes in subcellular location of the fusion protein resulting from changes in growth conditions, such as carbon source or temperature. An example of this type of analysis is shown in Figure 2.4, which demonstrates the time-course of Mig1p GFP-fusion protein exit from the nucleus after cells are shifted from a glucose-containing medium to a medium lacking glucose. To control for the possibility that fusion to GFP inactivates some or all of the functions of the protein under study, it is essential that the GFP-fusion construction be tested for its ability to complement the mutant phenotypes of a null allele of the gene of interest.

The introduction of a limited number of amino acid changes in the wild-type GFP gene has allowed for improvements in the fluorescence characteristics of GFP, such as increased emission of light or spectral resolution, and has even produced mutant products that absorb and emit light at slightly different wavelength ranges (for example, blue fluorescent protein or BFP). The coexpression of GFP- and BFP-fusion genes allows the researcher to compare the subcellular localization of these two proteins *in vivo*. Several different variants are now available.

CONFOCAL SCANNING MICROSCROPY

Most of the high-resolution subcellular localization studies done using fluorescence microscropy would not have been possible without the development of an improved imaging technique called confocal scanning microscropy. Fluorescence microscropy is generally done using whole cells because the embedding media used for sectioning often is fluorescent and obscures the fluorescence derived from the sample. Since eukaryotic cells, even *Saccharomyces*, have a thickness, the fluorescence one observes is coming not only from the molecules in the plane of focus but also from molecules above and below. The greater the thickness of the sample the greater the problem one will have in resolving specific structures.

The confocal microscope uses a laser light source to produce the excitation light beam that can be focused into a narrow focal plane allowing only a thin optical section of the sample to be illuminated. The laser beam set at a specific excitation wavelength rapidly scans the sample. The position and intensity of the emitted light is recorded and the information stored for computer analysis. The results of these scans are combined to generate a composite digital image of the fluorescence from a sample. Because only information from a narrow focal plane is used this method produces a high-resolution map of the subcellular position of the fluorescent molecule.

Other forms of image analysis can also be carried out. The amount of fluorescence can be quantified by computer analysis of the digital image and used as a measure of gene expression. Depending on the laser source and the filter sets available for the particular microscope, one can create images of the fluorescence produced by different antibody–dye conjugates. These images, when superimposed by the computer software system, can very accurately demonstrate whether the two antibodies are colocalized in the cell. Confocal imaging is also used to create a three-dimensional image by a method referred to as optical sectioning. The microscope stage is moved vertically in small steps thereby moving the focal plane through the cell. At each step an image is generated and these are then combined to create a single three-dimensional image that can be rotated in space on the computer screen.

NOMARSKI INTERFERENCE MICROSCOPY

Nomarski interference microscopy (sometimes called DIC) can be used to visualize live unstained cells or tissue samples. It makes use of the differences in thickness and refractive index of different parts of the cell and gives a three-dimension-like image. Light moves more slowly through material with a higher refractive index. Nomarski imaging requires a microscope equipped with special polarizing lenses and prism. A

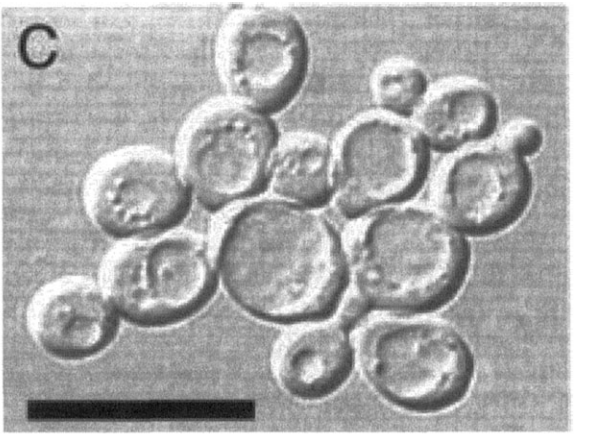

Figure 2.5 Nomarski optics view of *S. cerevisiae*. Nomarski optics can be used to visualize live or fixed cells. The vacuole is by far the most predominant organelle of *S. cerevisiae* and is very clearly observable by this method. The multiple punctate structures in the cytoplasm surrounding the vacuole are vesicles of various types. Taken from Lang *et al.* (2000). Reproduced by permission of the American Society for Microbiology

plane-polarized light beam is split into two and allowed to pass through the sample at nearly adjacent sites after which the two beams are rejoined. If the two sites differ in refractive index, then the beams of light will be out of phase when they exit the sample and when joined will interfere with each other thereby reducing the intensity of the light beam. If there is no difference between the two sites, then the intensity of light will be high. If the difference is substantial, then the beams will completely interfere and a dark region will result. When observed by Nomarski optics, the most prominent organelle in *Saccharomyces* is the vacuole (Figure 2.5).

ELECTRON MICROSCROPY

The electron microscope uses an electron beam instead of a light beam to visualize the cell and its components. The transmission electron microscope passes the electron beam through the sample and to do so the sample must be sliced into very thin sections using special tools and an embedding material. In order to see cellular structures the sample must be stained with an electron dense agent such as osmium. The electron beam is focused with magnetic lenses and this is projected onto a viewing screen or photographed.

The major problem with EM analysis is sample preparation. EM is not often used for *Saccharomyces* but it has proved to be very useful for some studies, particularly on the cytoskeleton. The development of antibody localization techniques for EM work has encouraged more researchers to attempt this difficult method of analysis. The method is referred to as immuno-gold localization because it uses antibodies that are bound to gold particles via *Staphylococcus aureus* Protein A. The surface of the gold particle (about 4 nm in diameter) is covered with Protein A, which also binds very tightly to the constant region of IgG antibodies. The EM sectioned sample is treated with the antibody–gold complex under conditions that allow

binding to the specific antigen. The gold particles appear as black dots in the transmission electron microscope and their subcellular location can be determined in osmium stained samples. Figure 2.6 shows an example of the immuno-gold labeling method to localize Ste2p, the α-factor receptor to endocytic vesicles forming at the plasma membrane. Double label methods are now available for the colocalization of proteins at the EM level.

The surface of a *Saccharomyces* cell can be studied using scanning electron microscropy (SEM). Cells are fixed, allowed to adhere to a solid support, and coated with a heavy metal film, such as platinum. The coating process is carried out in a vacuum chamber and the vaporized metal is allowed to deposit on the sample while the sample is rotated for even coating. The electron beam scans the sample and excited secondary electrons are released and visualized on a monitor. This method gives a three-dimensional appearance to the sample.

FLOW CYTOMETRY

Cells can be sorted into different classes using a fluorescence activated cell sorter (FACS). For this, cells are selectively labeled with a fluorescent dye and sorted into classes based on the extent of labeling. As described above, fluorescence labeling can be done using an antibody-conjugated fluorescent dye or a fluorescent dye such as DAPI that binds a specific cellular component. For example, an antibody specific to a particular cell surface protein is conjugated to a fluorescent dye. This will bind to the surface of cells that express the surface protein and these cells will be fluorescent. Cells lacking this surface protein will not be fluorescent. The extent of the fluorescence can also be quantified and the cells sorted based on the amount of fluorescence.

In the FACS, cells treated with the fluorescent dye label flow past a laser beam and will or will not fluoresce based on the level of bound dye. The cells are then dispersed into droplets containing no more than one cell. Droplets containing a fluorescent cell are made negatively charged and the charge is used to separate the droplets into categories. The number of cells in a category can be quantified and those cells falling into a specific category can be separated from the remainder of the cells and used for further analysis by other methods. DNA staining with a fluorescent dye is used to distinguish cells in G1 of the cell cycle containing the haploid amount of DNA (1 C amount of DNA) from cells in G2 (2 C) from cells in the S phase (1–2 C). Figure 2.7 shows such an analysis. Cells containing the temperature-sensitive mutation *mcm2-1* arrest at the nonpermissive temperature with 2 C DNA content while wild-type cells continue to proceed through the cell cycle (evidenced by a distribution of cells with 1 C or 2 C DNA content or amounts in between the two). Also, Figure 2.7 shows that normal cell cycling is restored in the *mcm2-1 dbf4-6* double mutant strain. This is an example of suppression (see Chapter 8).

PROTEIN EXTRACTION AND PURIFICATION

Standard analytical methods for protein purification and characterization are routinely used in the study of *Saccharomyces* proteins. It is important to have a

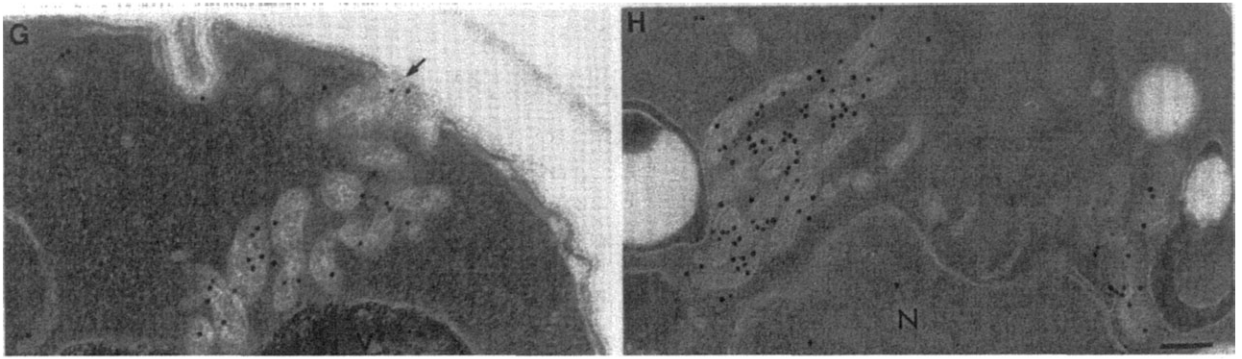

Figure 2.6 Immuno-gold localization. α-Factor receptor Ste2p is localized to the plasma membrane of **a** mating type cells. In response to the binding of **a**-factor, Ste2p is internalized in the form of vesicles by a process called endocytosis. Immunogold labeling methods are used here to visualize the subcellular location of Ste2p during this internalization process. The cells are fixed, sectioned, stained with osmium to visualize subcellular structures, and treated with anti-Ste2p antibody followed by the gold-labeled secondary antibody. One can see a series of black spheres (the electron dense gold beads) concentrated over a tubular–vesicular membrane compartment that appears to span the region from the plasma membrane to the vacuole (labeled V). Taken from Mulholland *et al.* (1999). Reproduced by permission of the American Society for Cell Biology

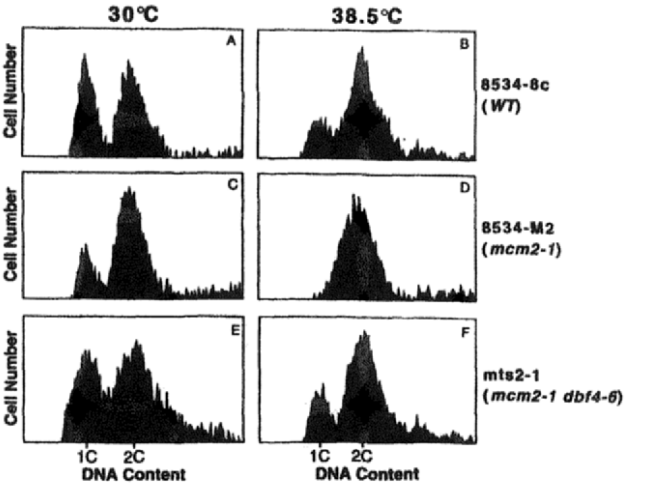

Figure 2.7 FACS analysis of DNA content. Strains carrying the temperature-sensitive mutation *mcm2*-1, encoding a protein required for the initiation of DNA replication at chromosomal origins of replication, and the mutation *dbf4*-6, a component of a regulatory kinase, and the wild-type strain are analyzed for DNA content by FACS analysis. Cells were grown at the permissive temperature and exposed to the nonpermissive temperature for at least one cell cycle. The DNA was stained with propidium iodide and the cells subjected to FACS analysis to determine the number of cells containing different amounts of DNA. The results are presented graphically, as seen in the figure. A 1C DNA content is the amount found in a haploid cell during G1 of the cell cycle and a 2C DNA content is the amount found in a haploid cell in G2 following the completion of DNA replication. Cells having DNA levels between 1C and 2C are in the S phase and in the process of replicating their DNA. As can be seen, the effect of the *mcm2*-1 mutation is to arrest cells in G2 but the *dbf4-6* mutation suppresses (bypasses) the *mcm2-1* alteration. Taken from Lei *et al.* (1997). Reproduced by permission of Cold Spring Harbor Laboratory Press

basic understanding of these methods and be familiar with the terminology since one will undoubtedly encounter many of these while reading the literature whether on *Saccharomyces* or other systems.

Saccharomyces can be cultured in liquid in large volume sufficient to provide milligram quantities of many proteins that then can be purified by standard methods such as gel filtration, affinity chromatography, and ion exchange chromatography. A thorough description of the variety of protein purification techniques available to the researcher is beyond the scope of this book. Instead the reader is referred to the several reference texts on protein purification, particularly Marshak *et al.* (1996), Ausubel *et al.* (2001), Spector *et al.* (1998a,b,c) and certain volumes of *Methods in Enzymology* edited by Abelson and Simons and others.

In *Saccharomyces* these protein purification techniques may be used in conjunction with overexpression vectors that place the ORF of any cloned gene under the control of high-level constitutive promoters in multicopy plasmids (see the discussion of expression vectors in Chapter 1). In this way, one can achieve sufficiently abundant levels of expression to allow the purification of almost any desired protein. Overexpression of a particular protein makes possible its purification but

may not be appropriate for the *in vivo* analysis of function. Artifacts such as mislocalization to unusual subcellular compartments may occur. The protein may have toxic effects when produced in abundance. For example, overproduction of an integral membrane protein may have serious consequences for the transit of other proteins through the secretory pathway. In fact, overproduction of any protein that relies on a saturable process for its synthesis and localization may lead to unforeseen effects. Examples of saturable processes are nuclear import via import proteins and the nuclear pore complex or protein modification and processing events. Additionally, many proteins are components of larger functional complexes or interact in a highly regulated manner with several different competing regulatory components. Overproduction of one member of a complex may impact the level of expression of the other components of the complex or may alter their interactions so as to cause aberrant regulatory patterns. Caution must be used in the interpretation of any result obtained by the overproduction of a single protein.

WESTERN ANALYSIS

Western analysis is very powerful technique for protein characterization and expression studies. The method is used for analysis to evaluate the amount of a particular protein for which protein-specific antibodies are available. Because of the antibody specificity, proteins can be identified even when present in a complex mixture such as a cell extract.

A special section of Ausubel *et al.* (2001) is devoted solely to *Saccharomyces* and describes in detail several extraction methods for protein studies. Many other methods are described in the literature. A major difference in these methods is whether the conditions of the extraction buffer are denaturing or nondenaturing. Nondenaturing conditions are similar to *in vivo* conditions and are designed to maintain the integrity of subcellular structures and protein complexes. One would use this type of condition if experimental interest were in identifying proteins found in association with one another. For example, is a particular protease found in a proteasome? Denaturing conditions, 5% sodium dodecyl sulfate or 8 M urea included in the extraction buffer, disrupt all protein–protein interactions. Denaturing conditions would be used if one simply wanted to compare the amount of a particular protein in a sample.

The proteins of a sample under analysis are size-separated by SDS polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred from this gel to a sheet of nitrocellulose (membrane) by electrophoresis. The proteins in the sample are now fixed in place in this two-dimensional membrane and represent the position to which the protein migrated in the gel. The position of a protein on this membrane is visualized using an antibody specific to that protein, referred to as the primary antibody or probe. The membrane is incubated in a solution containing the primary antibody giving sufficient time for the antibody to bind to the protein that is itself bound to the membrane. A secondary antibody specific to the constant region of the primary antibody is used as a probe to localize the position of the primary antibody on the membrane. This secondary antibody is also conjugated to horseradish peroxidase, biotin, or some other compound whose presence can be assayed easily.

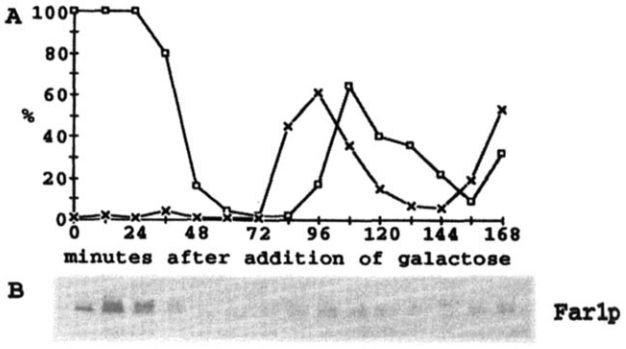

Figure 2.8 Western analysis. This figure follows the relative level of the cell-cycle-regulated protein Far1p during the course of about two cell divisions. Cells were synchronized in G1 (by depletion of an essential cyclin). The addition of galactose allows the cells to initiate a new round of cell division by restoring the expression of the cyclin. Panel (A) follows cell division by monitoring the level of unbudded cells (□) and binucleate cells (×), both determined by microscopic observation. Protein extracts were prepared from the cells in panel (A) and subjected to Western analysis using anti-Far1p antibody. The results of the Western analysis are shown in panel (B) and indicate that Far1 protein accumulates in G1 prior to Start. Far1p runs as a close doublet of bands suggesting that the protein is found in a phosphorylated form. Taken from McKinney *et al.* (1993). Reproduced by permission of Cold Spring Habor Laboratory Press

Typically, the detection assay produces a colored, luminescent, or fluorescent precipitate on the surface of the membrane. This is then photographed, detected by X-ray film, or scanned by some form of densitometer for a permanent record and quantification where possible. Figure 2.8 illustrates the results of a Western analysis of Far1p during a cell division cycle (McKinney *et al.*, 1993). The reader should note that often in addition to the level of the protein of interest the level of another protein, one considered to be relatively constant in amount during the cell cycle such as actin, is also determined using the same membrane. This protein serves as a control demonstrating that equal amounts of extract were loaded into each lane of the SDS-PAGE gel.

The different assay methods vary in their ability to be evaluated quantitatively. Moreover, determination of amounts is always relative to a standard sample run (for example the control condition) in the same gel. Some methods, such as chemiluminescence, are linear over only a single log while others, such as those using a fluorescent secondary antibody, can be linear over several logs. Primary antibodies, secondary antibodies, and detection kits are commercially available and a wide variety of products for all aspects of Western analysis can be obtained from several suppliers.

For detailed information on Western analysis Ausubel *et al.* (2001) is an excellent starting point and provides updated procedures, theoretical discussions, and additional references. Others references listed below are also valuable resources. In addition, there is an abundant technical literature available from the commercial suppliers (Molecular Probes for example) evaluating the capabilities of the various

products on the market as well as technical information from instrumentation manufacturers like Molecular Dynamics that manufactures densitometer, phosphorimager, and fluorimeter equipment.

EPITOPE TAGGING AND IMMUNODETECTION OF EPITOPE-TAGGED PROTEINS

An **epitope** is a structural feature of a molecule that is specifically recognizable by an antibody and to which the variable region of the antibody binds. Short amino acid sequences, branched-chain carbohydrate groups, or even the phosphorylation site on a peptide can serve as an epitope. Most large molecules like proteins contain several epitopes and, when such an antigenic molecule is injected into an animal, it will induce the production of several different antibodies to many or all of these epitopes in that individual animal. Serum isolated from this individual will contain a mixture of several secreted IgG antibody proteins each produced by a different line of antibody-producing B lymphocytes and each capable of recognizing a different epitope of the antigen. This type of antibody is referred to as a **polyclonal antibody** because several different clones of antigen-producing B lymphocytes produce the several serum antibodies.

The advent of **monoclonal antibody** technology solved the problems of antibody heterogeneity and made possible the method of epitope tagging for protein detection. Monoclonal antibodies are produced by mouse lymphocytes. An antigen having multiple epitopes is injected into the mouse and B lymphocyte differentiation is allowed to initiate. Multiple B lymphocyte clones each producing a different antibody specific to a particular epitope of the antigen will begin to undergo this differentiation process. The immunized mouse is sacrificed, the B-lymphocyte-containing spleen removed, and the developing B lymphocytes fused with an 'immortal' line of B lymphocyte tumor cells. Normally, B lymphocytes are able to undergo only a very limited number of divisions in culture, but these tumor cell fusions divide indefinitely. Individual hybrid cell clones are cultured separately and allowed to produce their unique antibody. This immortalized antibody-producing B lymphocyte cell line is called a **hybridoma**. The antibody product of each hybridoma line is then tested against the antigen, and fragments of the antigen, and the specific epitope recognized by the antibody determined. The antibody product is referred to as a monoclonal antibody because it is produced by a single hybridoma clone of B lymphocytes.

A variety of epitope-specific monoclonal antibodies are commercially available but not all are equally useful for different protein analysis techniques. Some are excellent as the primary antibody in Western analysis while others are poor for this application but work extremely well in immunoprecipitation. One should be guided by the methods used by published journal articles and by the recommendations of colleagues and commercial suppliers. Commercial suppliers provide technical assistance for customers and detailed information can be found in the product descriptions of most catalogues. Since all monoclonal antibodies are murine antibodies, all contain the murine constant region. For techniques such as Western analysis that often use a monoclonal antibody as the primary antibody, the secondary antibody

must be produced in an animal other than the mouse and directed against the mouse IgG constant region.

The most common type of epitope used by molecular biologists is the peptide epitope. This is because one can easily attach such an epitope to any protein of interest using recombinant DNA technology. This is referred to as **epitope tagging**. For this one simply needs to construct an in-frame fusion of the ORF of the gene encoding the protein of interest to an oligonucleotide encoding the epitope sequence. Most often, the epitope is placed at the N-terminal or C-terminal end of the ORF. It is essential to test the epitope-tagged allele for function to ensure that the presence of the epitope does not interfere with the functional activity, subcellular localization, or stability of the protein. In *Saccharomyces* this is done by determining whether the tagged allele is capable of complementing all of the mutant phenotypes of the null allele.

Vectors specific for constructing these in-frame epitope fusions can be obtained commercially or from colleagues. Many are described in the literature. These typically contain the epitope sequence located immediately upstream or downstream of a multiple cloning site (MCS). The ORF of interest is simply amplified using appropriate PCR primers and inserted into the MCS thereby placing the epitope at the N-terminus or C-terminus, respectively, of the encoded fusion protein. The resulting protein is said to be epitope tagged. Expression of the epitope-tagged gene product in these constructions is usually from a promoter such as the *ADH1* promoter.

One need not use these vectors, particularly if one wants to use the native promoter. Because the epitope sequence is frequently quite short, one can synthesize oligonucleotide primers containing the sequence and use these for PCR amplification of the ORF of interest. This product can then be inserted into any vector containing any desired promoter sequence (see the discussion of expression vectors in Chapter 1). The sequence of the epitope also may be inserted into any desired site in the ORF of a gene using *in vitro* mutagenesis techniques or by PCR-based methods. This is important if the N-terminal or C-terminal versions of the epitope-tagged protein are not functional and another insertion site for the epitope must be found.

Longtine *et al.* (1998) constructed a series of modules for use as PCR templates for the creation of tagged fusion genes at genomic sites by one-step gene replacement. The modules allow for C-terminal fusion of GFP, three copies of the HA epitope, 13 copies of the Myc epitope, or GST or for N-terminal fusion of GFP, three copies of the HA epitope, or GST to the gene of interest. The N-terminal fusions replace the native promoter with that of *GAL1*. Each module contains the protein tag and a selectable marker. This is amplified using primers containing 40 bp of genomic sequence at the 5' end and sequence complementary to the module template and the 3' end. The amplified product is then transformed into the host strain and *in vivo* recombination between the amplified DNA fragment and the genomic site creates the desired tagged fusion gene.

The researcher has a choice of any of several peptide epitopes. The most common are listed below. Monoclonal antibodies specific to each is commercially available. For some, the researcher has a choice of different monoclonal antibodies based on their performance in a particular technique such as Western analysis or immunoprecipitation or even whether the epitope is at the N-terminal or C-terminal end of the protein or in the middle.

HEMAGGLUTININ (HA) EPITOPE

The HA epitope is a nonapeptide (YPYDVPDYA) derived from the influenza virus hemagglutinin protein. Anti-HA antibody is quite specific and cross reaction to other yeast proteins is not seen. The HA-tag can be used for Western analysis, immunocytochemistry, and immunoprecipitation (see below). Often, the HA epitope sequence is repeated up to three times or more to improve antibody binding and make this tag more useful for immunoprecipitation.

FLAG EPITOPE

The FLAG epitope (DYKDDDDK) is recognized by three commercially available monoclonal antibodies, M1, M2, and M5. M1 and M5 require that the epitope be placed at the N-terminus of the tagged protein and M1 will even bind to a shorter version of the FLAG sequence. M2 is able to recognize the epitope at all locations in the protein. Moreover, M2 can be used for both Western analysis and immunoprecipitation.

Myc EPITOPE

The Myc epitope (EQKLISEEDL) is derived from the human Myc protein, the product of the *myc* oncogene. A number of different monoclonal antibodies are available from commercial sources. Some are suitable for Western analysis and immunoprecipitation while others are more suitable for immunocytochemistry.

IMMUNOPRECIPITATION AND RELATED TECHNIQUES

These methods are used to physically separate a particular protein from a cell extract. They rely on a high-affinity, sequence-specific interaction between the protein and another molecule capable of specifically binding to that protein, such as an antibody. To achieve physical separation, the molecule providing the recognition specificity is bound to an inert substrate that can be separated physically from the binding reaction mix. Once purified by one of these methods, the protein can be characterized further or its level of expression determined, often by Western analysis. Moreover, these methods can be scaled up for large samples and can thus be used for protein purification.

IMMUNOPRECIPITATION

Immunoprecipitation uses protein- or epitope-specific antibodies for protein recognition. The complex is then removed from the mixture because it is bound to small beads, usually composed of Sepharose (a form of starch), that are themselves bound to protein A, protein G, or a mixture of both. Protein A is a product of the bacterium *Staphylococcus aureus* and binds to immunoglobulin constant region with very high affinity. Protein G is also a product from a *Streptococcus* bacterial species and also binds to IgG constant region. Proteins A and G differ slightly in their

affinity for the IgG constant region of the different species commonly used to produce antibodies, like horse, mouse, or goat. Thus, researchers often use Sepharose beads bound with a mixture of proteins A and G in order to achieve high-affinity binding to a broad range of antibodies that they might be using in different experiments.

An antibody specific to the protein of interest is added to the cell extract (prepared under the appropriate conditions) and allowed to bind. Protein A/G-bound Sepharose beads are added and incubated to allow binding of the antibody–protein complex to the beads. Centrifugation is used to pellet the beads with the bound protein. The sample is then analyzed by Western analysis. If a protein-specific antibody is not easily available but the gene encoding the protein is, then researchers often choose to epitope-tag the protein of interest using the methods described above.

Immunoprecipitation is most often used to identify other proteins that may be found complexed with the protein of interest. For this, the cell extract must be prepared under nondenaturing conditions. Any proteins that interact with the protein of interest should **coimmunoprecipitate** (so-called co-IP). For example, if one's genetic analysis indicates that proteins X and Y form a complex, then protein Y should co-IP with protein X when anti-protein X antibody is used. Western analysis of the pelleted sample should detect protein X and, if the two proteins co-IP, should also be able to detect protein Y when anti-protein Y antibody is used as the probe.

METAL CHELATE AFFINITY PURIFICATION

Many natural proteins have metal binding sites for ions such as Ni^{2+} and Zn^{2+}. Metal chelate affinity purification makes use of this to purify proteins. If the protein of interest does not contain a metal binding site, then one can be added by epitope-tagging. By far the most common metal chelate tag is a series of six histidine residues that specifically binds to Ni^{2+} ions and is referred to as a **His-tag**. The His-tag is short enough to be inserted by PCR-based methods, but a variety of vectors are available commercially for the construction of His-tagged alleles of the protein of interest.

Metal chelate affinity purification uses resin beads to which a metal chelating group has been bound. Ni^{2+} ions are then bound to this chelating group in such a way as to allow the Ni^{2+} ion also to be available for binding by the metal binding group of the protein, the His-tag of the protein of interest. The Ni^{2+}-bound resin beads can be used either as a slurry or packed in an affinity column. First, the His-tagged protein is allowed to bind to the Ni^{2+}-bound resin by incubating the resin with cell extract. After washing off all the unbound protein, the His-tagged protein is released from the beads using an excess of imidazole (an analogue of histidine).

Proteins purified in this manner from cell extracts are frequently contaminated with other cellular proteins that normally contain Ni^{2+} binding sites. Snf1 kinase of *Saccharomyces* is an example. Therefore, to obtain a strictly pure product one will have to carry out a second purification step. This may not be necessary for many of the characterization methods to be undertaken in follow-up studies, particularly

Western analysis that can detect the protein of interest alone by the use of specific antibodies.

GST-TAGGED AND MalB-TAGGED PROTEINS

Other affinity purification methods are available and can also be used like immunoprecipitation for studies of protein complexes or for protein purification. Two of the most common affinity purification methods use GST and MalB protein fusions. GST is glutatione S-transferase, is a product of *Schistosoma japonicum* and binds glutathione with high affinity. GST fusions to the protein of interest can be made by standard epitope-tagging methods and many fusion vectors are commercially available to simplify these constructions. Cell extract containing the GST-tagged fusion protein is passed over a resin-bound glutathione affinity column and immobilized. The GST portion is cleaved from the protein of interest with enterokinase or thrombin both of which act on the sequence at the fusion junction site. This releases purified protein from the resin along with any associated proteins.

MalB protein is an *E. coli* product involved in maltose transport. It is localized to the periplasmic space and is used as a maltose carrier. Its high-affinity binding to maltose and amylose is the basis of this purification method. Using methods such as those described above, one constructs an in-frame fusion of the protein of interest to MalB. Vectors for these constructions are commercially available. The MalB portion will provide specific binding to a column-bound amylose and the MalB fusion protein will be retained by the column. The fusion protein is released from the amylose column by excess maltose. The purified fusion protein is then cleaved at the junction site with a site-specific protease and the protein of interest further purified by passage over an amylose column to remove the free MalB protein and any uncleaved residue.

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3 *Saccharomyces* Cell Structure

Saccharomyces cerevisiae is a eukaryote and as such contains the subcellular organelles commonly found in eukaryotes. The structure and function of these organelles is fundamentally the same as it is in other eukaryotes with less versatile systems for genetic analysis, and for this reason *Saccharomyces* is the organism of choice for many cell biologists. For a truly in-depth review of *Saccharomyces* cell structure and function, the reader is referred to *The Molecular and Cellular Biology of the Yeast Saccharomyces*: Vol. 3: *Cell Cycle and Cell Biology* (Broach *et al.*, 1997). The discussion here will provide a very brief overview of the cell structure and will focus on certain unique features of *Saccharomyces* cell structure in order to facilitate reading of the literature.

CELL SHAPE AND GROWTH PATTERNS

Under usual culture conditions, *Saccharomyces* is ellipsoidal/ovoid in shape and approximately 5–10 μm long by 3–7 μm wide. This is referred to as the yeast form. Figure 3.1 shows a scanning electron micrograph (SEM) of a cell in the **yeast form**. Cell division is by budding; that is, a smaller ovoid daughter cell forms as a projection from the surface of the mother cell. Haploid cells are generally about one-half the volume of diploid cells. The characteristic shape is maintained by a rigid cell wall that completely surrounds the plasma membrane of *Saccharomyces*. Changes in this shape involve remodeling of the cell wall and occur during budding, mating, and pseudohyphal differentiation. Under nutrient stressed conditions certain strains undergo a shape change, forming filaments consisting of short chains of individual cells called **pseudohyphe**. In the pseudohyphal form the cells are elongated rather than ovoid in shape, and form chains of cells because the daughters do not detach from the mother even though cytokinesis is complete (Figure 3.2). Pseudohyphae penetrate into the solid substrate growth medium making them difficult to wash off, so-called **invasive growth**.

The plasma membrane surrounds a cytoplasm that is organized structurally by a cytoskeleton and is divided into membrane-bound compartments including a nucleus, mitochondria, pexoisomes, a vacuole, a Golgi complex, vesicles of various types, and the endoplasmic reticulum (ER). In many respects the ER, Golgi, vacuole, secretory vesicles and endocytic vesicles, and plasma membrane should be considered to be part of an interconnected system. Newly synthesized membrane and membrane proteins move from the ER to the Golgi and then to either the plasma membrane or the vacuole via secretory vesicles. Additionally, plasma membrane is internalized to form endocytic vesicles that fuse eventually with the vacuole. These combined processes of secretion (or exocytosis) and endocytosis are often referred to as membrane trafficking processes. Various particulate structures are found in the cytosol including ribosomes and proteasomes (essential protease complexes involved in the degradation of soluble proteins).

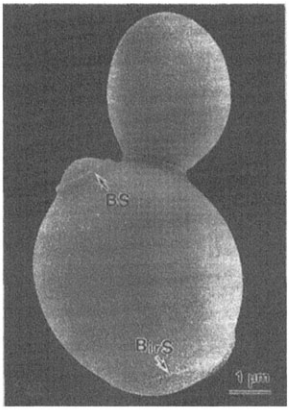

Figure 3.1 SEM of *S. cerevisiae*. A dividing cell exhibiting the yeast form is shown using SEM. Arrows point to the birth scar (BirS) and bud scar (BS), indicating that this cell has formed one bud prior to the one that is currently forming. Taken from Walker (1998). Reproduced with permission of John Wiley & Sons Limited

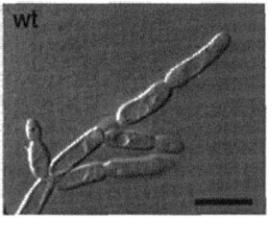

Figure 3.2 Pseudohyphal growth pattern. In starvation conditions, certain *Saccharomyces* strains alter their shape and budding pattern. This altered growth pattern, shown in this figure, is referred to as the pseudohyphal morphology. An elongated cell shape and a unipolar pattern of budding characterize the pseudohyphal growth. In the unipolar budding pattern, new buds form only at the end of the cell opposite to the birth end (the distal pole) in both the mother and the daughter cell. Adapted from Taheri *et al.* (2000) by permission of Oxford University Press

CELL WALL, CELL SURFACE MORPHOLOGY, AND MORPHOLOGICAL VARIATION

The *Saccharomyces* cell wall is about 200 nm thick and completely surrounds the cell. Its function is to preserve the osmotic integrity of the cell and define morphology but it has several other roles. Proteins involved in cell–cell recognition and adhesion, such as occurs during mating, are found in the cell wall. Other proteins are immobilized or retained in the periplasm, the space between the plasma membrane and the cell wall, by the cell wall. These are secreted proteins and localize specifically to this region. Finally, the cell wall may also be a partial permeability barrier. The cell wall is a very dynamic structure that is synthesized during bud

growth but must also undergo remodeling during cell division, mating, and sporulation. For a thorough review of the cell wall and cell wall biogenesis the reader is referred to Orlean (1997).

CELL WALL COMPOSITION AND SYNTHESIS

The major component (80%–90%) of the cell wall is polysaccharide. This includes β-glucans, mannoproteins, and chitin. β-Glucan is a glucose homopolymer. In *Saccharomyces*, one finds β-1,3 straight chains up to 1500 residues long with some β-1,6 branches. These long polymers are intertwined to form microfibrils that are interwoven into the meshwork that makes the basic support structure of the cell wall, much like the steel rods in reinforced concrete. Imbedded into this meshwork are the mannoproteins or mannans. These are secreted proteins with large, highly branched, covalently bound carbohydrate side groups consisting mostly of mannose residues but also including glucose and N-acetylglucosamine residues. Some of these glycoproteins are also attached to lipids of the plasma membrane via a GPI anchor (glycosyl phosphatidylinositol) at the C-terminus of the protein. Cell wall proteins include the agglutinins and flocculins that play important roles in cell adhesion. Enzymes such as invertase, a heavily glycosylated protein, are found in the periplasmic space. Chitin is a homopolymer of β-1,4-linked N-acetylglucosamine residues. It is a minor component of the cell wall (1%–2%) and is largely found at the site of bud formation and in bud scars. Many of the cell wall components are cross-linked to one another to form this very complex and interconnecting rigid structure. Figure 3.3 shows the organization of the *Saccharomyces* cell wall.

Synthesis of the major cell wall components takes place in the ER and Golgi, and transit to the cell surface is achieved via secretory vesicles. Glycosylation and mannosylation of cell wall proteins initiates in the ER and is completed in the Golgi. The same is true for the formation of the GPI anchor. Polymerization of β-glucan initiates in the ER but continues in the Golgi and completes at the plasma membrane and involves some membrane-bound protein components of these compartments. Chitin synthesis is different. Chitin synthase is a membrane enzyme. It uses an intracellular precursor, UDP-N-acetylglucosamine, to synthesize extracellular chitin by some type of transmembrane process that is as yet not well understood. Since chitin is found in selected regions of the cell wall, chitin synthase must be active only in certain sites and is apparently regulated by the processes of bud site selection.

BUD SCARS, BIRTH SCARS, AND BUDDING PATTERNS

Bud scars are chitin-rich turtleneck-like raised rings that form at the site of bud formation and surround the bud neck. The bud scar remains on the cell wall surface on the mother cell even after the bud has detached. Since an individual cell can divide 25 or more times, the surface of a mature cell will be studded with multiple bud scars. The site on the daughter cell that had been the attachment to the mother is also visible on the surface and is called the birth scar. Both can be seen in Figure 3.1.

Budding patterns differ in haploid versus diploid cells. In haploid cells, both **a**- and α-mating type, the new bud forms near the site of the previous bud scar on the

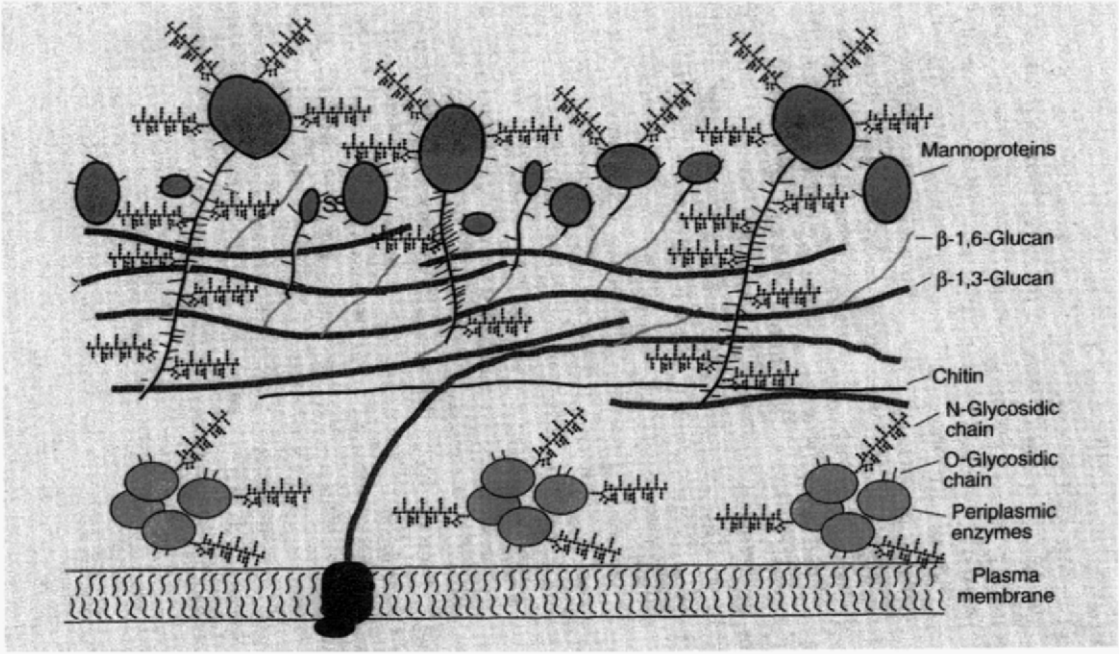

Figure 3.3 Composition and structure of the cell wall. The various components of the *Saccharomyces* cell wall and their complex intermolecular interactions to form a mesh-like organization are depicted. Taken from Schreuder *et al.* (1996) with permission from Elsevier Science

mother and the birth scar on the daughter, as is shown in Figure 3.1 and depicted in Figure 3.5. This is referred to as an **axial budding pattern**. In **a**/α diploid cells, the bud forms at or near either pole in the mother but only at the opposite pole in the daughter giving what is referred to as a **bipolar budding pattern** (Figure 3.4 and depicted in Figure 3.5). A third type of budding pattern, **unipolar budding**, is seen in cells undergoing pseudohyphal growth. Here the bud always forms in the mother at a site near the attachment of mother and daughter and always at the opposite pole in the daughter cell (Figure 3.4 and depicted in Figure 3.5). In budding yeast, the daughter cell (bud) is smaller than the mother cell at the time of cell separation. Therefore, before it can produce its own bud it must grow to a certain minimal size and does this by lengthening the time spent in G1 of the cell cycle. This G1 delay is evident from the finding that mother cells bud before daughter cells. This is indicated in Figure 3.5.

SCHMOO FORMATION AND MATING

In response to mating pheromone in the medium, haploid cells will arrest cell division in G1 and begin to form a protrusion on the side of highest pheromone concentration. This gives the cell a pearlike shape, jokingly called a **schmoo** after a defunct syndicated newspaper cartoon character, and the process is called schmooing. The shmoo is seen in Figure 3.6 and one should note that schmoos are unbudded. Schmoos of opposite mating type will attach to one another at the schmoo tip, a region of the cell wall containing a high concentration of agglutinin proteins. As mating progresses, the cell wall breaks down in the region of cell–cell contact and the plasma membranes and the cytoplasms of the two cells fuse (reviewed in Marsh & Rose, 1997). This is followed by fusion of the two nuclei to form the diploid nucleus that then migrates into a new bud produced at the junction of the two cells, as shown in Figure 3.7. Production of this diploid bud does not involve DNA replication or chromosome segregation.

BUD SITE SELECTION AND POLARIZED CELL GROWTH

As can be inferred from the above description, budding patterns and changes in cell morphology such as mating and pseudohyphal differentiation are highly regulated processes. Each requires polarized growth, i.e. preferential growth at a defined position on the cell surface. The site of growth must be selected and materials such as cell wall components and membrane lipids must be directed to the site to allow the growth to occur (reviewed in Madden & Snyder, 1998, and Johnson, 1999).

Axial and bipolar budding are controlled by two separate pathways. Interestingly, bipolar budding appears to be the default pathway because haploid strains carrying mutations in axial budding genes undergo bipolar budding. The function of these proteins is to mark the incipient growth site; they are sometimes referred to as the cytokinesis tag. The 10 nm neck filament system is the cytokinesis tag required for axial budding. It consists of several septin proteins and other proteins including Bud3p, Bud4p, Axl1p, and Axl2p. Genetic analysis has identified several components required for bipolar budding but the process is more complex, particularly because it differs in the mother and the bud. It is suggested that the G1 delay in

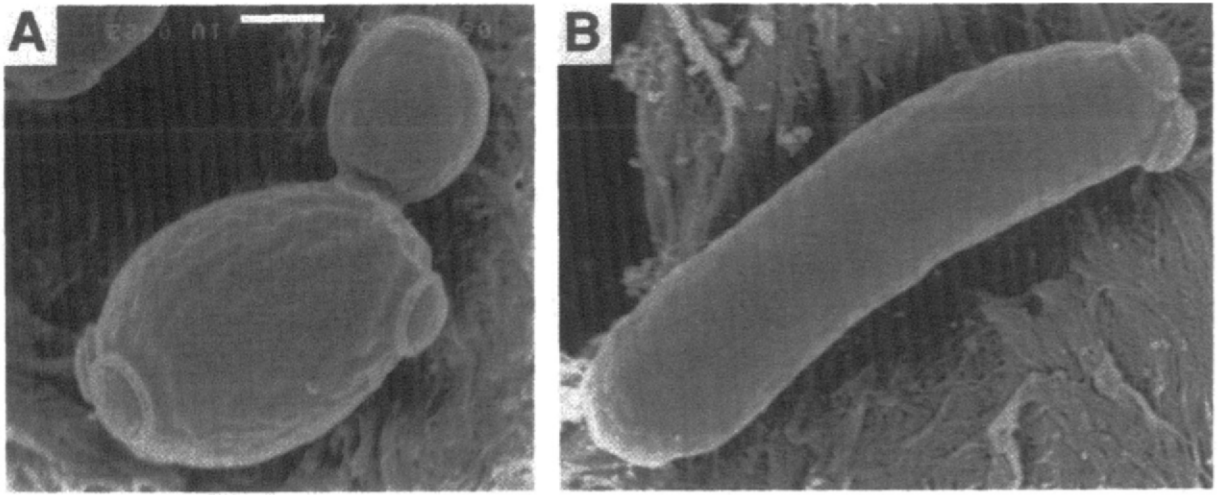

Figure 3.4 Bud scars in diploid cells. SEMs of diploid cells growing in the yeast form (A) and in the pseudohyphal form (B) is used to demonstrate the different patterns of bud site selection. The cell in panel A is undergoing an axial pattern of bud site selection. It is producing its fourth bud at a site in the proximal pole (adjacent to the birth scar). A bud scar is visible in a proximal pole location and two other bud scars are positioned in the distal pole. The cell in panel B has the elongated shape typical of pseudohyphal cells and bud site selection is unipolar. A birth scar is apparent at one end of the cell (to the left) and two bud scars can be seen at the opposite end. Reprinted from Gimeno *et al.* (1992) with permission from Elsevier Science

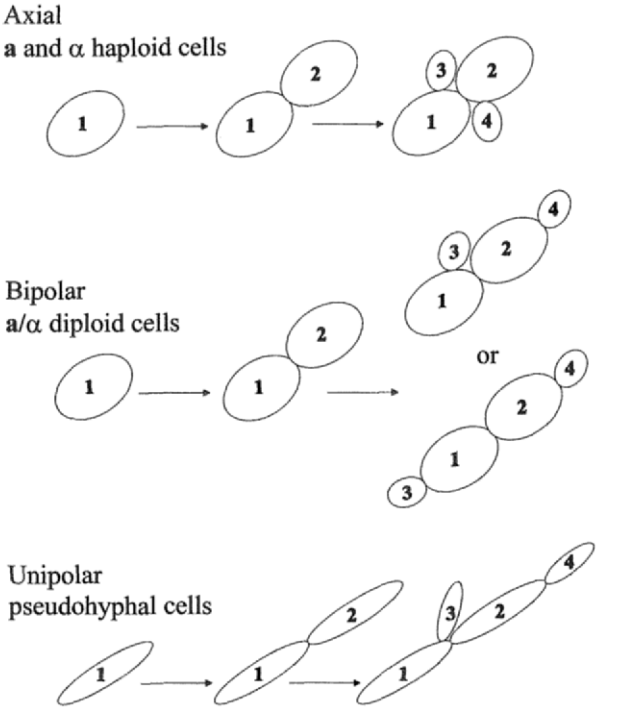

Figure 3.5 Bud site selection patterns in *Saccharomyces*. The three types of bud site selection patterns observed in *Saccharomyces* are depicted. Yeast form cells bud by an axial (haploid cells) or a bipolar (diploid cells) pattern. Cells growing in the pseudohyphal form have a unipolar budding pattern. The numbers indicate the order of formation of the particular cell

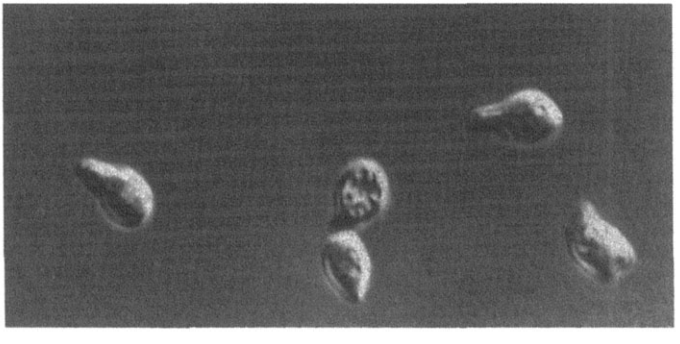

Figure 3.6 The schmoo morphology. Nomarski optics is used to demonstrate the characteristic pear-shaped schmoo morphology formed by haploid cells exposed to the peptide pheromone of the opposite mating type. The cells shown are **a**-mating type and have been exposed to α-factor, the peptide pheromone produced by cells of the α-mating type. The cells carry another genetic alteration that enhances the effect of pheromone treatment (*MAT***a** bar1). Taken from Sprague (1991). Reproduced with permission from Academic Press

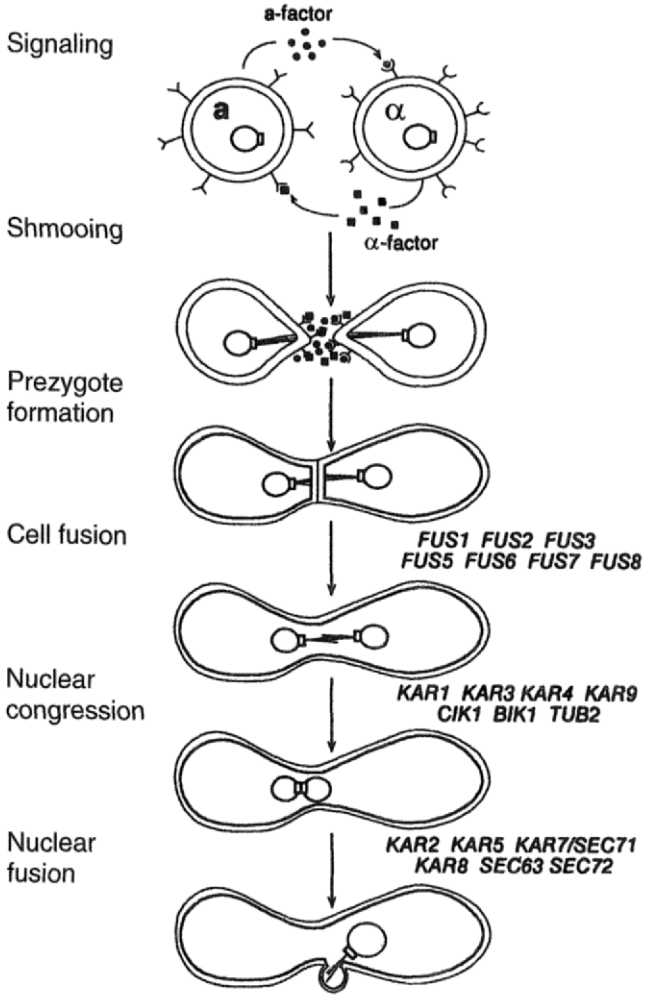

Figure 3.7 Mating and nuclear fusion in *Saccharomyces*. The pathway of events followed by the nuclei of mating cells and the zygote is depicted. The spindle pole bodies with their attached cytoplasmic microtubules orient toward each other and toward the schmoo projection, which has formed at this position in response to the higher concentration of pheromone. Fusion of the cytoplasms allows the cytoplasmic microtubules to make contact and to draw the two nuclei together. This is followed by nuclear fusion and the migration of the zygote nucleus into the newly forming bud by the cytoplasmic microtubules. Several of the genes involved in this process are listed. Taken from Rose (1996), with permission from Annual Reviews

daughter cells is a significant factor in this differing response. Little is reported about bud site selection in unipolar budding.

Following the establishment of the position of the growth site, the general bud site selection proteins, Rsr1p, Bud2p, and Bud5p, position themselves at the site. Rsr1p is a Ras-related GTPase. These proteins allow for assembly of the polarity-

establishment proteins that include Cdc42p, a Rac/Rho-type GTPase, Cdc24p, a GTP exchange factor, and Bem1p. Several other proteins, including kinases, are in this complex and it is this complex that interacts with the actin cytoskeleton.

The actin cytoskeleton plays a major role in all aspects of polarized cell growth in *Saccharomyces*, whether bud growth, pseudohyphal differentiation, or formation of a mating projection (reviewed in Botstein *et al.*, 1997; Madden & Snyder, 1998; Johnson, 1999). During budding, cortical actin patches (see below) with attached actin cables localize first to a ring surrounding the incipient site of bud emergence. Then they move to the bud tip before becoming evenly distributed throughout the bud, and finally positioning at the bud neck for cytokinesis. Secretory vesicles (see below) are directed via the actin cytoskeleton to these sites to allow the distribution of materials required for growth of the plasma membrane, elaboration of the cell wall, and chitin synthesis where appropriate. Thus, initially and in small buds growth is limited to the bud tip (apical growth) and in larger buds cell wall growth is more uniform (isotropic growth).

The mating projection is formed at the site of highest concentration of mating pheromone. Pheromone binding to the receptor stimulates the MAP kinase pheromone response pathway and also stimulates the binding of the polarity-establishment proteins (Cdc42p, Cdc24p, and Bem1p) to the site of the activated receptors. This then allows for the redistribution of the actin cytoskeleton to the region and provides the materials required for remodeling of the cell wall.

It is important to note that all of these processes of polarized cell growth must be tightly coupled with the cell cycle. Bud site selection occurs in G1. Prior to the formation of a mating projection, cells are arrested in G1 and bud site selection is repressed elsewhere.

SPORE FORMATION

The end product of *Saccharomyces* meiosis is an ascus containing four haploid ascospores, the four meiotic products. Meiosis results in a four-lobed nucleus, each lobe having a haploid set of chromosomes and a spindle pole body. When sporulation is complete, the four lobes are separated into four distinct haploid spore nuclei. Spore wall synthesis initiates as a thickening of the outer 'cytoplasmic' plaque of the spindle pole body (see below) and grows out from this site. The mature spore wall completely surrounds the contents of the nucleus and a small amount of cytoplasm and protects the contents from desiccation and other stresses (reviewed in Kupiec *et al.*, 1997). The ascus is approximately the size of the diploid cell from which it developed and the four ascospores are so tightly packed that they appear to form a pyramid.

NUCLEUS

The *Saccharomyces* nucleus is an ovoid structure localized off to one side of the vacuole in the unbudded cell. It contains the chromosomes and in the electron microscope one can observe a nucleolus. For an in-depth review of nuclear structure and function the reader is referred to Wente *et al.* (1997). The *Saccharomyces*

nuclear envelope that separates the contents of the nucleus from the cytoplasm consists of a double membrane, each having a lipid bilayer. One unique feature of *Saccharomyces* is the fact that the nuclear envelope remains intact throughout all stages of cell division and mating. This necessitates several adaptations to ensure proper chromosome segregation and karyokinesis. One of these is the spindle pole body described below.

NUCLEAR ENVELOPE

Numerous nuclear pore complexes span the double membranes of the nuclear envelope and create channels through which proteins, RNAs, and even larger complexes like ribosomal subunits can pass in both directions, often with the aid of specialized import and export protein chaperons. In *Saccharomyces*, the outer nuclear membrane faces the cytoplasm and is continuous with the endoplasmic reticulum (see below). Much of the outer membrane is covered with bound ribosomes and is in essence functioning as the rough ER.

SPINDLE POLE BODY

In addition to the nuclear pore complexes, the spindle pole body (SPB) spans the nuclear envelope (see Figure 3.8). It is a complex structure consisting of three plaques, platelike structures, bound together by cross-bridging proteins (reviewed in Botstein *et al.*, 1997). The middle plaque is embedded in and spans both membranes of the nuclear envelope. Microtubules (see below) are attached to the plaques that lie on the cytoplasmic and nuclear sides of this central plaque. It is believed that the SPB serves as a microtubule-organizing center and allows for the polymerization of tubulin in a polarized fashion. During cell division it also functions as a centrosome-like element (to be discussed below) and as the initiating site of spore wall formation in meiosis.

CYTOSKELETON

The cytoskelton is a meshwork of protein polymer filaments found in all eukaryotes (reviewed in Botstein *et al.*, 1997). In *Saccharomyces*, as in other cell types, it serves to organize the cytoplasm, to provide mechanisms for movement of structures such as vesicles or the nucleus during mating, and to maintain cell shape. Three types of filament consisting of distinct proteins are present. Microfilaments (5–7 nm in diameter) are long flexible polymers of actin hundreds of monomers long. Microtubules (25 nm in diameter) consist of tubulin subunits polymerized to form hollow tubes plus other associated proteins. A number of intermediate filaments (about 10 nm in diameter) consisting of fibrous proteins called septins are found at the bud neck. As discussed above, these neck filaments function as a cytokinesis tag or location signal.

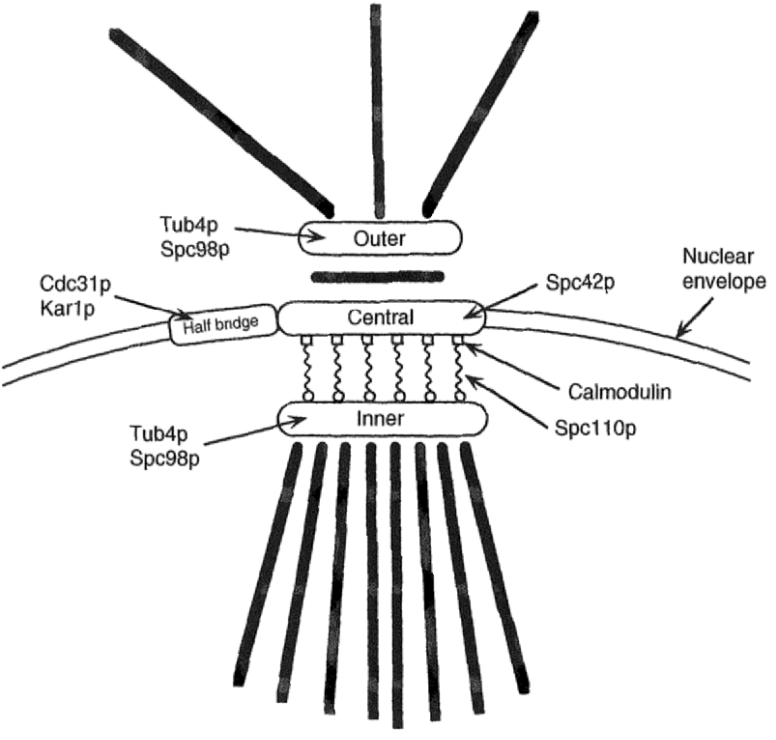

Figure 3.8 Representation of the *Saccharomyces* SPB. The three plate-like plaques of the SPB are shown in cross-section. The central plaque is imbedded in and spans the nuclear envelope. The outer plaque is on the cytoplasmic side of the nuclear envelope and is the attachment site of the cytoplasmic microtubules. It is also the site at which spore wall formation is initiated. The inner plaque lies within the nucleus, and the intranuclear microtubules, those that compose the spindle in a dividing cell, are attached here. A few of the known protein components and their location in this complex structure are indicated. Taken from Botstein *et al.* (1997). Reproduced with permission from Cold Spring Harbor Laboratory Press

ACTIN CYTOSKELETON

Saccharomyces actin is an approximately 42 kD protein encoded by *ACT1* and exhibits high sequence homology to actin from other eukaryotes. It is a globular protein found as a monomer and in polymerized form, i.e. microfilaments. In *Saccharomyces* polymerized actin is localized to several so-called cortical patches and to long fibers sometimes referred to as actin cables. The cortical patches are associated with the small invaginations of the plasma membrane and the actin cables often appear to extend from these cortical patches and orient parallel to the long length of the cell. The distribution of cortical actin patches changes during the cell cycle and this is beautifully illustrated in Figure 3.9. Similarly, changes in the distribution of cortical actin patches are seen prior to schmoo formation (Marsh & Rose, 1997). These changes in the distribution of cortical actin patches strongly

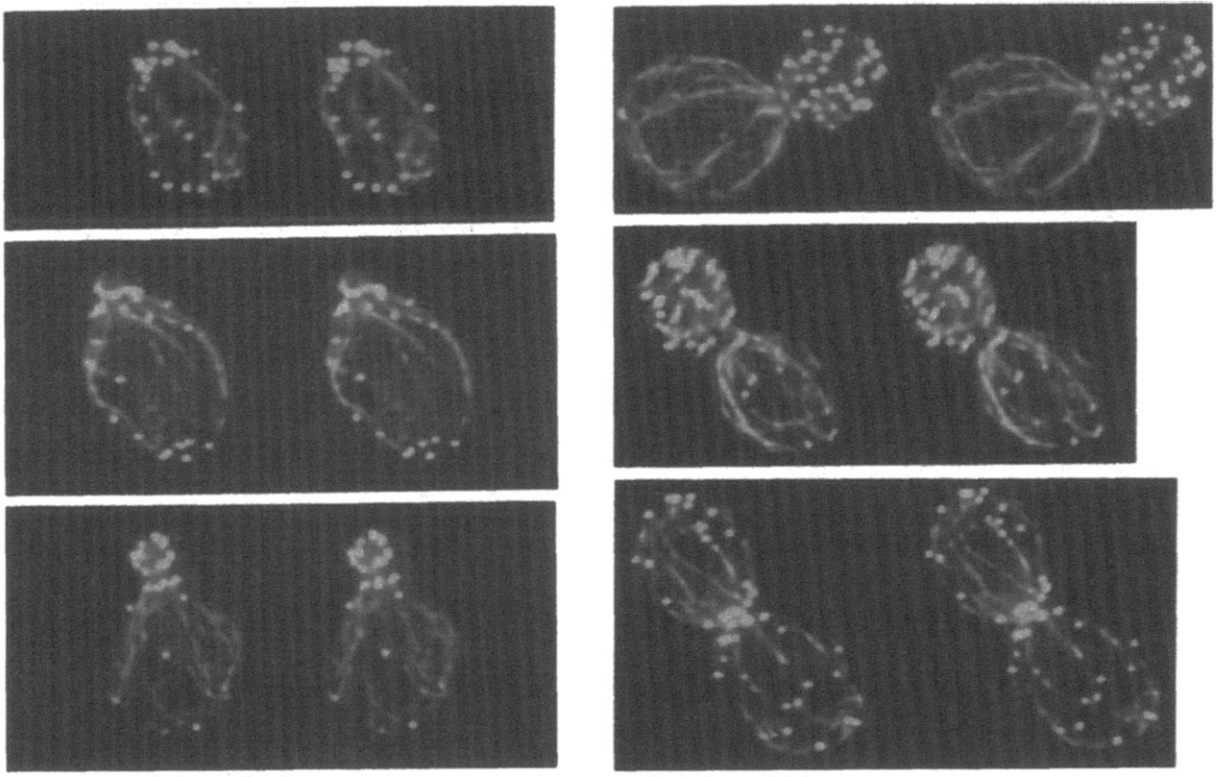

Figure 3.9 Confocal imaging of the actin cytoskeleton in budding *Saccharomyces*. Cells from a dividing population at different stages of the cell cycle are shown stained with rhodamine-conjugated phalloidin, which binds to polymerized actin cortical patches (bright spots) and actin cables (thread-like structures). Localization to the site of bud formation, to the growing bud, and to the bud neck during cell separation is clearly evident. Taken from Botstein *et al.* (1997). Reproduced with permission from Cold Spring Harbor Laboratory Press

suggest an association with polarized cell growth that includes remodeling of the cell wall, activation of chitin synthase, and microtubule localization.

MICROTUBULE CYTOSKELETON

Microtubules are hollow tubes composed of two highly homologous proteins called α-tubulin (50 kD and encoded by *TUB1* and *TUB3*) and β-tubulin (51 kD and encoded by *TUB2*). Two classes of microtubules are found in yeast, namely cytoplasmic microtubules and intranuclear microtubules. Both classes are attached to the spindle pole body and extend either into the cytoplasm or the nucleoplasm.

Microtubules are used for the movement of subcellular organelles, most particularly the nucleus. Motor proteins that attach to and move along the microtubule use the microtubule as a tracklike substrate. Two classes of motor proteins have been identified in *Saccharomyces*, i.e. dyneins and kinesins. Dynein is a large multisubunit protein complex involved in nuclear movement during cell division and mating. The kinesins are smaller protein complexes consisting of a heavy chain with motor activity and a light chain. At least five genes encoding kinesin components have been identified in *Saccharomyces*. Motor proteins can bridge between two microtubules and move these past one another thereby creating movement potentially in either direction depending on the motor proteins involved. Dynein moves toward the SPB direction (negative direction) whereas different kinesins are capable of moving in either the plus or minus direction. It is also likely that some lengthening of the microtubules themselves occurs during spindle elongations in anaphase.

MICROTUBULE MORPHOLOGY IN CELL DIVISION AND MATING

In the unbudded cell, several microtubules can be seen to extend from the SPB into the nucleus and in various directions into the cytoplasm. During budding, both cytoplasmic and intranuclear microtubules undergo dramatic changes beginning with the formation of a second SPB in early G1 that is complete by the start of S. The array of intranuclear microtubules extending from the two SPBs is collectively called the **spindle**. Detailed structural analysis of the *Saccharomyces* spindle during mitosis has been carried out by Mark Winey and coworkers and is reviewed in Winey & O'Toole (2001). During the S phase the two SPBs slowly move apart and by G2 are located at opposite poles of the nucleus. Short intranuclear microtubules attach to the inner plaque of the SPB at one end and to the chromosomal kinetochore. Other longer intranuclear microtubules extend from one SPB to the other. Chromosome separation, i.e. anaphase, is achieved in some degree by the shortening of the kinetochore-bound microtubules but mostly by an increase in the pole-to-pole distance between the two SPBs. This lengthening of the spindle is dependent on microtubule growth processes and motor proteins.

Throughout these intranuclear events of mitosis, the cytoplasmic microtubules are involved in localizing the nucleus to the bud neck and directing the future daughter cell nucleus into the bud. In Figure 3.10 one can see the nuclear oscillations that occur in the mother cell prior to anaphase and the apparent searching process is carried out by the bud-directed cytoplasmic microtubules, perhaps for an

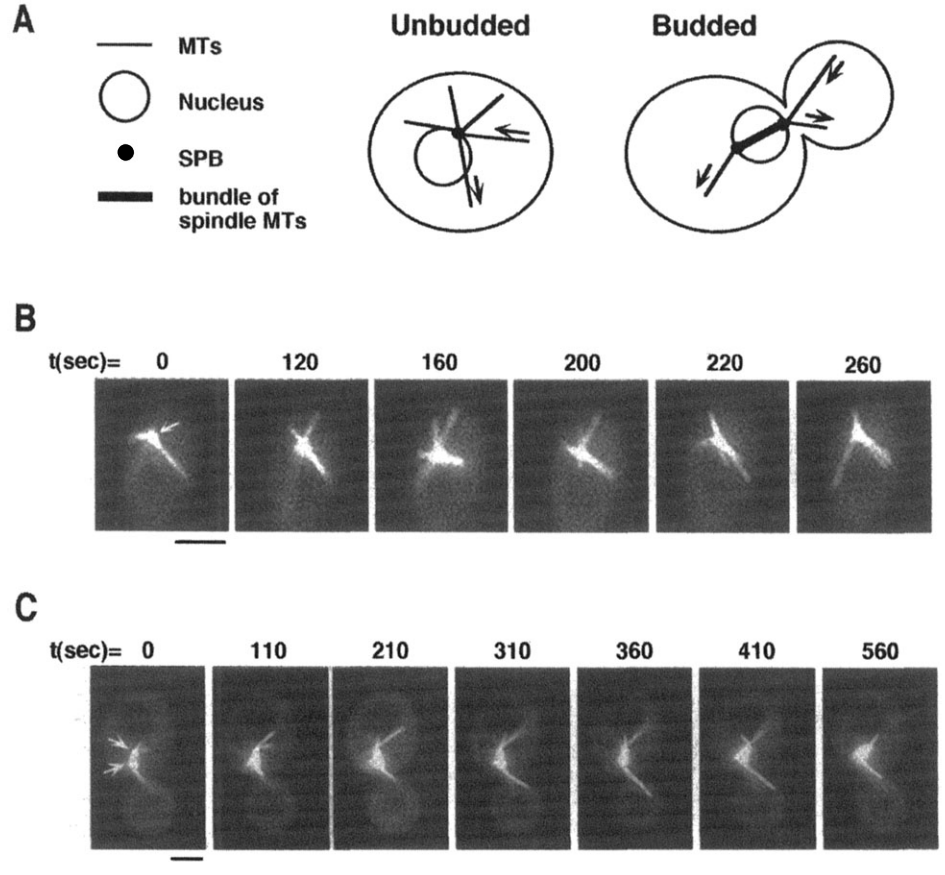

Figure 3.10 Microtubule orientation in dividing and nondividing living cells. The cells shown are expressing a fully functional GFP-tubulin fusion protein allowing microtubules to be visualized in living cells. A time course of changes in microtubule shape and orientation is shown for an unbudded cell (panel B) and for a large-budded cell (panel C). The photographs demonstrate the process of cytoplasmic microtubule searching that ultimately localizes the nucleus to the bud neck. Taken from Carminati and Stearns (1997). Reproduced with permission of the Rockefeller University Press

attachment site in the bud. These microtubules determine the orientation of the spindle and ensure that spindle lengthening occurs through the bud neck (Rong, 2000). As a result of spindle lengthening the nucleus acquires an elongated hourglass shape that extends through the bud neck and places one end in the mother cell and the other in the bud. Separation into two nuclei, the two-budded stage, marks the end of the division phase of the cell cycle and the beginning of G1 of the next cell cycle.

Various cytological events are used as mileposts to assess progress through the cell cycle. These events include SPB duplication, localization of cortical actin patches, initiation of bud growth, size of bud, localization of the nucleus to the bud neck, nuclear shape or spindle length and shape, and number of nuclei. These events

have been carefully correlated with the stages of the cell cycle and thus can be used to determine the timing of another process, such as the requirement for a particular gene function, in the cell cycle without the need to measure DNA levels or other more cumbersome assays.

Rose (1996) reviews the role of microtubules in nuclear fusion during mating. Figure 3.7 shows this process. The SPB of each parental nucleus orients toward the growing schmoo projection and, as the cytoplasms of the two mating cells fuse, the cytoplasmic microtubules attached to the two SPBs intertwine. Motor proteins associated with the cytoplasmic microtubules move the two nuclei together and allow fusion to occur. Following fusion, the cytoplasmic microtubules of the newly formed diploid nucleus direct it into the new bud forming at the junction of the parental cells.

PLASMA MEMBRANE, ENDOPLASMIC RETICULUM, GOLGI COMPLEX, VACUOLE, AND MEMBRANE TRAFFICKING

The endoplasmic reticulum (ER), the Golgi complex, the plasma membrane, and the vacuole must be considered together. These physically separated subcellular compartments are intimately interconnected as a result of the movement of membrane that occurs from one compartment to the next via membrane-bounded vesicles carrying a cargo of proteins and other macromolecules such as cell wall components. This movement of membrane and proteins is sometimes referred to as **membrane trafficking**. The secretory pathway flows from the ER to the Golgi complex where it branches, sending vesicles either to the plasma membrane (**exocytosis**) or to the vacuole. It is estimated that from 10% to 20% of a putative 6000 *Saccharomyces* proteins are either residents or passengers of the secretory pathway (Kaiser *et al.*, 1997). Membrane and selected proteins are also removed from the plasma membrane and directed to the vacuole via a process referred to as **endocytosis**. For an in-depth discussion of the structure, function, and biosynthesis of these compartments and pathways the reader is directed to reviews by Kaiser *et al.* (1997) and Jones *et al.* (1997). Figure 3.11 is a transmission EM image of *Saccharomyces* showing the various organelles involved in the secretory pathway.

ENDOPLASMIC RETICULUM

The endoplasmic reticulum is the site of protein synthesis of secreted and integral membrane proteins localized to the various compartments of the secretory pathway. The ER is contiguous with the outer membrane of the nuclear envelope where it is studded with ribosomes involved in the synthesis and translocation of secreted proteins across the ER membrane into the lumen of the ER or the insertion of newly synthesized integral membrane proteins into the ER membrane. Extensions of the ER can be seen extending in from the outer nuclear membrane into the cytoplasm out as far as the edges of the cytoplasm and these can be visualized as a discontinuous

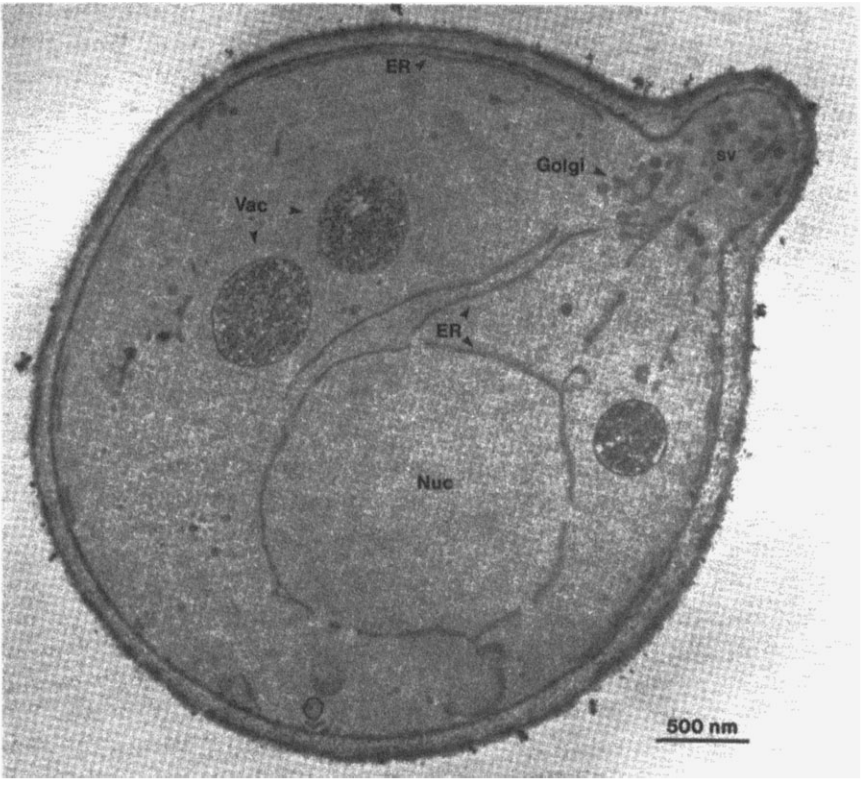

Figure 3.11 The organelles of the secretory pathway. A transmission electron micrograph of a cell with a newly forming bud illustrates the various subcellular organelles of the secretory pathway and their organization within the cytoplasm. The ER can be seen to extend from the outer membrane of the nuclear envelope (Nuc indicates the nucleus) in projections toward the surface of the cell. The ER also underlies the plasma membrane. Plasma membrane and cell wall growth is concentrated in the bud, which is packed with secretory vesicles (sv). The Golgi is localized to the bud neck region of the mother cell. *Saccharomyces* has only a single large vacuole (Vac) but this appears to be doubled in this micrograph because of a sectioning artifact. Taken from Kaiser *et al.* (1997). Reproduced by permission of Cold Spring Harbor Laboratory Press

cisterna underlying the plasma membrane (see Figures 3.11 and 3.12). Fluorescence microscropy of cells expressing a GFP fusion to the ER-localized protein Gsf2p illustrates the cellular position of the *Saccharomyces* ER (Figure 3.12).

Various protein modifications occur in the ER. Cleavage of the signal peptide occurs during protein translocation. The addition of N-linked and O-linked carbohydrate groups is initiated in the ER as well as the addition of GPI anchors. Protein folding and disulfide bond formation occur here. This is particularly important for certain membrane proteins and for the formation of multiprotein complexes in the ER lumen. Improperly folded integral membrane proteins are targets of proteolysis via quite specific pathways (Kaufman, 1999) and the ER is a site of regulated protein degradation.

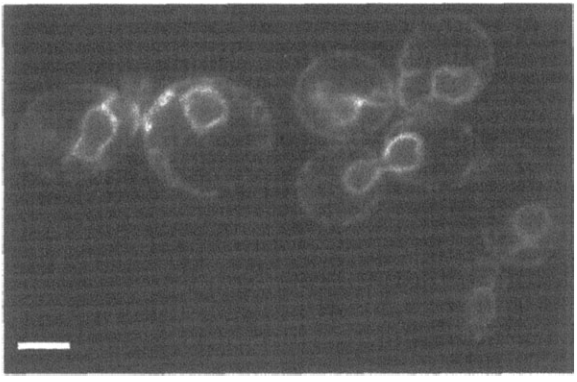

Figure 3.12 GFP-fluorescence imaging of the endoplasmic reticulum. The cells shown are expressing a GFP fusion to the ER-localized protein Gsf2p. The subcellular location of the fully active GFP–Gsf2p fusion is visualized by confocal image analysis. Fluorescence is observed in the region closely surrounding the nucleus, in cytoplasmic threads extending from the nucleus, and in the region underlying the plasma membrane. Taken from Sherwood & Carlson (1999). Reproduced by permission of the National Academy of Sciences, USA

GOLGI COMPLEX

The Golgi complex in *Saccharomyces* is a grouping of several membrane-bound cisternae. Despite the fact that the different cisternae cannot be distinguished microscopically, functional analysis clearly indicates that the *Saccharomyces* Golgi consists of three compartments referred to as the *cis*-, *medial*-, and *trans*-Golgi. Proteins enter the *cis*-Golgi from the ER and proceed to the *medial*- and *trans*-Golgi where they receive sequential modifications, including the addition of outer-chain mannose residues. Mature secretory proteins are selectively packaged in the *trans*-Golgi into vesicles and proceed from here to either the plasma membrane or the vacuole (with an intermediate stop in a prevacuolar compartment called the **endosome**).

VACUOLE

The *Saccharomyces* vacuole is a large centrally located single membrane-bound organelle easily visualized by Nomarski optics and is the equivalent of the mammalian lysosome (see Figure 2.5). Jones *et al.* (1997) provides an excellent overview of vacuolar function and biosynthesis. The vacuole contains a variety of proteases and hydrolases for the degradation of polysaccharides and RNA. It serves as a storage site for ions such as Ca^{2+} and amino acids and the vacuolar membrane contains pumps for these ions/molecules as well as a proton pump for regulating vacuolar pH. The vacuole plays a very important role in the physiological transition from one growth state to another such as occurs during changes in nutritional conditions or entry into the stationary phase.

Proteins get to the vacuole by a number of different pathways. Already mentioned is the movement of protein cargo-containing vesicles from the Golgi. Some of

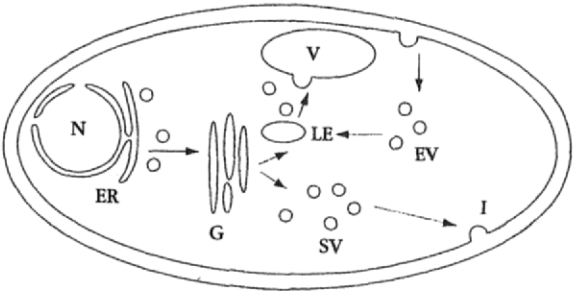

Figure 3.13 Overview of exocytosis and endocytosis. The movement of membrane through the secretory pathway and from the membrane to the vacuole via endocytosis is shown. N, nucleus; ER, endoplasmic reticulum: G, Golgi complex; LE, late endosome (prevacuolar compartment); EV, endocytic vesicle; SV, secretory vesicle; I, invagination of the plasma membrane (clathrin-coated pit); V, vacuole. Note that the forward and retrograde movement within the Golgi complex (*cis*-, *medial*-, and *trans*-Golgi compartments) as well as retrograde movement from the Golgi to the ER described in the text also involves very specialized vesicles, but these are not illustrated in the diagram for simplicity. Taken from Walker (1998). Reproduced with permission from John Wiley & Sons, Limited

these vesicles contain vacuolar resident proteins on their way to their final destination. More recently it appears that some plasma membrane proteins may be redirected to the vacuole for degradation in response to physiological changes (Roberg *et al.*, 1997). Vesicles formed at the plasma membrane by endocytosis are targeted to the vacuole for degradation by the vacuolar enzymes. Both vesicles from the Golgi and those from the plasma membrane do not go directly to the vacuole but go first to a prevacuolar compartment called the endosome. The endosome may go through a maturation process before finally fusing with the vacuolar membrane and delivering its contents to the vacuole for degradation. **Autophagy** is another route to the vacuole. Cytoplasmic proteins and even organelles are surrounded by membrane and these autophagosomes are taken into the vacuole where they are degraded and recycled. Finally, a so-called **cytoplasm-to-vacuole** targeting pathway, or cvt pathway, which at least partially overlaps with the autophagy pathway has been described (Lang *et al.*, 2000).

MEMBRANE TRAFFICKING

An overview of the **secretory pathway** and **endocytosis** is shown in Figure 3.13 and reviewed in Kaiser *et al.* (1997). This figure illustrates only forward movement through the secretory pathway but it should be noted that retrograde movement of vesicles also occurs from the Golgi to the ER as well as from the late Golgi to the *cis*-Golgi. The purpose of this retrograde movement is to return proteins that were carried along with the cargo from one compartment to the next back to their home compartment for reutilization.

It is most important to keep in mind that movement from compartment to compartment, whether forward or retrograde, is carried out by specialized **vesicles**. Vesicles are classified based on the protein 'coat' found on the cytoplasmic side of the vesicle. Vesicles directed to the vacuole, i.e. **endocytic vesicles** and vesicles

formed by the *trans*-Golgi, are **clathrin-coated vesicles**. **COPI vesicles** that are coated by a protein complex called **coatomer** are involved in intra-Golgi transport and retrograde transport from the Golgi to the ER. Vesicles from the ER to the Golgi are coated with a set of proteins called COPII and are referred to as COPII vesicles.

The specificity of the interaction between the vesicle and the target compartment is controlled by large protein complexes called SNARE complexes located within the membranes of the vesicle, called the **v-SNARE**, and the target membrane, called the **t-SNARE**. These can be thought of as a lock and key mechanism to ensure that a particular vesicle carrying a cargo intended for a specific compartment fuses with that compartment and only that compartment (reviewed in Kaiser *et al.*, 1997).

MITOCHONDRION

As in other eukaryotes, the *Saccharomyces* mitochondrion is a double membrane organelle. Enzymes of lipid metabolism are located in the outer membrane. The inner membrane, called the **cristae**, is highly convoluted and contains the enzymes involved in respiration and ATP synthesis. The enzymes of the citric acid cycle and of fatty acid oxidation are found in the matrix along with the mitochondrial DNA and the mitochondrial protein synthetic machinery. Biosynthesis of the mitochondrion involves both nuclearly encoded proteins and RNAs and proteins encoded by the mtDNA.

Under different growth conditions, such as growth on fermentable versus non-fermentable carbon source or the presence or absence of oxygen, the number of *Saccharomyces* mitochondria per cell will vary as well as the number of copies of the mtDNA. Figure 3.14 shows a three-dimensional reconstruction of the mitochondria produced by confocal analysis of *Saccharomyces* cells grown in different carbon sources. *Saccharomyces* is a facultative anaerobe and when grown on fermentable carbon sources like glucose does not require an active mitochondrion for ATP production. Under these conditions, the extent of the crista development is greatly reduced and the mitochondria are sometimes referred to as ghosts.

PEROXISOME

Peroxisomes are single-membrane organelles that contain at least one enzyme for the production of hydrogen peroxide, for example catalase, and enzymes to catalyze its decomposition. The reader is directed to Lazarow & Kunau (1997) for detailed information on the structure, function, and biosynthesis of peroxisomes. The expression of genes encoding peroxisomal proteins is repressed by growth on glucose. Therefore the number of peroxisomes is usually quite low in glucose-grown cells. A major function of peroxisomes is the β-oxidation of fatty acids and peroxisomes are best visualized in cells grown in oleic acid or other fatty acids that induce the expression of peroxisomal proteins.

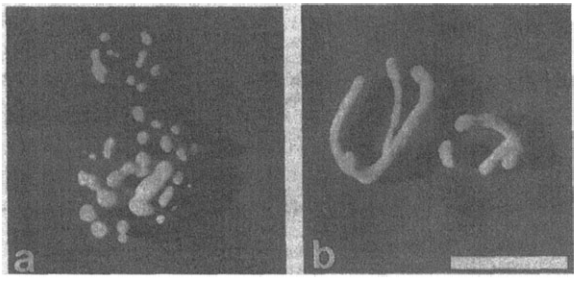

Figure 3.14 Three-dimensional images of mitochondria. Scanning confocal microscropy was used to create the three-dimensional images of the shape and distribution of mitochondria in *Saccharomyces*. The cell shown in panel (a) was grown in ethanol, a nonfermentable carbon source requiring active mitochondria for utilization. The cell in panel (b) was grown in a high concentration of glucose. Under this condition *Saccharomyces* ferments the sugar producing ethanol, carbon dioxide, and ATP, and does not require fully elaborated mitochondria. Taken from Visser *et al.* (1995). Reproduced with kind permission from Kluwer Academic Publishers

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II Techniques of Genetic Analysis

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4 Mutant Hunts — To Select or to Screen (Perhaps Even by Brute Force)

The first step in a genetic analysis of a process it to isolate mutant individuals that are unable to carry out that process or carry it out in an aberrant way. The researcher must hypothesize what characteristics, changes in growth capabilities, morphology, etc. will be exhibited by this individual and to develop a means of identifying such individuals from among a larger group of normal individuals.

A **mutation** is a heritable change in the genetic material, and **mutagenesis** is the process of producing such changes. **Spontaneous mutations** are the by-product of natural processes, particularly errors resulting from DNA replication and repair and the movement of transposable elements. The spontaneous rate of mutation varies from species to species and the rate of spontaneous production of different classes of mutation (point mutation, insertion, deletion) varies. The geneticist also has available a variety of methods for enhancing the rate of mutagenesis above the spontaneous, or background, rate including treatment with various chemical agents, ultraviolet light, X-rays, and transposon-mediated events. Different mutagens produce different types of genetic change. The best choice of mutagenic agent and the best method of delivering that mutagen will depend on one's particular experimental needs and the organism under study.

Regardless of whether the mutation is spontaneous or **induced** and regardless of which type of mutagen is used, the researcher is unable to direct mutations to specific sequences or particular genes. To a first approximation, mutagenesis is a random process, and any particular gene has an equal chance of being damaged by a mutagen or a spontaneous error in a cellular process such as DNA replication or repair. So how does one find those individuals carrying mutations in a gene of interest, i.e. a gene involved in the process under study? In other words, how does one distinguish the interesting mutant individuals from all the other individuals in the pool of potential mutant individuals? One **searches** for them or carries out what geneticists sometimes call a **mutant hunt**. To do this, one must develop a method of identifying mutants. This is the first job of the geneticist and, in many respects, it is the most difficult job.

An individual carrying a heritable genetic change is referred to as a **mutant** and the process of identifying mutants of interest is called either a **selection** or a **screen**, depending upon how it is carried out. Note that one identifies mutant individuals by observing their phenotype. **Phenotype** is an indirect expression of **genotype** and is the product of the biochemical and physiological interplay among all the genes of an individual's genome. For this reason, geneticists prefer to work with **isogenic** strains, i.e. strains that are genetically identical at all but perhaps one or a limited number of known genes. In this way the effect of a newly induced mutation on the phenotype of the individual can clearly be associated with that specific single genetic alteration. If isogenic strains are not available, then efforts should be made to work

with very closely related strains such as a congenic strain. A **congenic** strain is one obtained by repeated backcrosses to a common parent. The products of the tenth backcross should be better than 99.9% identical.

In the earlier yeast literature, prior to the early 1970s, researchers did not seem to pay a great deal of attention to working with genetically related strains. The influx of bacterial and phage geneticists into the yeast research community brought an awareness of the importance of working with isogenic strains where possible. In the prokaryotic systems the technology for constructing isogenic strains is rather straightforward. In yeast the ability to construct isogenic strains came with the development of *Saccharomyces* DNA technology. One can now change the mating type of a strain by the simple use of a plasmid expressing the *HO* gene.

To identify mutant individuals carrying alterations in a gene of interest, the geneticist will hypothesize the probable phenotype of these mutant individuals and develop a method of identifying them based on their phenotypic difference from the starting strain. The best method is **to select** for the mutant individuals. A **selection method** is one in which only individuals with the desired phenotype are able to grow, or move toward a light source, or carry out a function. For example, one is interested in studying the mechanism of action of an antibacterial drug, and the drug-resistant mutant bacteria capable of growing in the presence of the drug is isolated. This is a selection because millions of bacteria will be spread on the surface of a petri dish containing nutrient medium with the drug. Almost all the bacteria will die because of the action of the drug, but a few will grow to form a colony because these individuals carry a mutation that makes them resistant to the drug.

A **screen** is where the individuals of a population are observed one by one following growth in a particular condition and individuals with a particular phenotype are chosen. For example, one is interested in tryptophan synthesis, and needs to isolate mutant strains unable to synthesize tryptophan. Such mutants would be unable to grow in the absence of added tryptophan in the medium because tryptophan is an essential amino acid. Colonies (clones of cells) are grown on the surface of a solid (agar-containing medium in a petri dish or petri plate) medium containing tryptophan. **Clones** are genetically identical individuals. Each clone is tested by a simple test called **replica plating** to see if it grows on the same medium only lacking tryptophan. (Replica plating is a method of making duplicate copies of the pattern of colonies growing on the surface of solid media in a petri dish. Colonies are grown on the media in a petri dish; these colonies are transferred to a sterile velvet cloth by pressing the cloth to the surface of the plate. Then the colonies are transferred to other plates by pressing the cloth to the surface of new plates.) Colonies grown on a medium plus tryptophan are replica plated to plates containing the same medium with and without tryptophan. Those colonies that do not grow on the plate lacking tryptophan but do grow on the plate containing tryptophan have a tryptophan minus phenotype.

Only when all else fails will the geneticist resort to **a brute force screen** in which individuals are screened for a complex phenotypic trait by a difficult procedure. These are few and far between, and the geneticist would have to be desperate. Before undertaking a brute force screen, the geneticist will attempt to **enrich** for mutants of the desired type. Penicillin enrichment for *E. coli* mutants is an example. Penicillin kills only growing cells because it blocks cell wall growth causing the expanding cytoplasm to burst the cell. Mutagenized cells asked to grow in penicillin-

containing medium lacking histidine should be enriched for histidine minus mutants because these would have been unable to grow in the absence of histidine and therefore would have survived penicillin treatment.

Geneticists refer to the original strain isolated from nature and prior to any mutagenesis as the **wild-type strain** and the allele of each gene in this strain as the **wild-type allele**. Any alteration in a gene, whether associated with an altered phenotype or not, is a **mutant allele**. Most often, the strain used in a selection or screen will carry one or more known mutations that are for the use of the geneticist. These mutant genes are often referred to as **genetic markers** or **marker genes** because they mark the existence and position of the gene in the genome and are used to expedite the genetic analysis. Marker genes with easily identifiable phenotypes are generally chosen, such as nutritional mutations or drug-resistant alleles. Thus, because the original strain used to isolate new mutation is not truly wild-type, it is usually referred to as the **parental** strain.

A mutant allele may be **dominant** or **recessive** to the wild-type, and this can only be determined by making the heterozygous diploid individual and determining its phenotype. If the mutant phenotype is exhibited by the heterozygous diploid, then the mutation is dominant. If the wild-type phenotype is exhibited by the heterozygous diploid, then the mutation is recessive, i.e. the wild-type allele dominates the phenotype. A recessive mutation is a **loss of function mutation**; that is, the gene either no longer produces a product or the product produced is so abnormal that it is unable to carry out the function. A dominant mutation is a **change of function** mutation; that is, the product of the gene has retained all or part of its original functional characteristics but also has acquired new functions or carries out its usual functions differently.

The more rare a particular type of mutation, the more aggressively one must look. Selection is more aggressive than screening because more individuals can be tested. This statement does not contradict the fact that mutagenesis is a random process. What it does say is that the genetic alteration needed to produce a certain phenotype can be more or less difficult to achieve. For example, to knock out all function of a gene one can delete it, insert a DNA fragment into it, or change its sequence in any of a variety of ways. One does not care because no function need be retained. But if one wants a mutant with a slightly changed phenotype, such as resistance to a drug, then the product of the altered gene must be able to carry out its normal function but with different characteristics. Alterations capable of achieving this are few and far between and only certain select changes will do the trick. In other words, it takes a scalpel to make a dominant mutation but you can use a grenade to make a recessive mutation. A priori, the geneticist does not know how rare the searched-for defects will be so, ideally speaking, one tries to devise a selection. This is not always feasible, and so a screen may have to be used. The geneticist will often repeat a mutant search using subtle variations in the selection/screening method. The particular method used undoubtedly biases the types of mutation isolated. By varying the isolation method, it is hoped that the additional genes involved in the process will be identified or that different alleles of an already identified gene with subtly different phenotypes will be obtained.

If several genes are expected to be involved in a particular process or pathway, large numbers of mutant individuals must be obtained so as to ensure that

mutations in all of these genes are isolated. This is referred to as **saturating** the system. Mutations in different genes involved in the same process may have the same or slightly different phenotypes. Sometimes different mutations in the same gene may have different phenotypes. A great deal may be learned about the normal function of a gene by carrying out a careful phenotypic analysis of the different mutant alleles obtained.

A **forward mutation** is a genetic alteration of a wild-type gene. It produces a **mutant strain** with a mutant phenotype and mutant genotype. A **reverse mutation**, also called a **reversion**, is an alteration that produces a **revertant** strain with a wild-type or pseudo wild-type phenotype. If the reverse mutation exactly reproduces the original wild-type genetic sequence, then the revertant strain is a **true revertant** and has the wild-type phenotype and genotype. As we will see in Chapter 8, it is possible for a mutation at a different site, even in a different gene, to produce a revertant strain with what appears to be a wild-type phenotype even though the genotype is not wild-type but is actually a double mutant. In this situation the second mutation interacts with the original mutation to suppress its mutant phenotype, and the second mutation is called a **suppressor mutation**. Suppression is an important, interesting, and very valuable tool for the molecular geneticist, and we will explore it in great detail in future chapters.

A **null mutation** is one in which the alteration completely eliminates gene function. Null mutations result from deletions of the gene that include large sections of the ORF or the 5' end of the gene, nonsense mutations near the 5' end of the ORF, transposon insertions, or other changes that block gene expression or production of the gene product. Null mutations are always recessive.

A strain carrying a **conditional mutation** is one that exhibits the mutant phenotype under some conditions and the wild-type phenotype under other conditions. The strain is a mutant strain but whether the strain exhibits the mutant phenotype or the wild-type phenotype depends on the growth conditions. The condition that is most often varied is the growth temperature. Most organisms prefer to grow at a particular temperature. For example, the ideal growth temperature of *Saccharomyces* is 30°C, but wild-type strains are able to grow at temperatures from 14°C to 42°C. Mutant strains that exhibit the mutant phenotype at temperatures above the preferred growth temperature are called **temperature sensitive**, and the mutant gene is referred to as a **temperature-sensitive** or **ts allele**. Mutant strains that exhibit the mutant phenotype at temperatures below the preferred growth temperature are said to be **cold sensitive**, and the mutant gene is referred to as a **cold-sensitive** or **cs allele**. Since there is a condition where the mutant gene functions somewhat normally, conditional alleles are not null alleles and most often are changes of a single amino acid residue in the encoded protein. The growth condition at which the mutant phenotype is exhibited is called the **nonpermissive** or **restrictive** condition. The **permissive** or **nonrestrictive** condition is the one in which the wild-type phenotype is expressed.

Researchers interested in essential cellular processes like cell division or transcription must isolate conditional mutations. An **essential gene** encodes a product required for normal growth and cell division and its function cannot be by-passed by nutrient supplementation of the medium. A cell carrying a mutation in a gene encoding a necessary component of RNA polymerase II cannot survive if the

mutant exhibits the mutant phenotype under all conditions. There is no way of adding a nutrient or drug to the medium or altering the growth conditions that will enable this cell to grow. Such a mutation in an essential gene is a **lethal mutation**. A conditional mutation in an essential gene allows the researcher to culture the mutant strain under the permissive condition and to observe the mutant phenotype under the nonpermissive condition. This type of mutation is referred to as a **conditional lethal mutation**. The use of conditional mutations is a very powerful tool of the geneticist and its many uses will become obvious as we read the literature.

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5 Complementation Analysis: How Many Genes are Involved?

Complementation analysis is used to determine whether two independent mutations are alterations in the same gene; that is, they are **alleles**, or are alterations in different genes. In essence, a complementation analysis is a functional test used to define a gene. If a researcher has isolated a number of mutants with a similar phenotype, the next question asked is: 'How many genes have I identified?'. If there are 10 mutant strains, are they each in different genes, does each mutant carry a different mutation (allele) in the same gene, or something in between such as two genes one with six alleles and the other with four alleles? Complementation analysis will help answer this question.

Seymour Benzer's study of the *rII* locus of phage T4 is a most elegant example of the power of complementation analysis (Benzer, 1955). Benzer had several hundred mutations that gave the same phenotype, large plaques on one host strain of *E. coli* and no plaques on another host strain, and mapped to the same region of the T4 chromosome. Using the host strain in which *rII* mutants formed no plaques, he found that when host cells were coinfected with different mutant pairs some pairs produced a normal phage burst while others did not. Those that produced a normal burst he concluded were in different functional genetic units that he called **cistrons**, a term that is synonymous with **gene**. In contrast, those pairs of mutants that rarely or never produced a normal burst he concluded were in the same cistron. The rare productive infections Benzer proposed resulted from recombination between the different mutations in the same cistron thereby creating a wild-type genotype in a few coinfected cells. Using this method, he placed all of his *rII* mutations into two cistrons that he called *rIIA* and *rIIB*. Moreover, he made a detailed genetic map based on recombination frequency between the different mutations (a **fine structure map**) that also indicated the frequency at which a mutation was isolated at that position. In this way he demonstrated that genes are not indivisible units but consist of many mutable sites that can recombine. Geneticists working with other organisms soon followed Benzer's lead and adapted complementation analysis to their systems.

To carry out a complementation analysis, both mutant genes must be expressed in the same cell so that their gene products are synthesized in the same cytoplasm and can functionally interact. Only loss of function (recessive) mutations can be used for a complementation analysis. The theory behind the complementation analysis is simple. If both mutations are loss of function alterations of the same gene, then the diploid cell carrying these two mutant genes will not contain a functional allele and will have the mutant phenotype. If both mutations are loss of function mutations but in different genes, then the diploid cell will have one mutant allele and one functional wild-type allele of each gene and, since the mutant alleles are loss of function alleles, the diploid will have the wild-type phenotype.

To express both mutant genes in the same cytoplasm a heterozygous diploid must be constructed. The way the researcher establishes the diploid state varies with the organism under study. In *Saccharomyces*, this is accomplished by mating a *MAT***a** strain containing mutation #1 to a *MAT*α strain containing mutation #2. The **a**/α diploid will be heterozygous for the mutant genes. The phenotype of the heterozygous diploid is then observed. If the diploid has a wild-type phenotype, then the mutations are said to **complement** and this is strong evidence that the mutations are in different genes. A geneticist might also say, 'The mutations are in different **complementation groups**'. If the diploid has a mutant phenotype, then the mutations do not complement and are said to be in the same complementation group. This is considered strong evidence that the mutations are alleles. The definition of complementation group is a set of noncomplementing mutations. The term complementation group is synonymous with gene.

Two conditions must be met before one can carry out a complementation analysis on a series of mutants. First, the mutant strain can only contain a single mutation compared with the parental strain. Particularly when mutagenesis had been used to obtain the mutants, it is possible that more than one DNA alteration was induced in an individual and these are involved in producing the mutant phenotype. Therefore, each mutant strain must be tested to demonstrate whether one or more genetic alterations are required to produce the mutant phenotype. To test this the mutant strain is crossed to a wild-type strain and tetrad analysis of the heterozygous diploid is carried out. If only a single alteration is required, then only tetrads with two mutant spores and two wild-type spores will be produced, as shown in Cross 1 in Chapter 1. But if two or more alterations are present, tetratype and nonparental ditype tetrads will be produced, as shown in Cross 3 in Chapter 1. What would be the phenotypes of the spores of a tetratype tetrad if the mutant strain contained two altered genes and both alterations were required to produce the mutant phenotype? What would be the phenotypes of the spores of a tetratype tetrad if the mutant strain contained two altered genes and either mutation alone were sufficient to produce the mutant phenotype?

Of course to carry out a cross between the mutant and wild-type strains the strains must be of opposite mating type and should carry different nutritional mutations to facilitate the selection of diploids. But in other respects the two strains should ideally be isogenic except for any alterations required to produce the mutant phenotype. Usually, before undertaking a mutant hunt, the geneticist will construct an appropriate pair of isogenic (or congenic) haploid strains to be used as **parental** strains. The mutants isolated in one strain can then be mated to the parental strain of the opposite mating type to determine the number of mutant genes involved.

As described above, the second requirement for a complementation analysis is that the mutations be loss of function alleles. In other words, only mutations that are recessive to the wild-type allele can be used. So, as a second step in the genetic analysis of mutants, mutant strains carrying a single mutant gene are crossed to a parental strain carrying the wild-type allele. If the mutant carries a recessive loss of function mutation, then the heterozygous diploid (*GEN1*/*gen1-34*) will have the wild-type phenotype. This mutant allele can then be used for complementation analysis.

Cross 4 shows a complementation test for two mutant strains. Preliminary genetic analysis has shown that each strain contains only a single mutant gene and that the mutant allele is recessive.

Cross 4: Mutant strain 5 × Mutant strain 14
Diploid phenotype: Mutant

The result shown in Cross 4 indicates that the mutation in strain 5 and the mutation in strain 14 do not complement and thus are mutations in the same gene. If we call the gene *GEN1*, then these mutations are alleles and one could now name them *gen1-5* and *gen1-14*. This cross could be depicted as shown below.

Cross 4: *gen1-5* (mutant) × *gen1-14* (mutant) (genotypes of parental strains) (phenotype of parental strains)

Diploid: $\frac{\textit{gen1-5}}{\textit{gen1-14}}$ (genotype of diploid)
(mutant) (phenotype of diploid)

As a second test of whether or not the mutations are alleles, the researcher can determine the segregation pattern of the alleles in the meiotic products of the diploid. If the two mutations are in the same gene, then recombination between the mutations will be relatively rare because they map so close to one another. Therefore, 100% of the time (or close to it) the two mutant genes will segregate to different spores producing a tetrad with four mutant spores (two mutant #5 spores and two mutant #14 spores). This situation is depicted in Cross 2 of Chapter 1).

Cross 5 shows another complementation test between mutant strain 5, which carries the mutation *gen1-5*, and another mutant strain. Mutant strain 4 contains only a single mutant gene and the mutant allele is recessive.

Cross 5: Mutant strain 5 × Mutant strain 4
Diploid phenotype: Wild-type

The result shown in Cross 5 indicates that the mutation in strain 5 and the mutation in strain 4 complement and thus are mutations in different genes. We can then say that a different gene, *GEN2*, is mutant in strain 4. This cross could be depicted as shown below.

Cross 5: *gen1-5 GEN2* (mutant) × *GEN1 gen2-4* (mutant) (genotypes of parental strains) (phenotype of parental strains)

Diploid: $\frac{\textit{gen1-5}}{\textit{GEN1}}\ \frac{\textit{gen2-4}}{\textit{GEN2}}$ (genotype of diploid)
(wild-type) (phenotype of diploid)

If *GEN1* and *GEN2* are not linked, then the mutant genes will recombine producing recombinant meiotic products with the wild-type (*GEN1 GEN2*) and double mutant (*gen1-5 gen2-4*) genotype. This is exhibited by the presence of tetratype and nonparental ditype tetrads when this diploid is subjected to tetrad analysis (see Cross 3 in Chapter 1). The frequency of each type of tetrad will depend on the frequency of recombination. If the two genes are completely unlinked, that is 50% recombination, the frequency of PD : TT : NPD tetrads will be 1 : 4 : 1. If there is any linkage, then the frequency of recombination is less than 50% and the relative number of PD tetrads will increase to greater than the expected 1/6 of the total number of tetrads analyzed. Ultimately, for crosses between two alleles, the number of PD tetrads will closely approach 100%, as is shown in Cross 1. One can calculate the map distance between two mutations (the frequency recombination multiplied by 100) using the following formula, which is correct for map distances up to 35 cM (Sherman & Wakem, 1991):

$$\text{map distance in cM} = \frac{100}{2}\left[\frac{6 \times \#\text{ NPD tetrads} + \#\text{ TT tetrads}}{\text{total \# of tetrads}}\right].$$

The combination of these two methods, complementation analysis and tetrad analysis, should clearly indicate whether one is dealing with mutations in one or more genes. Either method alone is not as powerful, and therefore researchers do both tests. For example, if there are mutations in two very tightly linked genes, the mutations will complement, but recombination will be rare and most, if not all the tetrads will be PD. Such results would strongly suggest that one is dealing with mutations in two closely linked genes.

In a complete complementation analysis, all the mutants are crossed to all of the other mutants. Often as a complementation group containing several mutant alleles is identified, one allele will be chosen as the representative of that complementation group and only this allele will be crossed to the other mutants. At the end of this process, all the mutants isolated in a particular mutant selection/screen will be placed into complementation groups, i.e. genes. The researcher will have made a good start at determining the number of genes involved in the process of interest. If only a few genes have been identified with several mutant alleles, then the researcher will have some degree of confidence that the analysis saturated the genome and that new genes are not likely to be identified by the same selection/screening method. If many genes have been identified, several with only one mutant allele each, then it is likely that new genes will be identified if the same selection/screen is repeated.

There are some special situations in which straightforward interpretation of a complementation test is misleading and the geneticist must be on the alert for such possibilities. Infrequently, mutant alleles of the same gene are able to complement, and produce a heterozygous diploid with a wild-type like phenotype. This is referred to as **intragenic complementation**. Intragenic complementation can occur if the encoded polypeptide forms a multiple subunit protein composed of like subunits, such as a homodimer, or if it encodes a single polypeptide that carries out several distinct functions. In the case of the homomultimeric protein, mutant subunits encoded by different mutant genes associate with one another in the multimeric protein and are able to accommodate each other's mutant alteration in the mixed

multimer. When this happens the mixed-mutant multimer complex has some functional activity, although it may not be completely normal.

Another mechanism of intragenic complementation is possible if the protein product of the gene has several distinct functions, such as two different enzyme activities. In this situation it is possible to obtain mutations that affect one of these activities while leaving the other function intact. In a heterozygous diploid, cells carrying two different mutations, each one affecting only one of the two functions, proteins capable of carrying out both enzyme activities will be produced, albeit in different molecules, and the cell should have the wild-type phenotype.

In contrast to intragenic complementation where mutations in the same gene complement, in a few instances mutations in different genes which are expected to complement do not. This phenomenon is referred to as **nonallelic noncomplementation**. One explanation for this noncomplementation is that the two genes encode subunits of a heteromultimeric protein, and that the presence of a mutant alteration in either subunit destroys all function of the multimeric protein. Sort of, 'one bad apple spoils the whole barrel'.

Careful and thorough genetic analysis involving both complementation tests and genetic mapping of several mutant alleles is necessary to avoid the pitfalls of these potentially misleading situations.

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6 Epistasis Analysis

OVERVIEW

The *Random House Dictionary of the English Language*—Unabridged Edition (1966) defines epistasis as a genetic term describing the 'interaction between nonallelic genes in which one combination of such genes has a dominant effect over other combinations'. The key word to remember is 'nonallelic', i.e. different genes. **Epistasis** (from the Greek meaning 'stand above') is the masking of the phenotype of a mutation in one gene by the phenotype of a mutation in another gene (Huang & Sternberg, 1995). One gene is said to be **epistatic** to another when the double mutant strain exhibits the phenotype of that mutant gene. This is in clear contrast to the terms dominant and recessive, which describe the relationship between different alleles of the same gene. It is very important not to confuse these concepts.

Epistasis analysis is used to determine if genes with related mutant phenotypes act in the same or different pathways, and, if in the same pathway, to place them in a linear order relative to one another based on the step in the pathway controlled by that gene. In other words, one uses epistasis analysis to construct an **order-of-function** map that reflects the sequence of events in a pathway controlled by several genes.

The use of epistasis analysis for the study of complex pathways was suggested more or less simultaneously by two independent research groups working in different fields. Jarvik & Botstein (1973) reported the isolation of temperature-sensitive and cold-sensitive mutations that block phage P22 morphogenesis, i.e. assembly of the phage particle. They used a combination of double mutant studies and reciprocal temperature shifts (made possible by their use on conditional ts and cs mutants) to determine the order of events in phage assembly. Their work demonstrated that head and tail assembly were independent processes but that both were dependent on phage DNA replication. Hereford & Hartwell (1974) used epistasis analysis to order events in the *Saccharomyces* cell division cycle. They used temperature-sensitive mutants that blocked the cell cycle at morphologically distinguishable points. The execution points of these genes were ordered relative to the block produced by the cell cycle inhibitor α-factor by temperature-shift experiments and relative to each other by double mutant studies.

To determine the epistatic relationship between two genes, mutations in these genes must have distinguishable phenotypes. Epistasis analysis is undertaken only after the initial steps of genetic analysis. Mutations are isolated and placed into complementation groups. Then, representative alleles are selected for a detailed characterization of phenotype so as to reveal subtle differences in phenotype not obvious from initial characterization. This could be a complete morphological analysis, such as in secretory pathway mutants or cell division cycle mutants, or intensive biochemical characterization, such as for DNA replication mutants or mutants affecting metabolic pathways. Occasionally, different alleles exhibit somewhat different

phenotypes. The researcher can now capitalize on these phenotypic differences in the epistasis analysis.

For the purposes of epistasis analysis, there are two types of pathway in living systems, **substrate-dependent pathways** and **switch regulatory pathways** (Huang & Sternberg, 1995). A **substrate-dependent** pathway consists of an obligate series of steps or reactions that are required to produce a final outcome. The outcome can be as simple as the synthesis of a nutrient such as an amino acid or a macromolecule, or can be as complex as the formation of a ribosome. The substrate-dependent pathway can be thought of as a progression of events or even as a river flowing downstream with separate tributaries joining at different points and finally flowing into the lake. Another view is as a series of **positive** reactions each dependent on a source of substrate and a functional gene product for the successful completion of each step in the pathway. Moreover, the product of the more upstream step is used as the substrate of the downstream step. If there is no substrate available or if any one of the enzymes is missing or inactive, then the pathway will be blocked at that step. A production line at a factory would be considered to be this type of pathway. It is dependent on the input of parts (substrates) and workers (gene products) to assemble these parts.

A **switch regulatory pathway** consists of a series of genes or gene products that alternate between two states, 'on' and 'off'. The components of this pathway are usually acting directly on each other as opposed to on substrates, as occurs in a substrate-dependent pathway. The activity of a switch regulatory pathway is regulated by an upstream **signal** that stimulates the pathway and produces the downstream **response**. Environmental changes, cell–cell interactions, zygote formation, and mitogenic signals are only a few of the signals that can act as initiators of a switch regulatory pathway. The downstream response can be altered gene expression, cell division, or the initiation of a developmental process such as pattern formation.

Mutations in the genes encoding components of a switch regulatory pathway can lock the component into a permanently 'on' or permanently 'off' state. This has the effect of separating the downstream response from the initiating signal. Mutations that allow the response to be produced even in the absence of a stimulatory signal or despite the presence of an inhibitory signal are referred to as **constitutive mutations**. The isolation of constitutive mutations is a strong indicator that one is dealing with a switch regulatory pathway.

In a switch regulatory pathway the action of a particular component, or **regulatory factor**, can be either **positive** or **negative**. The function of a **positive regulatory factor** is to activate the next component in the pathway (its downstream component) when it is in its active form. A recessive (loss of function) mutation in a gene encoding a positive regulatory factor blocks the pathway. A dominant (gain of function) mutation in a gene encoding a positive regulatory factor produces a protein capable of functioning constitutively even in the absence of upstream activation of the pathway. A **negative regulatory factor** in the activated state inactivates the next downstream component in the pathway. Therefore, a recessive, loss of function mutation in a gene encoding a negative regulatory factor allows the next step in the pathway to be constitutively active. A dominant, gain of function mutation in a gene encoding a negative regulatory factor produces a gene product capable of constitutively inhibiting the pathway even in the absence of the signal. By determining whether the

mutation is dominant or recessive, constitutive or blocks the response, the geneticist can decide whether the gene product is a negative or positive regulatory factor.

Determining whether the pathway under investigation is a substrate-dependent or switch regulatory pathway is not a simple task. Nonetheless, as will be seen below, it is important because the interpretation of the results of double mutant analysis depends on the type of pathway. The characterization of mutant alleles of pathway genes can provide some clues. As discussed above, if constitutive mutants are obtained in any of the pathway genes, then one can conclude that the pathway is a switch regulatory pathway. More often than not, a switch regulatory pathway controls more than one downstream response. As a result mutations in a switch regulatory pathway affect a number of phenotypic traits, such as the expression of several genes, and are said to be **pleiotropic**. The identification of pleiotropic mutants is suggestive of a switch regulatory pathway. If no constitutive mutations in pathway components have been identified, one can proceed with an epistasis analysis under the assumption that one is dealing with a substrate-dependent pathway. For complex processes this is unlikely to be the case. More likely, the initial characterization of mutations has not uncovered all the genes in the pathway or isolated a sufficiently varied array of mutant alleles. As the genetic analysis of the pathway proceeds new genes and/or new alleles will be identified and the true character of the pathway will be revealed in full detail. This will become clearer as examples from the literature are discussed.

EPISTASIS ANALYSIS OF A SUBSTRATE-DEPENDENT PATHWAY

Let us say that *GEN1* and *GEN2* are related because mutations in both genes decrease the production of Z. Mutations in *GEN1* give phenotype A, and mutations in *GEN2* give phenotype B. Only recessive loss of function alleles of *GEN1* and *GEN2* have been isolated. No constitutive alleles of *GEN1* or *GEN2* have been identified and mutations in these genes alter Z production but appear not to affect other phenotypes. We assume that this is a substrate-dependent pathway and proceed with the epistasis analysis.

The four mechanisms of genetic interaction between *GEN1* and *GEN2* are given in Table 6.1 (based on Hereford & Hartwell, 1974) and the resulting phenotype of the single or double mutation with regard to production of Z is indicated.

Models 1 and 2: Proteins Gen1p and Gen2p participate in different steps of the same pathway and protein Gen1p acts in a step that is upstream (Model 1) or downstream (Model 2) of the step catalyzed by protein Gen2p.

Model 3: Proteins Gen1p and Gen2p are components of two independent and parallel pathways for Z production.

Model 4: Proteins Gen1p and Gen2p act at the same step and in conjunction with one another.

To determine the epistatic relationship between these two genes, one constructs a strain that is mutant at both genes and observes the phenotype of the double mutant

Table 6.1 Epistasis analysis of a substrate-dependent pathway

		Phenotype of *gen1* single mutation	Phenotype of *gen2* single mutation	Phentotype of *gen1 gen2* double mutation
Model 1	*GEN1* → *GEN2* →	A	B	A
Model 2	*GEN2* → *GEN1* →	A	B	B
Model 3	*GEN1* → *GEN2* →	A	B	Unique
Model 4	*GEN1, GEN2* →	A	B	=A =B or unique

strain. If the double mutant phenotype is A, then *GEN1* is epistatic to *GEN2* and *GEN1* encodes the upstream component in the pathway. Alternately, if the double mutant phenotype is B, then *GEN2* is epistatic to *GEN1* and *GEN2* encodes the upstream component. In summary, in a substrate-dependent pathway, if the double mutant exhibits a phenotype identical to one or the other mutant genes, then that gene is epistatic and encodes the more **upstream** component in the pathway.

If Model 3 or 4 describes the relationship, the results can be more difficult to interpret. The term 'unique' used in Table 6.1 indicates that the phenotype is different from either phenotype A or B. It can be qualitatively related to phenotypes A and B but quantitatively more extreme. For example, mutations in two different *RAD* genes might partially reduce the rate of recombination but to different extents, while the double mutant completely blocks all recombination. This type of interaction is called enhancement and will be discussed in detail in Chapter 9. Another classic example of two parallel pathways affecting a single trait comes from *Drosophila*. The reddish brown eye color results from a mixture of two pigments synthesized by parallel pathways. Mutations in one of these pathways that blocks red pigment production produces brown eyes while mutations in the alternate pathway that blocks brown pigment production produces red eyes. Flies defective for the production of both pigments, that is double mutants, have white eyes. White eyes is a unique phenotype and could not have been predicted from observing the phenotypes of the single mutants. The double mutant in Model 4 might exhibit a unique phenotype or could be phenotype A or B. What will distinguish Model 4 from Models 1 and 2 is that the phenotype of the double mutant is likely to vary with the alleles. Thus, epistasis analysis has limitations. The researcher will have to proceed to other methods, such as suppressor and enhancer analysis or coimmunoprecipitation, to support a proposed model.

EPISTASIS ANALYSIS OF A SWITCH REGULATORY PATHWAY

In a switch regulatory pathway, the epistatic gene encodes the **downstream** component. Because this type of pathway involves both negative and positive

components, it is not possible to come up with a simple table describing all possible results. Instead, several examples of hypothetical switch regulatory pathways will be presented for the reader to ponder. Then some sample results will be described and the reader can practice analytical skills. In the pathways given below, an arrowhead indicates that the action of the protein or signal is positive and a vertical line indicates that the action of the protein or signal is negative. For each pathway the reader should determine the phenotype of mutants in each protein component. The classes of mutations to consider are alleles that put the component in the permanently 'on' state and those that put it in the permanently 'off' state. The possible phenotypes are constitutive or no response produced in the presence of signal.

Regulatory pathway 1
Signal ⟶ Protein A ⟶ Protein B ⟶ Protein C ⟶ Response

Regulatory pathway 2
Signal ⟶ Protein X ⟊ Protein Y ⟊ Protein Z ⟶ Response

The experiments listed below present the results of epistasis analysis of mutations in genes *GEN1*, *GEN2*, *GEN3*, and *GEN4*. Mutations in these genes affect the same process and may be in a common pathway. A number of alleles of each are available including constitutive alleles. Experiments 1–5 provide an example of an epistasis analysis of a switch regulatory pathway. Use these results as a practice exercise. Determine whether each gene encodes a positive or negative regulator and the order-of-function of the genes in the pathway. As a guide, the results of each experiment are followed by their interpretation. Regulatory pathway 3 synthesizes these conclusions into an order-of-function map of the pathway.

Experiment 1: A recessive mutation in *GEN1* does not respond to the signal. A recessive mutation in *GEN2* is constitutive. A strain carrying both mutations (*gen1 gen2*) is constitutive. (*Conclusions*: Gen1 protein is a positive regulator. Gen2 protein is a negative regulator. *GEN2* is epistatic to *GEN1* and the product of *GEN2* acts downstream of the product of *GEN1*.)

Experiment 2: A recessive mutation in *GEN2* is constitutive. A recessive mutation in *GEN4* blocks the response to the signal. A strain carrying both mutations (*gen2 gen4*) does not respond to the signal. (*Conclusions*: Gen4 protein is a positive regulator. *GEN4* is epistatic to *GEN2* and the product of *GEN4* acts downstream of the product of *GEN2*.)

Experiment 3: A dominant constitutive allele of *GEN1* is identified. A recessive mutation in *GEN3* blocks the response to the signal. A strain carrying both mutations (*GEN1-c gen3*) does not respond to the signal. (*Conclusions*: Gen1-c protein is an activated form of the protein that is locked in the 'on' state. Gen3 protein is a positive regulator. *GEN3* is epistatic to *GEN1* indicating that the product of *GEN3* acts downstream of the product of *GEN1*.)

Experiment 4: A dominant constitutive allele of *GEN4* is isolated. A recessive mutation in *GEN3* blocks the response to the signal. A strain carrying both mutations (*gen3 GEN4-c)* is unable to respond to the signal. (*Conclusion*: *GEN3* is epistatic to *GEN4* and the product of *GEN3* acts downstream of the product of *GEN4*.)

Experiment 5: A dominant constitutive allele of *GEN3* is isolated. A recessive mutation in *GEN4* blocks the response to the signal. A strain carrying both mutations (*GEN3-c gen4*) does not respond to the signal. (*Conclusion*: *GEN4* is epistatic to *GEN3*. This result taken together with the results of Experiment 4 suggests that the products of *GEN3* and *GEN4* could act at the same step.

Regulatory pathway 3
Signal ⟶ *GEN1* ⟶ *GEN2* ⊣ *GEN3*, *GEN4* ⟶ Response

EPISTASIS GROUP

Genes encoding proteins that function in the same pathway or process will exhibit epistasis, as defined in the discussion presented above. Such genes are said to be members of an **epistasis group**. A well-studied example of an epistasis group is the *RAD52* epistasis group (Paques & Haber, 1999). Mutations in genes such as *RAD50*, *RAD51*, *RAD52*, *RAD54*, *RAD55*, *RAD57*, *RAD59*, *SPO11*, and *MRE11* exhibit defects in recombination and double-strand break repair. Construction of double mutant strains demonstrates epistatic relationships among the various members of this group of genes. Mutations in *RAD3* or *RAD6*, which like *RAD52* were originally isolated because of their increased sensitivity to X-ray radiation, do not exhibit epistasis with the *RAD52* epistasis group or with each other. When paired with *RAD52* or other members of the *RAD52* epistasis group, the results are consistent with Model 3 in Table 6.1 and clearly indicate that *RAD3* and *RAD6* are in distinct pathways. Thus, despite certain similarities in phenotype, *RAD52*, *RAD3*, and *RAD6* are members of different epistasis groups.

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7 Gene Isolation and Analysis of Multiple Mutant Alleles

There are many ways to isolate a DNA fragment containing a specific gene. The methods vary depending on the organism and the genetic and recombinant DNA technology available for working with that particular organism. Moreover, the approach chosen will vary depending on several other factors. These include the phenotype of strains carrying the mutant allele, whether the mutation is dominant or recessive, and whether one has developed a selection or a screen for identifying strains with the wild-type or mutant phenotype. Additionally, cloned genes homologous to the gene of interest or sequence information might be available. Therefore, the particular circumstances will be different for different genes, different mutant alleles, and different organisms.

Broadly speaking, there are three basic approaches to cloning a gene. **Cloning by complementation** uses *in vivo* functional activity (phenotype) to isolate the dominant allele of a gene. **Positional cloning** locates a gene to a particular chromosomal region based on information on its map position. Conservation of the sequence of a protein or a particular protein domain, and the implied conservation of gene sequence, can be used to isolate a gene by a method we will refer to as **cloning by sequence homology**. All three methods require the construction of libraries. The type of library, its size, and the vectors chosen will vary with the organism under investigation and the intended cloning approach.

PREPARATION OF THE LIBRARY

A thorough review of the methods and available vectors for the construction of libraries suitable for different experimental purposes is beyond the scope of this book. Presented below are a few of the basic concepts underlying their use and construction.

In the preparation of a library it is important to be assured that the library contains representative clones covering, as closely as possible, the entire genome of the organism under study. The size of the library (that is, the number of different chimeric plasmids or virus clones in the library) depends on the average size of the insert fragments, the size of the genome, and/or the number of transcribed genes. For genomic libraries a random method is usually used to digest the total genomic DNA into fragments. Commonly in organisms with smaller genomes like *Saccharomyces*, other fungi, and most prokaryotes, a frequent-cutter restriction enzyme (such as one with a 4 bp recognition site) will be used and a partial digestion of the DNA will be done to cut just a small percentage of the available sites. The digested DNA is then size fractionated and only large (about 10–15 kbp) fragments are used as inserts. In this way a random and probably overlapping series of insert fragments

can be obtained. There is no guarantee that all regions of the genome will be equally represented, and it is possible that some will be absent, perhaps because they are toxic to the library host organism, usually *E. coli*. The researcher will try to get a library of sufficient size to represent the entire genome several times. Larger fragments are obtained by digestion with rare-cutters; that is, restriction enzymes that recognize longer and therefore rarer target sequences.

In *Saccharomyces* the researcher has the choice of a number of types of plasmid vector from which to construct a library. Plasmid libraries are the library of choice for researchers using the cloning-by-complementation method (see below). At this point the reader is advised to re-read the section on *Saccharomyces* vectors and libraries in Chapter 1. Investigators working with other eukaryotes having larger genomes require the construction of very large libraries or libraries in vectors capable of carrying larger genomic fragments. Several types of vector have been developed and Brown (1999) briefly reviews these in *Genome*. Bacteriophage λ vectors are used by those interested in constructing a large library, numbering in the millions or billions of clones, but these vectors only accommodate insert fragments of about 5–12 kbp. Cosmids and fosmids, specialized plasmids with λ *cos* sites, are able to carry 8–44 kpb DNA fragments; the fosmid form is more stable. Bacteriophage P1 vectors are similar in concept to the vectors developed for bacteriophage λ but, because the genome of P1 is larger than that of λ, these vectors carry up to 125 kpb DNA fragments. There are also a variety of artificial chromosome vectors available including P1-derived artificial chromosomes (PACs) that carry up to 300 kpb fragments, bacterial artificial chromosomes (BACs) that carry fragments of 300 kpb and greater, and yeast artificial chromosomes (YACs, described in Chapter 1) that are able to accommodate 600—1400 kpb fragments.

The vectors described above are used, for the most part, for genomic libraries. Other vectors, usually plasmid based, are used in situations where the focus is not the chromosomal organization of the gene but its function and in this case investigators often use cDNA libraries. This is particularly true when one is working with an organism whose genes are frequently interrupted by introns or if one is only interested in genes expressed under particular conditions or in specific tissues. If one is interested in using a cDNA library for the cloning-by-complementation approach, the cDNA fragments must be cloned into an expression vector that contains a promoter suitable for expression in the host organism in which the screening/selection will be carried out.

CLONING BY COMPLEMENTATION

The most commonly used method for gene isolation is referred to as **cloning by complementation**. In this method one clones a dominant allele of the gene of interest using a host strain carrying a recessive allele and selects/screens transformants for the phenotype of the dominant allele. Using a vector capable of stable transformation of the host strain, a library is constructed from total genomic DNA (or cDNA) isolated from a strain carrying the dominant allele. The library is transformed into a host strain carrying the recessive allele of the gene of interest, and transformants are selected. Sufficient numbers of transformants must be obtained to represent the

entire library several times. These transformants are then screened or selected for individuals expressing the phenotype of the dominant allele of the gene of interest. The plasmid carried by this transformant is then recovered from the transformant and the insert fragment characterized. If the library is a good one and a large number of transformants are screened/selected, then one can expect to isolate several overlapping fragments carrying the gene of interest.

Cloning by complementation does have certain pitfalls. In the earlier days of *Saccharomyces* cloning libraries were commonly made with the multicopy plasmid vectors (YEp and YRp). This meant that a transformant could contain as many as 50 copies of a plasmid making 50 times as much of the encoded gene products. Occasionally, abundant amounts of the product of one gene can compensate for the loss of another different gene product. This phenomenon is called multicopy suppression and will be discussed in detail in Chapter 8. As a result, the researcher using a multicopy library to clone by complementation might isolate more than one genomic region capable of complementing the mutant phenotype of the host and faced with determining which of these complementing fragments contained the gene of interest. This became less problematic when YCp vectors became available but in rare cases even one or two additional copies of a gene are sufficient for multicopy suppression.

A number of methods are used in *Saccharomyces* to demonstrate that a cloned fragment contains the desired gene and is derived from the same genomic site. The most common method is targeted integration (see Chapter 1). For this, a YIp or YRp plasmid carrying the cloned fragment is digested with a restriction enzyme at a site unique to the insert. This linearized DNA is transformed into a mutant host strain where it will integrate into the chromosome at the site of the genomic copy of the insert. One can then demonstrate that the site of integration is the same as the mutant gene by a simple genetic cross as follows. The strain carrying the integrated plasmid is mated to a strain carrying a mutant allele of the gene of interest. Tetrad analysis of this diploid should exhibit single gene segregation of the mutant allele versus the nutritional marker gene (from the plasmid) if, and only if, the plasmid integrated into the site of the gene of interest.

In organisms where methods such as those discussed above are not available, the researcher must clone the same gene from both mutant and wild-type strains and compare the sequences of these alleles to demonstrate the presence of a sequence alteration in the mutant allele. Typically several different mutant alleles will be sequenced. Whatever the situation, when one uses cloning by complementation it is essential to use methods in addition to complementation to demonstrate that one has indeed cloned the true gene of interest and not a suppressor.

POSITIONAL CLONING

Occasionally cloning by complementation is not a workable approach, even in *Saccharomyces* and other genetically tractable organisms. The desired sequence may contain a region or encode a product that is toxic to the library host organism, usually *E. coli*. Or the gene may be located in a chromosomal region that is under-represented or absent from the library. In some organisms, vectors that are useful

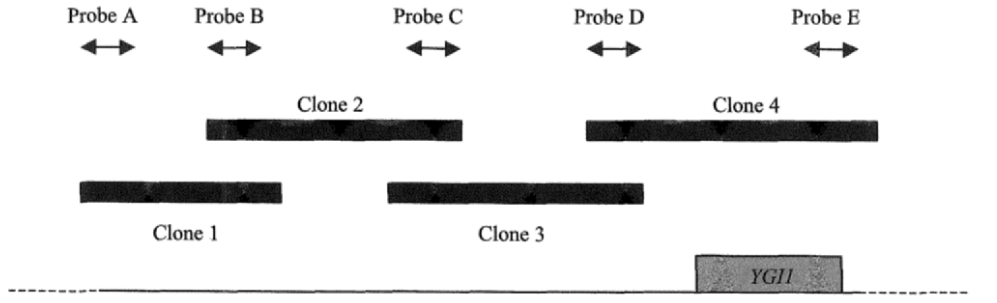

Figure 7.1 Chromosome walking and positional cloning

for cloning by complementation may not be available. When faced with these situations, investigators will attempt to clone the gene of interest by positional cloning methods.

Positional cloning uses the physical proximity of a cloned DNA fragment (or gene) to isolate the gene of interest. The cloned fragment is the starting point from which one moves, in a step-wise fashion, toward the gene of interest by a process called **chromosome walking**. The distance between the cloned fragment (or probe) and the gene of interest is measured genetically by recombination frequency. Because the probe is usually not associated with a phenotype, its position on the chromosome is marked by a tightly linked physical marker such as an **RFLP (restriction fragment length polymorphism)** or an **STD (short tandem repeat)** which can be detected by Southern analysis or PCR-based methods. The recombination frequency between the physical marker and the gene of interest must be determined for each probe. When the recombination frequency (genetic distance) decreases one is 'walking' in the correct direction, i.e. in the direction of the gene of interest.

Chromosome walking is illustrated in Figure 7.1. In many genetic model organisms, a series of overlapping clones, called contigs, are available covering large chromosomal regions or even entire genomes. Additionally, physical markers (RFLPs or STDs) have been associated with these clones and probes. If these are not available for the organism in which one is working, these tools will have to be developed, at least for the chromosomal region of interest. One starts with a cloned sequence, probe B which has been shown by genetic mapping is linked to the mutant allele of the gene of interest *YGI1*. Next probes A and C, located to either side of probe B, are tested and their map distance to the mutant allele of *YGI1* determined. As shown in Figure 7.1, probe C should be found to map closer to *ygi1*. Therefore, the next probe to be tested should be probe D. Eventually, when no recombination is observed between the probe and the mutant gene, as would be the case for probe E, *ygi1* is predicted to map very close to or to contain probe E. It would then be necessary to compare the sequences of each of the genes in the region of clone 4 in several *ygi1* mutant strains to the wild-type sequence to determine which of the genes is located in the region of probe E. If different sequence alterations of the same gene are found in several mutant alleles, then one can be reasonably sure that *YGI1* has been identified.

CLONING BY SEQUENCE HOMOLOGY

In its simplest form, **cloning by sequence homology** uses the evolutionary conservation of the sequence to isolate homologous genes from different species or homologous members of a repeated gene family from the genome of a particular species. Genes encoding highly conserved proteins like actin or ubiquitin are excellent examples of the use of cloning by sequence homology. The amino acid sequence of actin varies only slightly among different species and thus the actin gene from one species is expected to be highly sequence homologous to the actin gene from another species. Thus, one should be able to use the cloned actin gene from one species to identify a library clone containing the actin gene from a second species. The cloned actin gene is used as a probe for DNA–DNA hybridization to a library made from the second species. Hybridization is usually done under conditions of reduced stringency in order to accommodate some degree of sequence variation.

Often gene products may serve the same functional role in different species, but sequence homology may be limited to short highly conserved domains or functional motifs. In such cases, PCR-based methods can be used to identify functional homologues. Conserved domains or motifs are identified by sequence comparison and alignment of several functionally homologous proteins from different species or several members of a family of proteins from the same or different species. For example, a comparison of several cyclin-dependent kinases from many species has revealed sequence motifs that distinguish this class of kinases. When two conserved motifs are identified within the same protein, PCR-based methods can be used to amplify the region contained between them. Based on the amino acid sequence of the motifs, pairs of degenerate oligonucleotide primers are synthesized that would be expected to hybridize to the homologous regions encoding the motifs in a new member of this class of proteins. PCR with this primer pair, done under reduced stringency annealing conditions, should amplify the region between these paired primers. Genomic DNA, a cDNA library, or even total mRNA can be used at the PCR target. The amplified DNA product is then used as a probe to an appropriate library to identify clones containing the full-length gene.

ANALYSIS OF MULTIPLE MUTANT ALLELES

Molecular genetic analysis of a gene includes studies such as the sequencing of the gene, sequence comparison to other genes in the databases to see if clues to the function of the gene product can be obtained, analysis of transcript expression patterns, and subcellular location of the gene product. If available, several mutant alleles should be cloned and analyzed.

Mutation analysis, the detailed characterization of a number of mutant alleles, is an essential first step in determining the specific function(s) of a gene or gene product and the molecular mechanism(s) by which it carries out those functions. Often a gene product will be found to contain a certain consensus sequence suggesting a particular function. One must demonstrate that mutations in that consensus sequence expected to eliminate functional activity also produce a mutant

phenotype. If there is no effect on phenotype, then that sequence may be functionally irrelevant, at least in relation to the phenotype one is studying. In other words, having webbed feet does not make you a duck and even if you have webbed feet you do not necessarily use them for swimming.

Moreover, different alleles of a gene may have subtly different phenotypes suggesting that the encoded product may have more than one cellular function. Multiple functions are often associated with different structural regions of the protein. The analysis of several alleles with different phenotypes can be useful for assigning specific functions to particular regions of a protein. This type of analysis is referred to as a **structure–function analysis** and can identify functional domains of a protein. These domains could represent regions responsible for interaction with other proteins (such as in a heterodimer), or with enzymatic substrates or cofactors (such as ATP or heme), or DNA-binding domains (such as found in repressor proteins like LacI protein), etc. A single protein with multiple cellular functions is expected to have a complex structure.

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8 Suppression Analysis

OVERVIEW

Suppression analysis is an elegant and highly favored molecular genetic tool used to identify genes that are functionally related to the gene of interest. It dates back to the very earliest days of genetics and the work of Sturtevant (1920) and Beadle & Ephrussi (1936) but it was not until the 1960s that the variety of suppression mechanisms and the capabilities of suppressor analysis were fully appreciated. Increased use of suppressor analysis, particularly in model genetic organisms like *Saccharomyces*, has refined the method making it one of the two accepted genetic methods for identification of functionally related genes or gene products. The second method, enhancer analysis, will be described in Chapter 9. Functionally related genes encode products capable of carrying out the same or similar functions, are different components of the same metabolic or regulatory pathway, or function together in a structural or enzyme complex. Mutations in these genes might not have been revealed by the original mutant hunt for a variety of reasons. The mutant alleles might have a different and unpredicted phenotype or might not produce a detectable phenotype unless in conjunction with another mutation. Thus, a search for suppressor mutations has the potential for revealing an entirely new spectrum of genes.

A **suppressor mutation** is a mutation that counteracts the effects of the original mutation such that the double mutant individual containing both the original mutation and the suppressor mutation has a phenotype similar to that of the wild-type. Suppressors are isolated when a mutant strain is 'reverted' to restore the wild-type or wild-type-like phenotype. As with the isolation of the original mutation, one must devise a selection/screen that allows the identification of revertants having the 'wild-type' phenotype. One determines whether a revertant is a **true revertant**, that is restores the original gene sequence, or if it carries a suppressor mutation by crossing the revertant to a wild-type strain and seeing whether the original mutation can be recovered in the progeny. Recovery of the original mutation can only occur if there has been a recombination event separating the original mutation from the suppressor mutation. The suppressor mutation alone will frequently have a mutant phenotype, but this phenotype could be novel or could be similar to the phenotype of the original mutation.

INTRAGENIC SUPPRESSORS

If the suppressor mutation and the original mutation are in the same gene, this is referred to as **intragenic suppression**. Recombination between the original mutation and the intragenic suppressor is expected to be rare because the two alterations are very tightly linked. Intragenic suppression can occur by a variety of mechanisms. A classic example is found in Crick *et al.* (1961). The original mutation used in this

analysis was a +1 frameshift mapping to the 5' end of the *rIIB* ORF of the T2 phage of *E. coli*. Intragenic suppressors of this mutation were produced by a nearby −1 frameshift thereby restoring the correct reading frame. One can also get intragenic suppression of a missense mutation. A missense mutation might functionally inactivate a protein by altering its shape or by increasing its rate of degradation to such an extent that inadequate amounts of the protein are produced. An intragenic suppressor could restore function if it enables the doubly mutant protein to fold into a more active shape or reduces the rate of degradation sufficiently to provide adequate levels of the protein. Changes in the promoter could increase the level of expression of a partially active mutant protein to a level adequate to restore a wild-type-like phenotype.

INTERGENIC SUPPRESSION

If the suppressor mutation and the original mutation are in different genes, this is referred to as **intergenic suppression**. Recombination between the original mutation and the suppressor mutation is likely to occur at a high rate since with rare exceptions the suppressor mutation will be unlinked to the original mutation. Intergenic suppressors fall into two broad categories: **information suppressors** and what we will call **function suppressors**.

Information suppression

Information suppressors are mutations in genes involved in the transmission of information from DNA to protein. As such, they act by improving the expression of the mutant gene. Moreover, an information suppressor will suppress any other gene, even those that are functionally unrelated to the original mutant, so long as the mutation in that gene has the same effect on the information transfer process as the mutation in the original gene. The reader is already familiar with nonsense, missense and frameshift suppressors that are mutations in tRNA genes. These were among the first types of information suppressor identified. Mutations affecting ribosome components also can be information suppressors. This type of information suppression acts at the level of the translation process and alters the reading of the encoded information. Information suppression can also act by increasing the amount of the transcript, such as by altering components of the transcription machinery or complexes involved in post-transcriptional processing. In summary, information suppressors suppress particular types of mutational alteration affecting the information transfer process and can do this in any gene that has that type of alteration. In genetic terms, information suppressors are allele-specific but not gene-specific.

Researchers interested in the processes of information transfer can use suppressor analysis to obtain mutations in genes involved in these processes but it is important to choose the original mutant strain carefully. For example, if one is interested in translation start-site selection one should work with a mutation in the leader sequence of a gene that exhibits reduced protein production but not reduced transcript levels. Intergenic suppressors that increase translation should be obtained, for example, in translation initiation factors or the small ribosomal subunit. Starting

with a promoter mutation or an alteration in the coding region of the gene would not give the desired types of suppressor. As part of the initial characterization of the suppressors, one should test the ability of the suppressor to suppress a comparable mutation in the leader sequence of another unrelated gene. This should sort the suppressor mutations into the desired class of information suppressor from uninteresting mutations affecting some other aspect of the expression of the original mutant gene.

Function suppression

Function suppressors act to restore or replace the altered gene function. This is accomplished by several means. The activity of a gene product can be appropriately modulated by mutations that affect its post-translational modification, subcellular localization, degradation, or interaction with activators, inhibitors, or other regulatory factors. Mutational changes can substitute another gene product for the mutant gene product either by changing the specificity or abundance of that alternate protein. Or, if the mutant gene encodes a component of a switch regulatory pathway, suppression can occur by the constitutive activation of a downstream component.

The isolation of function suppressors provides a means of exploring the role of a gene product in a cellular process and identifies other functionally related gene products or other components of a pathway. Again, if one is interested in isolating function suppressors of a mutant gene, it is important to start the analysis with an appropriate mutation in that gene. The original mutation should affect the gene function, not expression, and thus should be confined to the coding region. Depending on the type of function suppressor desired, one could start with a null mutation, such as a deletion or an insertion, or with a missense mutation. Temperature-sensitive and cold-sensitive mutant alleles are frequently used for function suppression analysis because these types of mutation are almost always missense mutations.

There are three mechanisms of function suppression: by-pass suppression, allele-specific suppression, and suppression by epistasis.

BY-PASS SUPPRESSION

A by-pass suppressor by-passes the need for the mutated gene product by providing the function of that gene product by alternate means. Two mechanisms of by-pass suppression of mutations in hypothetical *GEN1* are shown in Figure 8.1. In Model 1, *GEN1* and *GEN2* carry out related but distinct processes that do not functionally overlap in the wild-type strain. The mutation of *GEN1* blocks the reaction thereby creating a mutant. An alteration in *GEN2* allows the *GEN2* gene product to acquire a new function that enables it to substitute for the *GEN1* product thereby by-passing the *GEN1* mutation. The short arrow indicates that the by-pass may be less effective than the wild-type function. In Model 2, expression of another gene is increased so that its product can substitute for the *GEN1* product. Gen3 protein is capable of carrying out the same or a similar function as the Gen1 protein but not at rates adequate for a wild-type phenotype. Perhaps the specific activity of Gen3p

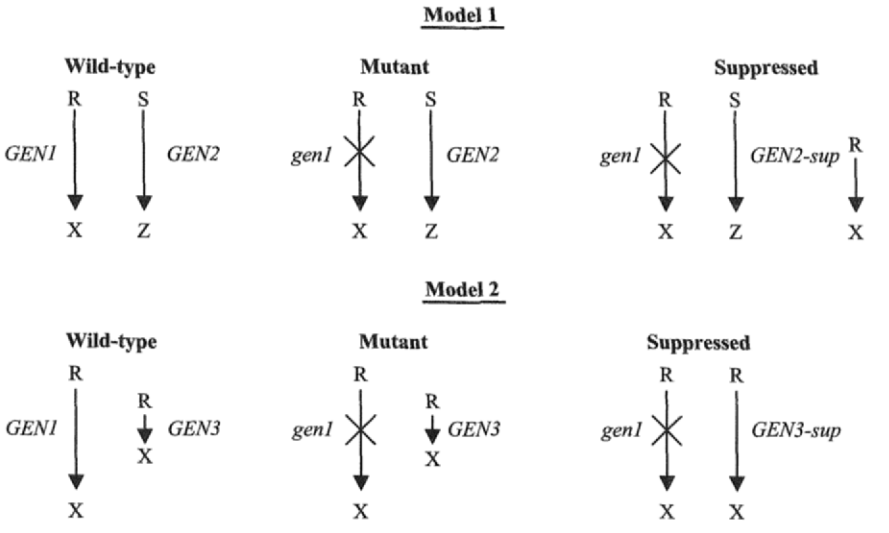


Figure 8.1 Models of by-pass suppression

for the Gen1p substrate R is very low. An increase in the expression levels of *GEN3* by the suppressor mutation provides levels of Gen3 protein sufficient to allow it to substitute for the loss of *GEN1*.

It is important to note that by-pass suppression is not dependent on the original gene product and thus by-pass suppressors are able to suppress **either** missense mutations or null mutations of the original gene. In addition, the suppressor allele is dominant in both Model 1 and Model 2.

ALLELE-SPECIFIC SUPPRESSION

In **allele-specific suppression**, a suppressor mutation in *GEN2* is able to suppress only a particular **non-null** allele or select a group of **non-null** alleles in *GEN1*. Experience has demonstrated that allele specificity of function suppressors implies that the products of the two genes **interact directly**, i.e. they make **physical contact** with each other. Both by-pass suppression and suppression by epistasis (see below) can occur with null alleles, i.e. alleles that produce no protein product. Therefore, if a suppressor is not capable of suppressing a null mutant allele but is only capable of suppressing missense mutations of a gene, then the mechanism of suppression is allele specific. Allele-specific function suppression is considered strong evidence that the gene products of the original mutant gene and the suppressor gene physically interact. This interaction could indicate an enzyme–substrate type of interaction (as between a protein kinase and its target protein), an interaction between components of a heterodimer or heteromultimeric complex, or an interaction between the catalytic subunit of an enzyme complex and its regulatory subunit(s).

To conceptualize allele-specific suppression, consider the fact that two proteins interact with each other only over a small portion of their surfaces, and that within

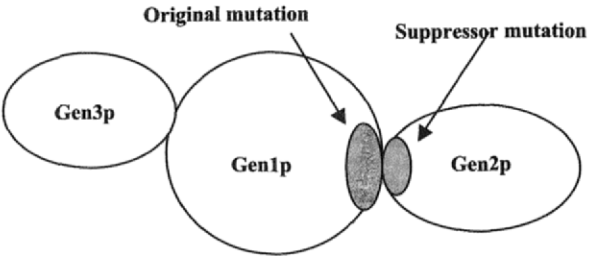


Figure 8.2 Model of allele-specific suppression

these defined regions of interaction specific residues on the surfaces of the proteins make the most significant contributions to the binding strength of the interaction. This is illustrated in Figure 8.2. Mutations that alter these residues affect the binding strength of the interaction either by weakening or strengthening it. If the original mutation in *GEN1* decreases the binding of the Gen1 protein to the Gen2 protein, a suppressor mutation in *GEN2* might act to restore strong binding of Gen1p to Gen2p. Suppression will occur if, and only if, the suppressor mutation in *GEN2* alters a residue mapping within the surface region of Gen2p that physically makes contact with Gen1p.

In the context of this explanation, the *gen2* suppressor mutation can only be expected to suppress *gen1* mutations that alter residues in the region of Gen1p involved in the Gen1p–Gen2p interaction; that is, they would be specific to alleles altering residues in this region. Suppressor mutations in *GEN2* would not be expected to suppress null mutations of *GEN1* (large deletions, 5' nonsense or frameshift mutations, insertions, or other rearrangements). Allele-specific suppressors also would not be expected to suppress mutations elsewhere in Gen1p outside of the surface region of Gen1p that interacts with Gen2p, for example in the Gen3p binding site. The suppressor mutation of *gen2* alone, that is when not in combination with the original *gen1* mutation, is likely to have a mutant phenotype similar to the *gen1* mutant phenotype because both affect the Gen1p–Gen2p interaction.

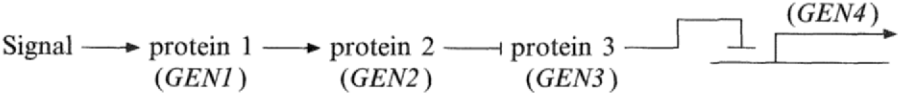
Allele-specific suppression is not limited to protein–protein interactions. It can be used to characterize DNA–protein or RNA–protein interactions. When two proteins (or a DNA sequence and a protein) are known to interact directly, allele-specific suppression can be used to explore the details of the binding, i.e. which specific residues and/or basepairs are involved. Alternately, one can identify novel proteins that interact with the product of a known gene by identifying allele-specific suppressors of a mutant allele of that gene.

SUPPRESSION BY EPISTASIS

Suppression by epistasis occurs between genes whose products are components of a switch regulatory pathway as described in Chapter 6 on epistasis. The component proteins of switch regulatory pathway alternate between the 'on' state and the 'off'

state. Mutations in these proteins can permanently shift them to either state. Such mutations act to separate the switch regulatory pathway from the upstream signal. The pathway can be constitutive (that is, unregulated) by activating the pathway in the absence of the stimulatory signal or despite the presence of inhibitory signal. Alternately, mutations can block the pathway despite the presence of the stimulatory signal or in the absence of an inhibitory signal. The ultimate effect of a mutation in a component protein depends on whether the component is a positive regulator or a negative regulator.

For example, in the switch regulatory pathway shown below, the signal activates protein 1, protein 1 activates protein 2, activated protein 2 inhibits protein 3, and protein 3 is an inhibitor of *GEN4* expression.



Recessive mutations in *GEN1* or *GEN2* lead to a lack of *GEN4* expression. Recessive mutations in *GEN3* lead to the constitutive expression of *GEN4*. A strain doubly mutant for recessive mutations in *GEN1* and *GEN3* or *GEN2* and *GEN3* would be constitutive for *GEN4* expression. Thus, a loss of function mutation in *GEN3* will suppress loss of function mutations in *GEN1* and *GEN2*, and restore *GEN4* expression to *gen1* and *gen2* mutant strains. This suppression of *gen1* and *gen2* mutations by a mutation in *gen3* is suppression by epistasis. Of course, in this case the suppression does not restore normal regulation but it does restore expression of *GEN4*, albeit constitutively.

Suppression by epistasis can occur with null or non-null alleles of either the original mutant gene or the suppressor gene. For example, in the above pathway, a deletion of *GEN3* will suppress either a missense mutation or a deletion mutation of *GEN1*. A dominant constitutive mutation in *GEN1* would activate this pathway in the absence of a signal. This constitutive activation would be suppressed by recessive mutations in *GEN2*.

OVEREXPRESSION SUPPRESSION

Because of the variety of plasmid vectors available for *Saccharomyces* it is possible to modulate the expression levels of a desired gene in a number of ways and not simply by *in situ* alterations in the promoter region of genes. This type of suppression is called multicopy suppression or suppression by overexpression or overproduction. Multicopy plasmids are frequently used to obtain overproduction of a particular protein. A gene carried by a YEp vector will be present at up to 50 copies/cell and thus one might expect a 50-fold overproduction of the protein. Vectors are available that allow one to fuse the coding region of a gene to any one of several different *Saccharomyces* promoters that have different expression levels and some of these are easily regulated by environmental signals such as nutrient availability. These are both high and low copy vectors and, as a result, a wide range of expression levels of the inserted gene can be achieved (see Chapter 1; Mumberg *et al.*, 1995; Labbé & Thiele, 1999). Libraries can be made with these vectors and

screened for suppression of mutant strains. The major strength of this type of suppressor hunt is that one can very simply identify the suppressing gene by recovering the suppression plasmid and sequencing its insert fragment. All three types of suppression can be obtained by multicopy or overexpression suppression.

BY-PASS SUPPRESSION BY OVEREXPRESSION

Overproduction of a gene product can lead to by-pass suppression. This is similar to Model 2 of by-pass suppression (above) except that the overproduction is from the plasmid-borne copy of the suppressing gene.

ALLELE-SPECIFIC SUPPRESSION BY OVEREXPRESSION

One might compensate for the reduced binding constant between mutant Protein 1 and wild-type Protein 2 by overproduction of wild-type Protein 2. Abundant amounts of Protein 2 could kinetically drive complex formation despite the lower binding constant by a mass action effect. This is a form of allele-specific suppression because it could not occur with null alleles of the gene encoding Protein 1. Mutant Protein 1 is necessary for the suppression. As is discussed above, overexpression allele-specific suppression of *gen1* mutant alleles by *GEN2* implies that the product of *GEN2* binds to the product of *GEN1* at the site of the mutant residue. Similar overproduction of Protein 3 should not suppress the same *gen1* mutations as overproduction of Protein 2.

OVEREXPRESSION SUPPRESSION BY EPISTASIS

In a switch regulatory pathway, overproduction of an activator or repressor could, respectively, activate or block a pathway regardless of the upstream signal. For example, overproduction of an activator (positive regulator) might activate a blocked pathway by overcoming an inhibitory regulator (perhaps by binding to it and titrating out its effects). Or, if the activator has a very low constitutive activity, its overproduction might be sufficient to restore progress through the pathway. Comparable situations can be proposed for repressors (negative regulator). The suppressing gene product necessarily would be downstream of the original mutant gene product. Moreover, the effect of overexpression of a particular component will depend on the role of the component in the pathway.

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9 Enhancement and Synthetic Phenotypes

OVERVIEW

Enhancement is the opposite of suppression. In suppression the two mutations act together to produce a phenotype that is similar to the wild-type. In enhancement the two mutations act together to produce a mutant phenotype that is more severe than that exhibited by either mutation alone. Examples of enhancement are often discovered by chance. An investigator carries out a selection procedure for a particular mutant phenotype, but, when the mutant strains obtained are analyzed, it is discovered that the mutant phenotype is dependent on alterations in two genes. For example, a researcher carries out a selection for a particular mutant phenotype in a *S. cerevisiae*. A mutant is isolated and crossed to an otherwise isogenic strain of the opposite mating type. The diploid is sporulated, and the four haploid meiotic products (the tetrad) is analyzed for the segregation pattern of the mutant phenotype. If all of the tetrads exhibit 2 wild-type : 2 mutant segregation, the mutant phenotype is the result of a single altered gene. If 4 wild-type : 0 mutant, 3 wild-type : 1 mutant, or some other such pattern is seen, one should consider the possibility that the mutant phenotype results from the interaction of mutations in two different genes and either mutation alone is insufficient to produce a mutant phenotype.

Investigators with a goal of identifying functionally interacting genes often will carry out searches for enhancers as well as for suppressors. As discussed for suppressor hunts, it also is important to use the appropriate type of starting mutation in a search for enhancer genes. If one is interested in gene function, one should start the enhancer search using a mutation in the coding region of a gene that affects function. Often mutant alleles with a modest effect on a phenotype are used and the enhancer search is for second site mutations that increase the severity of the mutant phenotype.

Enhancers can be intragenic or intergenic. Researchers interested in gene function will want to focus on intergenic enhancers. Mutant strains isolated from an enhancer search must be crossed to an otherwise isogenic wild-type strain of the opposite mating type and tetrad analysis undertaken. If the original mutant phenotype is recovered from the haploid progeny, then the enhancer mutation is in another gene. The enhancer mutation alone may or may not exhibit a phenotype and, if it does, the phenotype may be similar to the original mutation or it may be novel. This can all be determined from the results of the tetrad analysis. For example, if the cross produces tetratype tetrads with 1 wild-type : 2 mutant : 1 enhanced mutant spores, then one can conclude that the phenotype of the enhancer mutation has a phenotype and it is similar to that of the original mutation.

MECHANISMS OF ENHANCEMENT

Enhancement interactions can be complex, but three possible models for such interactions are described below. First, it is possible that the two genes encode

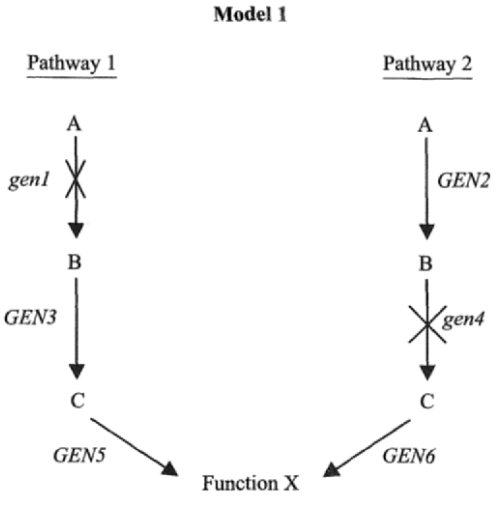

Figure 9.1 Enhancement by inactivation of a parallel pathway

components of parallel pathways with a common or overlapping function. The loss of one pathway can be tolerated, but not the loss of both. Therefore, a mutation blocking one pathway alone may have a slight mutant phenotype but two mutations each of which blocks one of the pathways will have a severe mutant phenotype. This is illustrated in Model 1 in Figure 9.1. Mutation of *GEN1* or *GEN4* may decrease the rate of function X only slightly but the double mutant is severely defective in the level of function X.

In the second model (shown in Figure 9.2), proteins Gen1p and Gen2p interact to form a heterodimer with a particular function. Non-null mutations in either *GEN1* or *GEN2* that alter residues involved in the interaction between Gen1p and Gen2p might slightly destabilize the interaction but have little or no effect on the function of the complex thus producing no phenotype or a modest phenotype. A combination of both of these mutations, that is a *gen1 gen2* double mutant strain, is likely to more fully destabilize the interaction and be detrimental to the functional activity of the complex thereby producing a mutant phenotype.

The third model involves a pathway of reactions such as the one illustrated in Figure 9.3. If the capacity of the pathway is greater than required for the wild-type phenotype, then the rate of reactions 1 or 2 can be reduced slightly without the overall rate of the pathway falling below a critical threshold rate. If the rates of both reactions are decreased, then the effect is multiplied and the impact on the activity of the pathway is likely to be reduced sufficiently to produce a mutant phenotype.

SYNTHETIC ENHANCEMENT

One mechanism for exploring the functional relationship between two known mutant genes with similar phenotypes is to test for enhancement. The mutants are crossed and double mutants isolated. If the double mutant exhibits a more severe

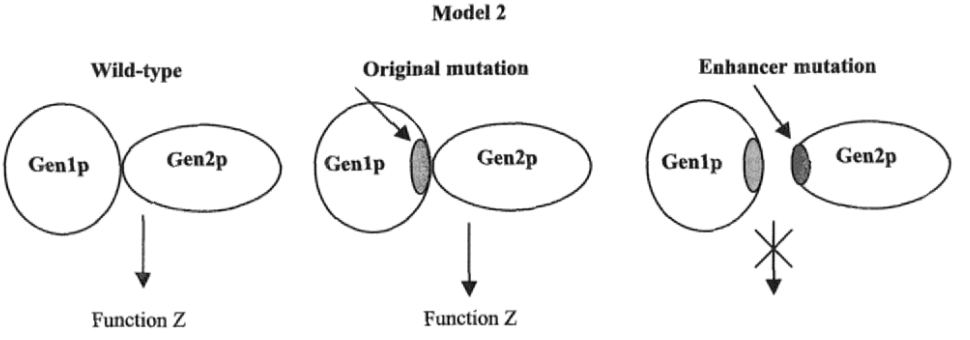

Figure 9.2 Allele-specific enhancement

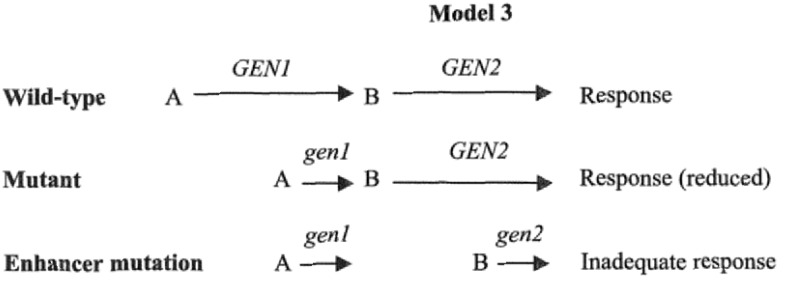

Figure 9.3 Enhancement within a common pathway

phenotype than either mutation alone, the mutations exhibit enhancement. This is referred to as **synthetic enhancement** because the researcher constructed the double mutant strain. One can then begin to explore the mechanism of enhancement, for example by testing null alleles of the genes for enhancement.

CONDITIONAL LETHAL MUTATIONS FOR THE ISOLATION OF ENHANCER MUTATIONS

Strains carrying conditional mutations, such as cold sensitive or temperature-sensitive mutations, are usually considered to exhibit the wild-type phenotype at the permissive temperature but, in reality, the function of these proteins is often somewhat compromised even at the permissive temperature. For example, if two proteins interact as in Model 2 above, a conditional mutation in either protein in the sites of interaction most likely will weaken the interaction even at the permissive temperature. The effect of either mutant gene alone may not be sufficient to disrupt function at the permissive temperature. However, if both proteins carry alterations at their sites of interaction, it is possible that the combination of the two mutant alleles will decrease the binding constant of the proteins even at the permissive temperature to a degree that is sufficient to disrupt interaction. In such a situation,

strains carrying both conditional mutations will have the mutant phenotype. If the proteins carry out an essential function, then the combination of these two mutations will be lethal even at the permissive temperature. This is referred to as **synthetic lethality**. Allele-specific synthetic lethality is strong evidence of physical interaction between two proteins.

GENETIC INTERACTION

When genetic analysis of two or more genes with similar mutant phenotypes identifies mutations in these genes that suppress one another or exhibit synthetic enhancement with one another, the genes are said to **interact genetically**. The implication of this statement is that the encoded proteins function in the same, overlapping, or related pathways or that the proteins interact physically to form a multimeric complex. A thorough examination of the evidence from studies of genetic interaction like suppression analysis, enhancement studies, and other so-called **synthetic phenotypes** will provide information on the role of these proteins in the process under investigation.

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10 Two-Hybrid Analysis

The two-hybrid method was conceived and developed by Stan Fields and coworkers as a technique to detect binding (physical interaction) between two proteins under *in vivo* conditions (Fields & Song, 1989). The concept underlying the two-hybrid method is based on the modular structure of some transcription activators and on the use of reporter genes. The reader should be sure to carefully review these subjects before attempting to understand the two-hybrid method.

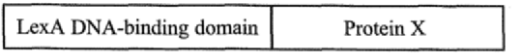
TWO-HYBRID ANALYSIS

Stan Fields conceived of the idea for two-hybrid analysis based on studies of transcription activators that indicated that the DNA-binding region and the region required for transcription activation consist of distinct domains of the protein that one could mix and match. He surmised that these domains may not even have to be part of the same protein. Instead, maybe they only have to interact with one another. If this interaction is strong enough to be capable of tethering the activation domain to the DNA-binding domain, then this might be enough for the complex to function as a transcription activator. So he tried it out with two proteins that he knew bound to one another. Let us call them proteins X and Y.

Two fusion genes are constructed using the genes encoding proteins X (gene X) and protein Y (gene Y) as follows. Gene X is fused, in-frame to a short sequence encoding the DNA-binding domain of either lexA repressor (residues 1–87) or Gal4 activator (residues 1–147) creating a hybrid gene encoding a LexABD–protein X fusion protein (see Figure 10.1) or Gal4BD–protein X. Gene Y is fused, in-frame to *GAL4* codons 768–881 (containing the Gal4p transactivation domain) creating a hybrid gene encoding a Gal4AD–protein Y fusion protein. Both fusion gene constructions are expressed from high-level constitutive promoters so that the proteins are abundantly made under commonly used growth conditions. The fusion containing the DNA-binding domain is often referred to as the 'bait' and the fusion containing the activation domain is referred to as the 'prey'. Both fusion gene constructions are carried on plasmid vectors with different nutritional selection genes. This allows both constructions to be maintained together in the same yeast host strain. Two-hybrid kits are commercially available that contain bait and prey plasmids with a multiple cloning site positioned to facilitate construction of the in-frame fusions.

To assay protein X–protein Y interaction, both hybrid fusion genes are expressed in the same yeast host cell that also contains a reporter gene appropriate to the bait construction. That is, the promoter of the reporter gene contains the DNA sequence to which the bait fusion protein binds. If the bait fusion uses the LexABD, the reporter must contain the short LexA operator sequence, which is often in several tandem copies to ensure strong binding of the bait to the reporter. Similarly, if the bait fusion uses the Gal4BD, the reporter must contain the Gal4p DNA-binding site.

LexA DNA-binding domain–protein X fusion (bait fusion)



Gal4p transactivation domain–protein Y fusion (prey fusion)

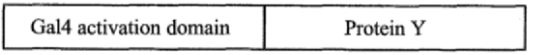


Figure 10.1 Construction of bait and prey fusions

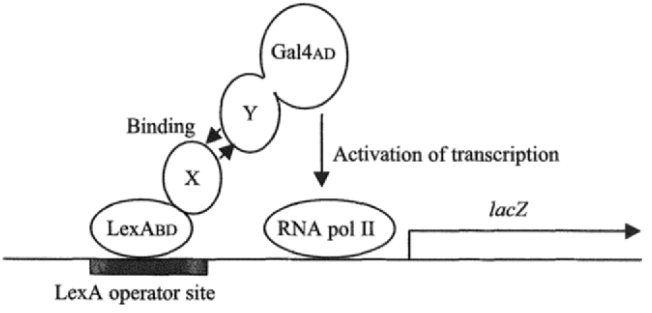


Figure 10.2 The two-hybrid interaction

As shown in Figure 10.2, the bait fusion protein will bind to its binding site in the promoter of the reporter gene. If proteins X and Y bind to one another, then the interaction will bring the activation domain of the Gal4p–protein Y fusion protein, the prey, into proximity to the transcription start site of the reporter gene and activate the transcription of the reporter gene. Assaying the level of expression of the reporter gene product monitors the rate of transcription of the reporter gene and provides a semi-quantitative evaluation of the strength of the binding between proteins X and Y.

The two-hybrid method can be used to demonstrate whether two proteins interact and under what conditions they interact. One can also test whether mutations in either protein affect the interaction and thereby identify the specific residues involved in the interaction. By using only short sections of the proteins in the hybrid constructions, one can also map the portion of each protein involved in the interaction.

The two-hybrid method can also be used to screen a library for novel proteins that interact with the bait fusion protein. (Of course, the bait fusion protein cannot itself have activation activity.) To make the prey library, random genomic DNA fragments (or cDNA fragments) are fused to the Gal4 activation domain using the prey vector. The library is then transformed into a yeast host cell containing the reporter gene and the bait fusion gene. Transformants are selected and then all are screened for expression of the reporter gene. If the reporter gene is *lacZ*, one can screen for blue colonies on X-gal plates.

Initial characterization of the positive transformants must be carried out to eliminate 'false positives'. Much of the literature on two-hybrid analysis discusses false positives and other pitfalls of the method. An example of a false positive is a prey fusion protein that binds, either directly or indirectly, to the promoter region of the reporter gene. Before undertaking a two-hybrid screen it would be very valuable to thoroughly read this literature. After the researcher is convinced that the interaction detected by the two-hybrid method is biologically relevant, the prey plasmid is recovered and the insert fragment sequenced and characterized.

The interaction detected by a positive two-hybrid result does not necessarily indicate that the two proteins being studied truly interact directly. It is possible that they both interact with the same protein as part of a multiprotein complex and a third protein might be needed to bridge the two fusion proteins thereby holding them together. To demonstrate direct interaction, one could use genetic approaches such as allele-specific suppression or enhancement or biochemical approaches such as coprecipitation. Other pitfalls to the proper interpretation of the significance of the interaction exist, particularly for researchers using the yeast two-hybrid system to study proteins from other organisms. Two proteins might be capable of interacting under the conditions of the yeast two-hybrid assay (abundant expression and localization to the host cell nucleus) but not under normal conditions. The two proteins in their normal host cell may be found in different compartments or expressed in different cell types. Therefore, in these situations the interaction in yeast is an artifact. A positive two-hybrid result is just the beginning and a great deal more genetic and biochemical analysis must be done to demonstrate the biological significance of the result.

Several variations of the basic two-hybrid method have been developed to improve the efficiency of introducing the library plasmid into the host tester strain and the detection of an interaction. This is particularly important when screening a large library such as a mammalian cDNA library. *HIS3* or other nutritional genes can be used instead of *lacZ* as the reporter gene. In this way one can select for the expression of the nutritional gene rather than screen for the expression of *lacZ*. *HIS3* is especially good for detecting weak interactions because only very low levels of *HIS3* expression are needed for the His+ phenotype. The most significant modification to the two-hybrid method was conceived of by Bendixen *et al.* (1994) and Brent & Finley (1994). Instead of using transformation to introduce the 'prey' plasmid library into cells carrying the 'bait' plasmid, they used **a**/α mating between a large pool of cells transformed with the 'prey' library plasmids and cells carrying the 'bait' plasmid to construct a double transformant containing both plasmids.

ONE-HYBRID AND THREE-HYBRID ANALYSIS

Variations of the two-hybrid analysis method have been developed to explore DNA–protein and RNA–protein interactions.

One-hybrid analysis is used to identify proteins that interact with a specific DNA sequence. This sequence is inserted into the promoter of a reporter gene: for example, in place of the LexA operator site of the *lacZ* reporter shown in Figure 10.2. A fusion gene library similar to the 'prey' library described above that fuses

the library proteins to a transcription activation domain is introduced into a host strain carrying the reporter. Any hybrid proteins capable of binding to the DNA sequence of interest will activate the reporter gene expression.

Three-hybrid analysis was developed to study the RNA–protein interaction (reviewed in Kraemer *et al.*, 2000). The third hybrid construct in this method is an RNA hybrid molecule. The bait hybrid protein binds one portion of this hybrid RNA. The other portion of the hybrid RNA is the sequence of interest. Fusion proteins of the 'prey' library are screened for their ability to bind to this RNA sequence of interest that is tethered to the reporter gene promoter via the bait hybrid protein.

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11 Advanced Concepts in Molecular Genetic Analysis

REVERSE GENETICS

With the advent of recombinant DNA technology and the development of methods for the purification and sequencing of proteins, it became possible to clone the gene encoding any purified protein. With the cloned gene in hand, one can use any one of a number of techniques to introduce random mutations into a cloned sequence, or one can induce mutations in regions of interest, such as sites of putative functional motifs, using a variety of *in vitro* techniques. The mutant alleles are then tested *in vivo* for a change in phenotype. This approach is called **reverse genetics**. In the classic genetic approach one starts with a phenotype of interest and isolates mutants with an altered phenotype. No prior mechanistic understanding of the phenomena controlling the phenotype is required and a large array of genes is likely to be identified. The classic genetic approach casts a wide net and is capable of providing a broad view of a complex biological system. In the reverse genetic approach mutations are directed to a particular gene (protein) and the effects of these changes on the phenotype of the organism are then determined. Some of these phenotypic effects can be predicted based on the biochemical and/or biological information available about this protein. Some of the phenotypes might be novel and unexpected. Whatever the outcome, the reverse genetic approach provides detailed information regarding the function of a particular gene product.

Several methods of *in vitro* mutagenesis are available to the researcher. Some induce random changes; others are site directed and can induce specific changes in particular basepairs or short sequence regions. One should just be aware that there is tremendous flexibility in the type of mutation that can be induced. Protocols for these methods are available in the literature and are included with commercially available kits. These will not be discussed here.

Two very important considerations must be kept in mind when developing a strategy for testing the *in vivo* phenotype of the mutant alleles. First, mutations generated *in vitro* when tested *in vivo* may not have a detectable phenotype. If they do have a phenotype, the mutant alleles could be recessive. Therefore, analysis of the phenotype must be done in strains that carry only recessive mutant alleles of the gene under study. Preferably, the other copies of the gene present in the strain should be null alleles. This avoids any possibility of an interaction with the mutant gene product being tested. Construction of a null allele in *Saccharomyces* is extremely straightforward using the one-step gene replacement methods described in Chapter 1. Second, because subtle phenotypic variation among the mutant alleles is possible, it is important to test each mutant allele in isogenic strains. This is necessary in order to be able clearly to associate the phenotype of the mutant with that specific mutational alteration and not variations in the genetic background.

The need to use a null allele when testing the phenotype of a mutant gene becomes a problem if the gene product has an essential function. In *Saccharomyces* this difficulty is addressed quite simply, but in other organisms this presents a greater difficulty. To test whether a gene is essential in *Saccharomyces*, a null allele is constructed in a diploid strain. Tetrad analysis of this heterozygous diploid will give two viable and two nonviable spores if the gene is essential. If the diploid is transformed with an extrachromosomal plasmid carrying the wild-type allele of the gene of interest, all four spores should be viable. The two spores containing the chromosomal null allele are able to grow because of the plasmid copy of the gene and viability of these spores will be dependent upon continued presence of the plasmid.

To determine the phenotype of a mutant allele of the gene of interest, the heterozygous *GEN1/gen1*Δ diploid is transformed with the extrachromosomal plasmid carrying the mutant *gen1* gene, sporulated under selective conditions to maintain the plasmid, and tetrad analysis done. If the two spores with the null mutation are not viable, then the plasmid-borne mutant allele is not functional. But, if all four spores are viable, then the mutant allele is capable of providing function, i.e. it is complementing the chromosomal null allele. The function of the *gen1* mutant allele may not be entirely wild type and this can be determined by a detailed analysis of the phenotype of the mutant spores.

Another method is called the **plasmid shuffle**. Using the procedures described above, a strain containing the chromosomal null allele and a plasmid-borne wild-type allele (plasmid 1) is constructed. A second plasmid carrying the mutant allele of the gene and a different nutritional marker for selection (plasmid 2) is introduced into the same host cell. The doubly transformed host cell is grown under conditions that select for the maintenance of plasmid 2 but not plasmid 1. If plasmid 1 can be lost, then the mutant gene on plasmid 2 is functional and its phenotype, if any, can be studied. If the plasmid is never lost despite growth in nonselective conditions, then the mutant gene on plasmid 2 is nonfunctional. A very simple way of testing the ability to lose a plasmid uses the pink : white color change of *ade2* versus *ADE2* strains, respectively. This is called a **colony sectioning assay**. If the selective marker gene on plasmid 1 is *ADE2* and the host strain is *ade2*, then the colony formed by this strain will be white so long as the cells contain plasmid 1. If the colony is allowed to grow on a medium containing adenine, then plasmid 1 can be spontaneously lost so long as the transformant is not dependent on the wild-type gene also carried by this plasmid. When plasmid 1 is lost the cell will be *ade2*, will produce the pink pigment, and all the progeny will be pink forming a pink sector in the white colony. Pink sectors are easily observed in colonies. The researcher can simply scan a large number of colonies growing on an adenine-containing medium for sectoring. If this is observed, the host cell is not dependent on plasmid 1 and the mutant gene on plasmid 2 is functional.

A third method uses tightly regulated promoters, such as *CTR1*, to control expression of the wild-type allele carried by plasmid 1 (Labbé & Thiele, 1999). If the mutant allele carried by plasmid 2 is not functional, then viability of the double transformant will be dependent on the expression of the wild-type gene, which can be determined by comparing growth under conditions where the gene is expressed and not expressed.

COLD-SENSITIVE CONDITIONAL MUTATIONS

Cold-sensitive mutations are especially useful because previous experience has demonstrated that cold-sensitive mutations occur most often in genes encoding protein components of complex multimeric structures such as ribosomes, kinetochores, viral coats, and other large multiprotein complexes. Cold sensitivity often appears to be the result of a decreased ability of the components to assemble into functional structures at the lower temperature, while at normal growth temperature assembly occurs at adequate rates. Thus, if one were interested in isolating mutations in genes encoding components of such structures or investigating the assembly process, one should consider isolating cold-sensitive mutations. This is done using various methods of random mutagenesis on the gene carried on a plasmid vector. The mutagenized pool is transformed into a null mutant host strain and screened for a cold-sensitive phenotype. If the null allele is lethal, a method such as plasmid shuffle would have to be used.

As with temperature-sensitive mutations, cold-sensitive mutations are non-null mutations. Therefore, if one obtains allele-specific suppressors of a cold-sensitive mutation, this suggests a physical interaction between the protein with the cold-sensitive alteration and the suppressor gene product. If a cold-sensitive mutation weakens the interaction at the nonpermissive temperature, then the suppressor mutation is likely to strengthen the interaction at the nonpermissive temperature. It is impossible to predict the phenotype of the suppressor mutation in the absence of the original cold-sensitive mutation. If the interaction with the wild-type product is unaffected, then the suppressor mutation will have no mutant phenotype. On the other hand, if the interaction is disrupted, the suppressor mutation might have a mutant phenotype similar to the original mutation; or, if the interaction is strengthened, the suppressor mutation might have a novel phenotype. This information would have to be empirically determined.

DOMINANT NEGATIVE MUTATIONS

Widespread use of dominant negative mutant alleles became possible with the advent of gene cloning and DNA technology (Herskowitz, 1987). A **dominant negative mutation** is one that disrupts the function of the wild-type allele. Thus, the use of dominant negative mutations has become a very powerful technique for studies of protein function in organisms where the usual methods of producing mutant alleles are difficult or impossible. The dominant negative is a way of eliminating the function of a particular gene without having to isolate loss-of-function mutations in that gene. One often sees this method used in studies with mammalian cells.

The term dominant negative seems contradictory because a negative mutation, which implies loss-of-function, should be recessive not dominant. Most often, the dominant negative mutation requires overexpression to be dominant but occasionally examples are discovered where this is not necessary. The need for overexpression is explained as follows. Many proteins bind to other proteins and form multiprotein complexes in which only one component has the catalytic activity while the others function as regulators of the catalytic subunit. A mutation in the catalytic domain of

the protein will affect its catalytic activity but will not necessarily affect its binding to the regulatory components in the complex. If the other components are made in limiting amounts, then the overproduced mutant subunit will bind to and sequester (titrate) the available subunits and make them unavailable to the small amount of wild-type protein synthesized by the otherwise wild-type host cell. If the limiting regulatory subunit is a positive regulator a null-like phenotype will be produced. If the limiting subunit is a negative regulator a constitutive phenotype will be produced. In this manner, a loss-of-function mutation can dominate the phenotype of a wild-type host.

The Cdc28 protein is a cyclin-dependent protein kinase and binding of proteins called cyclins is necessary to activate the kinase activity of Cdc28p. Cdc28p kinase is an essential protein needed for the progress from G1 to S and G2 to M in the cell cycle. Cyclin proteins are present in limiting amounts at certain times in the cell cycle and if these are depleted the cell will be unable to enter the S phase or mitosis. If a mutation is introduced into *CDC28* at a site encoding an essential residue of the kinase activity, such as in the ATP binding site, then the encoded protein will be inactive as a kinase and the mutation will be recessive. However, the ability of the altered Cdc28p to bind cyclin has not been affected by this mutation. Therefore, if this mutant *cdc28* gene is overexpressed, the mutant protein product will be very abundant and the cyclin proteins will bind to this nonfunctional protein instead of to the much less abundant wild-type Cdc28p encoded by the chromosomal copy. By so doing, the mutant product blocks the activation of the wild-type Cdc28 protein thereby preventing it from functioning. Thus, when this recessive *cdc28* mutation is overexpressed the nonfunctional phenotype becomes 'dominant'. In this way, the geneticist can explore the phenotype of a *cdc28* null mutation without having to construct one. This is extremely useful in mammalian cells where constructing a deletion mutation is an extremely lengthy and expensive undertaking.

Occasionally, one finds a dominant loss-of-function mutation that does not need to be overexpressed. In these cases the gene product is part of a multiprotein complex of like subunits and the mutation has altered a functional activity of the protein but not its ability to form the multiprotein complex. The dominance results from the fact that even if one subunit of the complex is mutant the entire complex is nonfunctional. The tumor suppressor protein p53 is a noteworthy example (Ko & Prives, 1996).

The dominant negative mutation has additional uses. For example, one can use it to isolate the gene encoding the interacting protein using multicopy suppression. The mutant phenotype produced by the overexpression of a dominant negative mutant gene should be suppressed by the overexpression of the interacting protein. One would use some type of overexpression library, introduce this into the host cell carrying the overexpressed dominant negative mutation, and select/screen for wild-type-like suppressor-containing transformants. The suppressing plasmid can easily be recovered and the gene responsible for the suppression identified.

The dominant negative strategy can be used for structure–function analysis of the catalytic subunit of a multiprotein enzyme complex. It also could be used to characterize a multiprotein DNA-binding complex. Mutation of the DNA-binding domain but not the domains used for protein–protein binding should produce a dominant negative allele. Similar approaches could be used for studies of other multiprotein complexes.

CHARGED-CLUSTER TO ALANINE SCANNING MUTAGENESIS

Charged-cluster to alanine scanning mutagenesis is a 'semi-random' approach to choosing which residues to mutate so as to improve the probability of affecting the function in a meaningful way. Alterations that cause the gene product to be unstable are uninteresting and do not allow one to explore function. Charged-cluster to alanine scanning mutagenesis is a method that optimizes the generation of mutant alleles with stable gene products.

X-ray crystallography has been used successfully to reveal the three-dimensional structure of many proteins. Generally speaking, clusters of charged residues tend to be located at the surface of a protein while hydrophobic and nonpolar residues are often buried in the core of the protein. The charged-cluster to alanine approach to constructing *in vitro* mutations uses this finding to propose the following. First, if one scans a protein sequence those regions containing clusters of charged residues have a reasonable probability of being positioned at the surface of the folded protein. Second, alterations of surface residues should produce fewer of the types of structural abnormality that often make mutant proteins the target of proteolytic degradation. Third, changes in these charged clusters are likely to alter surface residues that are often involved in protein–protein interactions. These predictions have proven to be correct frequently enough to make charged-cluster to alanine mutagenesis a valuable tool and widely accepted as a method for genetically dissecting the different protein–protein interactions of a particular multifunctional protein or protein component of a multiprotein complex. The types of interaction that can be detected by this approach are interactions with substrates, activating subunits, inhibitory subunits, targeting subunits, and other components of complexes. For proteins that function in several processes, the charged-cluster to alanine scanning approach is able to dissect these different functions because the clustered alterations may affect only one of the several functions.

One scans the sequence of a protein usually using an overlapping window of five residues looking for the presence of two or more charged residues in the window. All the charged residues are then changed to alanine using *in vitro* mutagenesis techniques. The mutant allele is then tested for phenotype in a null mutant strain as described above in the section on reverse genetics.

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12 Genomic Analysis

With the completion of the sequence of the *S. cerevisiae* genome in April 1996 came a new challenge, namely functional analysis of the genome. Methods needed to be developed on a genome-wide scale to provide the tools for understanding the roles of the approximately 6000 gene products, their expression patterns, and how they interact to create a eukaryotic organism capable of complex processes like growth, cell division, and the response to extracellular signals. Several groups from throughout the world are working to meet this challenge and develop databases and research resources for the *Saccharomyces* scientific community. The most important of these is the *Saccharomyces* Genome Database (SGD) at Stanford University. Others include the European Functional Analysis Network (EUROFAN), the Yeast Technology Resource Center at the University of Washington in Seattle, Yale Genome Analysis Center at Yale University Medical School in New Haven, and the Center for Molecular Medicine and Therapeutics of the University of British Columbia. In addition, reagents specific for working with *Saccharomyces* are becoming available through commercial sources.

Why such attention to a simple microorganism? Despite the absence of a multicelled development, *Saccharomyces* is not so very different from other eukaryotes at the genetic level. Comparative genomic analysis of *Drosophila melanogaster* and *Caenorhabditis elegans* found that the numbers and types of proteins (termed protein sets) found in these organisms is similar in size and only about twice the size of that of *Saccharomyces* when redundancy is taken into account (Rubin *et al.*, 2000). Clearly, *Saccharomyces* is an excellent model for the more complex systems and one that is far more amenable to genetic analysis, at least for the near future. It is important to keep in mind that the gene functions identified in *Saccharomyces* define the components to 'build' a eukaryotic cell. Gene functions involved in the assembly of cells into multicelled organisms will be missing from the *Saccharomyces* protein set. Nonetheless, if the reader is interested in basic eukaryotic cell functions, *Saccharomyces* is a very valuable experimental tool.

Saccharomyces can be used to analyze the function of genes from other systems by complementing yeast mutations. The heterologous expression of human genes in *Saccharomyces* as well as genes from other organisms including plants has already proved to be a valuable tool for functional analysis and a rapidly expanding literature of such studies already exists. Heterologous expression in *Saccharomyces* will also be used for drug development and testing and for the commercial production of pharmaceuticals and other agents. Thus, completing the functional analysis of *Saccharomyces* will be a major step for the functional analysis of eukaryotes with larger genomes, like humans.

This chapter is not intended as an exhaustive study of *Saccharomyces* genome functional analysis. The approaches are many and are constantly being refined. New approaches are conceived and put into practice all the time. A brief survey of the

most important and/or widely used methods is presented. The reader might also refer to Kumar & Snyder (2001).

DATABASES

The most valuable site which the reader should be familiar with is the *Saccharomyces* Genome Database (SGD), maintained by Stanford University (http://genome-www.stanford.edu/Saccharomyces). At the site one can access the complete genomic sequence of *S. cerevisiae*, the complete physical and genetic maps of all of the chromosomes, and information on all of the known genes including references to the literature. Meetings and other items of importance to the yeast community are listed at the site. One can get help with technical questions or look up the address of a colleague. This site also has links to other major sites for yeast protein analysis including YPD and MIPS.

The Yeast Proteome Database (YPD) (http://www.proteome.com/YPDhome.html) is a comprehensive site for information on the approximately 6000 *Saccharomyces* proteins. It is maintained by Proteome, Inc. (http://proteome.com/) and provides several products and services including a detailed curation of the scientific literature from a wide array of research publications and precalculated sequence alignments for comparative genomic analysis. A scheme for protein classification is available that summarizes the function and role in the cell of specific proteins. The goal of YPD is to provide a framework for functional analysis. The BioKnowledge Library found at the Proteome site is a relational database and the website compiles published information about individual proteins, including function, subcellular location, expression patterns, and interactions with other proteins, and presents it in an easy-to-use format. Information on the protein sets from *S. cerevisiae* (YPD), *Schizosaccharomyces pombe* (PombePD), *Casnorhabditis elegans* (WormPD), and *Candida albicans* (CalPD™) is available (Costanzo *et al.*, 2001).

Another important site is the Munich Information Center for Protein Sequences (MIPS) (http://mips.gsf.de/). MIPS is maintained by the bioinformatics section of the National Research Center for Environment and Health of the Max-Planck Institute for Biochemistry and is a member of PIR International (Protein Identification Resource) and of the European Molecular Biological Network (EMBNET). The MIPS Yeast Genome Database contains extensive information regarding the *Saccharomyces* ORFs. Other projects include genome analysis of *Arabidopsis thaliana*, the model dicot plant, and *Neurospora crassa*, another fungal genetic model organism. MIPS is involved in the development of active database systems for the efficient use of sequence data, particularly for human genome analysis. MIPS's Protfam project is for protein classification into families and superfamilies, for motif searches, and identification of homology domains.

A site for the prediction of protein function based on sequence and structural information has been established at UCLA (http://www.doe-mbi.ucla.edu). The site includes a database for yeast proteins and has many other interesting features.

BIOCHEMICAL GENOMIC ANALYSIS

Martzen *et al.* (1999) reported a genome-wide strategy for identifying genes encoding products with specific biochemical activities (reviewed in Carlson, 2000). This method fuses the complete library of yeast ORFs to glutathione S-transferase (GST), expresses these GST fusions from the high expression copper-regulated *CUP1* promoter, and introduces the constructions into an appropriate yeast host where abundant expression can be induced. The GST fusion proteins can then be partially purified using standard methods and tested for specific biochemical activity, such as cAMP activated kinase activity.

A collection of over 6000 yeast strains each expressing a different yeast ORF is available from Research Genetics (Huntsville, AL) (http://resgen.com/) for those interested in this biochemical screening method. Using a pooling approach, one can screen large numbers of transformants expressing the GST fusions. When an activity is detected in a pool, the pools are deconvoluted, and the identity of the specific transformant expressing the GST fusion protein with the desired activity is determined. The usefulness of this method was demonstrated by the identification of the genes encoding cyclic phosphodiesterase and cytochrome c methytransferase (Martzen *et al.*, 1999).

DNA MICROARRAY ANALYSIS

DNA microarray analysis is essentially a method for carrying out thousands of hybridizations at one time using small samples. DNA probes representing each of the *Saccharomyces* ORFs are irreversibly attached to a solid substrate such as a glass slide or a nylon membrane. The unique sequence fragments are made by PCR using carefully selected primer pairs internal to the transcribed regions. Detailed information on the production of DNA arrays for *Saccharomyces* can be found in Eisen & Brown (1999). Research Genetics is a commercial source of the complete set of *Saccharomyces* primer pairs. The DNA fragments are spotted onto the substrate using specialized devices capable of producing an 80×80 array of 6400 samples in an area of about 18 mm^2. Newer technology that synthesizes the single-stranded oligonucleotide on the substrate is now available and will be most useful for organisms with larger genomes and protein sets than *Saccharomyces* (Ramsay, 1998).

The DNA sample under analysis is incubated with the DNA chip under conditions that allow hybridization. Initially, the sample DNA was radioactively labeled and phosphorimaging was used to detect positions in the array at which hybridization had occurred. Currently, the sample DNA is labeled with a fluorescent tag and laser scanning or a fluorescent confocal microscope detects positions of hybridization. The results are expressed quantitatively relative to a control condition and changes of twofold or more are considered significant. Various methods on how best to display the results of genome-wide analysis are in development since the wealth of information produced by microarray analysis can be quite daunting (Eisen *et al.*, 1999; Zhang, 1999; Aach *et al.*, 2000; Brown *et al.*, 2000). To carry out a DNA microarray analysis, the researcher must affiliate with a facility that has the equipment to prepare the DNA chips and obtain and analyze the data.

Sample DNA preparation depends on the experiment. Typically, DNA microarray methods are used to compare transcription expression patterns under different growth conditions, in different mutant backgrounds, or different cell and tissue types. RNA samples are purified from cells grown in the experimental and control conditions. cDNA is made using fluorescently tagged primer that anneals to the oligoT sequence at the 3' end of mRNA. The cDNA sample produced is then used to hybridize to the DNA microarray chip. If the mRNA for a particular gene is represented in the RNA extract, then this method should detect a hybridization signal. The expression level in the experimental culture is compared with that in the control condition and changes in expression level are quantified.

DNA microarray analysis is being used for an increasing variety of studies. Spellman *et al.* (1998) used this technique to identify and characterize the expression of cell-cycle-regulated genes. Genome-wide comparisons of glucose repression of transcription in *mig1* and *mig2* null mutant strains identified many genes controlled by these repressors (Lutfiyya *et al.*, 1998). Similar studies of *snf/swi* mutants and multidrug-resistant yeast mutants have been reported (Sudarsanam *et al.*, 2000; DeRisi *et al.*, 2000). A method referred to as comparative genomic hybridization has been developed to study genomic copy-number changes using the DNA microarray methodology (Pollack *et al.*, 1999). These and other techniques for the study of genomic changes in tumor cells will be valuable tools for design of specific treatment therapies. Information on microarray analysis data sets can be found at the SGD database (http://genome-www4.stanford.edu/MicroArray/SMD/).

GENOME-WIDE TWO-HYBRID SCREENS

The basics of two-hybrid analysis were described in Chapter 10. Stan Fields and colleagues have expanded upon this method to develop reagents for improved genome-wide studies of protein–protein interaction. These methods are described in detail in Uetz *et al.* (2000) and more information is available on the Washington University Yeast Technology Resource Center web site (http://depts.washington.edu/yeastrc), at the Stan Fields laboratory website (http://depts.washington.edu/sfields/projects/YPLM/) and at the Curagen Corporation site (http://portal.curagen.com/).

Each of the approximately 6000 *Saccharomyces* ORFs has been cloned into a Gal4 transcription activation domain vector and a Gal4 DNA-binding domain vector using PCR-based approaches. Each of these bait and prey fusion sets is available as transformants of an appropriate pair of yeast host strains of different mating types. Interaction is tested by mating the appropriate transformant pairs and screening/selecting for a positive protein–protein interaction. *HIS3*, *URA3*, and *lacZ* reporters are available and often more than one is used in each test.

Methods for genome-wide analysis of all potential test pairs (6000 baits by 6000 fish) are under development and the efficacy of two of these approaches has been reported (Uetz *et al.*, 2000). Researchers interested in using this method to explore interactions involving a specific protein of interest should contact Stan Fields to set up a collaboration.

GENOME-WIDE GENERATION OF NULL MUTATIONS

GENE DISRUPTION STRAINS

The most straightforward method of creating null mutations in a gene uses one-step gene disruption similar to the methods described in Chapter 1 (Winzeler *et al.*, 1999). With the complete *Saccharomyces* sequence in hand it became possible to use PCR-based methods to delete each of the approximately 6000 ORFs. A consortium of European and American research laboratories is in the process of generating the complete set of knock-out strains in a number of isogenic haploid strains and in an **a**/α diploid. The complete list of available knock-out strains can be found at the SGD website (sequence-www.stanford.edu/group/yeast_deletion_project/deletions3.html) and strains can be obtained from Research Genetics (check their website for pricing).

TRANSPOSON MUTAGENESIS

For researchers interested in obtaining null mutations in specialized strain backgrounds, another method based on transposon mutagenesis developed by Michael Snyder of Yale University might better suit one's needs (Burns *et al.*, 1994; Ross-Macdonald *et al.*, 1997, 1999). Specific details of the method and protocols are available on the Yale Genome Analysis Center website (http://www.ygac.med.yale.edu/). Pools of mutagenized plasmid DNA are provided upon request along with amplification procedures.

The transposon used to randomly mutagenize the yeast genome is a Tn3 derivative that contains the *lacZ* gene, yeast *LEU2* and the *E. coli* ampicillin-resistance gene. The *lacZ* gene is at one end of the Tn3 sequence just after the inverted repeat and transposition into an ORF will create a *lacZ* fusion. Theoretically, one in six transposition events will create a gene fusion to *lacZ* in the proper orientation and reading frame. The yeast genomic library used for the mutagenesis contained 10^5 recombinant plasmids (about 20 genomes) with the yeast insert fragment bounded by *Not*I sites so that the fragment may be released from the vector by simple digestion.

Insertional mutagenesis of the library is achieved by a shuttle approach. A plasmid carrying the transposon is introduced into pools of *E. coli* host cells carrying the library plasmids. Transposition of the specially constructed Tn3 is activated, and transposition into the yeast insert will occur. Library plasmid DNA is then prepared separately from each pool. All insertions into the ORF of the yeast gene should create a null mutation with very rare exceptions.

Mutagenized library DNA is digested with *Not*I to release the Tn3-mutagenized yeast fragment, transformed into the yeast host cell, and Leu+ transformants selected. The Tn3-containing yeast fragment replaces the genomic copy of the region by homologous recombination, i.e. one-step gene replacement. The Leu+ transformants can be selected or screened for additional phenotypes, such as suppression of a mutant phenotype of interest. Using this method, one can create null mutations in genes throughout the *Saccharomyces* genome. The site of the Tn insertion can be readily identified using genomic PCR methods using a primer internal to the transposon. In addition, included among the null mutation will be some that create

lacZ fusions and these can be used for subcellular localization of the fusion product or studies of its expression pattern. Analysis has found that the Tn3-based system is not as random as suggested and a new method is under development.

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III Case Studies from the *Saccharomyces* Genetic Literature

INTRODUCTION TO THE CASE STUDIES

The case studies in Section III are an integral part of the learning experience to be gained from this book. The role of the case study is to reinforce the theory presented in each chapter of Section II by providing the reader with actual bench experiments that put the theory into practice. Unlike much of biological research that tends to be quite descriptive, genetic analysis techniques are abstract and in many ways similar to mathematics. In molecular genetics, one studies genes and proteins that one can only observe indirectly and in the mind's eye. The case study makes the theory concrete and demonstrates that the tools developed in theory actually do work in the laboratory. Hopefully readers will become more comfortable with these research tools by working through the case studies, and thus will more likely make use of them in their own research. In today's world of genomics and proteomics, it is unfortunate that so many still adhere to descriptive methods when the tools of genetic analysis would enable them to probe more deeply and uncover mechanisms rather than correlations.

Each case study is a developmental series of research articles that, taken together, tell a story. The cohesive thread of that story is broken if only a few articles are analyzed or if one picks and chooses articles from different case studies. Therefore, it is urged that the reader choose one case study and work only from that case study. Specific articles can be deleted from each case study but a minimum of six articles are needed if one is to experience at least one example of each of the essential genetic tools: mutant hunts, complementation analysis, epistasis analysis, suppression, enhancement, and gene isolation techniques. Adequate coverage of the complexities of suppression and enhancement requires analysis of more than one article for each of these areas. Moreover, given the broad-based uses of two-hybrid analysis and dominant negative mutations, it is strongly suggested that articles using these methods also be included. Thus, 10 articles is a reasonable selection but certainly the more the better.

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Case Study I
Glucose Sensing and Signaling Mechanisms in *Saccharomyces*

READING LIST

Mutant Hunts: To Select or to Screen (Perhaps Even by Brute Force)

Article 1

Carlson, M., B.C. Osmond, & D. Botstein (1981) Genetic evidence for a silent *SUC* gene in yeast. *Genetics* **98**: 41–54.

Complementation Analysis: How Many Genes are Involved?

Article 2

Carlson, M., B.C. Osmond, & D. Botstein (1981) Mutants of yeast defective in sucrose utilization. *Genetics* **98**: 25–40.

Article 3

Neigeborn, L. & M. Carlson (1984) Genes affecting the regulation of *SUC2* gene expression by glucose repression in *Saccharomyces cerevisiae*. *Genetics* **108**: 845–958.

Epistasis Analysis

Article 4

Neigeborn, L. & M. Carlson (1987) Mutations causing constitutive invertase synthesis in yeast: genetic interactions with *snf* mutations. *Genetics* **115**: 247–253.

Gene Isolation, Characterization, and Multiple Alleles

Article 5

Neigeborn, L., P. Schwartzberg, R. Reid, & M. Carlson (1986) Null mutations in the *SNF3* gene of *Saccharomyces cerevisiae* cause a different phenotype than do previously isolated missense mutations. *Mol. Cell. Biol.* **6**: 3569–3574.

Article 6

Celenza, J.L., L. Marshall-Carlson, & M. Carlson (1988) The yeast *SNF3* gene encodes a glucose transporter homologous to the mammalian protein. *Proc. Natl Acad. Sci. USA* **85**: 2130–2134.

Suppression

Article 7

Bisson, L.F., L. Neigeborn, M. Carlson, & D.G. Fraenkel (1987) The *SNF3* gene is required for high-affinity glucose transport in *Saccharomyces cerevisiae*. *J. Bacteriol.* **169**: 1656–1662.

Suppression

Article 8

Carlson, M., B.C. Osmond, L. Neigeborn, & D. Botstein (1984) A suppressor of *snf1* mutations causes constitutive high-level invertase synthesis in yeast. *Genetics* **107**: 19–32.

Enhancement

Article 9

Vallier, L.G. & M. Carlson (1994) Synergistic release from glucose repression by *mig1* and *ssn* mutations in *Saccharomyces cerevisiae*. *Genetics* **137**: 49–54.

More Suppression and Enhancement

Article 10

Marshall-Carlson, L., L. Neigeborn, D. Coons, L. Bisson, & M. Carlson (1991) Dominant and recessive suppressors that restore glucose transport in a yeast *snf3* mutant. *Genetics* **128**: 505–512.

Article 11

Vallier, L.G., D. Coons, L.F. Bisson, & M. Carlson (1994) Altered regulatory responses to glucose are associated with a glucose transport defect in *grr1* mutants of *Saccharomyces cerevisiae*. *Genetics* **136**: 1279–1285.

Molecular Genetic Analysis of Suppressor Genes

Article 12

Ozcan, S., T. Leong, & M. Johnston (1996) Rgt1p of *Saccharomyces cerevisiae*, a key regulator of glucose-induced genes, is both an activator and a repressor of transcription. *Molec. Cell. Biol.* **16**: 6419–6426.

Article 13

Ozcan, S., J. Dover, A.G. Rosenwald, S. Wolfl, & M. Johnston (1996) Two glucose transporters in *Saccharomyces cerevisiae* are glucose sensors that generate a signal for induction of gene expression. *Proc. Natl Acad. Sci. USA* **93**: 12428–12432.

Two-Hybrid Analysis

Article 14

Tu, J. & M. Carlson (1995) REG1 binds to protein phosphatase type 1 and regulates glucose repression in *Saccharomyces cerevisiae*. *EMBO J.* **14**: 5939–5946.

Analysis of Site-Directed Mutations

Article 15

Tu, J., W. Song, & M. Carlson (1996) Protein phosphatase type 1 interacts with proteins required for meiosis and other cellular processes in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **16**: 4199–4206.

Article 16

Baker, S.H., D.L. Frederick, A. Bloecher, & K. Tatchell (1997) Alanine-scanning mutagenesis of protein phosphatase type 1 in the yeast *Saccharomyces cerevisiae*. *Genetics* **145**: 615–626.

Genome-Wide Analysis

Article 17

Lutfiyya, L.L., V.R. Iyer, J. DeRisi, M.J. DeVit, P.O. Brown, & M. Johnston (1998) Characterization of three related glucose repressors and genes they regulate in *Saccharomyces cerevisiae*. *Genetics* **150**: 1377–1391.

ARTICLE 1

Carlson, M., B.C. Osmond, & D. Botstein (1981) Genetic evidence for a silent *SUC* gene in yeast. *Genetics* **98**: 41–54.

1. Describe how sucrose is fermented (catabolized, utilized) by *Saccharomyces*.

2. Describe the *SUC* genes of *Saccharomyces*.

3. Figure 2 describes a cross between strains DBY473 (*MATα SUC2 suc7^0 his4 URA3*) and DBY631 (*MAT***a** *suc2^0 SUC7 HIS4 ura3*).
   (a) Diagram this cross in detail using the format outlined in Chapter 1. Be sure to show the genotype and phenotype of the following: the parental strains; the heterozygous diploid produced by the mating; and the haploid spores of PD, NPD and TT tetrads. **Only the** *SUC/suc* genotype and Suc+/Suc- phenotype of the haploid spores needs to be indicated in the tetrads.
   (b) How do the authors use the difference in the *HIS4* and *URA3* genotypes of the two parental strains to obtain the diploid product of the cross?

4. The S288C *suc2^0* strain used to isolate the Suc+ revertants is 'congenic' to the original S288C *SUC2* strain obtained from G. Fink.
   (a) How many backcrosses to S288C were made to construct this strain?
   (b) In each backcross, what Suc phenotype was selected for from among the haploid segregants?
   (c) Because of independent segregation of chromosomes and recombination, each backcross of an individual progeny strain resulting from a cross to the same parental strain increases by 50% the percentage of the subsequent progeny's genome that is identical to that parent's genome. Based on this, calculate the average percent identity between the S288C *suc2^0* strain and the original S288C *SUC2* strain.

5. Sucrose fermenting revertants of the S288C *suc2^0* strain were obtained.
   (a) Were these Suc+ mutants identified by a selection method or by a screen? Discuss the method.
   (b) Was mutagenesis used to increase the frequency of Suc+ revertants or were these spontaneous? What was the frequency of reversion to Suc+?
   (c) How did the authors demonstrate that the mutation in the revertant strains represented a change in a single gene? (Give details of any crosses.)
   (d) How did the authors demonstrate that the Suc+ reversion mutations were in the *suc2^0* gene?

6. Sucrose fermenting revertants of S288C were obtained only at *suc2^0* and never at any of the other *SUC* loci.
   (a) What possible explanations do the authors give for this result?

(b) Which explanation do they prefer and what experimental evidence do the authors present in support of this explanation?

7. Describe the fundamental difference between the *suc2*^{0} locus found in strain S288C and the other *suc*^{0} loci found in this strain.

ARTICLE 2

Carlson, M., B.C. Osmond, & D. Botstein (1981) Mutants of yeast defective in sucrose utilization. *Genetics* **98**: 25–40.

ARTICLE 3

Neigeborn, L. & M. Carlson (1984) Genes affecting the regulation of *SUC2* gene expression by glucose repression in *Saccharomyces cerevisiae*. *Genetics* **108**: 845–858.

1. (a) Describe the method used to identify sucrose nonfermenting mutants. Would you consider this a selection or a screen and why?
   (b) Chemical mutagenesis with ethylmethane sulfonate was used to enrich for the desired class of mutant. Was this necessary or do you think that the authors could have worked with spontaneous mutations and why?
   (c) Three 'isogenic' strains were used. Are they genetically identical, and if not, how do they differ? What is the purpose for including the *his4* and *lys2* nonsense mutations? [You may have to read about nonsense mutations and nonsense suppressors (sometimes called information suppressors) to answer this question].

2. In your own words, explain why complementation analysis can not be done with dominant mutations.

3. The authors crossed each mutant strain to an otherwise isogenic parental strain (of course of opposite mating type).
   (a) What conclusion can be drawn if only PD tetrads are obtained?
   (b) If TT or NPD tetrads are obtained, what does this indicate?

4. Diagram the cross between two sucrose nonfermenting mutants isolated from strains DBY782 (*MAT*α *ade2 SUC2*) and the otherwise isogenic strain DBY916 (*MAT***a** *his4-86oc lys2-802oc*) in which the mutations in the two strains are in the same complementation group (i.e. gene). Call the gene *FER1* and show the genotype and phenotype of the mutant parents, the diploid, and the resulting tetrads.

5. With regard to complementation Group 1:
   (a) Why do the authors place the Group 1A, 1B, and 1C mutations in a single complementation group and not three different complementation groups?
   (b) How does the tetrad analysis data support this conclusion?

6. What is the evidence that complementation Group 1 (the *SUC2* gene) encodes invertase?

7. What reasons are given to support the hypothesis that complementation Group 2 (the *SNF1* gene) encodes a regulator of *SUC* gene expression?

8. What is the difference between the *SUC* genotype of the parental strains used in Articles 2 and 3? Why did Neigeborn & Carlson make this change for the second study?

9. (a) Define the term pleiotropic.
   (b) *SNF1*, *SNF2*, *SNF4*, and *SNF5* are pleiotropic. What phenotypes were scored?
   (c) What does this pleiotrophy suggest about the functions of these genes?
   (d) This suggestion is consistent with the finding that internal invertase is made in these mutant strains but not secreted invertase. Why?

10. (a) What is unique about the *snf3* mutants compared with strains carrying mutations in the other *SNF* genes?
    (b) Comment on the authors' hypothesis regarding the function of Snf3 protein.

ARTICLE 4

Neigeborn, L. & M. Carlson (1987) Mutations causing constitutive invertase synthesis in yeast: genetic interactions with *snf* mutations. *Genetics* **115**: 247–253.

1. The constitutive expression of a gene indicates that the expression is unregulated. The authors isolated mutant strains that express invertase constitutively.
   (a) Describe the normal regulation pattern of invertase expression.
   (b) Define repressed conditions.
   (c) Define derepressed conditions.
   (d) Define the term constitutive as it relates to invertase expression.

2. Describe the method used to obtain constitutive invertase mutants.
   (a) Is this a selection or a screen and why?
   (b) What is the role of the 2-deoxglucose in the medium, and what other carbon sources are in the medium?
   (c) What is the mutation rate? On average, if a brute force screen had been used, how many individual clones would have to have been tested to find one mutant?

3. A total of 210 mutant strains were obtained which produce >10% of the wild-type derepressed level of invertase when grown in repressed conditions.
   (a) Diagram the genetic cross carried out to determine whether each mutation is recessive or dominant to the wild-type allele. Include in

your answer the genotype and phenotype of the parental and diploid strains. The phenotype should include resistance versus sensitivity to 2-deoxyglucose and whether invertase is expressed in repressed and derepressed conditions.

(b) All of the mutations were recessive to the wild-type allele. What is the evidence?

(c) Do these genes encode positive or negative regulatory factors and why?

4. Next, the authors placed the mutations into complementation groups.

(a) Diagram the genetic cross used to determine whether two constitutive mutations (call them mutant 23 and mutant 152) were in the same or different complementation groups. Include in your answer the genotype and phenotype of the parental and diploid strains.

(b) Four of the genes identified in this article had been identified in previous studies. List them.

(c) Of the 210 mutant strains, 140 were found to contain mutant alleles of *REG1*. Select one of these 140 mutants and diagram the genetic cross used to demonstrate that it is an allele of *REG1*. Include the specific allele name of the *reg1* tester strain.

5. *CID1* is a new gene defined by this mutant hunt.

(a) How many mutant alleles of *CID1* were obtained?

(b) *cid1* mutations are pleiotropic. What results demonstrate this?

(c) The most downstream effect of the *cid1* mutations is on the transcription of *SUC2*. How do the authors demonstrate this? (Please read the section of Chapter 1 on Reporter Genes.)

6. The authors carried out an epistatic analysis of *cid1*, *hxk2*, *reg1*, and *ssn6* mutations.

(a) Are the phenotypes of strains carrying mutations in these genes distinguishable and if so how?

(b) What do the authors mean when they say, 'Segregants carrying combinations of *cid1*, *reg1*, and *hxk2* showed no unexpected phenotypes'?

(c) What results indicate that *ssn6* is epistatic to *cid1*, *reg1*, and *hxk2*?

7. Since *snf* mutations also affect the regulation of *SUC2* the authors were interested in carrying out an epistasis analysis with the *snf* mutations and constitutive mutations in *cid1*, *hxk2*, *reg1*, and *ssn6*. Double mutant strains were constructed by mating single mutant strains, sporulating the diploid, dissecting the haploid segregants, and determining the phenotype of the double mutant strain with regard to invertase expression. The results are shown in Tables 5 and 6.

(a) Except for the *snf3* mutant (which is too complex to consider here), 'all of the *snf cid1* and *snf reg1* combinations exhibited the phenotype of the *snf* parent'. Give one example from Table 5.

(b) In this analysis, which genes are epistatic to which?

(c) Is *SNF1* upstream or downstream of *REG1*? Of *CID1*?

(d) Is *SSN6* epistatic to *SNF1* or *vice versa*, and why?
(e) Is *SSN6* upstream or downstream of *SNF1*, and why?

8. Diagram the regulatory pathway showing the epistatic relationships of the following genes and indicate whether their action is as a positive (arrowhead) or negative (vertical line) regulatory factor controlling *SUC2* expression: *CID1*, *HXK2*, *REG1*, *SNF1*, *SSN6*, and *TUP1*. In your diagram, *SUC2* expression is the downstream response and, if the relationship between two or more genes is unknown but at a similar step, list the genes separately.

ARTICLE 5

Neigeborn, L., P. Schwartzberg, R. Reid, & M. Carlson (1986) Null mutations in the *SNF3* gene of *Saccharomyces cerevisiae* cause a different phenotype than do previously isolated missense mutations. *Mol. Cell. Biol.* **6**: 3569–3574.

1. The following questions relate to the construction and use of the library to isolate *SNF3*.
   (a) What vector was used to construct the genomic library used to isolate *SNF3*?
   (b) Name the *SNF3* mutant allele used in the host strain.
   (c) *URA3* is the nutritional selection marker on the library plasmid. The host strain used was MCY657. Describe the details of how the transformants were selected and how those containing *SNF3* were identified.

2. Plasmid pLN185 (Figure 1) contains the indicated *Bam*HI fragment in the *Bam*HI site of vector YIp5, a pBR322 derivative containing the yeast *URA3* gene. Plasmid pLN185 was used to demonstrate that this fragment is derived from the *SNF3* locus by targeted integration. Describe the targeted integration of pLN185 in detail. Include the method of integration (*Hint*: the *Bgl*II site in the insert fragment is unique to the plasmid), a diagram of the *SNF3* locus before and after the plasmid integration, and diagram the cross showing that pLN185 integrated at the *SNF3* locus and not elsewhere in the genome.

3. Two library plasmids complemented the *snf3* mutation in the host strain, pPSC4 and pPSC5.
   (a) How were these two plasmids helpful in localizing the position of *SNF3* in this region?
   (b) The fragments in plasmids pPSC4 and pRRC5 do not differ significantly in their ability to complement *snf3* mutations. How do you know that from the data in Table 2?

4. Northern analysis was used to characterize the transcription of *SNF3*.
   (a) Briefly, what is a Northern analysis and how does it differ from a Southern analysis?

(b) Is the expression of *SNF3* transcription regulated, and if so how?
(c) Why do the authors include *URA3* in Figure 2?

5. Diagram the one-step gene disruption used to construct *snf3-Δ4::HIS3*.

6. Table 3 presents data indicating that a *snf3* null allele differs from the mutations isolated in previous mutant hunts.
   (a) Describe the different phenotypes of *snf3-Δ4::HIS3* and *snf3-217*.
   (b) Which allele is dominant in the following gene pairs: *SNF3* and *snf3-217*; *SNF3* and *snf3-Δ4::HIS3*; *snf3-217* and *snf3-Δ4::HIS3*?
   (c) Why does this information indicate that *snf3* missense mutations exhibit abnormal function rather than a complete loss of function?
   (d) How do the authors explain the finding that growth on sucrose and raffinose is poor despite normal derepression of secreted invertase?

7. The authors evaluated the pleiotropic effects of the *snf3* mutations on other glucose-regulated genes. (Please ignore all data on *snf3-39* which later turned out to carry multiple mutations.)
   (a) Diagram the reporter gene used to assess the effects on *GAL10* expression.
   (b) What pieces of data indicate that *snf3-Δ4::HIS3* has little effect on galactose induction and glucose repression of *GAL10*?

8. 'We speculate that the aberrant glucose repression observed in *snf3* missense mutants results from a defect in sensing or signaling the availability of glucose in the environment.' What reasons are given for this conclusion?

ARTICLE 6

Celenza, J.L., L. Marshall-Carlson, & M. Carlson (1988) The yeast *SNF3* gene encodes a glucose transporter homologous to the mammalian protein. *Proc. Natl Acad. Sci. USA* **85**: 2130–2134.

1. Bioinformatics is a rapidly expanding field that includes computer scientists and molecular biologists working together to improve methods of storing and accessing sequence and structure information in biological databases. The authors of this article use sequence analysis to identify possible functions of the Snf3 protein.
   (a) What is a hydrophobicity profile and why does this analysis of Snf3 protein suggest that it is an integral membrane protein?
   (b) This study and others showed that Snf3p exhibits structural and functional homology to glucose, arabinose, xylose, maltose, and galactose transport proteins from various organisms such as humans, *E. coli*, and several different species of yeast. All of the genes encoding these proteins could be said to fall into a multigene family of transporters of what class of molecules?

2. Diagram the three fusion genes *SNF3(3)–lacZ*, *SNF3(321)–lacZ*, and *SNF3(797)–lacZ*. Be sure to clearly indicate how the three differ from one another.

3. Describe the two methods (cofractionation and indirect immunofluorescence) used to show that Snf3 protein localizes to the plasma membrane. What is the 'probe' used to detect the Snf3–LacZ fusion protein in indirect immunofluorescence?

4. The following questions explore the structure/function analysis carried out on Snf3p.
   (a) What portion of the Snf3 protein is responsible for membrane localization?
   (b) What portion of the Snf3 protein is unique compared with the human glucose transporter?
   (c) Is this unique region required for Snf3p function and how do the authors test this? Be careful. Note the location of codon 797 in Snf3p!

ARTICLE 7

Bisson, L.F., L. Neigeborn, M. Carlson, & D.G. Fraenkel (1987) The *SNF3* gene is required for high-affinity glucose transport in *Saccharomyces cerevisiae*. *J. Bacteriol.* **169**: 1656–1662.

Neigeborn *et al.* (1986) (Article 5) describe the cloning of *SNF3* using a low-copy vector library. In Article 7, they use a high-copy vector library. Fragments from six different genomic regions were isolated. They all cannot be the real *SNF3*! Thus, they must be suppressors of *snf3*.

1. Define the terms high-affinity glucose transport and low-affinity glucose transport. You may have to read the enzyme kinetics section of a biochemistry text.

2. Discuss the genes encoding hexose kinases in *Saccharomyces* and compare them functionally.

3. The library used in this study was constructed using the vector YEp24.
   (a) What is the yeast origin of replication of this vector and what is the average copy number per cell?
   (b) What is the nutritional selection marker carried by this vector?
   (c) How were suppressed transformants identified?
   (d) What methods were used to distinguish the 11 different plasmids that resulted from this selection and place them into groups?

4. Strain MCY657 (*snf3-72*) transformants carrying plasmids pSC2 and pSC7 had a different phenotype compared with those carrying plasmids pSC8 and

pLN133-7, and the pSC2 and pSC7 transformants were more similar to transformants carrying the actual *SNF3*. Discuss.

5. In your own words, explain why the authors propose that 'the complementing genes carried by pSC2 and pSC7 are perhaps the prime candidates for actually specifying a glucose carrier involved in high-affinity uptake'.

6. What caveats do the authors mention with regard to the other complementing genes that increase low-affinity glucose uptake?

7. Several of the complementing genes carried by the plasmids isolated in this study were characterized by molecular genetic analysis in future studies by these authors. They encode proteins that fall into the same multigene family as the Snf3 protein but are not highly sequence homologous to Snf3p. *HXT1* encodes a protein which is 30% identical to Snf3p and appears to function as a high-affinity glucose transporter (Lewis & Bisson, 1991). *HXT2* is another of the multicopy suppressors of *snf3*. *HXT2* encodes a protein which is 69% identical to Hxt1p and 66% identical to Gal2p, the galactose transporter (Kruckeber & Bisson 1990). Based on these statements, would you or would you not be surprised if I told you that one of these *snf3* complementing genes is *GAL2*? Explain your answer.

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Kruckeberg, A.L. & L.F. Bisson (1990) The *HXT2* gene of *Saccharomyces cerevisiae* is required for high-affinity glucose transport. *Mol. Cell. Biol.* **10**: 5903–5913.

ARTICLE 8

Carlson, M.B., C. Osmond, L. Neigeborn, & D. Botstein (1984) A suppressor of *snf1* mutations causes constitutive high-level invertase synthesis in yeast. *Genetics* **107**: 19–32.

1. The authors isolated 30 revertants of sucrose nonfermenting mutant strains DBY934, DBY1052, and DBY1053, each of which carries the *snf1-28* allele.
   (a) Describe the selection method used to isolate the revertants.
   (b) 'To test for dominance, each revertant was crossed to a *snf1 SSN* strain.' Diagram one example of such a cross showing only the *snf1* and *ssn* genes. Give the genotype and phenotype of the parental strains and the diploid.
   (c) If the revertant had been a true revertant to wild-type *SNF1*, what would have been the phenotype of the diploid from the cross in part (b) above?
   (d) Were any of these revertants true revertants or were they all suppressors of *snf1*, that is a suppressor mutation in an *ssn* gene? How do the mapping results shown in Table 4 support the conclusion that these revertants were intergenic suppressors?

2. Diagram the cross used to demonstrate that:
   (a) *ssn6-3* and *ssn6-2* are in the same complementation group.
   (b) *ssn6-2* and *ssn5-1* are in different complementation groups.
   For both of these crosses give the *SSN* and *SNF1* genotype and the phenotype of both parental strains and the resulting diploid.

3. Answer the following questions based on the results reported in Table 3.
   (a) Which *ssn* mutations are epistatic to *snf1*?
   (b) Which *ssn* mutations cause constitutive expression of invertase?
   (c) What do the authors feel is unique about the *ssn6* mutations?

4. Based on the results reported in Table 4, are strains of the *ssn SNF1* genotype sucrose fermenters or nonfermenters and how did you determine this?

5. Answer the following questions based on the results reported in Table 5.
   (a) Which result(s) indicate that *ssn6-1* is recessive to the wild-type allele?
   (b) Which result(s) suggest that *cyc8-1* might be partially dominant to the wild-type allele?

6. Present evidence to support the following statements.
   (a) Ssn6 protein has multiple functions in the cell.
   (b) Ssn6 protein acts as a repressor of *SUC2* expression.

7. Mutations in *SSN6* have been identified in several laboratories from different mutant hunts. List the four other names for *SSN6* and the mutant phenotype used to identify these mutant alleles.

ARTICLE 9

Vallier, L.G. & M. Carlson (1994) Synergistic release from glucose repression by *mig1* and *ssn* mutations in *Saccharomyces cerevisiae*. *Genetics* **137**: 49–54.

1. Answer using the results in Table 2.
   (a) Which piece(s) of data allow the authors to conclude '*mig1* is much less effective than *ssn6* in relieving glucose repression'.
   (b) Which mutation is epistatic to the other *ssn6*Δ*9* or *mig1*Δ*2::LEU2* and why?

2. Diagram the cross (MCY1773 × MCY1943) used to demonstrate that *SSN1* and *MIG1* are different names for the same gene. Show the genotype and phenotype of the parents and the diploid.

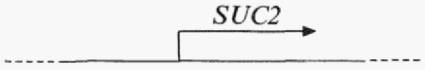
3. Use the data shown in Figure 2 to answer the following questions.
   (a) For this experiment, the cultures were grown in repressing conditions. What does the term 'repressing conditions' mean?

(b) Why were the cultures grown in minimal medium lacking uracil (SM-Ura or SC-Ura)?
(c) What is the level of invertase expression in the wild-type, *mig1*, *ssn4*, and *mig1 ssn4* double mutant strains? Give numbers.
(d) Describe why this is an example of enhancement.

4. Answer the following questions using the results reported in Table 4.
(a) How do the strains used in Table 4 differ from those used in Figure 2?
(b) Summarize the evidence that *mig1* and *ssn4* exhibit enhancement with regard to the ability of these mutations to suppress *snf1*.

5. The authors examined the relationship of the Mig1 and Ssn6 proteins by examining the genetic interaction of mutant alleles of *MIG1* and *SSN6*. Their model is that Mig1p binds to the *SUC2* promoter and to Ssn6p and Tup1p thereby 'tethering' the Ssn6p–Tup1p repressor complex near the transcription initiation site and inhibiting transcription.
(a) This model suggests that Mig1p and Ssn6p function in the same signaling pathway. What evidence do the authors present in support of this? *Hint*: look in Table 2.
(b) What evidence indicates that *mig1* and *ssn6* mutations do not exhibit enhancement of the glucose insensitive phenotype? *Hint*: look in Table 4.
(c) What evidence indicates that *mig1* and *ssn6* mutations do not exhibit enhancement with regard to their ability to suppress *snf1*? *Hint*: look in Figure 2.

6. The genetic interaction between *mig1* and *ssn6* is functionally different from the genetic interaction between *mig1* and the other *ssn* mutations.
(a) Discuss.
(b) Using the diagram below and arrows (⟶) to represent positive regulation and perpendicular lines (—|) to represent negative regulation, diagram the genetic relationship of *SNF1*, *SSN6*, *MIG1*, and *SSN4*.



ARTICLE 10

Marshall-Carlson, L., L. Neigeborn, D. Coons, L. Bisson, & M. Carlson (1991) Dominant and recessive suppressors that restore glucose transport in a yeast *snf3* mutant. *Genetics* **128**: 505–512.

1. In the last paragraph of the Introduction, the authors say, '. . . we have used a different approach to identify genes that are functionally related to *SNF3*'.
(a) How does the approach used in this article differ from that used in Bisson *et al.* (1987) (Article 7)?
(b) What classes of suppressor mutations are anticipated?

2. Thirty-eight UV-induced revertants of strains carrying either *snf3-72* (a Gly-153 to Arg missense mutation) or *snf3-217* (also believed to be a missense mutation) capable of growth on raffinose were isolated.
   (a) All were recessive. Diagram the cross used to demonstrate this.
   (b) All fell into a single complementation group that the authors called *RGT1* for **r**estores **g**lucose **t**ransport. Diagram the cross between mutant 24 (*rgt1-24*) isolated from strain MCRY168 and strain MCY1520 showing only the *SNF3* and *RGT1* genotype. You must indicate the specific allele of each gene. Give the phenotype of the parents and the diploid with regard to growth on raffinose. There is no need to do a tetrad analysis of this diploid.

3. What evidence indicates that the *rgt1* mutations are not allele-specific suppressors of *snf3*?

4. What evidence indicates that these suppressor mutations are not:
   (a) intragenic suppressor mutations in *SNF3*?
   (b) alleles of *HXT2*, encoding a high-affinity glucose transporter?

5. The authors next set out to isolate dominant suppressors of *snf3*. Again, they selected for the ability to ferment raffinose.
   (a) What critical difference between the strain used in this selection and the strains used to obtain the *rgt1* suppressor mutations enables the authors to select for dominant suppressor mutations?
   (b) In addition, the strain was chosen so as to isolate only by-pass or epistasis suppressors and not allele-specific suppressors. How?
   (c) All their dominant suppressors fell into a single complementation group that the authors called *RGT2*. Diagram the cross between mutant 4 (*RGT2-4*) and strain MCY1710 demonstrates that the mutations fall into a single complementation group. Show only the *SNF3* and *RGT1* genotype of the parents and the diploid. You must indicate the specific allele. Give the phenotype of the parents and the diploid with regard to growth on raffinose.
   (d) What would you expect from a tetrad analysis of the diploids in (c)? In other words, would the tetrads be PD, NPD, and/or TT and what phenotypes would you expect of the haploid spores?

ARTICLE 11

Vallier, L. G., D. Coons, L. F. Bisson, & M. Carlson (1994) Altered regulatory responses to glucose are associated with a glucose transport defect in *grr1* mutants of *Saccharomyces cerevisiae*. *Genetics* **136**: 1279–1285.

Article 11 introduces a new player in the glucose sensing/signal transduction pathway called *GRR1* for **g**lucose **r**epression **r**esistant (see Article 11, References section, for references). *grr1* mutations are pleiotropic. Strains carrying *grr1*

mutations exhibit a number of phenotypic defects. These include glucose-insensitive transcription of the normally glucose repressed *GAL*, *MAL*, and *SUC2* genes; an extremely elongated shape rather than the normal ovoid morphology; slow growth on glucose; increased sensitivity to osmotic stress and nitrogen starvation; decreased divalent cation transport; and growth defects in aromatic amino acid auxotrophs. These pleiotropic defects suggest quite strongly that the Grr1 protein plays a regulatory role in many cellular pathways.

The Grr1 protein is quite large (132 kDaltons) and contains 12, 26-residue leucine-rich repeats. X-ray crystallographic analysis of other leucine-rich repeat proteins demonstrates that the overall structure is that of a thick horseshoe with each of the 12 repeats forming a beta-sheet and these arranged so that they are exposed on the outer surface of the horseshoe curve. Grr1p and other proteins in this class are known to interact with several other proteins by means of these repeats to form large multiprotein complexes. Recent evidence (Li & Johnston, 1997) shows that Grr1p is part of a large protein complex with ubiquitin-protein ligase-like activity and is involved in the degradation of certain proteins, including cell cycle regulators.

1. The results in Figure 1 demonstrate that *grr1*Δ mutant strains are deficient in high-affinity glucose transport. *rgt1-1* partially restores the level of high-affinity glucose transport in the *grr1*Δ mutant strain. The results in Figure 2 are consistent with this finding.
   (a) What phenotype is being observed in Figure 2?
   (b) Why are these results consistent with those in Figure 1 regarding the *grr1*Δ strain and the *grr1*Δ *rgt1-1* double mutant strain?
   (c) Figure 2 shows that the *snf3*Δ mutation enhances the *grr1*Δ phenotype. What result demonstrates this?
   (d) Is *rgt1-1* epistatic to *grr1*Δ? To *snf3*Δ? What data support your conclusion?

2. Specifically, what result reported in Table 3 indicates that *rgt1-1* restores the glucose repression sensitivity of *grr1*Δ mutants?

3. Does *rgt1-1* suppress all *grr1* phenotypes?

4. Briefly, why do the authors say that *grr1* mutations do not by-pass regulation by *SNF1*, *SNF2*, *SNF4*, and *SNF5*?

5. Specifically, what evidence is presented in Table 4 to indicate that *ssn6*Δ mutations suppress the glucose-repression resistance of invertase expression in *grr1*Δ mutation strains?

REFERENCE

Li, F. & M. Johnston (1997) Grr1of *Saccharomyces cerevisiae* is connected to the ubiquitin proteolysis machinery through Skp1: coupling glucose sensing to gene expression and the cell cycle. *EMBO J.* **16**: 5629–5638.

ARTICLE 12

Ozcan, S., T. Leong, & M. Johnston (1996) Rgt1p of *Saccharomyces cerevisiae*, a key regulator of glucose-induced genes, is both an activator and a repressor of transcription. *Mol. Cell. Biol.* **16**: 6419–6426.

1. Describe the method used to clone *RGT1*. Be sure to include the genotype of the host strain, a brief description of the library (vector and genotype of strain providing the insert fragments), the structure of the reporters, and the phenotype used to identify transformant clones containing *RGT1*.

2. How did the authors use targeted integration to demonstrate that they had cloned the real *RGT1* gene?

3. What interesting structural feature of Rgt1p was revealed by its sequence?

4. According to their previous publications, Rgt1p repressed expression of several *HXT* genes encoding glucose transporters. How did they demonstrate that Rgt1p repression of *HXT1* is direct, i.e. results from the binding of Rgt1 protein to the *HXT1* promoter?

5. Figure 3, Panel A, lines 1 and 2 demonstrate that Rgt1p is both a repressor (in the absence of glucose) and an activator (in 4% glucose). Discuss these data.

ARTICLE 13

Ozcan, S., J. Dover, A.G. Rosenwald, S. Wolfl, & M. Johnston (1996) Two glucose transporters in *Saccharomyces cerevisiae* are glucose sensors that generate a signal for induction of gene expression. *Proc. Natl Acad. Sci. USA* **93**: 12428–12432.

1. How was the *RGT2* gene identified?

2. Give the specific data from Table 2 demonstrating that *RGT2* is required for an approximately fivefold increase in *HXT1* expression in 4% glucose.

3. What is the alteration in *RGT2-1*? What is the effect of this same alteration in *SNF3*?

4. How did the authors demonstrate that Rgt2p is upstream of Grr1p?

5. Compare *RGT2* and *SNF3* transcription in cells grown in a medium containing different concentrations of glucose. How was this determined?

ARTICLE 14

Tu, J. & M. Carlson (1995) REG1 binds to protein phosphatase type 1 and regulates glucose repression in *Saccharomyces cerevisiae*. *EMBO J.* **14**: 5939–5946.

1. The results shown in Table I are the meat of this article.
   (a) Diagram the reporter gene used.
   (b) There are eight lines of data shown in Table I. Why are the results in lines 1 and 2 shown?
   (c) Which result indicates that Glc7p and Reg1p bind to each other?
   (d) Which result indicates that the *glc7-T152K* mutation alters a residue in the Reg1p binding site of Glc7p?
   (e) Which result indicates that Gac1p and Glc7p interact? Is the binding strength of this interaction as strong as that between Glc7p and Reg1p and if so why?
   (f) Does the *glc7-T152K* mutation affect Gac1p binding to Glc7p?
   (g) What do the results in lines 7 and 8 indicate?
   (h) Draw a model showing Glc7p indicating the position of the Gac1p and Reg1p binding sites and probable location of the altered residue in the proteins encoded by *glc7-1* and *glc7-T152K*.

2. What other evidence is presented to support the hypothesis that Reg1p and Glc7p bind to each other?

3. Which result in Table II indicates that overexpression of *REG1* suppresses *glc-T152K*? Discuss a possible mechanism of this suppression.

4. Neither glucose repression nor glycogen synthesis is an essential function for yeast cells. Nevertheless, strains carrying null mutations of *GLC7* are inviable. What does this suggest and how might you use the two-hybrid method to explore your hypothesis?

ARTICLE 15

Tu, J., W. Song, & M. Carlson (1996) Protein phosphatase type 1 interacts with proteins required for meiosis and other cellular processes in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **16**: 4199–4206.

1. Diagram the bait fusion gene, the library fusion gene, and the reporter gene constructs.

2. Gip1 protein is a novel protein identified in this study.
   (a) What part of the 573-residue Gip1p is responsible for binding to Glc7p?
   (b) List all of the phenotypes of the *gip1* null mutation.
   (c) At what time during sporulation (which takes about 18–24 hours) is *GIP1* expressed?

(d) Is this consistent with the phenotype of *gip1* mutations? In what way?
(e) Why do the authors suggest that Gip1 protein is a regulator of late meiotic gene expression?

3. Several *GIP* genes were identified in this study.
(a) List the genes other than *GIP1* and *GIP2* that were identified by this two-hybrid analysis as Glc7p interacting proteins.
(b) These genes were not novel but had been identified previously by genetic analysis. What cellular function is proposed for each?
(c) What results in Table 3 indicate that Gip1p, Red1p, and Scd5p (poorly) compete with Reg1p but not Gac1p for an overlapping binding site on Glc7p?

ARTICLE 16

Baker, S.H., D.L. Frederick, A. Bloecher, & K. Tatchell (1997) Alanine-scanning mutagenesis of protein phosphatase type 1 in the yeast *Saccharomyces cerevisiae*. *Genetics* **145**: 615–626.

1. The alanine-scanning *glc7* mutations were generated by *in vitro* site-directed mutagenesis of the cloned *GLC7* gene. These mutant genes were then transferred into the vector pNC160-PP1, a *TRP1* CEN shuttle vector.
(a) How was the dominance/recessiveness to *GLC7* determined?
(b) How did the authors determine whether each mutant allele complemented a *glc7* null mutation?
(c) Several phenotypes were examined for each *glc7* mutation. What were they? Which one is novel and had never been identified as a function of Glc7p?

2. Why was it so important to determine the stability of each of the mutant proteins?

3. Does the inability to detect the *glc7-126*, *glc7-128* or *glc7-295* gene product impact on the interpretation of the finding that these alleles are lethal mutations? What information do the authors present to indicate that this gene product is stable enough in the *in vivo* situation to have been able to complement the *glc7* null if any residual function had been retained?

4. What information was obtained from the *gly7-299°* mutant allele regarding the function of the conserved lysine (K) residues at the C-terminal end of Glc7p? What is the lesson to be learned from this result?

5. (a) Describe the growth phenotype of strains carrying *glc7-109*, *glc7-129*, and *glc7-131*.
(b) Do strains carrying *glc7-129* and *glc7-131* just grow more slowly or is there a specific event in the cell cycle that proceeds more slowly in these mutant strains? Discuss.

6. Alleles *glc7-131* and *glc7-T152K* are both insensitive to glucose repression. Comment on the position of these alterations in the Glc7 protein molecule.

7. In contrast to the clustering of the alterations producing glucose repression resistance and mitotic cell cycle control, the alterations producing a glycogen metabolism defect are scattered over the surface of Glc7p. How do the authors explain this?

8. How are the phenotypes of the *glc7-129* and *glc7-131* alleles similar and how are they different? What does this suggest regarding the role of PP1 in the G2 to M transition?

9. If you were interested in identifying the gene encoding the regulatory protein that binds at the altered site in the *glc7-129* protein product, how might you use suppressor or enhancer analysis to accomplish this goal?

ARTICLE 17

Lutfiyya, L.L., V.R. Iyer, J. DeRisi, M.J. DeVit, P.O. Brown, & M. Johnston (1998) Characterization of three related glucose repressors and genes they regulate in *Saccharomyces cerevisiae*. *Genetics* **150**: 1377–1391.

Two proteins with homology to the Cys_2 His_2 zinc finger DNA-binding domain of Mig1 repressor are found in the *Saccharomyces cerevisiae* genome. Mig2p was identified as a regulator of *GAL* genes (Lutfiyya & Johnston, 1996). Yer028p was found by a homology search of the *Saccharomyces* genome sequence. Previous studies had identified a number of glucose-repressed genes whose promoters contain putative Mig1p binding sites but surprisingly their regulation is unaffected by deletion of *MIG1* (Ronne, 1995). Article 17 tests the possibility that Mig2p and Yer028p control the expression of these genes and identifies genes in the *Saccharomyces* genome that are regulated by Mig1p, Mig2p, and Yer028p.

1. The results in Figure 2 demonstrate that Mig1p and Mig2p are differentially regulated.
   (a) Which specific results indicate that Mig2p is a glucose-responsive repressor of *SUC2*?
   (b) Which specific results indicate that the Mig2 repressor is not regulated by Snf1 kinase.
   (c) Why were Hsl1p, Ycl024p, and Gin4p tested as possible regulators of Mig2p?
   (d) In Table 3, what results would have been expected if Gin4p were 'X', the upstream negative regulator of Mig2p?

2. Describe the experiments showing that Yer028p is a glucose-responsive repressor that represses via interaction with Tup1p and Ssn6p.

3. Describe the analysis carried out to define and compare the sequence of the preferred Mig1p and Mig2p DNA-binding site.

4. The *Saccharomyces* genome was searched for potential Mig1 repressor binding sites.
   (a) Describe the search procedure.
   (b) How were candidate promoters tested for regulation by Mig1p, Mig2p, and Yer028p?

5. DNA microarray analysis was used to identify genes regulated by Mig1p, Mig2p, and Yer028p. Describe the strains used for this analysis (control and experimental) and the criteria used to select candidate target genes.

6. Several novel glucose-regulated genes were identified by this study (see Table 5). You wish to explore the function of these genes and determine whether they are simply targets of glucose regulation or if they participate as glucose regulatory factors. List the biochemical, cell biology and genetic characterizations you would carry out on one gene from Table 5. Explain your reasons for selecting this particular gene for further study.

REFERENCES

Lutfiyya, L.L. & M. Johnston (1996) Two zinc-finger-containing repressors are responsible for glucose repression of *SUC2* expression. *Mol. Cell. Biol.* **16**: 4790–4797.

Ronne, H. (1995) Glucose repression in fungi. *Trends Genet.* **11**: 12–17.

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Case Study II
Secretion, Exocytosis, and Vesicle Trafficking in *Saccharomyces*

READING LIST

Mutant Hunts: To Select or to Screen (Perhaps Even by Brute Force)

Article 1

Novick, P. & R. Schekman (1979) Secretion and cell-surface growth are blocked in a temperature-sensitive mutant of *Saccahromyces cerevisiae*. *Proc. Natl Acad. Sci. USA* **76**: 1858–1862.

Complementation Analysis: How Many Genes are Involved?

Article 2

Novick, P., C. Field, & R. Schekman (1980) Identification of 23 complementation groups required for post-translational events in the yeast secretory pathway. *Cell* **21**: 205–215.

Mutant Hunts and Complementation Analysis (Continued)

Article 3

Deshaies, R.J. & R. Schekman (1987) Yeast mutant defective at an early stage in import of secretory protein precursors into the endoplasmic reticulum. *J. Cell Biol.* **105**: 633–645.

Epistasis Analysis

Article 4

Novick, P., S. Ferro, & R. Schekman (1981) Order of events in the yeast secretory pathway. *Cell* **25**: 461–469.

Article 5

Kaiser, C.A. & R. Schekman (1990) Distinct sets of *SEC* genes govern transport vesicle formation and fusion early in the secretory pathway. *Cell* **61**: 723–733.

Gene Isolation, Characterization, and Multiple Alleles

Article 6

Normington, K., K. Kohno, Y. Kozutsumi, M-J. Gething, & J. Sambrook (1989) *S. cerevisiae* encodes an essential protein homologous in sequence and function to mammalian BiP. *Cell* **57**: 1223–1236.

Article 7

Sadler, I., A. Chiang, T. Kurhara, J. Rothblatt, J. Way, & P. Silver (1989) A yeast gene important for protein assembly into the endoplasmic reticulum and the nucleus has homology to DnaJ, an *Escherichia coli* heat shock protein. *J. Cell Biol.* **109**: 2665–2675.

Article 8

Feldman, D., J. Rothblatt, & R. Schekman (1992) Topology and functional domains of Sec63p, an endoplasmic reticulum membrane protein required for secretory protein translocation. *Mol. Cell. Biol.* **12**: 3288–3296.

Suppression

Article 9

Kurihara, T. & P. Silver (1993) Suppression of a *sec63* mutation identifies a novel component of the yeast endoplasmic reticulum translocation apparatus. *Mol. Biol. Cell* **4**: 919–930.

Article 10

Elrod-Erickson, M.J. & C.A. Kaiser (1996) Genes that control the fidelity of endoplasmic reticulum to Golgi transport identified as suppressors of vesicle budding mutations. *Mol. Biol. Cell* **7**: 1043–1058.

Enhancement

Article 11

Rothblatt, J.A., R.J. Deshaies, S.L. Sanders, G. Daum, & R. Schekman (1989) Multiple genes are required for proper insertion of secretory proteins into the endoplasmic reticulum in yeast. *J. Cell Biol.* **109**: 2641–2652.

More Suppression and Enhancement

Article 12

Scidmore, M.A., H.H. Okamura, & M.D. Rose (1993) Genetic interactions between *KAR2* and *SEC63*, encoding eukaryotic homologues of DnaK and DnaJ in the endoplasmic reticulum. *Mol. Biol. Cell* **4**: 1145–1159.

Article 13

Roberg, K.J., S. Bickel, N. Rowley, & C.A. Kaiser (1997) Control of amino acid permease sorting in the late secretory pathway of *Saccharomyces cerevisiae* by *SEC13*, *LST4*, *LST7* and *LST8*. *Genetics* **147**: 1569–1584.

Gene Isolation and Characterization

Article 14

Espenshade, P., R.E. Gimeno, E. Holzmacher, P. Teung, & C.A. Kaiser (1995) Yeast *SEC16* gene encodes a multidomain vesicle coat protein that interacts with Sec23p. *J. Cell Biol.* **131**: 311–324.

Two-Hybrid Analysis

Article 15

Gimeno, R.E., P. Espenshade, & C.A. Kaiser (1996) COPII coat subunit interactions: Sec24p and Sec23p bind to adjacent regions of Sec16p. *Mol. Biol. Cell* **7**: 1815–1823.

Article 16

Shaywitz, D.A., P.J. Espenshade, R.E. Gimeno, & C.A. Kaiser (1997) COPII subunit interactions in the assembly of the vesicle coat. *J. Biol. Chem.* **272**: 25413–25416.

ARTICLE 1

Novick, P. & R. Schekman (1979) Secretion and cell-surface growth are blocked in a temperature-sensitive mutant of *Saccahromyces cerevisiae*. *Proc. Natl Acad. Sci. USA* **76**: 1858–1862.

Secretion is an essential process. It provides a mechanism for releasing proteins to the extracellular space and for the insertion of integral membrane proteins into the

lipid bilayer of cellular membranes. The process of secretion directs proteins to compartments such as the plasma membrane and vacuole, and provides a pathway for the movement of phospholipids from their site of synthesis to various cellular membranes for membrane growth. Article 1 describes the isolation and phenotypic characterization of *sec1*, the first *Saccharomyces* mutation identified involved in secretion.

The authors follow the synthesis of two enzymes, invertase and acid phosphatase, in their effort to identify secretion mutants. Both are secreted proteins and are found outside the plasma membrane of the yeast cell but are retained in the space between the plasma membrane and the cell wall, called the periplasmic space. The dyes used to assay invertase and acid phosphatase are available to the secreted periplasmic forms of the enzymes but are unavailable to the internal enzyme because the dyes cannot pass across the plasma membrane. Thus, whole cell enzyme assays measure only secreted protein levels. Spheroplasts are made by stripping the cell wall using various enzymes, and this releases all secreted forms of invertase and acid phosphatase. Intracellular levels of these enzymes can be measured using spheroplast lysate extracts.

1. Describe the details of the method used to isolate *sec1*.
   (a) Name the wild-type strain from which *sec1* was obtained.
   (b) Were these mutations spontaneous or induced and why?
   (c) Is this a selection or a screen and why?
   (d) Why were conditional mutants isolated as opposed to mutants that express the mutant phenotype under all conditions?

2. What was the purpose of screening first for temperature-sensitive mutants?

3. Why did the authors screen for the secretion of both acid phosphatase and invertase?

4. How was the rate of protein synthesis determined? Why did the authors focus on mutants that exhibited normal protein synthesis?

5. The results reported in Figures 1 and 2 are consistent with a **reversible** block **late** in the secretory pathway. Discuss.

6. The authors note the accumulation of small vessicles at the nonpermissive temperature in the *sec1-1* mutant strain. What result(s) support their hypothesis that these vessicles are an intermediate in the secretory process?

7. List other phenotypes observed in *sec1-1* mutant strains. What is the significance in the finding that cell division is blocked at the nonpermissive temperature but not at any particular stage?

ARTICLE 2

Novick, P., C. Field, & R. Schekman (1980) Identification of 23 complementation groups required for post-translational events in the yeast secretory pathway. *Cell* **21**: 205–215.

In this article the authors use *sec1-1* mutant cells to improve the isolation method and obtain additional *sec* mutants. Characterization of the new mutants is begun by placing them into complementation groups (genes) and making phenotypic comparisons among strains carrying these different mutant genes.

1. Figure 1 shows the results of a test of the ability of the Ludox density gradient to separate *sec1-1* cells from the wild-type.
   (a) Describe this experimental test.
   (b) What does the open-circle line indicate?
   (c) What does the closed-circle line indicate?
   (d) How were the *sec1-1* cells distinguished from the *SEC1* cells?
   (e) What result in this Figure allows the authors to say that this method allows '*sec1* cells to be separated completely from the *SEC1* cells'?
   (f) Which fraction would you collect in order to isolate potential *sec* mutants?
   (g) Do you expect this method to yield *sec* mutants with a vesicle accumulation phenotype similar to that of *sec1-1* and if so why?
   (h) In using this enrichment method, why is it essential to incubate the mutagenized cells at 37°C for 3 h prior to sedimentation in the Luxor gradient?
   (i) How well does this enrichment method work (see Table 1)?

2. The enrichment method was used to isolate *sec* mutants in strains NF-1R and SF 182-3B. While we are not given this information, NF-1R and SF 182-3B most likely carry different nutritional mutant genes to allow for the selection of diploids. For the purposes of this analysis let us assume that NF-1R has the genotype *MAT***a** *his3* and SF 281-3B has the genotype *MATα leu2*.

   Parents: NF-1R mutant (*MAT***a** *SEC his3*) × SF 281-3B mutant (*MATα sec leu2*)
   sec$^+$ phenotype sec$^-$ phenotype

   (a) Describe the sec$^-$ phenotype.
   (b) What medium would you use to select for the diploid?
   (c) All the *sec* mutants were recessive. What is the Sec phenotype of the diploid?

3. Class A mutants from these two strains were crossed to place them into complementation groups. The mutant cross is shown below.

   Parents: NF-1R mutant (*MAT***a** *sec his3*) × SF 281-3B mutant (*MATα sec leu2*)
   sec$^-$ phenotype sec$^-$ phenotype

   (a) If the *sec* mutants in the cross are in the same complementation group,
      (i) What would be the phenotype of the diploid, sec$^-$ or sec$^+$?

(ii) What results would you expect from tetrad analysis of this diploid? (Give only the sec phenotype of the meiotic products.)

(b) If the *sec* mutants are in different unlinked complementation groups,
(i) What would be the phenotype of the diploid?
(ii) What results would you expect from tetrad analysis of this diploid? (Give only the sec phenotype of the meiotic products.)

4. How many mutant alleles of *SEC5* were isolated?

5. Which *SEC* genes are represented by only a single mutant allele? Do you think that additional new *SEC* genes might be identified if this screen were repeated and if so why?

6. Describe the three classes of organelles that accumulate in the different *sec* mutants at the nonpermissive temperature.

7. What is unique about *sec11* mutants with regard to invertase secretion and secretory organelle accumulation? Why is *sec11* considered a *sec* mutation?

ARTICLE 3

Deshaies, R.J. & R. Schekman (1987) Yeast mutant defective at an early stage in import of secretory protein precursors into the endoplasmic reticulum. *J. Cell Biol.* **105**: 633–645.

The authors of this article are interested in identifying the genes involved in the first step in the secretory pathway, i.e. insertion into the endoplasmic reticulum (ER). *In vitro* studies of protein translocation in the ER using mammalian cells had been quite extensive and productive and several components of the translocation machinery had been identified (for references see Article 3). Moreover, an *in vitro* yeast ER protein translocation assay had recently become available. For a complete analysis of this process and determination of the mechanism of translocation, these authors undertook a genetic approach that would complement the biochemical tools.

The class A *sec* mutations identified in Articles 1 and 2 all appeared to affect late stages in secretion. Only mutations in *SEC53* and *SEC59*, also isolated by this screen, affected earlier steps altering the addition of the oligosaccharide core but not translocation into the ER. In Article 3 the authors develop a new selection method specifically designed to isolate translocation-defective mutants. Their method evolved from a similar protocol used to identify such mutants in *E. coli* that looked for mutants that retained a normally secreted protein in the cytoplasm.

Before reading this article, it would be helpful to learn some basics regarding the genes used in the selection. Some details are reviewed in the text of Article 3 and here, but more information is available in the references listed below. *HIS4* encodes a large multifunctional polypeptide that catalyzes the third (domain A of His4p), second (domain B of His4p), and tenth (domain C of His4p) steps in the synthesis of histidine. Domain 4C is histidinol dehydrogenase. This enzyme is normally cytoplasmic.

SUC2 encodes invertase, a glycosylated secreted enzyme that hydrolyzes sucrose to glucose and fructose. *SUC2* produces two different sized mRNAs depending on the growth condition because different transcription start sites are used. In low glucose (<0.5%) the mRNA is longer at the 5' end and encodes an invertase with an N-terminal signal sequence. In high glucose (1%–5%) the mRNA is shorter and the encoded invertase lacks the signal sequence and remains cytoplasmic. The cytoplasmic species of invertase is synthesized in both growth conditions while the secreted form is present only in low glucose, so-called derepressed, conditions. Glycosylation of secreted invertase occurs in several steps carried out in the ER and Golgi. Thus, monitoring the extent and type of oligosaccharide additions to invertase allows one to follow its transit through the secretory process.

MFα1 encodes the secreted mating pheromone α-factor. The nacent product of *MFα1* is called prepro-α-factor and consists of an N-terminal signal sequence for secretion, an approximately 60-residue region containing three potential glycosylation sites, and four tandem copies of the 13-residue α-factor. Between these four copies are spacer residues that are the sites of proteolytic cleavage used to separate the individual pheromone molecules.

Cells that are blocked in their ability to translocate protein into the ER (tunicamycin or *sec* mutants) accumulate this primary translation product. Core glycosylated forms of prepro-α-factor are formed in the ER and the signal sequence is still present. Proteolytic processing begins in the Golgi but mature α-factor is seen in late secretory vesicles.

1. Figures 2A and 3A show the three protein products expressed by the two constructions *SUC2–HIS4* and *MFα1–SUC2–HIS4*. The results shown in Figures 2B and 3B test the premise of the selection strategy.
   (a) What allele of *HIS4* and *HOL1* is present in the host strain DYFC2-12B and why?
   (b) Which of the two fusion proteins shown in Figure 2A (the one with or the one without the N-terminal signal sequence) is synthesized in DYFC2-12B transformed with *SUC2–HIS4* and why? To which subcellular compartment is it localized and why? Why is this test essential in their evaluation of the potential of the selection method?
   (c) The result in Figure 3B indicates that the prepro-α-factor–invertase–His4 fusion protein is unable to complement *his4Δ*. The authors propose that this is because the fusion protein is localized to the ER. How do the results in Figure 4 support this conclusion?

2. Describe the growth conditions used to select for mutants defective in ER translocation at 30°C.
   (a) Why was 30°C used?
   (b) In round I, 440 histidinol prototrophs were obtained and these were screened for temperature-sensitive growth on a rich medium at 37°C. Why?
   (c) Why was plasmid pαSHF8 cured from the five isolates which were then only to be retransformed with unmutagenized pαSHF8?

3. Genetic analysis of these five isolates was complicated by the fact that, unknowingly, all were diploid. Haploid *MAT***a** and *MAT*α strains carrying each mutant were isolated by genetic methods.
   (a) Diagram the cross between a mutant *MAT***a** strain and a wild-type *MAT*α strain. Give the genotype and phenotypes of the parents and the diploid if the mutant allele is recessive. Or dominant.
   (b) Give the results of tetrad analysis of the diploid (genotype and phenotype of the four meiotic products). Assume that each mutant strain contains only a single recessive alteration and all phenotypes segregate with the single mutant allele.
   (c) How might the results of the tetrad analysis differ if the mutant strain carried two different unlinked alterations if only the double mutant exhibited the mutant phenotype? If either mutant alone exhibited the mutant phenotype?

4. All the isolates carried single recessive mutations.
   (a) Diagram a cross between mutants 2 and 5 showing that both are in the same complementation group. Give the genotype and phenotype of parents and diploid.
   (b) Diagram a cross between mutants 2 and 5 showing that they are in different complementation groups. Give the genotype and phenotype of the parents and the resulting diploid.
   (c) For the cross in part (a), show the results of tetrad analysis of the diploid.

5. The five mutants isolated in round I did not complement. Thus, they are mutations in the same gene that the authors called *sec61*. Round II produced an additional seven isolates all of which complemented the *sec61* mutants. The authors conclude that a second gene, *SEC62*, has been identified. Could there be more genes with a similar mutant phenotype and how would you determine this? What is unusual about these results? Are these necessarily independent mutations?

6. Describe the phenotypes of *sec61* with regard to the following proteins: α-factor, invertase, carboxypeptidase Y. Where is CPY localized in yeast cells?

REFERENCES

Carlson, M. & D. Botstein (1982) Two differentially regulated mRNAs with different 5' ends encode secreted and intracellular forms of yeast invertase. *Cell* **28**: 145–154.

Donahue, T.F., P.J Farabaugh, & G.R. Fink (1982) The nucleotide sequence of the *HIS4* region of yeast. *Gene* **82**: 47–59.

Julius, D., R. Schekman, & J. Thorner (1984) Glycosylation and processing of prepro-alpha-factor through the yeast secretory pathway. *Cell* **36**: 309–318.

Keesey, J.K. Jr, R. Bigelis, & G.R. Fink (1979) The product of the *HIS4* gene cluster in *Saccharomyces cerevisiae*. A trifunctional polypeptide. *J. Biol. Chem.* **254**: 7427–7433.

Kurjan, J. & I. Herskowitz (1982) Structure of a yeast pheromone gene (*MF*α): a putative α-factor precursor contains four tandem copies of mature α-factor. *Cell* **30**: 933–943.

ARTICLE 4

Novick, P., S. Ferro, & R. Schekman (1981) Order of events in the yeast secretory pathway. *Cell* **25**: 461–469.

The secretory pathway can be viewed as a substrate-dependent pathway. Genetic analysis to date has not identified negative regulators. The secreted protein is the most upstream substrate. This is then acted on by a series of Sec proteins that act on the target protein in a stepwise fashion to translocate into the ER, move it to the Golgi where it traverses the various Golgi compartments, and then package for localization to either the cell surface or the vacuole. During this process signal sequences may be removed, the target protein may be glycosylated to varying extents, and other modifications may occur. Nonetheless, because each step must be successfully completed before the next step can occur, this is a substrate-dependent pathway. In Articles 4 and 5 the authors use epistasis analysis of this substrate-dependent pathway to determine if the *SEC* genes lie in a single pathway (called an epistasis group) and to order the *SEC* genes in this pathway.

1. To carry out an epistasis analysis one must be able to distinguish differences among the phenotypes of the different mutant genes. The authors used two *sec* mutant phenotypes in this study.
   (a) Describe the different intermediate secretory organelles accumulated in the different *sec* mutants.
   (b) Describe the intermediates in invertase glycosylation. Include the technique used to distinguish these intermediates.

2. The *SEC* genes are unlinked. The cross shown below was used to construct the *sec1-1 sec7-1* double mutant strain.
   (a) Give the *SEC* genotype and phenotype (temperature sensitivity) of the PD, NPD, and TT tetrads derived from this diploid.
   (b) Which of these haploid segregants would you choose in order to be certain that it is a double mutant?

   Parents: *MAT***a** *sec1-1 SEC7 his3* (sec$^-$ phenotype) × *MATα SEC1 sec7-1 leu2* (sec$^-$ phenotype)

   Diploid: *MAT***a**/*MATα sec1-1*/*SEC1 sec7-1*/*SEC7* (sec$^+$ phenotype)

3. Based on the results reported here, the authors conclude that the *SEC* genes are in a single pathway and not separate parallel pathways.
   (a) Discuss, referring to the four models of epistasis described in Chapter 6.
   (b) If two of the *sec* mutants were in parallel pathways, a unique phenotype would have been exhibited. What do the authors suggest as this unique phenotype?

4. Which *sec* mutant is epistatic in each of the following gene pairs:
   (a) *sec2-56* and *sec7-1*?

(b) *sec9-4* and *sec7-1*?
(c) *sec14-3* and *sec7-1*?
(d) Why did the authors place *SEC7* and *SEC14* at the same Golgi to late secretory vesicle step and *SEC2* and *SEC9* at the post late secretory vesicle step?

5. Which *sec* mutant is epistatic in each of the following gene pairs:
   (a) *sec19-1* and *sec7-1*?
   (b) *sec19-1* and *sec18-1*?
   (c) Why do you think the authors did not include *SEC19* in Figure 6?
   (d) Where might you place it in the pathway and why?

ARTICLE 5

Kaiser, C.A. & R. Schekman (1990) Distinct sets of *SEC* genes govern transport vesicle formation and fusion early in the secretory pathway. *Cell* **61**: 723–733.

1. The authors of Article 5 uncovered a subtle phenotypic difference among the nine *sec* mutants described by Novick *et al.* (1981) (Article 4) as acting at the ER to Golgi step in secretion.
   (a) Describe this phenotype.
   (b) Which four *sec* mutants fall clearly into Class I and define this phenotype?
   (c) Which three *sec* mutants fall clearly into Class II and define this phenotype?
   (d) What do the authors mean when they say that the *sec* mutant phenotype was 'not allele specific'?

2. 'Double mutants were constructed by genetic crosses.' Give an example.

3. The data in Figure 3 indicate that Class I mutants are epistatic to Class II mutants. Discuss.

4. These results (Figure 3) are consistent with the hypothesis that Class I genes are involved in the formation of 50 nm vesicles and that Class II genes are involved in their consumption. Discuss.

The last part of Article 5 involves a genetic interaction study. We will return to this section of Article 5 later in the series after Article 10.

ARTICLE 6

Normington, K., K. Kohno, Y. Kozutsumi, M-J. Gething, & J. Sambrook (1989) *S. cerevisiae* encodes an essential protein homologous in sequence and function to mammalian BiP. *Cell* **57**: 1223–1236.

BiP is an ER localized member of the HSP70 (heat shock protein 70) family. Studies of BiP function in mammalian cells showed that it bound transiently to a number of nascent secreted proteins and strongly to improperly folded proteins blocking their exit from the ER. As such, it was hypothesized that BiP played a very important, possibly essential cellular function and would likely be evolutionarily conserved. The mammalian BiP gene had been cloned, and this article describes the use of this gene to identify the yeast BiP gene.

Table 1 describes the results of quantitative mating assays between strains carrying mutations in *KAR2*. The assay is described in detail in Rose & Fink (1987). Matings involving *kar2* mutants are defective in nuclear fusion, karyogamy (see Chapter 3). The cytoplasms of the mating partners fuse and cytoplasmic organelles such as the mitochondria become distributed throughout the common cytoplasm. However, the resulting zygote contains two haploid nuclei and cell division results in haploid products containing one or the other of these two nuclei.

In a quantitative mating assay, cytoplasmic fusion is monitored by following the status of the mitochondrial DNA (mtDNA). A [*rho*+] strain contains wild-type mtDNA, has functional mitochondria, and forms large (*grande*) creamy-colored colonies on YPD media. A [*rho*–] strain has lost its mtDNA, lacks functional mitochondria, and forms small (*petite*) colonies on YPD because it cannot utilize the products of glucose fermentation. Ethidium bromide interferes with mtDNA replication and growth in ethidium bromide is commonly used to produce [*rho*–] strains. In addition to colony size, one can distinguish [*rho*–] from [*rho*+] by its interaction with the *ade2* mutation. An *ade2* [*rho*+] strain is pink in color while an *ade2* [*rho*–] strain is white.

The genotypes of the strains used in a typical quantitative mating assay are given below:

*MAT***a** *kar2 ade2 LYS2* [*rho*–]	×	*MAT KAR2 ADE2 lys2* [*rho*+]
white colonies		pink colonies

If mating is complete, both nuclear and cytoplasmic fusion occurs and diploid ade+ lys+ prototrophs will be produced that form creamy-white colonies on YPD. If only cytoplasmic fusion occurs, no prototrophs will be produced and the process is referred to as cytoduction. When these zygotes divide by mitosis they produce some *MAT***a** *kar2 ade2 LYS2* [*rho*+] progeny, called cytoductants, which are pink. In a typical mating such as the one shown above nuclear fusion is usually not 100% defective but is only partially defective. This is quantified by calculating the ratio of cytoductants to prototrophs resulting from the mating. The number of cytoductants is determined by the number of pink colonies and the number of prototrophs is determined by the number of colonies growing on the appropriate selective medium. In the case shown above this would be SM lacking adenine and lysine.

1. The authors tested a number of hybridization conditions using Southern analysis to adjust the stringency at which they would screen their library. They settled on conditions that detected one strongly hybridizing fragment plus several weakly hybridizing fragments.
   (a) What were these conditions?
   (b) Why were they not able to use the typical high stringency conditions?

(c) What did they hope was the single strongly hybridizing fragment? The several weak fragments?

2. The yeast BiP gene was initially isolated from a cDNA library.
   (a) Describe a cDNA library. Why is this type of library usually not used for *Saccharomyces* studies?
   (b) Describe the first probe used to screen the library.
   (c) Two hundred potential BiP gene clones were obtained from the initial screening of 20 000 lambda clones. These were screened at high stringency with a second probe. Describe the probe and the reason for doing this second screen.
   (d) Of the 20 clones that passed the second screen, 12 were sequenced and only one was novel. What were the other 11 and why were these not eliminated by the second screen? Other than sequencing, how might these 11 clones be excluded and found to contain other known HSP70 genes?
   (e) How was a full-length copy of the yeast BiP gene obtained (Figure 2C)?

3. What sequence features of yeast BiP suggest that it is an ER localized protein and thus differs significantly from its close homologues Ssb1p and the other HSP70 proteins that are found in the cytoplasm?

4. What does it mean when the authors say that yeast BiP exhibits 67% identity and 84% similarity to mammalian BiP?

5. How did the authors determine that their yeast BiP gene was identical to *KAR2*? What is *KAR2*?

6. Describe how the authors demonstrated that *KAR2* is an essential gene.

7. Describe the experiment used to demonstrate that the N-terminal 42 residues of yeast BiP function as a signal sequence to localize BiP to the lumen of the ER.

8. Diagram the mammalian BiP expression constructs in plasmids YEpCup-MB and YEpCup-FusB.
   (a) Why is the *CUP1* promoter used?
   (b) How do the protein products of these two constructs differ?

9. Strains carrying mutations in *KAR2* are temperature sensitive for growth.
   (a) Does mammalian BiP complement the ts phenotype of *kar2-1*?
   (b) Does it complement as well as the cloned yeast BiP gene and what is the evidence?

10. Strains carrying mutations in *KAR2* exhibit a defect in karyogamy; that is, mating cells fuse their cytoplasms but not their nucleii. True diploids are not formed but mitochondria are exchanged.

(a) Diagram the cross from Table 1 between W303-2/none and SEY6210. [Note that there is a typo in the table legend. SEY6210 is a '*MATα*' tester strain.]
(b) What do the numbers presented in Table 1 represent?
(c) Strains W303-2 and KNH2/YEpCup-YB are positive controls in this experiment but differ. Discuss.
(d) Strain KNH2/YEpCup is a negative control. Discuss.
(e) What data demonstrate that reduced expression of yeast BiP only partially complements *kar2-1*?
(f) What data demonstrate that expression of mammalian BiP partially complements *kar2-1*?

11. What is the significance of showing that mammalian BiP complements *kar2-1*?

12. What unique features (sequence, expression, localization, etc.) are shared by Kar2p and mammalian BiP that distinguish them from the other HSP70 proteins?

REFERENCE

Rose, M. & G. Fink (1987) *KAR1*, a gene required for function of both intranuclear and extranuclear microtubules in yeast. *Cell* **48**: 1047–1060.

ARTICLE 7

Sadler, I., A. Chiang, T. Kurhara, J. Rothblatt, J. Way, & P. Silver (1989) A yeast gene important for protein assembly into the endoplasmic reticulum and the nucleus has homology to DnaJ, an *Escherichia coli* heat shock protein. *J. Cell Biol.* **109**: 2665–2675.

This article describes the cloning of yeast *SEC63*. It also illustrates how the same gene can be identified in different selection/screens that use different phenotypes as the basis of the selection/screen. The authors had developed a selection method for mutations in genes involved in nuclear protein localization. They constructed a gene encoding a hybrid cytochrome c1 protein fused in-frame to the nuclear localization signal (NLS) from Gal4 protein or the SV40 T antigen, and this fusion was expressed from the high-level constitutive *ADH1* promoter. Cytochrome c1 is encoded by *CYC1*, synthesized by cytoplasmic ribosomes, and post-translationally enters the mitochondrion for functions in the electron transport chain. Mutants in *CYC1* are unable to grow on nonfermentable carbon sources. Gal4p is the transcription activator of the *GAL* genes and is normally found in the nucleus. SV40 T antigen is also a nuclear protein.

The NLS cytochrome c1 fusion protein localized to the nucleus was so abundantly expressed that it was toxic. The authors reasoned that mutants with alterations in the nuclear transport system might be resistant to the toxic effects of the fusion protein and also might allow some entry into the mitochondrion by default. As a result these mutant strains would be able to grow on a nonfermentable

carbon source. Mutants were selected based on their ability to grow on glycerol at 30°C where partially functional mutant proteins could lead to reduced rates of nuclear localization. Since nuclear localization is an essential function the mutants were secondarily screened for temperature-sensitive growth at 37°C where the altered product would be completely nonfunctional. Mutations in three so-called *NPL* genes were isolated.

1. The immunofluorescence studies of the NLS cytochrome c1 fusion proteins suggested that they were found 'at the nucleus and opposed to the nuclear envelope'. Based on this, the authors suggested that the *npl* mutant genes could be involved in the early stages of ER localization. This was tested and *NPL1* was found to be *SEC63*.
   (a) Diagram the cross showing that *npll-1* and *sec63-1* are alleles. Give the genotype and phenotype of the parent strains, the diploid, and the results of tetrad analysis.
   (b) What phenotype was assayed in the tetrads?

2. *NPL1* was cloned by complementation of the ts-lethal phenotype of *npl1-1*.
   (a) Describe the library.
   (b) Give the genotype of the host strain.
   (c) What phenotype was used to select for transformants carrying a library plasmid?
   (d) What phenotype was used to screen transformants for those potentially carrying the *NPL1* allele?
   (e) Why is it essential to recover the plasmid from these transformants and reintroduce it into the *npl1-1* strain?

3. Deletion analysis of plasmid pTK1 localized *NPL1* within the 6 kbp yeast insert. Describe how this was done.

4. Targeted integration was used to demonstrate that the cloned complementing fragments truly contained *NPL1/SEC63* and not a suppressor gene.
   (a) Describe what was done. Be sure to include a diagram of the integrating plasmid and diagram the cross to the *npl1-1* strain.
   (b) Why was this process repeated with a *sec63-1* strain?

5. Describe the method used to demonstrate that *NPL1/SEC63* is essential.

6. Do *npl1-1*, *npl1-2*, and *sec63-1* exhibit a similar phenotype with regard to CPY maturation? Are these null alleles? Discuss the significance with regard to the finding that *SEC63* is an essential gene.

7. Does *sec63-1* exhibit an *npl* phenotype?

8. Discuss the following 'striking' sequence features of Sec63 protein.
   (a) The three potential membrane-spanning domains.

(b) The homology to the DnaJ protein of *E. coli*.
(c) The C-terminal 52 residues.

ARTICLE 8

Feldman, D., J. Rothblatt, & R. Schekman (1992) Topology and functional domains of Sec63p, an endoplasmic reticulum membrane protein required for secretory protein translocation. *Mol. Cell. Biol.* **12**: 3288–3296.

Sequence analysis of Sec63 protein suggested that this is an integral membrane protein and, given its function in the early stages of ER translocation, Sec63p is probably localized to the ER. This must be experimentally determined. In addition the functional importance of the DnaJ-like domain needs to be demonstrated as well as its subcellular location. That is, is the DnaJ domain exposed to the cytoplasmic or lumenal side of the ER membrane? Article 8 resolves these questions using some noteworthy molecular genetic techniques.

1. Describe briefly how the anti-Sec63p antibody was obtained. Would this method be possible without the cloned *SEC63* gene? Can it, or a related method, be applied to any cloned gene?

2. Describe the position of the ER in yeast.

3. Why is it important for the authors to demonstrate that overproduction of Sec63p does not result in mislocalization?

4. Discuss the results in Figure 3.
   (a) Indicate the effect of each of the different treatments on soluble, membrane-associated, and integral membrane proteins.
   (b) Why was Sec62p included in this experiment?
   (c) Why was Sec23p included in this experiment?

5. The topology of Sec63p was explored using sensitivity to protease digestion and constructing Sec63–Suc2 fusion proteins. Both methods lead to the same conclusion.
   (a) Diagram the protein products produced by the fusion constructions Δ28, Δ529, Δ608, Δ610, and the vector construct. Use the diagram in Figure 5A as a guide.
   (b) Sequence analysis in Article 7 indicated that there are three potential glycosylation sites just after (C-terminal to) the third hydrophobic region. Based on the results in Figure 5B lanes 1 and 2, is Sec63p protein glycosylated at these sites?
   (c) Could this analysis have been done with invertase if it were not known to be glycosylated?
   (d) How might this analysis been done with *SEC63–HIS4* fusions?

6. *SEC63* mutations of two types were constructed. The first consisted of a series of in-frame *SEC63–SUC2* fusions to different sites in the *SEC63* ORF. The second series were point mutations in *SEC63* altering a single residue in the DnaJ domain. Both were tested for their ability to complement *sec63* mutations. The results are given in Figure 5.
   (a) Describe the method used to test complementation of the *sec63-1* temperature-sensitive allele.
   (b) *SEC63* is an essential gene. Thus, strains carrying the *sec63*Δ null allele are inviable. Complementation of *sec63*Δ by the mutant allele, which could be null alleles, using the method described in part (a) is not possible. Describe the method used to test complementation of the *sec63*Δ null allele.
   (c) Just prior to the publication of this article, the genes encoding several DnaJ domain-containing proteins were sequenced. What role did this play in the mutation analysis described here?

7. The authors propose that the DnaJ domain of Sec63p is required for its function in protein translocation. Why is it important for their argument to demonstrate that Sec63p complex formation is normal in the DnaJ domain mutants?

ARTICLE 9

Kurihara, T. & P. Silver (1993) Suppression of a *sec63* mutation identifies a novel component of the yeast endoplasmic reticulum translocation apparatus. *Mol. Biol. Cell* **4**: 919–930.

The Sec63 protein is part of an ER-localized complex required for the translocation of secreted proteins into the ER. Additional components of this complex were identified along with *SEC63* from genetic selections for mutants defective for translocation. Article 9 uses suppressor analysis to identify new components that interact physically with Sec63p. To be specific, they interact with the cytoplasmic domain of Sec63p.

1. What is unique about *sec63-1* compared with the other *sec63* alleles (*sec63-101*, *sec63-106*, *sec63-107*, *sec63-108*)?

2. The authors give two reasons for using *sec63-101* in this selection.
   (a) Why is it important to have a 'tight' ts-lethal phenotype? By 'tight' the authors mean that there is no leak-through growth at the nonpermissive temperature.
   (b) What is the significance to knowing that *sec63-101* alters a single residue in the cytoplasmic domain?

3. Describe the library used to isolate multicopy suppressors of *sec63-101*. Include the type of library (genomic or cDNA), the vector, the estimated copy number

of these plasmids in yeast, the site of insertion of the yeast DNA fragments, and the selection yeast marker.

4. The size of the yeast genome is about 12.6 Mbp. The average insert size of the yeast inserts in the Nasmyth library is 10 kbp.
   (a) What is the minimum number of library plasmids needed to represent the entire genome?
   (b) Statistically, why do you really need more than this minimum?
   (c) The authors screened 6000 transformants, or by their estimate about three genomes worth. Two plasmids were isolated containing *SEC63* in the insert fragment. Based on this information, is their estimate reasonable?

5. Plasmid pHSS20 contains an approximate 8 kbp yeast DNA insert which, on average, could contain three or more genes. Describe how the suppressing gene, called *HSS1*, was localized within this DNA fragment.

6. Figure 1B demonstrates that multicopy *HSS1* specifically suppresses *sec63-101*. For each mutant host strain, two transformants carrying either vector, pHSS1, or 'wild-type' were tested for growth at the nonpermissive temperature.
   (a) What is the purpose of the 'vector' transformants?
   (b) What is meant by 'wild-type' plasmid and why was this included?
   (c) What result demonstrates that *HSS1* only suppresses *sec63-101*?

7. Strains carrying a null mutation of *HSS1* are temperature sensitive for growth. How was this demonstrated?

8. Describe the phenotype of the *hss1*Δ mutant versus the *HSS1* strain with regard to invertase and Kar2p precursor accumulation. How does this compare with *sec63-101* mutant strains?

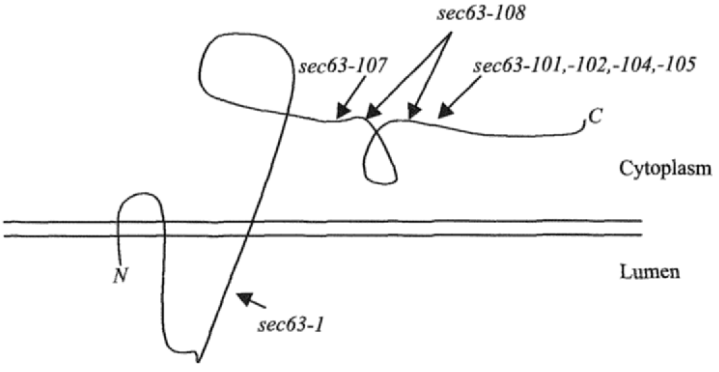
9. Does the *hss1*Δ mutant affect nuclear localization? How does this compare to *sec63-101* mutant strains?

10. Define epitope tagging. Prior to this method, what methods were used to obtain antibody to a protein of interest for the purpose of subcellular localization studies or Western analysis? Discuss some of the technical drawbacks to each method.

11. Describe how the authors used *in vitro* mutagenesis to demonstrate the topology of Hss1 protein in the ER membrane.

12. What is the mechanism of suppression (allele-specific, by-pass, or by epistasis) by which high-copy *HSS1* suppresses *sec63-101*? Draw a model showing Sec63p and Hss1p (Sec66p) that demonstrates the basis of this suppression.

13. The diagram below (adapted from Nelson *et al.*, 1993) indicates the location of each alteration in Sec63 protein in the *sec63* mutations. The topology of Sec63p is shown. Mutations *sec63-101*, *sec63-102*, *sec63-104*, and *sec63-105* all have the same change, Gly511Arg. Allele *sec63-108* contains two alterations, Ile431Asn and Pro503Ala. The alteration in mutation *sec63-1* is Ala179Thr and in *sec63-107* is Pro426Asn.



Based on this diagram, are you surprised that *sec63-1* is not also suppressed by *HSS1* in high copy? Explain.

REFERENCE

Nelson, M.K., T. Kurihara, & P.A. Silver (1993) Extragenic suppressors of mutations in the cytoplasmic C terminus of *SEC63* define five genes in *Saccharomyces cerevisiae*. *Genetics* **134**: 159–173.

ARTICLE 10

Elrod-Erickson, M.J. & C.A. Kaiser (1996) Genes that control the fidelity of endoplasmic reticulum to Golgi transport identified as suppressors of vesicle budding mutations. *Mol. Biol. Cell* **7**: 1043–1058.

Article 10 looks at the issue of sorting secreted proteins from ER and Golgi resident proteins during the secretion process. ER localized proteins such as Sec63p and Kar2p are targeted to the ER during their synthesis and maturation and are retained in the ER. At the same time, secreted proteins such as invertase that also localize to the ER remain there only transiently before being segregated to vesicles and moved progressively through the secretory pathway. Evidence outlined in the Introduction to this article suggests that secreted proteins are actively concentrated into vesicles while ER proteins are selectively removed. A short ER retention signal sequence, HDEL, has been identified. The proteins involved in cargo selectivity and fidelity are unknown.

Article 5 reported that Sec12p, Sec13p, Sec16p, and Sec23p are involved in an early step in ER to Golgi vesicle formation. Sec24p, Sec31p, and Sar1p have

recently been added to this list. All are components of the vesicle coat referred to as COPII and it is thought that assembly of this complex drives vesicle formation. Both Sec13p and Sec23p have mammalian homologues suggesting that they play a central evolutionarily conserved role. Mutation of *SEC13* blocks vesicle formation. The hypothesis of this study is that the *sec13* block is not structural; that is, not because COPII with mutant Sec13p is structurally defective or does not assemble properly. The authors suggest the possibility that vesicle formation is blocked because some other protein(s) senses that cargo selectivity is defective and is blocking the formation/release of vesicles containing improper cargo. Such proteins would be negative regulators of vesicle formation and loss of this function should by-pass *sec13* mutants.

1. Why do the authors choose *sec13*Δ rather than a *sec13-ts* for this suppressor selection scheme?

2. *SEC13* is an essential gene. Thus, the authors could not simply construct a *sec13*Δ strain and select for suppressors. Describe the strain used for this selection and the method designed to identify *sec13*Δ by-pass suppressors. (*Note*: 5FOA is 5-fluoro orotic acid. In strains carrying the wild-type *URA3* gene, the Ura3p enzyme converts 5FOA to a toxic compound and thus *URA3* strains die on a medium containing 5FOA. In strains in which the *URA3* gene is carried on a plasmid, cells that have lost the plasmid, and thus the *URA3* allele, will survive. Therefore, researchers use growth on 5FOA for selecting *ura3* strains.)

3. How were the potential by-pass suppressor strains screened to determine the following?
   (a) That the plasmid-borne *SEC13* gene was not needed for growth; that is, the suppressor mutation by-passed *SEC13*.
   (b) That the *sec13*Δ mutation was still present.
   (c) That the suppressor mutations were recessive.
   (d) That the by-pass suppressor mutations identified fell into three complementation groups. Give the genotype (for *BST* and *SEC13* genes) and phenotype (viable/nonviable) of the parents and the resulting diploid.

4. Strains carrying the *bst* mutants alone were constructed by crossing the *bst sec13*Δ mutants to wild-type (*BST SEC13*).
   (a) Give the genotype and phenotype (leu+/leu– and viable/nonviable) of the spores in a PD, NPD, and TT tetrad. (Since the phenotype of a *bst SEC13* strain would not be known at the outset of this experiment, use a ?.)
   (b) If a *SEC13 bst* strain is viable, what would you expect to find in a PD, NPD, and TT tetrad regarding the number of viable spores and their leucine phenotype?
   (c) If a *SEC13 bst* strain is *not* viable, what would you expect to find in a PD, NPD, and TT tetrad regarding the number of viable spores and their leucine phenotype?

(d) Given that a *SEC13 bst* strain is viable, and the viable spores in a TT tetrad were crossed to a *bst sec13*Δ strain, what results would you expect with each of the different genotypes?
(e) Given that a *SEC13 bst* strain is viable, and the viable spores in a TT tetrad were each transformed with an integrating plasmid that knocks out the *SEC13* gene, what results would you expect with each of the different genotypes?
(f) Were both tests necessary or would just (d) or just (e) have been sufficient? Explain.

5. What novel phenotype is exhibited by the *bst* and *bst sec13*Δ strains?
   (a) How was this assayed?
   (b) How was this novel phenotype used to clone *BST1*?
   (c) Describe the integrative disruption of *BST1*.
   (d) What is the significance of the finding that 'the alleles of *bst1* isolated as suppressors of *sec13*Δ have the same phenotype as the disrupted allele'?

6. How did the authors use their analysis of Bst1p glycosylation patterns to predict the topology of this integral membrane protein?

7. Residues of HDEL are a signal recognized by the Erd2p-dependent retention system. What result suggests that the Bst proteins are involved in a distinct retention system that is independent of the HDEL-dependent retrieval pathway?

8. Double *bst* mutant strains were constructed and the Kar2p secretion phenotype of the double strains was compared with the single *bst* mutant strains. Using the epistasis models described in Chapter 3 for a substrate-dependent pathway, suggest a functional relationship for Bst1p, Bst2p, and Bst3p.

9. The authors 'tested the ability of the *bst* mutations to suppress null alleles of other *SEC* genes in crosses segregating both a *bst* mutation and a null *SEC* allele'. Given the fact that all of the *SEC* genes are essential, suggest a straightforward way of demonstrating this.

10. Discuss the preferred model for Bst protein function in ER protein sorting.

ARTICLE 5 (continued)

The questions that follow are based on the last portion of Article 5.

11. List all synthetic lethal combinations revealed by the results in Table 3. Are these results consistent with the epistasis analysis that placed *SEC12*, *SEC13*, *SEC16*, and *SEC23* in the Class I epistasis group, and *SEC17*, *SEC18*, and *SEC22* in the Class II epistasis group? Discuss.

12. The authors do not feel that the results in Table 4 support allele-specific synthetic lethal interactions. What results would have supported allele-specificity? What would this have suggested with regard to the function of these gene products modeled in Figure 5 that eludes the authors, at least for the time being?

ARTICLE 11

Rothblatt, J.A., R.J. Deshaies, S.L. Sanders, G. Daum, & R. Schekman (1989) Multiple genes are required for proper insertion of secretory proteins into the endoplasmic reticulum in yeast. *J. Cell Biol.* **109**: 2641–2652.

Several of the previous articles describe the structure and function of Sec63 protein demonstrating it to be an ER-localized integral membrane protein required for an early step in the translocation of secreted proteins into the lumen of the ER. This article presents evidence that the *SEC61*, *SEC62*, and *SEC63* gene products act at a similar step in the translocation process.

1. The authors modified the selection method presented in Article 3 (Deshaies & Schekman) to identify additional genes, if any, that affect an early step in ER translocation. Why? What was the modification?

2. Table II presents the results of this revised and repeated selection.
   (a) How many mutant alleles of *SEC61*, *SEC62*, and *SEC63* were obtained?
   (b) Do you think it likely that additional *SEC* genes of this type will be identified if the selection is repeated? Explain your answer.

3. Based on the results presented in this article, make a list that compares the phenotypes of sec*61*, sec*62*, and sec*63* mutants with regard to α-factor accumulation and processing, CPY processing, and invertase and acid phosphatase modification and signal sequence cleavage. Can you clearly distinguish mutations in these genes based on their processing defects?

4. The authors looked for genetic interactions between mutant alleles of *sec61*, *sec62*, and *sec63* in the form of synthetic enhancement. Crosses between *sec61* and *sec62*, *sec61* and *sec63*, and *sec62* and *sec63* were made and dissected. The results are shown in Tables III and IV.
   (a) What results indicate that the double mutants (*sec61 sec62*, *sec61 sec63*, and *sec62 sec63*) are inviable at 24°C and exhibit reduced viability at 17°C?
   (b) Do the double mutant strains exhibit an enhancement of the processing defects (CPY, invertase, acid phosphatase) compared with the single mutant strains? If so, how is this enhancement manifested?

5. The authors propose two interpretations of their results. First, Sec61p, Sec62p, and Sec63p form a complex or act on each other to carry out a common

function. Second, these proteins act in parallel pathways and carry out similar functions.

(a) Why do the authors suggest that their second model is unlikely?

(b) Which allele of *sec63* was used in these crosses? Would using *sec63-101* potentially provide interesting information and if so why?

ARTICLE 12

Scidmore, M.A., H.H. Okamura, & M.D. Rose (1993) Genetic interactions between *KAR2* and *SEC63*, encoding eukaryotic homologues of DnaK and DnaJ in the endoplasmic reticulum. *Mol. Biol. Cell* **4**: 1145–1159.

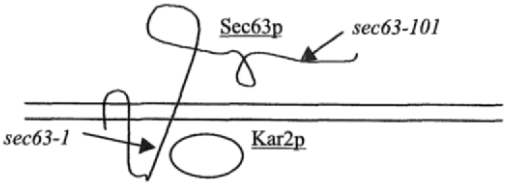
The *E. coli* DnaJ and DnaK (an HSP70 homologue) proteins interact physically. The authors of this article ask the question: Do Sec63p (DnaJ homologue) and Kar2p (DnaK homologue) also interact physically? The DnaJ domain of Sec63p is located in the lumen of the ER and Kar2p is the only yeast DnaK homologue localized to the ER lumen. The authors undertake a genetic approach to this question. They will use allele-specific genetic interactions, suppression, and enhancement to reveal a direct physical interaction.

1. *sec63-1* is the only mutation available that affects a residue in the lumenal DnaJ domain of Sec63p. *sec63-101* alters a residue in the C-terminal cytoplasmic domain of Sec63p. Table 2 shows the results of tetrad analysis of crosses between *kar2-159* and mutant alleles of *SEC61*, *SEC62*, and *SEC63*.
   (a) What is the purpose of crosses 1 and 2?
   (b) What results indicate an allele-specific synthetic lethal interaction between *kar2-159* and *sec63*? Include in your answer the genotype and expected phenotype (spore viability at 23°C) of a PD, NPD, and TT tetrad where synthetic lethality is exhibited.
   (c) What is the significance of the result obtained at a germination temperature of 13°C?
   (d) What results demonstrate the lack of enhancement between *kar2* and *sec61* or *sec62*? From this result can you conclude that Kar2p does not interact with either Sec61p or Sec62p and why?
   (e) The authors state, '. . . none of the *kar2* Ts-alleles tested were synthetically lethal with *sec61-1*, *sec62-1*, *sec65-1*, *sec63-101* nor *sec63-106*. . .'. What additional information regarding the interaction (or potential interaction) between Kar2p and Sec61p, Sec62p, Sec65p, and Sec63p is provided by this statement?

2. Table 4 presents the results of crosses between *sec63-1* and several *kar2* alleles to test the allele-specificity of the interaction.
   (a) Examine the results of crosses 1, 2, 3, 4, 6, 8, 10, 12, and 14 in which the spores were germinated at 23°C. Do you find evidence of an allele-specific synthetic lethal interaction? Why or why not?

(b) Comparison of the results of crosses 6, 8, 10, and 12 in which the spores were germinated at 13°C does suggest allele-specificity. Discuss. Which *kar2* alleles exhibit synthetic lethality with *sec63-1* and which do not?

(c) Complete the diagram below proposing the location of the altered residues in *kar2-133*, *kar2-203*, *kar2-157*, and *kar2-191*.



3. Describe the genetic screen designed to isolate dominant mutations in *KAR2* capable of suppressing the temperature-sensitive growth phenotype of *sec63-1*. The authors showed that *KAR2* in high copy does not suppress *sec63-1*. How would it have complicated the screening procedure if they had found that *KAR2* overexpression did suppress *sec63-1*?

4. Suggest a method of determining the phenotype of these *KAR2* dominant alleles in combination with wild-type *SEC63*. If some of these alterations affect Sec63p–Kar2p binding, what phenotypes might you expect for these *KAR2* alleles? Into what two classes does the *KAR2* dominant suppressor fall? Might you expect different phenotypes for these two classes of suppressors in this analysis and if so why?

5. Table 5 demonstrates that the dominant *KAR2* suppressors of *sec63-1* are allele specific. These results taken together with the results shown in Table 4 indicating partial allele-specific synthetic lethality between *sec63-1* and *kar2* led the authors to propose that, like other DnaJ/DnaK pairs, Sec63p and Kar2p interact physically. Moreover, based on studies of other DnaJ/DnaK pairs, they suggest that this interaction stimulates the ATPase activity of Kar2p. These conclusions would be greatly strengthened by some biochemical assays of Sec63p–Kar2p binding and Kar2p ATPase activity using the different mutant alleles of these proteins.

   (a) In the Discussion the authors allude to a copurification technique developed by Brodsky and Schekman that was able to demonstrate the complex formation between Sec63p and Kar2p but not Sec63-1p and Kar2p. Using this method, you explore the physical interaction between the proteins encoded by *sec63-1* and the different dominant *KAR2* suppressor. What result would you expect if the hypothesis of a direct interaction between Sec63p and Kar2p and the authors' explanation of the mechanism of suppression by the *KAR2* dominant suppressors were correct? Use the table below for your answer. The first two rows are controls to demonstrate that you are carrying out the binding assay correctly.

SEC63 allele	*KAR2* allele	Copurification with Sec63 protein	ATPase activity of purified Kar2 protein
SEC63	*KAR2*		Low
sec63-1	*KAR2*		Low
sec63-1	*KAR2-699*		Low
sec63-1	*KAR2-6199*		Low
sec63-1	*KAR2-6116*		Low
sec63-1	*KAR2-6139*		Very high

(b) Would the Kar2p dominant suppressor proteins be expected to copurify with wild-type Sec63p?

(c) Develop a method to purify Kar2 protein from strains expressing wild-type and each of the four mutant alleles listed in the above table and assay ATPase activity *in vitro*. Your results are shown in the table above. Propose a mechanism for suppression of *sec63-1* by *KAR2-6139*.

ARTICLE 13

Roberg, K.J., S. Bickel, N. Rowley, & C.A. Kaiser (1997) Control of amino acid permease sorting in the late secretory pathway of *Saccharomyces cerevisiae* by *SEC13*, *LST4*, *LST7* and *LST8*. *Genetics* **147**: 1569–1584.

The initial goal of this study was to identify new genes encoding proteins involved in ER to Golgi vesicle formation. Mutations in *SEC13* were found to produce reduced numbers of 50 nm vesicles (ER to Golgi intermediates) and to exhibit synthetic lethal interactions with mutations in *SEC12*, *SEC16*, and *SEC23* (Kaiser & Schekman, 1990). This approach might have identified nonessential components of the complex or factors involved in the assembly or stability of the complex whose function became essential when an essential component is defective.

1. Describe the genetic screen used to isolate mutations that exhibit synthetic lethality with *sec13-1*.

2. The 139 nonsectoring colonies were tested to identify clones carrying mutations of interest, i.e. those that contained *sec13-1* synthetic lethal mutations and exclude uninteresting or complex multiple changes.
   (a) Why were 82 colonies excluded from further analysis based on the fact that the introduction of a plasmid carrying *SEC13* did not allow sectoring?
   (b) The remaining 57 nonsectoring colonies were crossed to a *sec13-1* strain. Most, 52, showed 2:2 segregation of sectoring indicating a single *lst* mutation. Diagram the cross.
   (c) The five nonsectoring colonies eliminated in (b) had multiple alterations. Assuming two alterations to produce the nonsectoring phenotype, what would be the phenotype of the spores in a TT tetrad?

3. Diagram the crosses used to place the *lst* mutations into complementation groups.

4. Mutations in *SEC12, SEC16*, and *SEC23* were expected. How did the authors screen the *lst* mutant strains to determine if they carried alterations in these genes?

5. The authors wanted to determine whether *lst1* exhibited synthetic lethal interactions with other *sec* genes.
   (a) Diagram the cross used to separate *lst1* from *sec13-1*.
   (b) Diagram the cross and tetrad analysis used to test *lst1* for synthetic lethal interaction with *sec16-2*.

6. Based on the results shown in Table 2, the *lst* mutations were placed in two classes. Discuss why *LST1* and *LST6* were not considered further in this study.

7. Describe briefly the method used to clone *LST5*. How was it determined to be *THR4* encoding threonine synthetase?

8. Explain in your own words why a mutation in a gene encoding an enzyme for the biosynthesis of an amino acid would exhibit synthetic lethality in combination with *sec13-1*, a mutation affecting the late secretory pathway.

9. Which amino acid permeases are dependent on Sec13p for delivery from the Golgi to plasma membrane?

10. How do the results in Figure 2 distinguish *sec13-4* from *sec13-1*, *sec13-5*, and *sec13-7*?
    (a) If the authors had used *sec13-4* in their screen, would they have isolated synthetic lethal mutations in the same *LST* genes as with *sec13-1* and if so why or why not?
    (b) The authors examined this question: Does *sec13-4* cause synthetic lethality with *lst4-1* and *lst7-1*? What experiment was done and what were the results?

11. Figure 3 compares the effect of *sec13-1*, *sec12-4*, *sec16-2*, *sec23-1*, and *sec31-1* on citrulline uptake (a measure of Gap1 permease activity). Only *sec13-1* reduces Gap1p activity. Why would it have been valuable to test all the available mutant alleles of each gene? This is particularly true for *SEC16*. Why? (*Hint*: based on *lst* genes identified by their screen.)

12. The authors used the analogues listed in Figure 4 as a quick assay of specific permease activities. Why is resistance to the analogue an indicator of reduced transport activity?

13. How did the authors demonstrate that *lst4*, *lst7*, and *lst8* mutations specifically affect the secretion of certain amino acid permeases and do not cause general defects in secretion?

14. In the experiment shown in Figure 6, why did the authors determine the position in the gradient of Sec61p, GDPase, and plasma membrane ATPase? The results in Figure 6 are consistent with those in Figure 7 with regard to the subcellular distribution of Gap1p in *lst4*, *lst7*, and *lst8* mutant strains. Describe.

15. Why did the authors decide to test *pep12*Δ as a possible suppressor of *sec13-1*, *lst4*, *lst7*, and *lst8*? How might you use genetic analysis to identify additional mutations in genes involved in Golgi to vacuole transport?

16. The authors suggest that Lst4p, Lst7p, and Lst8p might play a direct role in Golgi to plasma membrane transport of Gap1p, that is, that these are components of a Sec13p-containing complex or are involved in the function or assembly of such a complex. An alternate hypothesis is that they are involved in nitrogen sensing. *LST7* and *LST8* have both been cloned. How might you use the tools of molecular genetics to explore these possibilities?

REFERENCE

Kaiser, C.A. & R. Schekman (1990) Distinct sets of *SEC* genes govern transport vesicle formation and fusion early in the secretory pathway. *Cell* **61**: 723–733.

ARTICLE 14

Espenshade, P., R.E. Gimeno, E. Holzmacher, P. Teung, & C.A. Kaiser (1995) Yeast *SEC16* gene encodes a multidomain vesicle coat protein that interacts with Sec23p. *J. Cell Biol.* **131**: 311–324.

SEC12, *SEC13*, *SEC16*, and *SEC23* are required for vesicle formation for ER to Golgi transport. This article begins to explore the role of the encoded proteins, in particular Sec16p.

1. Review the biochemical functions of Sar1p, Sec12p, and Sec23p. What evidence suggests that *SEC16* genetically interacts with *SAR1*?

2. Describe the strategy used to isolate *SEC16*. Include
   (a) a description of the library;
   (b) the genotype and phenotype of the host strain;
   (c) the phenotype used to select transformants containing possible *SEC16* plasmids.

3. Given the library used for the isolation, why is it unlikely that multicopy suppressors of *sec16-1* were isolated? Targeted integration was used to definitively demonstrate that the cloned 7.2 kbp *Bam*HI–*Sph*I fragment contained the real *SEC16* gene. Describe how this was done; include a diagram of the cross.

4. 'Each mutation was mapped by *in vivo* recombination tests with a nested set of *SEC16* deletions' according to the method of Falco *et al.* (1983).
   (a) Describe this method. Use diagrams where needed for clarity.
   (b) How was the nested set of deletions in *SEC16* constructed (see Methods and Materials)?

5. Gap repair was used to clone the *sec16* mutations. Describe how you would do this for *sec16-5*. Include in your answer the basis for you selection of the appropriate plasmid to use for the gap repair.

6. Given the size of Sec16p, 2194 residues, what can you conclude about its function based on the location of the five *sec16* mutations?

7. Sec16p is a very large protein with an essential function. The experiments presented in Figure 2 test whether all parts of this protein are essential by determining if truncated alleles are able to complement *sec16* mutations.
   (a) What important difference is there between the vectors in plasmids pPE8 and pPE129?
   (b) Different methods were used to test the ability of the plasmids to complement *sec16-1* and *sec16-1*Δ. Describe both.
   (c) Do these results demonstrate that the C-terminal 30 residues are essential or that they are essential in the absence of N-terminal residues 1–565?
   (d) Propose an explanation for the finding that pPE129 complements all three *sec16* mutant alleles but pPE130 only complements *sec16-1* and *sec16-2* and not *sec16-1*Δ. (*Hint*: think about intragenic complementation.)

8. The following questions relate to Figure 3.
   (a) Describe how Sec16p depletion was achieved. Include a description of the *GAL1pro–SEC16* fusion gene.
   (b) Lanes 7, 8, and 9 represent the positive control condition. Discuss. Include a description of the maturation of CPY.
   (c) Lanes 13, 14, and 15 represent the negative control condition. How do these results demonstrate that CPY does not exit the ER in *sec16-2* cells at the nonpermissive temperature?
   (d) *GAL1* is a glucose-repressed gene but lanes 1, 2, and 3 demonstrate that there is some expression of the *GAL1pro–SEC16* fusion gene in cells grown in 1% glucose and 1% galactose. How do we know this?
   (e) What results demonstrate that both depletion and overproduction of Sec16p block CPY maturation at the ER? What is the significance of this finding?

9. The following questions relate to Figure 5.
   (a) The constructs in Figure 2 do not provide a yeast promoter. Instead, 'transcription presumably was initiated within vector sequences'. How does the transcription expression of the *SEC16* constructs in Figure 5 differ from those in Figure 2?

(b) Based on the results with plasmids pPE46 and pPE27 where would you locate the dominant negative domain?

(c) Why was the result with plasmid pPE53 unexpected? How do the authors explain this? Propose another explanation based on your finding that overproduction of a fragment containing residues 650–1050 from the *GAL1* promoter does not allow growth on galactose.

10. Present a model explaining why both depletion of Sec16p and overproduction of Sec16p cause a block in secretion.

REFERENCE

Falco, S.C., M. Rose, & D. Botstein (1983) Homologous recombination between episomal plasmids and chromosomes in yeast. *Genetics* **105**: 843–856.

ARTICLE 15

Gimeno, R.E., P. Espenshade, & C.A. Kaiser (1996) COPII coat subunit interactions: Sec24p and Sec23p bind to adjacent regions of Sec16p. *Mol. Biol. Cell* **7**: 1815–1823.

In Article 14 two-hybrid analysis and copurification studies were used to demonstrate that Sec16p binds Sec23p. In an unpublished two-hybrid study, Gimeno and Kaiser identified a Sec24p homologue that bound to the central domain of Sec16p. Sec24p is required for vesicle formation and copurifies with Sec23p as part of a large protein complex (Hicke *et al.*, 1992). In this article, the authors explore the physical interactions of Sec16p, Sec23p, and Sec24p using two-hybrid analysis.

1. Diagram the *lexA–SEC24(34-926)* fusion gene construct in plasmid pRH286. Plasmid pEF202 uses the constitutive *ADH1* promoter to express the bait fusion gene. Diagram the protein product encoded by this fusion gene.

2. Diagram the *GAD–SEC16(565-1235)* fusion gene construct in plasmid pPE167. Plasmid pGAD-GH uses the constitutive *ADH1* promoter to express the Gal4p C-terminal transcription activation domain. The *Bam*HI cloning site is located at the 3'-end of the GAD sequence. Diagram the protein product encoded by this fusion gene.

3. Diagram the *LacZ* reporter gene carried on plasmid pSH18-34.

4. All three of these plasmid constructs must be introduced into the same host strain to test for the interaction of Sec24(34-926)p and Sec16(666-926)p. To do so, what nutritional gene mutations must be carried by the host strain?

5. Diagram the fusion genes bound at the promoter of the *LacZ* reporter.

6. The following questions are based on the results shown in Figure 1.

(a) What results indicate that the C-terminal residues of Sec24p bind to the central domain of Sec16p but not the N-terminal or C-terminal regions of Sec16p?
(b) What results indicate that the N-terminal but not the C-terminal domain of Sec24p binds Sec23p?
(c) What region of Sec16p binds Sec23p? What is the evidence?

7. How would you use two-hybrid analysis to explore the possibility that the *sec16* ts alleles described in Article 14 affect binding to Sec24p?

REFERENCE

Hicke, L., T. Yoshihisa, & R. Schekman (1992) Sec23p and a novel 105-kDa protein function as a multimeric complex to promote vesicle budding and protein transport from the endoplasmic reticulum. *Mol. Biol. Cell* **3**: 667–676.

ARTICLE 16

Shaywitz, D.A., P.J. Espenshade, R.E. Gimeno, & C.A. Kaiser (1997) COPII subunit interactions in the assembly of the vesicle coat. *J. Biol. Chem.* **272**: 25413–25416.

In this article, the Kaiser laboratory extends its analysis of the assembly of COPII using two-hybrid analysis. Genetic analysis identified *SEC12*, *SEC13*, *SEC16*, and *SEC23* as interacting genes involved in ER to Golgi vesicle formation. *SAR1* was identified as a multicopy suppressor of *sec12-4* (Nakano & Muramatsu, 1989). Sec12p is reported to be a guanine nucleotide exchange factor and activates GDP release from Sar1p (Barlowe & Schekman, 1993). Sec13p was found to copurify with a 105 kDa protein that was cloned by reverse genetics and shown to be *SEC31* (Salalma *et al.*, 1997). Gimeno *et al.* (1996) used two-hybrid analysis to model the interaction of Sec16p, Sec23p, and Sec24p. This article adds Sec13p and Sec31p to the complex.

1. Sequences from each of the genes listed in the table below were fused to *lexA* using vectors pBTM116 or pGilda and used for the experiments shown in Figure 1. For each indicate the residues contained in the fusions.

Gene	Residues in LexA fusion
SEC13	
SEC24	
SEC16	
SEC23	

2. Which residues of Sec31p interact with Sec13p? What type of protein–protein interaction domain is located in this region of Sec31p?

3. Which residues of Sec31p interact with Sec23p? With Sec24p? What data support your conclusion?

4. Which residues of Sec31p interact with Sec16p?

5. Redraw Figure 4 showing only Sec13p, Sec16p, Sec23p, Sec31p, and Sec24p. Indicate which is the N-terminal and C-terminal end of Sec31p. Include in the diagram the approximate residues of Sec31p involved in the interaction with each protein.

6. Based on the table above, what information do we have on the region(s) of proteins Sec13p, Sec16p, Sec23p, and Sec24p that interact with Sec31p?

7. Sec13p and Sec31p can be purified from cell extracts as a cytoplasmic factor. This is also true of Sec23p and Sec24p and of Sar1p. These three factors can be added to ER *in vitro* and stimulate vesicle production. Sec16p is never found free in the cytoplasm and is very tightly bound to ER. Propose a model for the assembly of COPII. How might you test whether the Sec23p–Sec24p complex was the first to bind to Sec16p?

REFERENCES

Barlowe, C. & R. Schekman (1993) *SEC12* encodes a guanine-nucleotide-exchange factor essential for transport vesicle budding from the ER. *Nature* **365**: 347–349.

Nakano, A. & M. Muramatsu (1989) A novel GTP-binding protein, Sar1p, is involved in transport from the endoplasmic reticulum to the Golgi apparatus. *J. Cell Biol.* **109**: 2677–2691.

Salama, N.R., J.S. Chuang, & R.W. Schekman (1997) *SEC31* encodes an essential component of the COPII coat required for transport vesicle budding from the endoplasmic reticulum. *Mol. Biol. Cell* **8**: 205–217.

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Case Study III
The Cell Division Cycle of *Saccharomyces*

READING LIST

Mutant Hunts: To Select or to Screen (Perhaps Even by Brute Force)

Article 1

Hartwell, L.H. (1967) Macromolecule synthesis in temperature-sensitive mutants of yeast. *J. Bacteriol.* **93**: 1662–1670.

Complementation Analysis: How Many Genes are Involved?

Article 2

Hartwell, L.H., R.K. Mortimer, J. Culotti, & M. Culotti (1973) Genetic control of the cell division cycle in yeast: V. Genetic analysis of *cdc* mutants. *Genetics* **74**: 267–286.

Epistasis Analysis

Article 3

Hereford, L.M. & L.H. Hartwell (1974). Sequential gene function in the initiation of *Saccharomyces cerevisiae* DNA synthesis. *J. Mol. Biol.* **84**: 445–461.

Gene Isolation, Characterization, and Multiple Alleles

Article 4

Patterson, M., R.A. Sclafani, W.L. Fangman, & J. Rosamond (1986) Molecular characterization of cell cycle gene *CDC7* from *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **6**: 1590–1598.

Article 5

Hollingsworth, R.R., R.M Ostroff, M.B. Klein, L.A. Niswander, & R.A. Sclafani (1992) Molecular genetic studies of the Ccd7 protein kinase and induced mutagenesis in yeast. *Genetics* **132**: 53–62.

Gene Isolation, Characterization, and Multiple Alleles

Article 6

Nasmyth, K.A. & S.I. Reed (1980) Isolation of genes by complementation in yeast: Molecular cloning of a cell-cycle gene. *Proc. Natl Acad. Sci. USA* **77**: 2119–2123.

Article 7

Hadwiger, J.A. & A.T. Lörencz (1985) Protein kinase activity associated with the product of the yeast cell division cycle gene *CDC28*. *Proc. Natl Acad. Sci. USA* **82**: 4055–4059.

Article 8

Lörencz, A.T. & S.I. Reed (1986) Sequence analysis of temperature-sensitive mutations in the *Saccharomyces cerevisiae* gene *CDC28*. *Mol. Cell. Biol.* **6**: 4099–4103.

Suppression

Article 9

Reed, S.I., J.A. Hadwiger, H.E. Richardson, & C. Wittenberg (1989) Analysis of the Cdc28 protein kinase complex by dosage suppression. *J. Cell Sci. Suppl.* **12**: 29–37.

Article 10

Kitada, K. L.H. Johnston, T. Sugino, & A. Sugino (1992) Temperature-sensitive *cdc7* mutations of *Saccharomyces cerevisiae* are suppressed by the *DBF4* gene, which is required for the G_1/S cell cycle transition. *Genetics* **131**: 21–29.

Article 11

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Enhancement

Article 12

Surana, U., H. Robitsch, C. Price, T. Schuster, I. Fitch, A.B. Futcher, & K.K. Nasmyth (1991) The role of *CDC28* and cyclins during mitosis in the budding yeast *S. cerevisiae*. *Cell* **65**: 145–161.

Two-Hybrid Analysis

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Advanced Concepts in Molecular Genetic Analysis

Article 14

Ohtoshi, A., T. Miyake, K. Arai, & H. Masai (1997) Analyses of *Saccharomyces cerevisiae* Cdc7 kinase point mutants: dominant-negative inhibition of DNA replication on overexpression of kinase-negative Cdc7 proteins. *Mol. Gen. Genet.* **254**: 562–570.

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More Suppression

Article 16

Lei, M., Y. Kawasaki, M.R. Young, M. Kihara, A. Sugino, & B.K. Tye (1997) Mcm2 is a target of regulation by Cdc7-Dbf4 during the initiation of DNA synthesis. *Genes Dev.* **11**: 3365–3374.

More Enhancement

Article 17

Dohrmann, P.R., G. Oshiro, M. Tecklenburg, & R.A. Sclafani (1999) *RAD53* regulates *DBF4* independently of checkpont function in *Saccharomyces cerevisiae*. *Genetics* **151**: 965–977.

Genome-Wide Analysis

Article 18

Iyer, V.R., C.E. Horak, C.S. Scafe, D. Botstein, M. Snyder, & P.O. Brown (2001) Genomic binding sites of the yeast cell-cycle transcription factors SBF and MBF. *Nature* **409**: 533–538.

ARTICLE 1

Hartwell, L.H. (1967) Macromolecule synthesis in temperature-sensitive mutants of yeast. *J. Bacteriol.* **93**: 1662–1670.

1. What is a conditional mutant? What is a temperature-sensitive conditional mutant?

2. Researchers interested in an essential process, such as cell division, isolate conditional mutants. Explain.

3. What is the name of the parental strain in which the mutations were isolated? Does it carry any mutations in known genes, and if so, what is its genotype?

4. The questions below relate to the approach used to isolate cell cycle mutants.
   (a) What is the initial phenotype used to identify the mutant strains?
   (b) Are these spontaneous mutants or induced?
   (c) How is replica plating used to identify the mutants?
   (d) Is this a selection or a screen and why?

5. Describe the methods used to separate the mutants into classes affecting protein synthesis, RNA synthesis, DNA synthesis.

6. What are the phenotypes of cell division mutants carrying alterations in an 'early' step and a 'late' step in cell division?

7. What percentage of the mutants could be clearly categorized as protein synthesis, RNA synthesis, DNA synthesis, cell division, cell wall formation mutants.

8. Of the 400 mutant strains analyzed, 396 were recessive.
   (a) Choose one mutant strain and diagram the cross carried out to demonstrate that the mutation in this strain is recessive. Be sure to give the genotype and phenotype of both parents and the diploid.
   (b) Are you or are you not surprised by the large percentage of recessive mutations and why?

9. In the next article, Dr Hartwell describes how sporulation of each heterozygous diploid (formed by mating the temperature-sensitive mutants to another strain of opposite mating type which is not temperature sensitive) produced two temperature-sensitive haploid spores and two normal haploid spores. What does this tell you about the alteration in each mutant that is causing the temperature-sensitive phenotype? Compare the genotypes of strains A364A and X1069-2D. Do we know that these strains are identical (that is isogenic) at all other genes, and why might this be important?

ARTICLE 2

Hartwell, L.H., R.K. Mortimer, J. Culotti, & M. Culotti (1973) Genetic control of the cell division cycle in yeast: V. Genetic analysis of *cdc* mutants. *Genetics* **74**: 267–286.

1. These questions relate to the process of isolating and identifying mutant strains.
   (a) The authors obtained the 148 cell division cycle mutant strains analyzed in this report from a collection of 1500 mutant strains that were temperature sensitive for growth. They looked for mutant strains which, at the nonpermissive temperature, arrested growth at a specific step in cell division or produced an aberrant multinucleated morphology as shown on page 270 of the article. Would you describe this as a selection or a screen and why?
   (b) The temperature-sensitive phenotype was used to enrich for the desired class of mutant. Explain the term 'enrich' as it is used here and discuss why this enrichment was helpful.
   (c) These mutations were obtained by chemical mutagenesis. Was this necessary or do you think that the authors could have worked with spontaneous mutations and why?

2. The pattern of bud emergence and growth in *S. cerevisiae* is the same in both haploid and diploid cells. Why is this an important advantage to the geneticist interested in studying the regulation of cell division?

3. Define the term 'execution point' as used by the authors. It is determined by calculating the percentage of cells . . . . (Complete the sentence.)

4. Define 'prototype phenotype'. What is the prototype phenotype for strains carrying the *cdc28-1* mutation?

5. The questions below are intended to reinforce the basic concepts of strain construction and genetic manipulation of experimental strains.
   (a) All the *cdc* mutations were isolated in strain A364A, an **a** mating type strain that has other nutritional mutations in its genome that facilitate genetic manipulation. Give the complete genotype of A364A. Indicate all the genes present as mutant alleles and the mating type allele. What nutrients need to be added to a minimal synthetic medium with glucose for A364A cells to grow?
   (b) The *cdc* mutants of A364A were mated to strain H79-20-3. Give the complete genotype of H79-20-3 including its mating type, and indicate what nutrients need to be added to a minimal synthetic medium with glucose for H79-20-3 cells to grow.
   (c) To mate A364A cells to H79-20-3 cells one only needs to mix the two cell types together without agitation for a few hours. A percentage (sometimes small) of the cells in the mating mixture will mate, and geneticists use nutritional requirements to select for the growth of only the diploids

and not the haploid parent strains. Give the complete genotype of the diploids from this cross, and list which nutrients should be added to a minimal synthetic medium with glucose for **only** these diploid cells to grow and **not** the haploid parents (A364A and H79-20-3).

(d) How did the authors construct an α mating type strain carrying each *cdc* mutation? Answer this question by diagramming the cross for *CDC28*. Show the genotype (for only the *CDC* and *MAT* genes) and Cdc phenotype of the two parent strains, the diploid, and the spores of a PD, TT, and NPD tetrad.

6. For almost all of the temperature-sensitive (ts) *cdc* mutant strains analyzed by the authors by crosses to the wild-type strain, the diploid cells had the wild-type phenotype and 100% of the tetrads contained two temperature sensitive spores and two wild-type spores. What information does this provide?

7. You wish to determine if the mutations in strains 438 and 601 are in the same or different genes. Both mutants arrest as unbudded cells when grown at high temperature. You have already shown that there is only a single, recessive mutation in both mutant strains, and have constructed strains of both mating types carrying the mutant genes.
   (a) How do you obtain a diploid heterozygous for both mutations?
   (b) What test(s) do you run on the diploid cells resulting from a cross between strains carrying the mutations from 438 and 601?
   (c) What alternate results might you expect from this test, and what would be your interpretation?
   (d) What would you do next to confirm your conclusion?

8. In four cases (mutants 104, 4028, 5011, and 23019) the original mutant strain harbored two independent temperature-sensitive mutations in two different genes, and only one of these was responsible for the cdc phenotype. Diagram the cross between one of these double mutant strains and the wild-type. PD, NPD, and TT type tetrads should result. Give the genotype, ts phenotype, and cdc phenotype of each of the four spores of each tetrad type.

9. In nine other cases, the authors found mutant strains that contained two independent mutations that each gave a Cdc mutant phenotype. Therefore, 13 of the 148 mutants strains contained mutations in two genes suggesting that the conditions of the chemical mutagenesis used to obtain the mutants were severe enough to produce multiple 'hits' in the genome. How could this be avoided? Does this make the mutant hunt process more or less difficult and why?

10. Of the 32 complementation groups identified by the authors, how many are represented by only one mutant allele? Do you think that the authors have identified all the *cdc* genes of *Saccharomyces*; that is, have they saturated the genome? Explain.

11. All the temperature-sensitive mutant alleles of the *cdc* genes arrest cell division at a particular terminal phenotype except *cdc7*. As described in Table 1, haploid cultures of some alleles display 20%–40% unbudded cells at the restricted temperature while other alleles show greater than 80% of the cells with the large-budded phenotype with a dividing nucleus. Those *cdc7* alleles arresting in the unbudded stage indicate an execution point in which stage of the cell division cycle? And those arresting in the large-budded stage? What might this be saying about the function(s) of the Cdc7 protein?

12. Some mutant alleles of a particular gene divide only once after the shift to the restrictive temperature while others divide several times before they arrest at the terminal phenotype. How do the authors explain this?

ARTICLE 3

Hereford, L.M. & L.H. Hartwell (1974) Sequential gene function in the initiation of *Saccharomyces cerevisiae* DNA synthesis. *J. Mol. Biol.* **84**: 445–461.

Hartwell and coworkers identified 32 *Saccharomyces CDC* genes required for progress through the cell division cycle. They also grouped these genes based on their execution point in the cell cycle, and to a first approximation each gene product is required at a particular time during the cell cycle. Temperature-sensitive mutations in three genes (*CDC4*, *CDC7*, and *CDC28*) do not initiate DNA synthesis. Cells treated with *Saccharomyces* α-factor, a peptide pheromone produced by cells of the α mating type, also do not initiate DNA synthesis and pheromone-treated cells arrest as unbudded cells. Thus, all act in the first portion of the cell cycle called G1 or Gap 1. The goal of this article is to determine whether or not the three gene products act independently on different processes, and if so can the process carried out by these gene products be temporally ordered in relation to one another and to the process blocked by the α-factor?

The authors use two methods to determine the temporal order of the events controlled by Cdc4p, Cdc7p, Cdc28p, and the α-factor. The first is called the reciprocal shift method. The second is referred to as the double-mutant method. Both are explained in the text of the article. The double-mutant method is also known as epistasis analysis. It is used to determine if genes with similar mutant phenotypes are in the same or different pathways, and, if in the same pathway, to place them in a linear order relative to one another based on the step in the pathway controlled by that gene.

1. Strains containing temperature-sensitive mutations in *CDC4*, *CDC7*, and *CDC28* will not initiate DNA synthesis at the elevated, or nonpermissive, temperature. *MAT***a** strains treated with α-factor also are unable to initiate DNA synthesis. The morphological effects of these blocks, however, are different.
   (a) Describe the morphology assumed by **a** mating-type cells treated with α-factor for approximately one cell cycle and by the cells of *cdc4*, *cdc7*, and *cdc28* mutant strains exposed to 36°C for approximately one cell cycle.

(b) Based on these phenotypes and on your knowledge of the morphological events of the *Saccharomyces* cell cycle, what preliminary hypothesis(es) might you have proposed at the outset of this experiment and why? (You may just be able to group these genes into early and late G1 events.)

2. The following questions are based on Figure 1a.
   (a) Why does the closed-circle line go smoothly up indicating a continuous DNA synthesis at a constant rate and the open-triangle curve look like steps?
   (b) Approximately how long (to the quarter hour) does it take for cells of strain H135.1.1 to complete one full division cycle? How did you determine this?
   (c) How long is the S phase? How did you determine this?
   (d) In the open-triangle curve, what is happening during the flat portion of the curve from about 3.75 h to about 5.0 h?

3. The following questions are based on Figure 1b.
   (a) Why does the open-square curve go through more than one step while the filled-square curve levels off after only one step?
   (b) Cycloheximide is a powerful inhibitor of protein synthesis. Are all the proteins required for completion of the cell cycle made prior to the α-factor or Cdc4p block in G1 and on what evidence do you base this conclusion?
   (c) Is it surprising that the filled-square cells arrest as unbudded cells? Explain.

4. In the *cdc7* mutant strain, cell separation (cytokinesis) was delayed. How was this manifested?

5. Mutant strains carrying a recessive temperature-sensitive alteration in a (fictitious) cell division cycles gene, *cdc108*, block initiation of DNA synthesis at a step that preceeds the α-factor block. Diagram the results you would expect if you were to run an experiment like the one shown in Figure 1.

6. Why do the authors put the *cdc4* block before the *cdc7* block? (Give two reasons.) Is *CDC4* epistatic to *CDC7* or *vice versa*?

7. It is interesting to note that in *cdc4* and *cdc7* mutants bud formation can be initiated in the absence of DNA synthesis while in *cdc28* mutants and α-factor-treated **a** mating type cells, which also do not initiate DNA synthesis, cells arrest as unbudded cells.
   (a) What does this suggest about the regulation of bud formation initiation and its relationship to the initiation of DNA synthesis?
   (b) Is the initiation of DNA synthesis epistatic to the initiation of bud formation? If not, which model of Table 2 best fits their relationship, and why?

8. The spindle plaque (now referred to as the spindle pole body) is an elaborate multilayered platelike structure embeded in the nuclear envelope of *Saccharomyces* (see Chapter 3). The *Saccharomyces* nuclear envelope never falls apart during cell division and the spindle pole body serves the role of the centriole.
   (a) Describe the terminal morphological phenotype of the spindle pole body of α-factor-treated cells and *cdc4*, *cdc7*, and *cdc28* mutant cells exposed to high temperature (work done by Beyers and coworkers).
   (b) Are their results consistent with those reported here, and, if not, how do their conclusions differ?

ARTICLE 4

Patterson, M., R.A. Sclafani, W.L. Fangman, & J. Rosamond (1986) Molecular characterization of cell cycle gene *CDC7* from *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **6**: 1590–1598.

1. The Introduction to this article summarizes the phenotype of strains carrying temperature-sensitive mutations of *CDC7* as follows. At the nonpermissive temperature *cdc7* mutants arrest prior to the initiation of DNA replication as budded cells with a single nucleus lacking an elongated spindle but having a divided spindle pole body. Several laboratories investigated other aspects of the *cdc7* phenotype and found a number of interesting roles for Cdc7p outside of its role in mitotic cell division. List these.

2. The authors cloned *CDC7* by complementation of the *cdc7-1* allele.
   (a) Explain in general terms (that is, do not specifically refer to *cdc7* or the *cdc7* phenotype) the concept behind this approach, namely cloning by complementation.
   (b) What changes would you have to make if you only had dominant mutant alleles available?

3. The libraries were made from partial digests using enzymes that cut frequently in the *Saccharomyces* genome. Moreover, different enzymes were used to make different libraries. Propose reasons why.

4. How does the isolation of several complementing clones help in localizing the gene of interest?

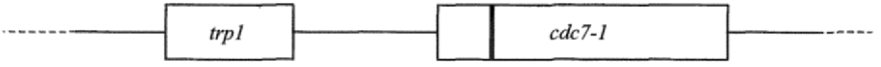
5. Why were URA+ colonies selected after strain 158 was transformed with the YEp24 library?

6. YIp5 is a pBR322-based vector containing the *URA3* gene and no ARS element is present on this vector. Plasmid pMP104 was constructed by inserting a DNA fragment containing the putative *CDC7* gene into this vector.
   (a) Diagram the integration of pMP104 into the *cdc7-1* gene of strain SB158. Use the diagram below to help you.

(b) Diagram the cross between a strain carrying an integrated copy of plasmid pMP104 and another strain of opposite mating type with the genotype *cdc7-1 ura3 TRP1* in transformants with the following two possible sites of integration. For each cross state whether PD, NPD, or TT tetrads would be expected and give the phenotype (TRP, CDC, URA) of the spores in each tetrad type obtained.

(i) Integration occurred at the *cdc7-1* gene. The vertical line indicates the position of the mutant alteration in *cdc7-1*. Show the integration event at a site to the right of the mutation.

(ii) Integration occurred at *ura3-52* instead. (*URA3* is unlinked to *CDC7*.)



7. Diagram the crossover(s) that are required for the 'rescue by recombination' method used to localize the alteration in *cdc7-1* to the left end of the map. Remember, the plasmid does not integrate.

8. How might you improve the vector used for the construction of the library so that you did not have to worry about multicopy suppression?

ARTICLE 5

Hollingsworth, R.R., R.M Ostroff, M.B. Klein, L.A. Niswander, & R.A. Sclafani (1992) Molecular genetic studies of the Ccd7 protein kinase and induced mutagenesis in yeast. *Genetics* **132**: 53–62.

This article is an example of mutation analysis, the detailed characterization of several mutant alleles of a gene. It is an important first sep in determining the specific function or functions of the gene product of a gene and the molecular mechanism by which it carries out those functions. Article 4 reported that Cdc7p exhibits sequence homology to Cdc28p, a known protein kinase. Hollingsworth & Sclafani (1990) and Yoon & Campbell (1991) purified Cdc7 protein and demonstrated that it has protein kinase activity *in vitro*. While these findings are suggestive, none proves that the essential function of Cdc7p is associated with its protein kinase activity. Penguins and chickens have wings but neither species flies. Just because an animal has wings one cannot assume it uses them to fly. Similarly, if a protein contains a particular sequence motif one cannot assume that this is functionally significant for this particular protein. It must be demonstrated by mutation analysis.

Moreover, different alleles of a gene may have subtly different phenotypes that could indicate that the encoded product has more than one cellular function. Multiple functions are often associated with different structural regions of the protein. Analysis of several alleles having different phenotypes can be useful for assigning specific functions to particular regions of a protein. This type of analysis is

referred to as a structure–function analysis and is used to identify functional domains of a protein. These domains could represent regions responsible for interaction with other proteins (such as in a heterodimer), or with enzymatic substrates or cofactors (such as ATP or heme), or DNA-binding domains (such as is found in some activator and repressor proteins). A single protein with multiple cellular functions is likely to have a complex multidomain structure and exhibit a pleiotropic phenotype.

This article reports on the sequencing of several *cdc7-ts* alleles. Other mutations in essential domains associated with protein kinase activity, such as the ATP-binding site, were introduced by *in vitro* mutagenesis, and these alleles are characterized as well. The analysis demonstrates that the protein kinase activity of Cdc7p is essential for its role in DNA replication and repair.

1. This article describes the cloning of several *cdc7* temperature-sensitive alleles using a method based on gene conversion of a plasmid-borne copy of *CDC7*.
   (a) Describe the method used, which must include the role of the herpes virus *TK* gene. Use a diagram to illustrate your answer that shows the chromosomal *cdc7-ts* gene, the plasmid construct, and the number and location of the recombination events needed for gene conversion of the plasmid.
   (b) What is the phenotype of the transformant before and after the conversion event described above with regard to 5-fluorodeoxyuridine resistance and temperature-sensitivity?

2. Table 2 and Figure 1 summarize the results of sequence analysis of the nine *cdc7* mutant alleles. Seven of these *cdc7* alleles were isolated using procedures that ensure that independent, and presumably different alterations would be obtained. Despite this, alleles 1, 2, and 5 and alleles 7 and 90 are identical alterations. Hypothesize why particular changes were isolated multiple times. Remember that the mutagens used to induce the sequence alterations do not preferentially attack one guanine residue over another.

3. Define missense suppressor. In your own words, describe why *SOE1*, a tRNA mutation, suppresses the *cdc7-7* allele. (You will need to use information from the genetic code for your answer.) Would you expect the *soe1* suppressor mutation to be dominant or recessive and why?

4. Mutant allele *cdc7-10* was constructed by *in vitro* mutagenesis of codons 76 and 77 that code for the two essential lysines of the putative ATP-binding domain of Cdc7 kinase.
   (a) Describe the method used by the authors to demonstrate whether *cdc7-10* is a lethal mutation, that is, a loss of Cdc7p kinase activity causes a defect in cell division.
   (b) Describe a method that could be used to demonstrate that *cdc7-10* does not complement a *cdc7*Δ null allele.
   (c) Are you surprised that *cdc7-10* is not a conditional mutation? Explain.

5. The authors conclude that the results obtained with *cdc7-10* provide strong evidence that the kinase activity of Cdc7 protein is essential for its function in cell division. Why is it necessary to demonstrate that sufficient Cdc7-10 protein is present? In other words, could they have come to the same conclusion if they had found that Cdc7-10 protein levels were undetectable?

6. Table 3 shows the results of a study to determine if *cdc7* mutants exhibit a different rate of mutation than wild-type.
   (a) How was the mutation rate measured?
   (b) Do *cdc7* mutations exhibit different rates of spontaneous mutation compared with wild-type and how was this determined?
   (c) Do the different *cdc7* mutant alleles exhibit differences in mutation rate? Discuss.
   (d) Do the different *cdc7* mutant alleles exhibit differences in mutation rate when UV light is used as the mutagenic agent? What might be the significance of this finding?

7. Define 'segregation lag' according to the authors' usage. What do the results in Table 5 indicate with regard to the functional activity of these temperature-sensitive alleles at the permissive temperature? Are they truly normal at the permissive temperature or are there indications of the mutational defect?

8. The mutant allele *cdc7-23* was made by *in vitro* mutagenesis.
   (a) What is linker scanning insertional mutagenesis?
   (b) List the phenotypes of *cdc7-23* mutant strains.

9. Is there a structure–function relationship between the hyper- versus hypo-mutability phenotype?

10. What is the functional implication of the finding that the hypo- or hyper-mutability phenotype of *cdc7* mutations is recessive to wild-type?

11. What conclusions, if any, do the authors come to regarding the role of Cdc7 protein kinase in mutation and repair?

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Hollingsworth, R.E. & R.A. Sclafani (1990) DNA metabolism gene *CDC7* from yeast encodes a serine (threonine) protein kinase. *Proc. Natl Acad. Sci. USA* **87**: 6272–6276.

Yoon, H.Y. & J.L. Campbell (1991) The CDC7 protein of *Saccharomyces cerevisiae* is a phosphoprotein that contains protein kinase activity. *Biochemistry* **88**: 3574–3578.

ARTICLE 6

Nasmyth, K.A. & S.I. Reed (1980) Isolation of genes by complementation in yeast: molecular cloning of a cell-cycle gene. *Proc. Natl Acad. Sci. USA* **77**: 2119–2123.

1. These questions refer to the construction of the plasmid library.
   (a) List the essential features of the vector YRp7.
   (b) Which type of library is this (genomic, cDNA, other)?
   (c) Describe the preparation of insert DNA fragments. Include the genotype of the strain that was the source of the insert DNA.
   (d) The DNA fragments were inserted into the *Bam*HI site of YRp7. Would you expect to be able to release all of these insert fragments using *Bam*HI? Explain.
   (e) How large is the library? The *S. cerevisiae* genome is 1.2×10^7 bp (1.2×10^4 kbp or 12 Mbp). Is the authors' estimate that the library contains each gene, in whole or in part, in about ten independent library clones reasonable? Explain.

2. These questions refer to the identification of yeast transformants containing a library plasmid carrying the putative *CDC28* gene.
   (a) Why were Trp+ transformants selected and how were they selected?
   (b) What selection method was used to identify transformants with the Cdc+ phenotype?
   (c) Why is this cloning approach called 'cloning by complementation'?

3. Figure 1 shows the results of Southern analysis of several Trp+ Cdc+ transformants probed with pBR322 plasmid DNA.
   (a) What is the significance of the finding that some transformants exhibit only a plasmid-sized fragment while others had high molecualar weight forms or both?
   (b) Is it surprising that those transformants that exhibit only plasmid-sized fragments lose both the Trp+ and Cdc+ phenotypes when grown under nonselective conditions? Explain.
   (c) Describe the method used to recover the plasmid from the unstable transformants in *E. coli*.
   (d) Restriction mapping and Southern analysis were used to demonstrate that three of the recovered plasmids contained overlapping inserts. Describe how you would use Southern analysis to distinguish vector-derived from insert-derived fragments in a *Hind*III digest of YRp7-*CDC28*(1). Describe how you would use Southern analysis to demonstrate which *Hind*III fragments of YRp7-*CDC28*(3), the plasmid with the smallest insert, are also contained in the YRp7-*CDC28*(1) insert.
   (e) How is it helpful to have several complementing plasmids with overlapping inserts of different sizes for localizing the complementing gene within the insert fragment?

4. Unpublished genetic mapping studies determine the distance between *CDC28* and *TYR1* to be 7 map units (centamorgans). What is the significance of the finding that YRp7-*CDC28*(1) complements both *cdc28-4* and *tyr1*?

5. Diagram a hypothetical integration event between YRp7-*CDC28*(3) and the chromosomal *cdc28-4* gene. Use the diagram below of the region of

chromosome II of strain SR668-2 containing both *cdc28-4* and *tyr1* mutations. Include a diagram of the circular YRp7-*CDC28*(3) plasmid, the position of the recombination event between plasmid and chromosome, and the map of the chromosomal product of the integration event. (The vertical line marks the position of the mutant sequence alteration in *cdc28-4*.)

6. How does the product of the integration event described in question 5 differ if the position of the recombination site is to the right versus the left of the mutant alteration?

7. Table 2 presents the results of a cross between strain SR668-2 containing an integrated copy of YRp7-*CDC28*(3) (refer to this allele as *cdc28-4:CDC28-TRP1*) with strain KN-86.
   (a) Diagram the cross indicating the genotype and phenotype of the parent strains and the resulting diploid. Include the *TYR1*, *CDC28*, and *TRP1* genes.
   (b) Give the genotype of the four ascospores in a PD tetrad.
   (c) What is the origin of the small number of tetratype asci that were obtained?

8. The results in Table 2 determined the linkage of the integrated *TRP1* gene to *tyr1*. How would you determine the linkage of the integrated *TRP1* to *cdc28-4* using these same tetrads? Would you expect the map distance to be smaller, larger, or the same as the map distance to *tyr1*? Explain.

9. Show the calculation that the authors made to conclude that 1 map unit is equivalent to a maximum of 1.75 kbp in this region of chromosome II.

ARTICLE 7

Reed, S.I., J.A. Hadwiger, & A.T. Lörencz (1985) Protein kinase activity associated with the product of the yeast cell division cycle gene *CDC28*. *Proc. Natl Acad. Sci. USA* **82**: 4055–4059.

Lörencz and Reed (1984) describe the sequence analysis of *CDC28*. They found that the encoded protein exhibits sequence homology to several vertebrate oncogenes including *src* (21% homology), *fes* (25.5% homology), *fps* (24.5% homology), *yes* (25% homology), and *v-raf* (24% homology). Most of these oncogenes have been shown to encode protein kinases and so the authors of this article attempt to demonstrate that *CDC28* also encodes a protein kinase.

1. Polyclonal antibody directed against an in-frame LacZ–Cdc28 fusion protein had been prepared by Reed (1982) and this was used here to precipitate Cdc28 protein from yeast extracts. Figure 1 shows an analysis of the yeast proteins precipitated by this antibody.
   (a) Why were the yeast cells grown in the presence of ^{35}S-methionine?
   (b) Define preimmune serum.
   (c) About 7–10 different proteins are precipitated from the yeast cell extract by the antibody. Give two reasons (other than the molecular weight) why the authors conclude that the 36 kD protein is a Cdc28 protein and not the other precipitated proteins, particularly the 70 kD protein.

2. Briefly describe the kinase assay developed here to determine the protein kinase activity of the immune precipitate.
   (a) What is the basis for the authors' conclusion that this phosphorylation of the 40 kD protein is dependent on the presence of the Cdc28 protein in the immune precipitate?
   (b) How do the authors know that the phosphorylation of the 40 kD protein is occurring in the immune precipitate and not *in vivo* prior to preparation of the cell extract?

3. Extracts prepared from cells expressing *cdc28-4* lacked kinase activity even at the permissive temperature and with no decrease in Cdc28-4 mutant protein levels. Based on this finding alone the authors cannot conclude that it is the kinase activity of the Cdc28 protein and not some other as yet unidentified function that is required for its role in the cell cycle. Discuss why this finding is inconclusive.

4. The finding that the protein kinase activity of the immune precipitate prepared from cells expressing the *cdc28-13* mutant allele is temperature sensitive is the most compelling argument supporting the authors' conclusion that the kinase activity of Cdc28 protein is its essential function in regulating the cell cycle. Discuss.

REFERENCES

Lörencz, A.T. & S.I. Reed (1984) Primary structure homology between the product of yeast cell division control gene *CDC28* and vertebrate oncogenes. *Nature* **307**: 183–185.

Reed, S.I. (1982) Preparation of product-specific antisera by gene fusion: antibodies specific for the product of the yeast cell-division-cycle gene *CDC28*. *Gene* **20**: 255–265.

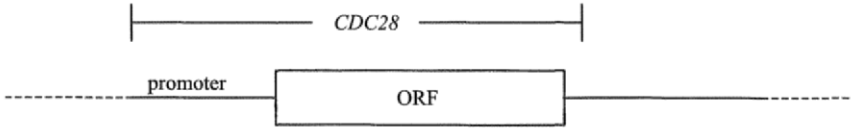
ARTICLE 8

Lörencz, A.T. & S.I. Reed (1986) Sequence analysis of temperature-sensitive mutations in the *Saccharomyces cerevisiae* gene *CDC28*. *Mol. Cell. Biol.* **6**: 4099–4103.

In this article the authors sequence several independent mutant alleles of *CDC28*. They find that these mutations cluster in two distinct regions of the Cdc28 protein.

In Article 12 we will see that two of these mutations, *cdc28-4* and *cdc28-1N*, that map to one of these two regions define different cell cycle functions of Cdc28 protein kinase.

1. Figure 1a shows how the authors synthesized the double-stranded DNA fragments of *CDC28* that they used to localize the sites of the alterations in the 11 *cdc28-ts* mutations. These laborious methods are no longer necessary given the advent of polymerase chain reaction (PCR). Describe how you would use PCR-based methods to synthesize a series of five overlapping DNA fragments spanning the *CDC28* open reading frame (ORF). Use the diagram below to illustrate primer pair location. Draw an arrow at the 3' end of each primer and number both members of each pair (1, 2, 3, 4, and 5).



2. Explain why it is probable that the *cdc28-ts* alterations are in the ORF and not likely to be in the promoter region.

3. Draw a diagram illustrating the gene conversion event between the DNA fragment of *CDC28* and the genomic *cdc28-ts* mutation in each of the following cases. Show the exchange sites and the chromosomal product.
   (a) The alteration lies within the fragment sequence.
   (b) The alteration lies outside of the fragment sequence.

4. Explain in your own words what the authors mean when they say that 'fragments spanning the mutation are able to rescue the temperature-sensitive mutant phenotype'.

5. Illustrate the gap repair method used to recover the mutation in the genome onto a gapped plasmid as follows.
   (a) Diagram the YRp7 plasmid construct used to create the 'gapped' plasmids.
   (b) How are the gaps created in the construct shown in (a)?
   (c) Diagram a gene conversion event between a gapped plasmid and the chromosome. Include the plasmid product of this conversion event.
   (d) Is the chromosomal copy altered as a result of this gene conversion event?
   (e) What is the Cdc phenotype of the transformed strain after the gene conversion event?
   (f) What happens to host cells if gene conversion of the gapped region does not occur?

6. The sequence alterations in 11 *cdc28-ts* mutants were determined but only 6 mutant alterations are reported in Figure 2. Explain.

7. The *ts* mutations clustered in two regions of the Cdc28 protein, codons 120–130 and the C-terminal 50 residues. Moreover, the same alteration was isolated independently many times. The authors suggest that this implies that these regions of the Cdc28 protein are uniquely important for the cell cycle function of Cdc28 protein kinase. Discuss their reasons for excluding the following explanations.
   (a) EMS mutagenesis is not random.
   (b) Only alterations to these restricted regions of the Cdc28 protein can cause temperature sensitivity.
   (c) These regions of the Cdc28 protein are involved in functions required of all kinases.

ARTICLE 9

Reed, S.I., J.A. Hadwiger, H.E. Richardson, & C. Wittenberg (1989) Analysis of the Cdc28 protein kinase complex by dosage suppression. *J. Cell Sci. Suppl.* **12**: 29–37.

This article describes the isolation of multicopy suppressors of two mutant alleles of *CDC28*. We will only concern ourselves with the suppressors of the temperature-sensitive allele *cdc28-4*.

1. Describe the strategy behind the authors' decision to search for multicopy suppressors of *cdc28-4*.
   (a) What multicopy suppressor genes encoding which types of protein do they expect to identify using this approach?
   (b) What are their reasons for proposing the existence of such genes?
   (c) Discuss the choice of the temperature-sensitive allele *cdc28-4* for this project.
   (d) Discuss the following statement. Implicit in the authors' approach is the hypothesis that the *cdc28-4* mutation alters the ability of Cdc28p regulators to specifically bind to and activate its kinase activity.

2. Describe the procedure used for the isolation of multicopy (dosage) suppressors of *cdc28-ts* mutations by answering the following questions.
   (a) Describe the construction of the library from which the suppressors were isolated. Give detailed information on the vector and the strain from which the yeast inserts were obtained. What type of library is it (genomic, cDNA, other)? Explain.
   (b) Specify the genotype of the host yeast strain.
   (c) What growth conditions were used to select for:
      (i) yeast transformants carrying a library plasmid?
      (ii) transformants that carry a plasmid that suppresses the temperature-sensitive phenotype of *cdc28-4*?

3. Three plasmids were isolated that suppressed *cdc28-4* (PSC1, PSC2, and PSC3).

(a) Did these plasmids **fully** restore the wild-type phenotype? Upon what evidence do you base your answer?
(b) What is the evidence that these are allele-specific suppressors?
(c) In addition to *cdc28-4*, what other mutant alleles of *cdc28* are also suppressed by these plasmids? (*Note*: read the Materials and Methods description of the isolation of these plasmids.)

4. Describe the method used to localize the suppressor gene contained in the yeast insert in plasmids (PSC1, PSC2, and PSC3).

5. Compare the sequence of the proteins encoded by the suppressor genes *PSC1* and *PSC2* (*CLN1* and *CLN2*) to each other and to Cyclin A of the clam.

6. In addition to their sequence homology and the finding that both *CLN1* and *CLN2* are dosage suppressors of *cdc28-4*, what other evidence is presented to support the suggestion that Cln1p and Cln2p have overlapping, possibly identical functions in the control of cell cycle progression?

7. What evidence suggests the existence of another *CLN*-like gene? Discuss the reasons why the authors suggest that this gene could be *DAF1/WHI1*.

8. Later articles from the Futcher, Cross, and Reed laboratories found that dominant alleles of *DAF1/WHI1* (now called *CLN3*) alter the C-terminal region of the Cln3 protein and make the mutant protein resistant to proteolytic degradation. Based on your knowledge of the role of cyclins in G1 to S progression, why should this type of alteration produce a mutant phenotype? Why is this mutation dominant to the wild-type allele?

9. Based on the model of allele-specific suppression discussed in Chapter 8 and the sequence analysis of *cdc28-ts* mutations described in Article 8, which region(s) of the Cdc28 kinase might be involved in interactions with cyclin? Explain.

10. Describe an alternate (not genetic) method that you would use to demonstrate that Cln1p and Cln2p are components of a Cdc28p complex.

11. Describe how you would use genetic analysis of the several *cdc28-ts* alleles to explore the possibility that Cln1p and Cln2p bind **directly** to Cdc28p and not indirectly; that is, via binding to another protein component in the Cdc28p complex.

ARTICLE 10

Kitada, K. L.H. Johnston, T. Sugino, & A. Sugino (1992) Temperature-sensitive *cdc7* mutations of *Saccharomyces cerevisiae* are suppressed by the *DBF4* gene, which is required for the G_1/S cell cycle transition. *Genetics* **131**: 21–29.

Temperature-sensitive mutations in *DBF4* had been isolated in an independent screen for *cdc* mutants (Chapman & Johnston, 1989). These *dbf4* ts alleles do not initiate DNA synthesis at the nonpermissive temperature and arrest with a dumbbell-shaped morphology similar to the *cdc7* alleles. Epistasis analysis placed *DBF4* downstream of *CDC4* but upstream of *CDC7*. *DBF4* was cloned by complementation (Chapman & Johnston, 1989). Their article describes the isolation of *DBF4* as a multicopy suppressor of *cdc7-1*.

1. The authors used Southern analysis to demonstrate that the library plasmid contained in transformants T33 and T34 carried an insert fragment that was not *CDC7*. Describe the analysis and draw a diagram showing the expected results from Southern analysis of transformant T33 compared with another transformant that contains a *CDC7* plasmid. Be sure to indicate the probes used.

2. *DBF4* suppresses mutations in *CDC7*.
   (a) Which experimental result(s) indicates that the suppression of *cdc7-1* by *DBF4* is not by-pass suppression?
   (b) Which experimental result(s) support a physical interaction between Cdc7p and Dbf4p?
   (c) What type of suppression is operating here?

3. Answer the following by referring to Table 3.
   (a) What percent sporulation was obtained in the *dbf4/dbf4* homozygous diploid strain containing *CDC7* on a high-copy plasmid at 25°C and 35°C?
   (b) What percent sporulation was obtained in the *cdc7/cdc7* homozygous diploid strain containing *DBF4* on a high-copy plasmid at 23°C and 32°C?
   (c) What is the purpose of the last column labeled 'YEp'?
   (d) What is the conclusion of this experiment?

4. How did the authors demonstrate that *DBF4* is an essential gene?

5. Propose a model for the suppression of *cdc7-1* by *DBF4*. Include an explanation for the difference between mitotic and meiotic suppression.

REFERENCE

Chapman, J.W. & L.H. Johnston (1989) The yeast gene, *DBF4*, essential for entry into S phase is cell cycle regulated. *Exp. Cell Res.* **180**: 419–428.

ARTICLE 11

Hardy, C.R.J., O. Dryga, S. Seematter, P.M.B. Pahl, & R.A. Sclafani (1997) *mcm5/cdc46-bob-1* by-passes the requirement for the S phase activator Cdc7p. *Proc. Natl Acad. Sci. USA* **94**: 3151–3155.

Jackson *et al.* (1993) isolated *bob1-1* as a suppressor of the temperature-sensitive growth defect of *cdc7-1*. They found that *bob1-1* can by-pass null mutations in *CDC7* and *DBF4* but not temperature-sensitive mutations in *CDC28* or *CDC4*. They conclude that *bob1-1* cannot suppress all events that occur in the G1 phase of the cell cycle and that Cdc7p and Dbf4p act at the same step, which the epistasis analysis of Hereford & Hartwell (1974) places downstream of *CDC28*. This article continues the characterization of *bob1-1* and finds that it is an allele of a gene known as *CDC46* (now officially called *MCM5*).

1. Describe how you would undertake the isolation of additional suppressors of *cdc7-1* such as *bob1-1*.

2. Diagram crosses to demonstrate the following. In each case give the genotype and phenotype of the parent strains, the diploid, and of each spore in a tetratype tetrad.
   (a) *bob1-1* is a suppressor of *dbf4-1* (demonstrated in Jackson *et al.*, 1993).
   (b) *bob1-1* does not suppress the temperature-sensitive *cdc28-1* mutation (demonstrated in Jackson *et al.*, 1993).
   (c) *bob1-1* does not suppress the temperature-sensitive *orc2-1* mutation (a conclusion stated here but the data are not shown).

3. *bob1* suppresses a *cdc7Δ::HIS3* null mutation (Jackson *et al.*, 1993). This was demonstrated by sporulating a diploid strain homozygous for *bob1* and heterozygous for *cdc7Δ::HIS3*.
   (a) Describe the construction of this diploid strain starting with strains of the following genotype of opposite mating types: *bob1-1 CDC7 his3*.
   (b) Give the genotype and phenotype of the spores resulting from the sporulation of this diploid. Include *bob1-1*, *cdc7*, and *HIS3* in your cross.
   (c) What would the tetrad analysis results be if *bob1-1* does not suppress *cdc7Δ::HIS3*?

4. From the results of the *CDC46* gene disruption experiments carried out prior to the cloning and sequencing of the *bob1-1* mutant allele, the authors had concluded that *bob1-1* is not a null allele. What is the evidence in support of this?

5. Which result in Table 1 indicates that *bob1-1* is recessive?

6. Describe the cloning of *BOB1*. How does genetic mapping provide evidence consistent with their finding that *BOB1* is *CDC46*?

7. Using the diploid strain (*dbf4-1/dbf4-1 bob1-1/BOB1*) the authors confirm their finding that *bob1-1* is a mutant allele of *CDC46*. Describe.

8. Deletion/disruption of *BOB1* is lethal but the Pro83Leu alteration of Cdc46 protein found in the *bob1-1* mutation produces only a modest phenotype.
   (a) What is the phenotype of the *bob1-1* mutation in the absence of *cdc7-1*?

(b) On what basis do the authors suggest that *bob1-1* cells enter the S phase prematurely?
(c) Suggest a model to explain the different phenotypes of these alleles that takes into consideration the fact that *bob1-1* is recessive.

9. This article demonstrates that *BOB1* is *CDC46*. It also shows that *cdc46-1*, isolated as a temperature-sensitive suppressor of *cdc45-1*, does not suppress a *cdc7*Δ null allele while the *bob1-1* allele does. Propose a model explaining these results that includes a discussion of the possible role of Cdc7–Dbf4 kinase in Cdc46p activation.

REFERENCES

Hereford, L.M. & L.H. Hartwell (1974) Sequential gene function in the initiation of *Saccarhomyces cerevisiae* DNA synthesis. *J. Mol. Biol.* **84**: 445–461.
Jackson, A.L., P.M. Pahl, K. Harrison, J. Rosamond, & R.A. Sclafani (1993) Cell cycle regulation of the yeast Cdc7 protein kinase by association with the Dbf4 protein. *Mol. Cell. Biol.* **13**: 2899–2908.

ARTICLE 12

Surana, U., H. Robitsch, C. Price, T. Schuster, I. Fitch, A.B. Futcher, & K.K. Nasmyth (1991) The role of CDC28 and cyclins during mitosis in the budding yeast *S. cerevisiae*. *Cell* **65**: 145–161.

In Article 3, the authors found that Cdc28 protein functions at START and that strains carrying the *cdc28-1* allele arrest with an unbudded phenotype and 1C DNA content. As demonstrated in previous articles in this case study, Cdc28p is a member of a class of protein kinases called cyclin-dependent kinases (Articles 7 and 9). In *Schizosaccharomyces pombe* the gene encoding the Cdc28p homologue is *CDC2*, and in mammals this protein kinase is called p34 kinase (based on its molecular weight of 34 kD). Cyclin-dependent kinases are functionally active only when bound to an appropriate cognate cyclin protein.

The levels of the cell cycle regulating cyclins vary throughout the cell cycle with different cyclins accumulating at different points in the cell cycle. While there are several species of cyclins, all organisms studied appear to have only one Cdc28p homologue. Thus, it was believed that the *S. cerevisiae* Cdc28 kinase carried out functions at both G1/S and G2/M but this had not been demonstrated experimentally until this report.

1. Strains containing the *cdc28-4* and *cdc28-1N* mutations exhibit distinct phenotypes.
   (a) What is the cellular morphology phenotype and DNA content of strains carrying these alleles at the restrictive temperature at the time of arrested growth?
   (b) What do the authors propose to explain this different phenotype?

2. Four of the seven multicopy suppressors of *cdc28-1N* were B-type cyclins (called *CLB1*, *CLB2*, *CLB3*, and *CLB4*).
   (a) What is a B-type cyclin?
   (b) The *CLB* genes suppressed *cdc28-1N* but not *cdc28-4*. What type of suppression is this? Explain a possible mechanism.
   (c) None of the suppressors obtained in this study was from the G1 class of cyclins (*CLN1*, *CLN2*, and *CLN3*). Propose an explanation.
   (d) Propose a molecular model regarding the interaction between Cdc28p and these two classes of cyclins keeping in mind the specificity of the suppression.
   (e) Other than activating the enzymatic function of Cdc28 kinase, what additional role is suggested for the cyclin subunit by these results?

3. Mutations in *CLB1* and *CLB2* show enhancement and the *clb1*Δ *clb2*Δ double mutant is synthetically lethal.
   (a) How was this demonstrated experimentally? Diagram the appropriate cross.
   (b) Of the models of enhancement presented, which type of enhancement best describes these results?

4. Table 1 shows that *cdc28-1N* and *clb2*Δ exhibit synthetic lethality but *cdc28-1N* and *clb1*Δ do not.
   (a) Diagram the crosses you would do to demonstrate this.
   (b) What evidence in Table 1 demonstrates this?
   (c) How might you have predicted this result from the phenotypes of the *clb1*Δ and *clb2*Δ strains?
   (d) Of the models of enhancement described in Chapter 9, which one best describes the *cdc28-1N* and *clb2*Δ enhancement?

ARTICLE 13

Dowell, S.J., P. Romanowski, & J.F.X. Diffley (1994) Interaction of Dbf4, the Cdc7 protein kinase regulatory subunit, with yeast replication origins *in vivo*. *Science* **265**: 1243–1246.

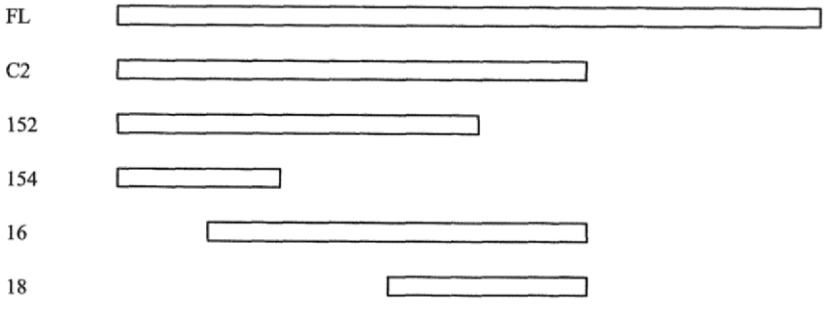
1. Describe the *ARS1-lacZ* reporter gene. A diagram would be helpful.

2. The authors propose to use the *ARS1-lacZ* reporter to identify proteins that bind to origins of replication.
   (a) Describe the concept of this approach which is sometimes referred to as 'the one-hybrid' method.
   (b) Does a positive result in this case indicate **direct** binding of the library fusion protein to ARS1, and if not, why?
   (c) Would you think differently if you were using a mouse ORI and a mouse fusion library? Explain.

3. Figure 1C suggests that Dbf4p interacts generally with yeast origins. What experimental result is the basis of this conclusion?

4. The results in Figure 2 indicate that Dbf4p binds preferentially to domain A of the ARS1. Why do the authors suggest that this binding is not directly to the DNA but is mediated by ORC?

5. The reporter gene in this study is *lacZ* and therefore expression can be monitored on plates using X-gal and blue color production. Based on the results reported in Figure 4, mark the regions containing the ARS1-binding site and the Cdc7p-binding site on the rectangular bar labeled FL in the diagram below. FL represents the full-length Dbf4 protein.



6. Draw a diagram showing the structure of the ARS1, ORC, Cdc7p, Dbf4p complex indicating clearly which components are interacting with which other components based on the results reported in this article.

ARTICLE 14

Ohtoshi, A., T. Miyake, K. Arai, & H. Masai (1997) Analyses of *Saccharomyces cerevisiae* Cdc7 kinase point mutants: dominant-negative inhibition of DNA replication on over-expression of kinase-negative Cdc7 proteins. *Mol. Gen. Genet.* **254**: 562–570.

1. State the specific amino acid changes made in each of the mutations listed in Table 1. (You will need to refer to a table of the single letter designations for the amino acids.) In each case, what reasons do the authors give for choosing to make the particular alteration?

2. The *CDC7* genes under analysis in Table 1 are expressed from the *GAL1–GAL10* promoter. In glucose-grown cells there is a **very** low rate of expression from this promoter and this is increased about 1000-fold by growth on galactose.
   (a) What evidence presented in Table 1 indicates that even the very low rate of the wild-type allele of *CDC7* expression is sufficient to complement the temperature-sensitive phenotype of *cdc7-3*?
   (b) What evidence indicates that each of the *in vitro* generated *cdc7* mutant alleles encodes a defective Cdc7 protein compared with wild-type?

(c) What evidence indicates that Cdc7p alterations T281A, D182N, and D163N lack kinase activity while T281A and T167E have reduced activity?
(d) Western analysis of the galactose-grown cells using anti-Cdc7p antibody would have been an important control and would have strengthened the authors' conclusions. Why?
(e) Correct the underlined word in the following statement from the text of the article. 'However, all the mutants, including T281A and T167E, had lost the ability to complement temperature-sensitive growth of *dbf4(ts)* even when fully induced (Table 1).' Explain.

3. In a number of experimental situations it has been shown that an acidic residue, such as glutamate, mimics a phosphorylated serine or threonine residue. Based on this and on the authors' suggestion that activation of Cdc7p protein kinase requires phosphorylation of T281, predict the phenotype of the T281A and T281E mutant alleles. Did these predictions hold up to experimental testing? Upon which specific results in Table 1 do you base your answer? Discuss.

4. These questions relate to the experiments and results described in Table 2.
(a) Which promoter is driving the expression of the *CDC7* alleles, the native promoter (i.e. the *CDC7* promoter) or the *GAL1–GAL10* promoter?
(b) Are all, some, or none of the transformed strains 209 (*cdc7-3*), KKY401-10B (*dbf4-1*), and 15Dau (*CDC7 DBF4*) carrying plasmids containing the indicated *cdc7* mutants able to grow on glucose at 25°C (data not shown but implied in the text)? If you answered some, which ones?
(c) What carbon source is used to culture the cells in Table 2 and what effect should this have on the expression of the plasmid-borne *cdc7* mutant genes?
(d) Based on the discussion of dominant negative mutations in Chapter 11, which of the two explanations best describes the results in Table 2? Why?
(e) Which results in Table 2 point to the depletion of titratable levels of Dbf4 protein as the basis of the dominant negative effect of Cdc7p-mutant overexpression? Discuss.

5. Describe the experiments demonstrating that the phenotype of overexpression of *cdc7-D182N* is similar to that of loss of Cdc7 kinase, that is, arrest at the G1/S boundary.

6. Figure 3 indicates that the dominant negative effect of each of the three *cdc7* mutants (D163N, D182N and T281E) is suppressed by overexpression of *DBF4*. Draw a diagram showing what the results would have looked like had overexpression of *DBF4* not suppressed the D182N mutation but did suppress the other two.

7. If the authors had had no clues as to the identity of the titratable Cdc7p activator, what experimental approach might they have used to identify the

gene encoding this activator? Be specific as to the type of library, the genotype of the host strain, and the phenotype (including growth conditions) to be selected.

8. Which results in Table 3 indicate that mutation T281E does not affect the ability of the mutant protein to bind Dbf4 protein?

9. If the Dbf4p binding site of Cdc7p had not been known, how might the dominant negative mutations been used to identify it?

ARTICLE 15

Hennessy, K.M., A. Lee, E. Chen, & D. Botstein (1991) A group of interacting yeast DNA replication genes. *Genes Dev.* **5**: 958–969.

The authors of this article make very elegant use of genetic analysis to explore the function of a new series of essential genes involved in the initiation of DNA synthesis. Strains carrying cold-sensitive mutations at two loci, *CDC45* and *CDC54*, arrest growth at low temperatures with a phenotype similar to *cdc7* mutants; that is, they arrest as large budded cells with a single nucleus (Moir *et al.*, 1982). Mutations that suppress the cold-sensitive phenotype of *cdc45* and *cdc54* alleles were isolated in *CDC46* (*MCM5*, *BOB1*) and *CDC47* and strains carrying only these suppressor mutations were found to exhibit a temperature-sensitive phenotype and arrested with the same phenotype as the cold-sensitive *cdc45* and *cdc54* mutants at the nonpermissive temperature (Moir *et al.*, 1982).

The finding that strains carrying mutations in any one of these genes exhibit an apparently identical phenotype that is similar to the *cdc7* mutant phenotype strongly suggests that they all participate in the same essential function, a step required for the initiation of DNA synthesis. The cold-sensitive phenotype of some of the mutant alleles also suggests that the encoded proteins might be components of a large multimeric protein complex. This suggestion is further supported by the results presented in this article.

1. Describe how Southern analysis of CHEF gel-separated chromosomes is used here to map a gene to a particular chromosome.

2. Table 1 lists the various alleles of *CDC45*, *CDC46*, *CDC47*, and *CDC54* used in this analysis and their growth phenotype, cold sensitive or temperature sensitive. The genetic interactions among these genes are summarized in Figure 1. Diagram the crosses demonstrating the following genetic interactions. For each give the genotype and phenotype of the parent strains, the diploid strain, and the spores of a parental ditype, tetratype, and nonparental ditype tetrads.
   (a) *CDC46* and *CDC45* exhibit allele-specific suppression. (Requires two crosses.)
   (b) *CDC46* and *CDC54* show allele-specific synthetic lethality.

3. Mutant strains carrying *cdc45-1*, *cdc46-1*, and *cdc47-1 cdc45*-1 double mutants all arrest at the same point of initiation of DNA replication at the non-permissive temperature. How was this demonstrated? Do *cdc7* and *dbf4* temperature-sensitive mutants exhibit this same phenotype? Be sure to reference the article from this case study series in which this was demonstrated.

4. Studies of the mechanism of recombination indicate that homologous DNA exchange events initiate at sites of double-strand breaks and single-stranded regions of chromosomes. Suggest a hypothesis explaining why *cdc46-1*, *cdc45-1*, and *cdc54-1* mutant strains might exhibit increased rates of recombination. Is your hypothesis consistent with the studies of chromosome integrity described in Figure 4? Explain.

5. PEST sequences are regions of a protein rich in proline, aspartate, glutamate, serine, and threonine.
   (a) What is believed to be the role of PEST sequences in proteins?
   (b) Why do the authors find it so interesting that several of these Cdc proteins contain PEST sequences?

6. What does the term 'immunologically related' mean and how is it demonstrated?

7. What is the significance of finding a mouse protein that is immunologically related to Cdc46p, particularly given the role of Cdc46p in a process common to all eukaryotic cells—the initiation of DNA replication?

REFERENCE

Moir, D., S.E. Stewart, B.C. Osmund, & D. Botstein (1982) Cold-sensitive cell division cycle mutants of yeast: isolation, properties, and pseudoreversion studies. *Genetics* **100**: 547–563.

ARTICLE 16

Lei, M., Y. Kawasaki, M.R. Young, M. Kihara, A. Sugino, & B.K. Tye (1997) Mcm2 is a target of regulation by Cdc7-Dbf4 during the initiation of DNA synthesis. *Genes Dev.* **11**: 3365–3374.

The results of Hardy *et al.* (1997) (Article 11) suggest that one or more components of the MCM complex, a complex of six proteins that is essential for the initiation of DNA synthesis at replication origins in *S. cerevisiae*, is downstream of Cdc7-Dbf4 protein kinase in the pathway that triggers DNA replication. Dowell *et al.* (1994) (Article 13) demonstrate that Cdc7-Dbf4 protein kinase binds to replication origins but their results suggest that binding is indirect and is mediated by other ORI binding proteins such as ORC, or possibly others. The experiments described in Article 16 suggest that the Mcm2 protein may be the protein to which

Cdc7-Dbf4 kinase binds and, along with other Mcm proteins, may be the target of phosphorylation by Cdc7-Dbf4p kinase.

MCM stands for mini-chromosome maintenance. Tye and coworkers isolated mutants with alterations in the *MCM* genes based on their poor retention of ARS-containing YCp plasmids (Maine *et al.*, 1984). Defective plasmid maintenance of *mcm* mutants results from impaired or absent initiation of replication at the plasmid ARS (Yan *et al.*, 1993). The severity of the initiation defect varies from origin to origin suggesting that the Mcm proteins are important regulators of the initiation of DNA replication at chromosomal origins. Mcm2-7 proteins physically interact to form a large multimeric complex found in both the cytoplasm and the nucleus (Chong *et al.*, 1995; Kubota *et al.*, 1995; Lei *et al.*, 1996; Thommes *et al.*, 1997; Young & Tye, 1997). Moreover, the Mcm complex binds to chromatin at origin sites. It is associated with chromatin during the G1 phase of the cell cycle but is removed during the S phase (Todorov *et al.*, 1995; Coue *et al.*, 1996; Donovan *et al.*, 1997; Young & Tye, 1997). The Mcm complex is considered to be a 'licensing factor' that plays an important role in limiting the initiation of DNA replication to the G1 phase. Demonstration that the Mcm proteins are downstream of Cdc7-Dbf4 protein kinase suggests a mechanism whereby this kinase regulates the initiation of DNA replication and restricts it to cells that have to pass through the G1 phase.

1. Describe the method used to isolate suppressors of *mcm2-1*.
   (a) Name the parental strain in which the suppressor mutations were isolated. Give the genotype and phenotype.
   (b) Were the suppressor mutations spontaneous or induced?
   (c) Describe the method used to identify mutant strains containing potential *mcm2-1* suppressor mutations. Is this a selection or a screen and why?
   (d) The potential suppressor mutant clones were then screened for a cold-sensitive phenotype, lack of growth at 14°C. Why? If a second screen had not been used, what would the authors have done to identify second site suppressor mutant strains and distinguish these from true revertants of the *mcm2-1* mutation?

2. Moir *et al.* (1982) obtained *mcm5-ts* (*cdc46-ts*) mutations as suppressors of cold-sensitive mutations in *CDC45* and *CDC30*. It is likely that the results of Moir *et al.* (1982) were instrumental in the decision of these authors to use cold sensitivity as a second screen. Describe how you might use enhancement rather than suppression to identify Mcm2p interacting proteins.

3. Summarize the results that indicate the following.
   (a) The suppressor mutation in strain mts2-1 is an alteration of *DBF4*.
   (b) The suppressor mutation *dbf4-6* is recessive.
   (c) Strains with the genotype *dbf4-6 MCM2* are inviable.

4. Tetrad analysis of diploids homozygous for *mcm2-1* and heterozygous at the *DBF4* locus, *DBF4/dfb4-ts* (*dbf4-1, -3, -4*, and *-5*) gave two viable and two inviable spores (even at the permissive temperature) leading to the conclusion that *mcm2-1* and these *dfb4-ts* alleles are synthetically lethal.

(a) What is the genotype of these inviable spores?
(b) Contrast this result with the phenotype of the *mcm2-1 dbf4-6* double mutant.
(c) Is the genetic interaction between *mcm2-1* and *dbf4* mutants allele specific? Discuss.

5. Describe the two methods used to demonstrate that *dbf4-6* restores the ability of *mcm2-1* mutant strains to complete DNA replication and initiate new rounds of DNA replication.

6. The following questions refer to Figure 4.
(a) Diagram the fusion product of plasmids GAD2F-Dbf4 and BTM116.Mcm2.
(b) Which plasmid pair in Figure 4A controls for the possibility that Mcm2p may be able to bind to the promoter region of the reporter gene and activate *lacZ* expression?
(c) The GAD2F-Dbf4 and BTM116.Mcm2 pair promotes high-level expression of the *lacZ* reporter. The combination of GAD2F-Cdc7 and BTM116.Mcm2 gives a pale blue color. What do these results suggest and are they supported by the results of affinity purification experiments presented in Figures 4B and 4C? Discuss.

7. Describe the *in vitro* kinase assay used to demonstrate that Mcm2, 3, 4, and 6 proteins are targets of Cdc7-Dbf4 protein kinase. Which results demonstrate that Mcm5 and Mcm7 are not phosphorylated by Cdc7-Dbf4 kinase?

8. Discuss the significance of the finding that mutant Mcm2-1 protein is poorly phosphorylated by Cdc7-Dbf4 kinase.

9. The following mutations affect the interaction between Mcm2p and Dbf4p: *mcm2-1*, *dbf4-1*, *dbf4-3*, *dbf4-4*, *dbf4-5*, *dbf4-6*. Based on the results reported in this article, circle the Mcm2–Dbf4 pair with the higher binding affinity given the choices listed below and indicate the result that led you to this conclusion.

(a)	Mcm2p–Dbf4p	versus	Mcm2p–Dbf4-1p
(b)	Mcm2-1p–Dbf4p	versus	Mcm2-1p–Dbf4-1p
(c)	Mcm2p–Dbf4-3p	versus	Mcm2-1p–Dbf4-3p
(d)	Mcm2-1p–Dbf4-6p	versus	Mcm2-1p–Dbf4p
(e)	Mcm2p–Dbf4-6p	versus	Mcm2-1p–Dbf4-6p

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ARTICLE 17

Dohrmann, P.R., G. Oshiro, M. Tecklenburg, & R.A. Sclafani (1999) *RAD53* regulates *DBF4* independently of checkpoint function in *Saccharomyces cerevisiae*. *Genetics* **151**: 965–977.

The eukaryotic cell cycle is a highly regulated process requiring dozens of proteins to coordinate the series of complex events, sometimes occurring in parallel, which must proceed in an orderly manner. After years of studying the genes encoding these regulators, Hartwell and coworkers realized that a second class of regulators existed whose function is to scrutinize the proper completion of each major event in the cell cycle. Hartwell termed these surveillance functions 'checkpoints' (reviewed in Hartwell & Weinert, 1989; Weinert, 1998; Russell, 1998; Lew, 2000, and elsewhere). The events that are monitored by checkpoint pathways include the completion of DNA replication, attachment of kinetochores to the spindle, sister chromatid separation, and cytokinesis.

Progress from one phase of the cell cycle to the next is blocked by the appropriate checkpoint pathway, which becomes activated when the events of the previous cell cycle phase are not properly completed. The checkpoint pathways ensure that DNA replication occurs only once per cell cycle and that replication alternates with sister chromatid segregation and cytokinesis. A checkpoint pathway includes sensor functions that detect whether or not the cell cycle event has been completed, transducer functions that respond to the sensor and act on target functions which block exit from that stage of the cell cycle. The checkpoint pathway thereby provides a delay that hopefully allows for the completion of the necessary steps.

RAD53 encodes a protein kinase involved in all three of the DNA damage checkpoints (reviewed in Weinert, 1998). Other evidence suggests that Rad53

protein kinase is also required for the initiation of DNA replication and Article 17 addresses this role of Rad53p.

1. Summarize the results of previous published studies that suggest a role for Rad53p in the initiation of DNA replication distinct from its checkpoint function.

2. Describe the screen used to identify the *lsd* (lethal with seven defect) mutations.
   (a) Give the name and genotype of the strain used to isolate the *lsd* mutations.
   (b) Describe the red/white adenine colony sectoring assay.
   (c) In your own words, describe why a colony carrying a mutation in a gene other than *CDC7* that is synthetically lethal with *cdc7-1* will not have red sectors.
   (d) How many nonsectoring clones were obtained?
   (e) All nonsectoring clones carried recessive alterations based on the cross to PDY093. Diagram the cross including the *cdc7* and *lsd* genotypes and sectoring phenotypes of both parental strains and describe the sectoring phenotype of the diploid. Explain why the phenotype of the diploid indicates that the *lsd* mutation is recessive.
   (f) If one of the mutations had been dominant, what would have been the phenotoype of the diploid?

3. Eighteen of the mutants showed single gene segregation when crossed to PDY093. These were placed into complementation groups.
   (a) Mutants 18 and 24 are in the same complementation group, *LSD6*. Diagram the cross between these mutants that demonstrates that they are alleles of *LSD6*. Show the genotypes of the parental strains with regard to *LSD6* and *CDC7*, and *ADE2* and plasmid p*ADE2*. Give the sectoring phenotype of the parents and the diploid.
   (b) Mutants 6 and 18 are in different complementation groups. What is the sectoring phenotype of the diploid heterozygous for these mutant genes?

4. Describe the cloning of *LSD1*. Include the genotype of the strain used to construct the library, the genotype of the host strain transformed with the library, and the phenotype used to identify the clone containing the plasmid-borne *LSD1*.

5. How did the authors demonstrate the *LSD1* was *RAD53*? (Two methods.)

6. Describe the methods used to demonstrate that the checkpoint function of Rad53p is not affected in the *rad53-31* allele.

7. Which model of enhancement best describes the genetic interaction between mutations in *CDC7* and *RAD53*? Why? (Use the results in Table 3 for your answer.)

8. Based in part on the results in Table 4, the authors conclude that *bob1-1* does not suppress *rad53-31* as it does *cdc7-1* and *dbf4* mutations. What interfering phenomenon complicated their analysis of these results?

9. The results of two-hybrid analysis shown in Figure 2 indicate that Rad53p and Dbf4 interact.
   (a) Approximately which residues of Rad53p are involved in the interaction?
   (b) The strain carrying pRad53iDB and pDbf4AD gives 15 Miller units of β-galactosidase activity. The authors conclude that there is no interaction. Why?

10. The results in Figures 3 and 4 indicate that *DBF4* expression at the level of transcription and translation is dependent on Rad53p.
   (a) These results do not explain the synthetic lethality of *rad53-31* with *cdc7-1* and *dbf4-1*. Why?
   (b) What explanation do the authors provide?
   (c) Which of the results presented in Figures 3 and 4 is consistent with this interpretation? (List at least two.)

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ARTICLE 18

Iyer, V.R., C.E. Horak, C.S. Scafe, D. Botstein, M. Snyder, & P.O. Brown (2001) Genomic binding sites of the yeast cell-cycle transcription factors SBF and MBF. *Nature* **409**: 533–538.

This article uses a combination of DNA microarray analysis and chromatin immunoprecipitation to identify potential binding sites of the transcription factors SBF and MBF. The method is interesting because it provides an example of how DNA microarray analysis can be used to identify sequences that are enriched in a particular sample. Here the sample is enriched for DNA fragments that are specifically bound by a particular protein. One could also use this method to identify sequences that are enriched or depleted from a genome because of duplications, deletions, or aneuploidy.

1. Describe the transcription factors SBF and MBF and their role in regulating the cell cycle in *Saccharomyces*.

2. Describe chromatin immunoprecipitation (often referred to as CHIP) and the specific method used in this article to isolate SBF- or MBF-binding DNA fragments.
   (a) The cross-linking step is done *in vivo* in whole cells. Why is this important?
   (b) What is the size range of the DNA fragments generated by their method? Why was this size range chosen instead of shearing the samples to produce smaller or larger fragments?
   (c) CHIP was done on unsynchronized cells, α-factor treated cells, and benomyl-treated cells. The last two treatments synchronize cells at which point in the cell cycle?

3. Describe the primer pairs used for the PCR amplification of the DNA fragments and synthesis of the intergenic DNA microarray.

4. Discuss the reason for each of the control experiments (Lanes 1, 2, and 3).

5. Describe the criteria used to evaluate the microarray data in order to identify potential SBF-regulated genes.

6. Describe the criteria used to evaluate the microarray data in order to identify potential MBF-regulated genes.

7. The authors present three lines of evidence in support of their conclusion that valid *in vivo* SBF- and MBF-binding sites have been identified. Describe this evidence.

8. The authors ask the question, 'Why are two different transcription factors used to mediate nearly identical transcriptional programmes during the cell-division cycle in yeast?' Summarize their reasons for making this statement and their answers to this question.

9. Many of the SBF- and MBF-binding sites identify potential novel genes involved in regulating the cell cycle. You wish to explore the function of these novel genes.
   (a) Give criteria that you might use to select a specific novel ORF for further study.
   (b) List the biochemical, cell biology, and genetic characterizations you would carry out to explore the cell cycle role of this novel gene.

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Case Study IV
Mating-Type Pheromone Response Pathway of *Saccharomyces*

READING LIST

Mutant Hunts: To Select or to Screen (Perhaps Even by Brute Force)

Article 1

MacKay, V. & T.R. Manney (1974a) Mutations affecting sexual conjugation and related processes in *Saccharomyces cerevisiae*. I. Isolation and phenotypic characterization of nonmating mutants. *Genetics* **76**: 255–271.

Article 2

MacKay, V. & T.R. Manney (1974b) Mutations affecting sexual conjugation and related processes in *Saccharomyces cerevisiae*. II. Genetic analysis of nonmating mutants. *Genetics* **76**: 273–288.

Complementation Analysis: How Many Genes are Involved?

Article 3

Hartwell, L.H. (1980) Mutants of *Saccharomyces cerevisiae* unresponsive to cell division control by polypeptice mating hormone. *J. Cell Biol.* **85**: 811–822.

Gene Isolation and Characterization

Article 4

Burkholder, A.C. & L.H. Hartwell (1985) The yeast α-factor receptor: structural properties deduced from the sequence of the *STE2* gene. *Nucleic Acids Res.* **13**: 8463–8475.

Article 5

Whiteway, M., L. Hougan, D. Dignard, D.Y. Thomas, L. Bell, G.C. Saari, F.J. Grant, P. O'Hara, & V.L. MacKay (1989) The *STE4* and *STE18* genes of yeast encode potential and subunits of the mating factor receptor-coupled G protein. *Cell* **56**: 467–477.

Epistasis Analysis

Article 6

Blinder, D., S. Bouvier, & D.D. Jenness (1989) Constitutive mutants in the yeast pheromone response: ordered function of the gene products. *Cell* **56**: 479–486.

Article 7

Cairns, B.R., S.W. Ramer, & R.D. Kornberg (1992) Order of action of components in the yeast pheromone response pathway revealed with a dominant allele of the 11 kinase and the multiple phosphorylation of the 7 kinase. *Genes Dev.* **6**: 1306–1318.

Gene Identification, Isolation, and Characterization (A New Selection Method)

Article 8

Chang, F. & I. Herskowitz (1990) Identification of a gene necessary for cell cycle arrest by a negative growth factor of yeast: *FAR1* is an inhibitor of a G1 cyclin, CLN2. *Cell* **63**: 999–1011.

Article 9

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Suppression

Article 10

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Article 11

Hasson, M.S., D. Bllinder, J. Thorner, & D.D. Jenness (1994) Mutational activation of the *STE5* gene product bypasses the requirement for G protein β and γ subunits in the yeast pheromone response pathway. *Mol. Cell. Biol.* **14**: 1054–1065.

Enhancement

Article 12

Akada, R., L. Kallal, D.I. Johnson, & J. Kurjan (1996) Genetic relationships between the G protein $\beta\gamma$ complex, Ste5p, Ste20p and Cdc42p: investigation of effector roles in the yeast pheromone response pathway. *Genetics* **143**: 103–117.

Two-Hybrid Analysis

Article 13

Choi, K-Y., B. Satterberg, D.M. Lyons, & E.A. Ellion (1994) Ste5 tethers multiple protein kinases in the MAP kinase cascade required for mating in *S. cerevisieae*. *Cell* **78**: 499–512.

Article 14

Whiteway, M., K.L. Clark, E. Leberer, D. Dignard, & D.Y. Thomas (1994) Genetic identification of residues involved in association of α and β G-protein subunits. *Mol. Cell. Biol.* **14**: 3223–3229.

Advanced Concepts in Molecular Genetic Analysis—Mutation Analysis

Article 15

Valtz, N., M. Peter, & I. Herskowitz (1995) *FAR1* is required for oriented polarization of yeast cells in response to mating pherones. *J. Cell Biol.* **131**: 863–873.

Two-Hybrid Analysis

Article 16

Butty, A.-C., P.M. Pryciak, L.S. Huang, I. Herskowitz, & M. Peter (1998) The role of Far1p in linking the heterotrimeric G protein to polarity establishment proteins during yeast mating. *Science* **282**: 1511–1516.

Genome-Wide Analysis

Article 17

Erdman, S., L. Lin, M. Malczynski, & M. Snyder (1998) Pheromone-regulated genes required for yeast mating differentiation. *J. Cell Biol.* **140**: 461–483.

ARTICLE 1

MacKay, V. and T.R. Manney (1974a) Mutations affecting sexual conjugation and related processes in *Saccharomyces cerevisiae*. I. Isolation and phenotypic characterization of nonmating mutants. *Genetics* **76**: 255–271.

Haploid *Saccharomyces* cells may be either mating type **a** or mating type α and mating type is determined by the *MAT* locus which maps to chromosome *III*. There are two alleles of *MAT*: *MAT***a** is present in cells of mating type **a** and *MAT*α is present in cells of mating type α. The *MAT* alleles encode different DNA-binding transcription regulators that control the expression of a large battery of unlinked genes involved in cell type determination (**a** mating type, α mating type, **a**/α diploid). Haploid cells mate with cells of the opposite mating type but do not undergo meiosis or form spores. Diploid **a**/α cells undergo meiosis and sporulate under the appropriate conditions but do not mate with any cell type. Mating and sporulation are both very complex processes and require dozens of gene functions. This Case Study focuses on mating and, more specifically, how haploid cells sense and respond to the presence of the mating pheromone produced by cells of the opposite mating type.

Haploid cells respond to the presence of cells of the opposite mating type as follows. First, both partners in the mating pair arrest as unbudded cells in G1 of the cell cycle at Start. Start is the point at which the cell becomes committed to division and is the execution point of Cdc28 cyclin-dependent kinase action. Second, both partners form a mating projection directed toward the mating partner. Cells with this projection are pear-shaped and are quite distinct from budding cells. The shape is referred to as a schmoo. Third, gene expression of a number of mating-specific genes is induced. One of the first of these genes to be identified was **a**-agglutinin, a surface protein involved in adhesion to the mating partner and produced by **a** mating type cells. It localizes to the schmoo tip. Most strains of mating type α produce α-agglutinin constitutively but in some strains α-agglutinin expression is induced by the presence of mating type **a** cells.

At the time that Article 1 was published mating type α cells were known to produce a secreted peptide hormone, or pheromone, called α-factor. Similarly, in Article 1 the authors demonstrate that mating type **a** cells produce a diffusible factor called **a**-factor that was later shown to also be a peptide hormone. Thus the mating response described above is likely to be the result of events initiated by the effects of these pheromones on cells of the opposite mating type. Mutations affecting the ability of haploid cells to mate could identify genes encoding components of the pheromone sensing pathway as well as functions required for pheromone production or the process of mating itself. In Article 1, the authors develop a strategy for isolating mutants that are unable to mate, which they call 'sterile' or *ste* mutants.

1. Using the table below, summarize and compare the phenotypes of **a** mating type, α mating type, and **a**/α diploid cells.

Phenotype	**a** Mating-type cells	α Mating-type cells	**a**/α Diploid cells
Cell cycle arrest in response to **a**-factor			
Cell cycle arrest in response to α-factor			
a-Factor secretion			
α-Factor secretion			
Schmoo formation in response to **a**-factor			
Schmoo formation in response to α-factor			
a-Agglutinin synthesis			
α-Agglutinin synthesis			
Sporulation under starvation conditions			

2. The *CAN1* gene encodes arginine permease. Canavanine is an analogue of the amino acid arginine and is utilized by *Saccharomyces* in place of arginine for protein synthesis. When this occurs it is toxic to the cells. Based on this description, explain the fact that recessive mutations in *CAN1* are resistant to the toxic effects of canavanine.

3. Explain the strategy behind the procedure used here to isolate nonmating clones.

4. Are these *ste* mutants spontaneous or induced? Explain.

5. Is this a selection or a screen? Explain.

6. Explain why the *can1* mutation must be in the strain that is mutagenized and not in the strain to which it is mated during the mutant isolation procedure.

7. Explain why it is necessary to screen the canavanine resistant potential *ste* mutant clones obtained for the following criteria.
   (a) The nutritional requirements of the potential mutant are the same as the mutagenized parental strain.

(b) The potential mutant is unable to mate with tester strains of both mating types.
(c) The potential mutant is unable to sporulate.

8. Explain why the mutants obtained from one isolation procedure are unlikely to be independent, i.e. different mutations that arose separately.

9. List the other phenotypes for which the *ste* mutants were tested.

ARTICLE 2

MacKay, V. & T.R. Manney (1974b) Mutations affecting sexual conjugation and related processes in *Saccharomyces cerevisiae*. II. Genetic analysis of nonmating mutants. *Genetics* **76**: 273–288.

In Article 1, MacKay & Manney isolated a series of sterile mutants that they placed into 16 different phenotypic classes. Most of these mutants were not conditional, i.e. they were sterile under all growth conditions. Thus, genetic analysis of these mutants would be difficult. Mating frequency was very low and some diploids did not sporulate. The greatest difficulty was encountered when the mutations were to be placed into complementation groups. It was not possible to do the traditional complementation test because the phenotype, ability to mate, is a characteristic of haploid cells and complementation tests are done in the diploid. So the authors did the next best thing, they mapped the mutations. If two mutations are unlinked, then they must be in different genes. If two mutations are tightly linked, then they **may** be in the same gene.

1. Ten of the mutants isolated in the *MATα* strain exhibit tight linkage to the mating-type locus and are probably alterations in *MATα*, which the authors refer to as *MT* or *STE1*. Diagram a cross of a Class 1 mutant (*matα/ste1*) to a wild-type *MAT***a**/*STE1* strain. Give the genotype and phenotype of the tetrads resulting from this cross (remember the *ste* mutation is tightly linked to *MATα*). Are your results consistent with those for mutant VC2 shown in Table 2? Discuss why the authors state that, 'the upper limit for the map distance between the *ste* mutation in VC2 and *MT* is 1.2 centimorgans'.

2. Class 4 mutants were isolated in a *MATα* strain. The five mutants in this class are unlinked to *MATα* and are α-specific. That is, only *MATα* strains exhibit the *ste* phenotype. The authors refer to these mutants as *ste3* mutants. Diagram the cross between a Class 4 mutant (*MATα ste3*) and a wild-type *MAT***a** *STE3* strain. Give the genotype and phenotype (mating type and ability to mate) of the spores in the tetrad types that result from such a cross. Are your projected results consistent with those shown in Table 2 for mutant VC3? Discuss. Include a calculation demonstrating that *MATα* and *ste3* mutations in VC3 are unlinked.

3. The results in Table 5 indicate that all five of the Class 4 α-specific mutants are tightly linked. Discuss these results and the authors' conclusion that all the Class 4 mutations are likely to be allelic.

4. Class 5 mutants were nonspecific for mating type. That is, both **a** and α mating-type strains carrying these *ste* mutations were sterile. Four mutants fell into Class 5. Based on the results shown in Table 6, the authors place these four mutants into two groups representing two *ste* genes, *ste4* and *ste5*. Discuss their reasoning.

5. Discuss the authors' conclusion that *STE2* and *STE3* encode the α-factor and **a**-factor receptors, respectively.

ARTICLE 3

Hartwell, L.H. (1980) Mutants of *Saccharomyces cerevisiae* unresponsive to cell division control by polypeptide mating hormone. *J. Cell Biol.* **85**: 811–822.

In this article Hartwell uses the G1 arrest response to isolate mutants in the pheromone response pathway. Since α-factor is easily purified from the culture medium of mating type α cells, Hartwell was able to add the pheromone directly to the culture medium. Hartwell avoided the problems in genetic analysis encountered by MacKay & Manney as follows. First, he isolated temperature-sensitive mutants. In this way, crosses could be carried out at the permissive temperature at which even the mutants were able to mate but phenotype testing could be done at the non-permissive temperature. Second, he used a genetic manipulation method to convert the *MAT***a**/*MAT*α diploids, in which the phenomone sensitivity of the *ste* mutations could not be determined, to *MAT***a**/*MAT***a** homozygous diploids. Despite the fact that these strains are diploid for all chromosomes and at all loci, they are mating type **a** because they are homozygous for the *MAT***a** allele and they exhibit the same phenotypes as haploid *MAT***a** strains. They arrest as unbudded cells in the presence of α-factor, they schmoo in the presence of α-factor, they mate with mating type α strains, etc. *MAT***a**/*MAT***a** diploids heterozygous for different *ste* mutations thus can be tested for all of these mating type **a** phenotypes and therefore can be used for complementation analysis.

The conversion of *MAT*α/*MAT***a** to *MAT***a**/*MAT***a** diploids relies on a type of gene conversion event or 'loss of heterozygosity' that occurs at low frequency at any site in the genome but perhaps is more common at the *MAT* locus because of the mating-type switching process that occurs in this region. The conversion of the *MAT*α locus to *MAT***a** involves a rather large section of the chromosome and clearly extends beyond the *MAT* locus to the linked *CRY1* gene. Hartwell cleverly uses the recessive *cry1* cryptopleurine resistant mutation to select for this 'loss of heterozygosity' event. The *MAT*α *CRY1*/*MAT***a** *cry1* diploid strain is sensitive to cryptopleurine but, if a conversion event occurs in this region that replaces the *CRY1* sequence with the *cry1* sequence from the homologous chromosome, then one can select these cells based on their resistance to cryptopleurine. Because of the

linkage of *MAT*α to *cry1*, these cells are frequently simultaneously converted from *MAT*α to *MAT***a**.

1. Describe the method used to isolate mutants resistant to α-factor arrest. Include in your answer:
   (a) List the complete genotype and phenotype of the parent strain 381G. Discuss the *SUP4-3* mutant allele.
   (b) Are these spontaneous or induced mutants and why?
   (c) Is this a selection or a screen and why?
   (d) Describe the conditions of this selection/screen.
   (e) Define the conditional nature of the phenotype exhibited by the mutants and describe the permissive and nonpermissive conditions.
   (f) How does the author ensure that the mutants are independent?

2. After the primary selection/screening of mutant clones, secondary testing was used. Describe this test and the desired phenotype. Give one reason why this secondary testing was carried out.

3. Before complementation analysis could be carried out, the author needed to demonstrate that each mutant clone contained only a single mutation. Describe the cross you would do to demonstrate this. Give the genotype and phenotype of the parents and the results of tetrad analysis of this diploid if the mutant contains a single alteration in a *ste* gene that is not mating-type specific.

4. Before complementation analysis could be carried out, the author needed to demonstrate that the mutation in each mutant clone was recessive. Describe a method Hartwell might have used to determine this. Base you answer on the methods presented in Figure 1.

5. Describe in detail the method for complementation analysis summarized in Figure 1.
   (a) List the complete genotype and phenotype of the 381G mutant parent, the 382-31 parent, and the diploid produced by mating these two strains.
   (b) Describe the conditions for mating these strains and for the selection of diploids.
   (c) Describe the conditions for selecting *MAT***a** *cry1*/*MAT***a** *cry1* homozygotes.
   (d) Describe how you would determine whether the two *ste* mutations in your cross are or are not complementing.

6. Hartwell (1980) tested his *ste* mutants for the following phenotypes: mating ability, biochemical synthesis of macromolecules, schmoo formation, budding patterns, agglutination, and mating factor production and destruction. List the different phenotypes of mutations in *ste2, 4, 5, 7, 8, 9, 11,* and *12*. Why are *ste8* and *ste9* mutants considered different from the rest? *SIR3* and *SIR4* encode proteins required for silencing the normally repressed copies of *MAT***a** and *MAT*α, called *HMR***a** and *HML*α, located near the telomeres of chromosome

III. Explain why mutations in *sir3* or *sir4* would give the demonstrated phenotypes of *ste8* and *ste9* mutations.

7. In Article 3, Hartwell (1980) identified mutations in *STE2, 4,* and *5* that were also identified by MacKay & Manney (Article 2). None was isolated in *STE3*. Explain.

ARTICLE 4

Burkholder, A.C. & L.H. Hartwell (1985) The yeast α-factor receptor: structural properties deduced from the sequence of the *STE2* gene. *Nucleic Acids Res.* **13**: 8463–8475.

In Article 2 MacKay & Manney suggest that *STE2* and *STE3* encode components of the pheromone receptors, **a**-factor receptor and α-factor receptor, respectively, because mutations in these genes are specific to one mating type. None of the other *ste* mutants was mating-type specific. Strains of the **a** mating type respond specifically to α-factor and thus must synthesize a receptor capable of recognizing this peptide pheromone. *STE2* is **a** mating-type specific and temperature-sensitive *ste2* mutant strains exhibit temperature-sensitive α-factor binding (Jenness *et al.* 1984). In Article 4, the authors clone *STE2* and the deduced sequence of the encoded protein is consistent with a membrane receptor function for Ste2 protein.

1. Describe the library used to clone *STE2*.
   (a) What type of library was used (genomic, cDNA, expression, etc.)?
   (b) What was the *STE2* genotype of the strain that was the source of the DNA fragments (*STE2* or *ste2*)? You should be able to deduce this without reading the source reference.
   (c) Describe the vector used. Give its name and average copy number in *Saccharomyces*. (This vector carries *LEU2* as the selection marker.)
   (d) Describe how the yeast DNA fragments were obtained and inserted into the vector.

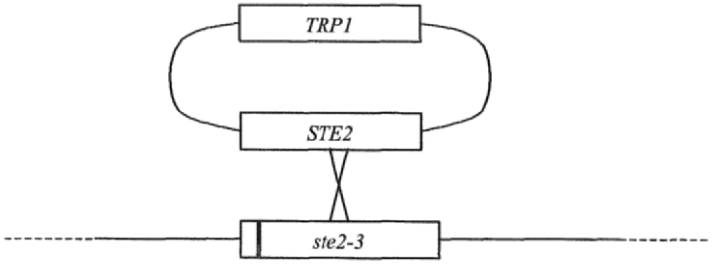
2. Describe the cloning method.
   (a) Give the complete genotype of the *Saccharomyces* strain used as the host strain for the library. What specific mutant allele of *STE2* does it contain and describe the phenotype?
   (b) Why were Leu+ transformants selected?
   (c) Why were transformants screened for their ability to mate at 34°C?
   (d) Why is this method referred to as 'cloning by complementation'?

3. Two transformants showed wild-type mating ability.
   (a) What is the significance of demonstrating that loss of the plasmid causes loss of both the Leu+ and mating ability+ phenotype simultaneously?
   (b) The isolation of two complementing plasmids helped to localize the *STE2* gene within the insert fragments contained in these plasmids. How?

(c) Describe the method used to further localize *STE2* within the 4.3 kbp *Bam*HI fragment shown in Figure 1.

4. Define the term 'allele specific'. Describe the method used to demonstrate that the plasmid-borne complementing gene is not allele specific.

5. Plasmid integration was used to demonstrate that the complementing fragment derives from the *STE2* sequence. Integration occurs via a recombination event between a sequence on the plasmid and a **homologous** sequence in the host cell's genome. Plasmid pAB503 contains two yeast sequences, *TRP1* and the presumed *STE2*, and therefore it should be able to integrate but **only** at either of these two positions in the genome. If the insert sequence is not *STE2*, then integration will occur at the genomic site of the homologous sequence and not at *STE2*. Tetrad analysis is used here to demonstrate the site of integration and the results are presented in Table 1.
   (a) Using the diagram below, draw the structure of the genomic region that would result from the integration of plasmid pAB503 at *ste2-3*.



   (b) Explain why the results for transformants B3-1 and B3-12-3 in Table 1 are consistent with integration at *ste2-3*.
   (c) How would these results differ if B3-1 had been crossed to a *MAT*α *ste2-3 TRP1* strain?
   (d) How could this plasmid have been targeted to integrate **only** at the homologous site in the genome?

6. What do the results in Table 2 demonstrate? Why were the results using strains 4277-7, YR-4-3, and 4345-32 included?

7. What is the significance of finding an α2 protein binding site in the promoter sequence of *STE2*?

8. Describe how a 'hydropathy plot' like the one shown in Figure 4 is caluculated. What is the significance of a 'peak' about 20 residues in length with a hydrophobicity of greater than +1?

9. How are the results of the sequence analysis of Ste2 protein consistent with its proposed role as the α-factor receptor? How does Ste2p differ from other known peptide receptors?

10. Ste2p and Ste3p (the **a**-factor receptor) have similar structures but exhibit no obvious sequence similarity. Nonetheless, the genetic evidence (Article 2, Article 3) suggests that both receptors signal via the same pathway. Discuss.

REFERENCE

Jenness, D.D., A.C. Burkholder, & L.H. Hartwell (1983) Binding of α-factor pheromone to yeast **a** cells: chemical and genetic evidence for a factor receptor. *Cell* **35**: 521–529.

ARTICLE 5

Whiteway, M., L. Hougan, D. Dignard, D.Y. Thomas, L. Bell, G.C. Saari, F.J. Grant, P. O'Hara, & V.L. MacKay (1989) The *STE4* and *STE18* genes of yeast encode potential β and γ subunits of the mating factor receptor-coupled G protein. *Cell* **56**: 467–477.

1. Describe the phenotype of the M200-6c *ste18* mutant strain (*MAT***a** *sst1 sst2 ste18-1*) shown in Figure 1.

2. The authors state, 'The original *ste18* mutant was transformed with a genomic yeast DNA library cloned in the centromere vector YCp50, and colonies were screened for the reappearance of the supersensitivity to α-factor exhibited by the parent strain of the mutant.'
   (a) Considering that 5000–10 000 transformants need to be screened, suggest a rapid screening method using replica plating that might be used.
   (b) YCp50 carries the *URA3* selectable marker. List the steps needed to clone *STE18* from this library in strain *MAT***a** *sst1 sst2 ste18-1*.

3. One-step gene disruption was used to demonstrate that the cloned fragment derives from and contains *STE18*. What segregation pattern of Ura+ to Ura–, mater to nonmater, would have resulted had the cloned fragment derived from a sequence unlinked to *STE18*?

4. Discuss the sequence homologies for Ste4p and Ste18p to other proteins in the databases. What sequence motifs of known significance are found in Ste4p and Ste18p?

5. In Table 1, the colum labeled '*STE18*' indicates whether a *ste18-LacZ* reporter is integrated at *STE18*.
   (a) Diagram this 'in-frame' *ste18-LacZ* reporter. You need to indicate the approximate position of the junction site between *STE18* and *LacZ* sequences and in what part of *STE18* (promoter, ORF, etc.) the junction site is located.

(b) Diagram the protein product produced.
(c) Summarize the results that indicate that *STE18* is transcribed in **a** and α mating type cells but not in **a**/α diploid cells.

6. Discuss the significance of the finding that strains carring the null *ste4*Δ and *ste18*Δ mutations have the same phenotypes as the previously isolated mutations that may be only single amino acid changes.

7. *GPA1*, *STE4*, and *STE18* appear to encode the components of a heterotrimeric G-protein complex similar to others characterized in mammalian cells. In those systems, *in vitro* biochemical evidence suggests that the α subunit acts separately from the $\beta\gamma$ subunits and that the disassociated state is the activated state. What genetic results reported in this article are consistent with these findings from the mammalian systems?

8. Summarize the genetic evidence reported in this article that allows the authors to conclude the following. (This is an epistasis analysis and was studied in detail in Chapter 6; see also Articles 6 and 7 of this Case Study.)
   (a) Ste4p and Ste18p are both required for activation of the mating type signaling pathway. That is, Ste4p and Ste18p have positive functions in the pathway.
   (b) Gpa1p inhibits signaling through the pathway. That is, Gpa1p has a negative function in the mating-type signaling pathway.
   (c) The inhibitory action of Gpa1p occurs at an earlier part of the pathway than the function of Ste4p or Ste18p (Table 2).

ARTICLE 6

Blinder, D., S. Bouvier, & D.D. Jenness (1989) Constitutive mutants in the yeast pheromone response: ordered function of the gene products. *Cell* **56**: 479–486.

1. The results reported in Dietzl & Kurjan (1987) and Whiteway *et al.* (1989) (Article 5) indicate that the *STE* genes encode components of a switch regulatory pathway as defined in Chapter 6. Discuss the evidence that supports this conclusion.

2. Explain why the anticipated phenotype of constitutive mutations in the mating-type signaling pathway is haploid lethal.

3. Based on your knowledge of the roles of Ste2p, Gpa1p (Scg1p), and Ste4p, would you expect dominant or recessive constitutive mutations in *STE2*, *GPA1*, and *STE4*? Explain.

4. Describe the red/white colony-sectoring assay. How is it used here to identify clones carrying potential haploid lethal mutations? Include in your answer an

explanation of why candidate haploid lethal mutants would have a non-sectoring phenotype.

5. Describe the procedures undertaken in the analysis of the 87 nonsectoring mutant clones that reduced this number to the final five mutants studied in this article. What phenotypes distinguish haploid lethal mutants affecting the mating-type signaling pathway from other haploid lethal mutants affecting other pathways?

6. For genetic analysis of the haploid lethal mutants, it is essential that cells that have lost the pDJ117 plasmid are capable of mating even though they are incapable of division. Discuss.

7. Describe the experimental procedure used to test dominance of the haploid lethal mutations.

8. Describe the two methods used to demonstrate that the four recessive haploid lethal mutations are alleles of *SGC1* (*GPA1*).

9. Diagram the cross *HPL-6* × *HIS3*. Give the genotype and phenotype (viability and ability to grow in the absence of histidine) of the parents, diploid, and PD, NPD, and TT tetrad spores. (Assume that the mating is between cells of strain 5680-4 (*his3*) and cells of mutant DB6 that have lost the pD117 plasmid.)

10. Table 3 presents the segregation pattern of the mutant phenotype.
    (a) What is the 'mutant phenotype'?
    (b) In the column labeled '3:1', what is the phenotype of the '3' spores?

11. In Table 3, the first two rows demonstrate that *scg1-4* is epistatic to *ste2-3^{ts}*.
    (a) What data allow the authors to make this conclusion?
    (b) Of 10 tetrads from the W.T. × *ste2-3^{ts}* cross, two give a 3:1 segregation pattern. What does this indicate and how does this help you in your evaluation of the results of the *scg1-4* × *ste2-3^{ts}* cross?
    (c) Would you have expected 'cold-sensitive segregants' given the segregation pattern observed? Explain.

12. The results of the cross of *STE4^{Hpl}* × *ste5-3^{ts}* in Table 3 indicate that *ste5-3^{ts}* is epistatic to *STE4^{Hpl}*.
    (a) Specify these results.
    (b) What is the phenotype (inviability, morphology, mating efficiency) of the *STE4^{Hpl} ste5-3^{ts}* double mutant segregants at 34°C and at 22°C?
    (c) Give the genotype and phenotype of the spores of a 3:1 tetrad.
    (d) What is the genotype of the 11 cold-sensitive segregants and is this the expected number given the number of tetrads analyzed? Explain.

13. The authors suggest one possible mechanism for the constitutive activity of the *Ste4^{Hpl}* mutant protein. Discuss. Given the same model of the pheromone

response pathway proposed in Figure 2, suggest an alternate mechanism for this Ste4p constitutive mutant. (Assume that some free $\beta\gamma$ is always present.)

REFRENCE

Dietzel, C. & J. Kurjan (1987) The yeast *SCG1* gene: a G α-like protein implicated in the **a**- and α-factor response pathway. *Cell* **50**: 1001–1010.

ARTICLE 7

Cairns, B.R., S.W. Ramer, & R.D. Kornberg (1992) Order of action of components in the yeast pheromone response pathway revealed with a dominant allele of the 11 kinase and the multiple phosphorylation of the 7 kinase. *Genes Dev.* **6**: 1306–1318.

1. Diagram the structure of the *GAL1promoter–STE11ΔN* fusion gene. Based on the information given in this article, indicate in your diagram the basepair numbers of the region of *STE11* included in the fusion, the basepair number of the proposed translation start site, and the predicted size of the protein product (number of residues). How did the authors experimentally demonstrate the expression of a Ste11 protein of the predicted size?

2. Describe the experiments used to demonstrate the following.
   (a) The Ste11ΔN protein is functional.
   (b) *STE11ΔN* is dominant to *STE11*.
   (c) *STE11ΔN* is constitutive.

3. Give the specific result(s) from Table 1 that demonstrate the following.
   (a) *STE11ΔN* is epistatic to *ste4*.
   (b) *ste7* is epistatic to *STE11ΔN*.
   (c) *FUS3* and *KSS1* have overlapping functions in the mating-type signaling pathway.
   (d) *fus3* and *kss1* are epistatic to *STE11N*.

4. Ste7 protein exhibits sequence homology with several serine/threonine protein kinases. The authors constructed the *ste7-A220* mutation to demonstrate that the postulated kinase activity is essential for Ste7p signaling in the mating-type pheromone response pathway. Describe this experiment. Include in your answer the reasons for mutating this specific residue.

5. Discuss the reasons why the authors cannot place the Kss1 and Fus3 kinases at a specific position downstream of Ste11 kinase. Describe a genetic approach (using epistasis analysis) that you might use to determine whether *KSS1* and *FUS3* are upstream or downstream of *STE7*.

ARTICLE 8

Chang, F. & I. Herskowitz (1990) Identification of a gene necessary for cell cycle arrest by a negative growth factor of yeast: *FAR1* is an inhibitor of a G1 cyclin, CLN2. *Cell* **63**: 999–1011.

Prior to this report, all the mutants isolated based on their resistance to α-factor arrest were also found to be sterile and did not induce *FUS1* or other genes regulated by the mating-type pheromone response pathway. In this article the authors search for mutants that separate these phenotypes. That is, they searched for mutants that were resistant to pheromone arrest but were still capable of signaling.

To understand the mechanism of cell cycle arrest by the mating-type pheromones, the reader must become familiar with the regulatory controls of the cell division cycle, particularly those controlling the G1 to S transition. The description in a cell biology text should be sufficient. In *Saccharomyces*, *CDC28* encodes the $p34^{Cdc2}$ kinase homologue that is the key regulator of both the G1 to S and G2 to M transitions. Cdc28 protein is a cyclin-dependent kinase and is activated by binding with a cyclin protein. *Saccharomyces* has two classes of cyclins used to regulate the cell cycle encoded by the *CLN* and *CLB* genes. *CLN1*, *CLN2*, and *CLN3* encode the G1 cyclins. That is, they are expressed during the G1 phase, although their expression patterns are distinct. The Cln proteins exhibit sequence homology but are not identical, and binding of any one of these to Cdc28 is sufficient to traverse START. Three different cyclins, encoded by the *CLB* genes, regulate Cdc28 during the G2 to M transition and are referred to as the G2 cyclins. A culture of unsynchronized cells when treated with pheromone will proceed through S, G2, and M and arrest in G1 at START. In earlier studies Hereford & Hartwell (1974) demonstrated that α-factor arrested cells at START and this was coincident with the requirement for Cdc28 activity. Thus, the G1 cyclins are the likely targets of α-factor arrest. For these reasons, Chang & Herskowitz investigated the *CLN* genes as targets of *FAR1*-mediated cell cycle arrest in response to α-factor.

1. Discuss the details of the mutant isolation screen that enabled the authors to isolate this novel class of mutants resistance to α-factor arrest. How are they novel?

2. Describe the following phenotypes of *far1* mutants **in the presence of α-factor**.
   (a) Changes in the cell cycle.
   (b) Colony formation.
   (c) Transcription of downstream target genes like *FUS1* and agglutinin.
   (d) Morphological changes.
   (e) Mating competency.

3. What results indicate that Far1p acts only in the α-factor response pathway that regulates cell division and not in other pathways regulating the cell cycle, such as those that respond to changes in nutrient levels?

4. Summarize the evidence that *FAR1* expression is regulated by pheromone via the pheromone response pathway.

5. Based on the results shown in Figure 6, the authors conclude that Far1p is a negative regulator of Cln2p and not Cln1p or Cln3p. These conclusions are summarized in Figure 9.
   (a) What evidence indicates that all three Cln proteins are inhibited in *MATα* cells exposed to α-factor?
   (b) Which mutant allele is epistatic, *cln2* or *far1*? Which gene is downstream?
   (c) What evidence indicates that Far1p is a **negative** regulator of Cln2p? (Remember that Cln2p is a positive regulator of the G1 to S transition.)
   (d) What evidence indicates that *cln1*Δ and *cln3*Δ are not downstream of *far1*?

6. Discuss the reasons why the authors suggest that the Far1 protein has additional roles in mating other than its role in G1 arrest.

7. Why is it necessary to use the *far1*Δ null mutation and not a *far1* point mutation to conclude that Far1p has no role in the mating-type pheromone response pathway itself?

8. Describe how you would select/screen for mutants in the α-factor dependent inhibitor of Cln1p, i.e. the one referred to as X in Figure 9. Be specific with regard to the genotype of the starting strain.

REFERENCE

Hereford, L.M. & L.H. Hartwell (1974) Sequential gene function in the initiation of *Saccharomyces cerevisiae* DNA synthesis. *J. Mol. Biol.* **84**: 445–461.

ARTICLE 9

Ramer, S.W. & R.W. Davis (1993) A dominant truncation allele identifies a gene, *STE20*, that encodes a putative protein kinase necessary for mating in *Saccharomyces cerevisiae*. *Proc. Natl Acad. Sci. USA* **90**: 452–456.

FUS1 encodes a product required for the fusion of haploid cells during mating. *FUS1* expression is induced by exposure to mating type pheromone and induction requires signal transduction via the pheromone response pathway defined by the Ste2, Ste4, Ste18, Ste5, Ste7, Ste11, Fus3/Kss1, and Ste12 proteins. Ste12p, a DNA-binding transcription activator, turns on *FUS1* transcription by binding to sites in the promoter. In Article 9 the authors use yet again another approach for the isolation of genes involved in mating-type signal transduction and successfully identify a new *STE* gene.

1. In this article the authors searched for genes that, when overexpressed, cause the constitutive expression of one of the downstream targets of the mating type pheromone pathway *FUS1*.
   (a) Diagram the library vector showing the structure of the insertion site of the yeast DNA fragments. At best, only one in six inserts will produce a product normally expressed in yeast. Explain why one in six is the maximum number.
   (b) Diagram the *FUS1* reporter construct.
   (c) Outline the steps used to identify 'positive' clones. Start with the selection of transformants (assume the selection marker on the vector is *LEU2*). Be sure to specify the carbon source at each step.

2. 'Preliminary sequence data suggested that this clone might not contain a full-length gene.' Based on the information in the text, diagram the fusion gene found in the novel clone. Indicate the translation start site of the fusion gene. Based on the sequence of *STE20* in Figure 3, what residues are present in the protein product of *ste20N*?

3. Is overexpressed *STE20N* dominant or recessive to *STE20*? What does this suggest with regard to the role of the N-terminal region of Ste20p? Which residues contain the putative kinase domain of Ste20p?

4. Describe the construction of *ste20-1* and list the complete phenotype.

5. Is Ste20p a positive or negative regulator of the mating-type response pathway? Explain.

6. Describe the expression pattern of *STE20*.

7. Epistasis analysis was undertaken to place *STE20* in the mating type response pathway in relation to the other *STE* genes.
   (a) When mating efficiency is measured, which is epistatic, *STE20N* or *ste4*, *ste5*, *ste11*, or *ste12*? Where does this place *STE20* in the pathway:
   STE4 ⟶ *STE5* ⟶ *STE11* ⟶ *STE7* ⟶ *STE12*
   (b) When growth arrest is measured, which is epistatic, *STE20ΔN* or *ste4*, *ste5*, *ste11*, or *ste12*? Where does this place *STE20* in the pathway:
   STE4 ⟶ *STE5* ⟶ *STE11* ⟶ *STE7* ⟶ *STE12*
   (c) Which is epistatic: *ste20-Δ1* or overexpression of *STE4*? What phenotype is monitored in this experiment?
   (d) Which is epistatic: *ste20-Δ1* or overexpression of *STE11ΔN*? What phenotype is monitored in this experiment?
   (e) Which is epistatic: *ste20-Δ1* or overexpression of *STE12*? What phenotype is monitored in this experiment?

8. Based on the conflicting results of their epistasis analysis the authors finally settle on the suggestion that *STE20* functions 'prior to *STE12*' in the mating-type signaling pathway. Discuss the possibility that these results suggest a dual

function for Ste20p in the mating response. One function is a positive one in the mating-type signaling pathway and the second function is in growth arrest.

ARTICLE 10

Leberer, E., D. Dignard, D. Harcus, L. Hougan, M. Whiteway, & D.Y. Thomas (1993) Cloning of *Saccharomyces cerevisiae STE5* as a suppressor of a Ste20 protein kinase mutant: structural and functional similarity of Ste5 and Far1. *Mol. Gen. Genet.* **241**: 241–254.

The results described in Article 9 do not clearly indicate the position of *STE20* in the mating type pheromone response pathway. The authors of this article hope to gain insight into this question by isolating multicopy suppressors of an *ste20* null mutation.

1. Describe the selection/screen designed by the authors for the isolation of multicopy suppressors of *ste20*.
   (a) Describe the genotype of the host strain in detail, specifically the *ste20* mutant allele used for the search.
   (b) Describe the library. YEp24 carries *URA3*.
   (c) What phenotype will be used to identify clones carrying a multicopy suppressor?
   (d) Outline the steps in the selection/screen starting with the selection of transformants.

2. List all the phenotypes of *ste20-1* that are suppressed by plasmid p24-1.

3. Describe how the authors demonstrated that the multicopy suppressor gene in plasmid p24-1 was *STE5*.

4. Ramer and Davis (1993) (Article 9) reported that overexpression of *STE11N* did not suppress a *ste20* null mutation. Leberer *et al.* report here that a hyperactive *STE11* allele suppresses the mating defect of *ste20* mutations. Both articles find that overexpression of *STE12* suppresses *ste20*-1. What type of suppression is this (by-pass, allele specific, or suppression by epistasis)? Where do these results place *STE20* in relation to *STE11* in the mating-type pheromone response pathway?

5. Compare the structure of the mutant alleles *ste20-1* and *ste20-2*. Use diagrams for your answer.

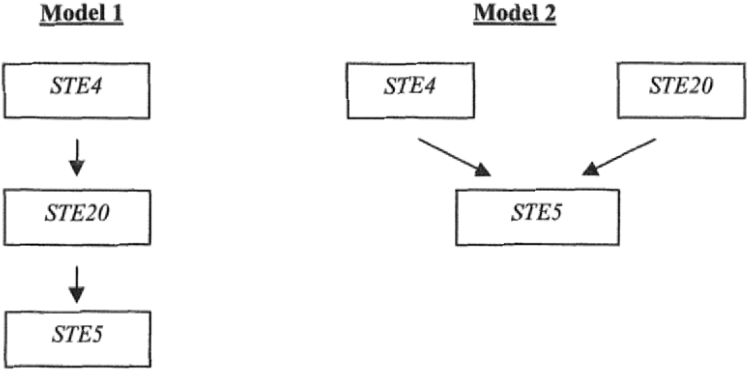
6. What experimental results suggest that *ste20-1* produces a partially functional product? What is the presumed product of *ste20-1* (based on your knowledge from Article 9)? Does expression depend on the *STE20* promoter? Explain.

7. Summarize the results that indicate the following.

(a) Activation of the mating-type pheromone response pathway by *STE5* overproduction is dependent on the partially functional *ste20-1* allele.
(b) Activation of the mating-type pheromone response pathway by *STE5* overproduction is dependent on *STE4* and *STE18*.

8. What type of suppression is the suppression of *ste20-1* by multicopy *STE5* (bypass, allele specific, or suppression by epistasis)?

9. Choose one of the following two models of the relationship of the *STE4*, *STE5*, and *STE20* genes as best explaining this portion of the mating-type pheromone response pathway. Support your choice using the results presented in this article.



10. Describe the structural and functional similarities of Ste5p and Far1p. How was the functional similarity demonstrated?

ARTICLE 11

Hasson, M.S., D. Bllinder, J. Thorner, & D.D. Jenness (1994) Mutational activation of the *STE5* gene product bypasses the requirement for G protein β and γ subunits in the yeast pheromone response pathway. *Mol. Cell. Biol.* **14**: 1054–1065.

This article describes the isolation of constitutive *STE5* mutations. Such mutations generate constitutive signaling via the mating-type response pathway and cause cell cycle arrest in haploids. Therefore, investigators isolating such mutations must do so in diploid cells. In previous articles, the *MAT***a**/*MAT*α 'diploid' genotype was reversibly maintained by the introduction of a plasmid-borne copy of the opposite mating-type locus. In this article this is accomplished by a different mechanism.

Chromosome *III* carries the expressed copy of the *MAT* locus and two additional but nonexpressed copies of *MAT*. These so-called silent copies of *MAT* are located near the telomeres of chromosome *III* and are referred to as *HML*α (left telomere,

copy of *MAT*α) and *HMR***a** (right telomere, copy of *MAT***a**). *HML*α and *HMR***a** are not expressed in wild-type cells because of the repressing effects of the *SIR1, 2, 3,* and *4* genes. The products of these genes silence the expression of *HML*α and *HMR***a** via chromosomal position effects (reviewed in Laurenson & Rine, 1992).

A mutation in any one of the *SIR* genes relieves the repression at the silent *HML*α and *HMR***a** loci. Both loci are expressed and the cell is functionally an **a**/α diploid. This article uses a temperature-sensitive *sir3* mutation to isolate constitutive *STE5* mutations. At the permissive temperature Sir3p is functional, the strain expresses only the information at the *MAT* locus, and the cell is genetically and phenotypically haploid. At the nonpermissive temperature Sir3p is inactive, *MAT*, *HML*α, and *HMR***a** are all expressed, and the cell is phenotypically diploid yet genetically haploid.

1. Describe the strategy outlined here for the isolation of haploid-lethal *STE5* mutations. Why are such mutations expected to be dominant to *STE5*? What is the growth phenotype of *sir3*^{ts} cells carrying a plasmid-borne *STE5*^{Hpl} mutation at the permissive and nonpermissive temperatures?

2. *STE5* is a large gene. Moreover, the mutagenesis method introduced multiple mutations. Describe how the phenotypically significant alteration in *STE5*^{Hpl}*-1* was localized.

3. Describe the growth phenotype of *STE5*^{Hpl}*-2*. Is this a generalized effect or specific to a particular phase of the cell cycle? Is this consistent with the expected phenotype of a constitutive *STE5* mutation?

4. Describe the method used to assay mating efficiency.

5. List the results in Table 2 that indicate that *STE5*^{Hpl}*-2* suppresses *ste2::LEU2*. Discuss whether multiple copies of *STE5*^{Hpl}*-2* are needed. What form of suppression is this (by-pass, allele specific, suppression by epistasis) and why?

6. Three alleles of *ste4* are tested in Table 2. Compare the mutational alteration in *ste4-3*, *ste4*Δ*::LEU2*, and *ste4::LEU2*. Compare the ability of *STE5*^{Hpl}*-2* to suppress each of these alleles. What form of suppression is this (by-pass, allele-specific, suppression by epistasis)? What does this result suggest with regard to the functional relationship between Ste4p and Ste5p?

7. List the results in Table 2 that indicate that *STE5*^{Hpl}*-2* does not suppress *ste7::LEU2*, *ste11*Δ*::hisG*, or the double *fus3-6::LEU2 kss1*Δ*::HIS3*. What two interpretations of this result are presented?

8. Based on the results in Tables 3 and 4 discuss the following statement. 'Together, the data indicate that the product of the *STE5*^{Hpl}*-1* gene can activate the pheromone pathway in the absence of the pheromone receptor and the G protein but that for full activity it requires G$\beta\gamma$' or overexpression.

REFERENCE

Laurenson, P. & J. Rine (1992) Silencers, silencing, and heritable transcriptional states. *Microbiol. Rev.* **56**: 543–560.

ARTICLE 12

Akada, R., L. Kallal, D.I. Johnson, & J. Kurjan (1996) Genetic relationships between the G protein $\beta\gamma$ complex, Ste5p, Ste20p and Cdc42p: investigation of effector roles in the yeast pheromone response pathway. *Genetics* **143**: 103–117.

1. Summarize the selection scheme designed to identify mutations that enhance the phenotype of *ste4-ts* mutant. Be sure to include the following.
   (a) The genotype of the parent strain(s).
   (b) Which alleles of *ste4* were used? Why did the authors use temperature sensitive alleles and not a *ste4*Δ mutation?
   (c) The growth conditions of the first step in the selection process (carbon source, temperature, etc.).
   (d) Potential mutants identified in the selection were screened for their ability to mate at the permissive temperature on galactose plates (step 2). What classes of unwanted mutants would be eliminated by this screen? Explain.
   (e) Mutants that passed the screen in 'c' were tested further for their ability to mate at the nonpermissive temperature on galactose plates (step 3). What is the purpose of this screen? Explain.

2. Define the term 'synthetic sterile'.

3. Diagram a cross that would allow the isolation of segregants carrying only the *ste-x* mutation from a *ste4-ts ste-x* double mutant isolated by this selection process.

4. Describe the cloning strategy used to isolate *ste-x* complementing plasmids.

5. Which of the secondary screens described above (step 2 or step 3) should have weeded out the *SIR* mutations? Why did it fail to do so?

6. The *ste18* and *ste21* mutations were isolated starting with the parental strain carrying *ste4-3510*. The *ste5* and *ste20* mutations were isolated starting with the parental strain carrying *ste4-299*. Nonetheless, the authors state that 'the synthetic sterile effects were not allele specific'.
   (a) Diagram a cross that would test whether the *ste18-14* mutation (isolated in the *ste4-3510* strain) is allele specific.
   (b) There is no specific information given as to the position of the alterations in *ste4-3510* or *ste4-299* nor are we informed as to which other *ste4* alleles were tested for the authors to conclude that none of the synthetic sterile mutations were allele specific. Discuss why this information would have been valuable.

(c) The original intent of the search for mutations that are synthetic sterile with *ste4-ts* mutations was to identify proteins that interact directly with Ste4p. Do the results reported here allow the authors to conclude that Ste4p physically interacts with Ste5p? Explain.

7. Discuss a model of enhancement, other than 'allele-specific enhancement', that might explain the results obtained here. That is, mutations in *STE18*, *STE5*, and *STE20* enhance the phenotype of a *ste4-ts* mutation. Keep in mind that epistasis analysis of these genes indicates that they could act at the same step.

8. In a recent study Blondel *et al.* (1999) identify *STE21* as *MSN5*, a member of the nuclear exportin family. They report that Msn5p (Ste21p) is responsible for the pheromone-stimulated export of Far1p from the nucleus. Discuss how a mutation in *MSN5* (*STE21*) could enhance a *ste4-ts* mutation. Which of the models of enhancement described in Chapter 9 does this represent?

9. Evaluate the results presented regarding the genetic interaction of *STE20* and *CDC42*, particularly in Table 6 and Figures 6 and 7. Of the models presented in Figure 8, which do you think is most consistent with the results presented in this and the other articles of this case study? Explain.

REFERENCE

Blondel, M., P.M. Alepuz, L.S. Huang, S. Shaham, G. Ammerer, & M. Peter (1999) Nuclear export of Far1p in response to pheromones requires the export receptor Msn5p/Ste21p. *Genes Dev.* **13**: 2284–2300.

ARTICLE 13

Choi, K-Y., B. Satterberg, D.M. Lyons, & E.A. Ellion (1994) Ste5 tethers multiple protein kinases in the MAP kinase cascade required for mating in *S. cerevisieae*. *Cell* **78**: 499–512.

Epistasis analysis, described in Articles 6 and 7 and elsewhere, places Ste5p upstream of the Ste11, Ste7, and Fus3 or Kss1 kinases in the mating-type pheromone response pathway. The results were consistent with a linear pathway as shown below.

Ste5 protein ⟶ Ste11 kinase ⟶ Ste7 kinase ⟶ Fus3/Kiss1 kinase

Nevertheless, evidence was accumulating that this simple pathway was not the full story. Kranz *et al.* (1994) found that overexpression of Ste5p suppressed point mutations (single residue alterations) in Fus3 kinase and did so in an allele-specific manner. Such a result strongly indicates that Ste5p and Fus3p directly physically interact and is not consistent with the proposed linear pathway that places Ste5p three steps upstream of Fus3 kinase. Kranz *et al.* (1994) also demonstrated that Ste5p and Fus3p associate with each other even in the absence of a pheromone and even if a catalytically inactive Fus3p mutant is used.

In view of the large size of Ste5 protein and the absence of any recognizable sequence motifs (other than homology to Far1p, another large protein of unknown multiple functions), the authors of this article propose to test the possibility that Ste5p serves as a 'scaffold protein', that is a protein to which other proteins attach in order to come into physical proximity with one another and thereby increase the efficiency of their functional interactions. This article also explores the relationship between Ste20 kinase and Ste5p. Epistasis analysis of Ste20 kinase places it downstream of Ste4p but its relationship to Ste5p remains unclear. Two-hybrid analysis and coimmunoprecipitation are used as complementary approaches to analyze the relationships among these proteins of a MAP kinase signaling cascade.

1. This article uses a '*lexA*-based' two-hybrid system. Define the term '*lexA*-based'. Include the following in your answer.
   (a) A diagram of the reporter gene.
   (b) A diagram of the structure of the *lexA* bait construction. Indicate the region encoding the DNA-binding domain, the insertion site for the sequence encoding the bait protein, and the structure of the fusion protein product.
   (c) What is B42?
   (d) A diagram of the structure of the B42 prey construction. Indicate the insertion site for the sequence encoding the prey protein, and the structure of the fusion protein product.
   (e) What is bicoid and what role does it play in this analysis?

2. The results in Table 1 are central to the hypothesis of the authors. That is, Ste5 protein is a scaffold protein capable of interacting with all three of the MAP kinases of the mating-type pheromone response signaling pathway. What evidence in Table 1 supports the following conclusions? Be sure to give the results of the control along with the results of the experiment.
   (a) Ste5p interacts with Ste11p.
   (b) The N-terminal domain of Ste11p is required for the interaction with Ste5p.
   (c) The C-terminal domain of Ste7p is required for the interaction with Ste5p.
   (d) The interaction of Ste5p with Ste11p, Ste7p, or Fus3p is not dependent on the genomic copies of *FUS3*, *STE11*, or *STE7*.
   (e) Ste11p interacts with Fus3p and the interaction is not dependent on Ste5p or Ste7p.

3. An interaction between Ste11p and Ste7p is suggested in Table 1 but does not hold up under detailed analysis.
   (a) Which initial result suggests an interaction between Ste11p and Ste7p?
   (b) Which result indicates that this interaction between Ste11p and Ste7p is indirect and dependent on the genomic copy of *STE5* and is mediated by Ste5p?
   (c) Draw a diagram of this interaction.

4. The authors conclude that Ste20p does not interact with Ste5p.
   (a) List the data for both the experiment and the control that support this conclusion.
   (b) Why do you think that the authors do not consider the 33 units or 61 units of activity seen with the Ste5 and Ste11 constructs, respectively, to be significant?
   (c) The authors state, '. . . LexA-Ste20 . . . functions to repress transcription of a *GAL1-LexAop-LacZ* gene with a LexA operator between the *GAL1* UAS and transcriptional initiation site'. Why is this an important control for this experiment?

5. Describe the results in Figure 1 that demonstrate that Ste11p, Ste7p, and Fus3p interact with distinct regions of Ste5p.

6. Describe the results in Figure 1 that demonstrate that Kss1p and Fus3p interact with the same or an overlapping region of Ste5p.

7. Describe the experiment that indicates that binding of Fus3p to Ste5p is essential for the activation of Fus3 kinase.

8. Discuss the functional significance of the finding that Ste5p binds to the N-terminal domain of Ste11 kinase.

REFERENCE

Kranz, J.A., B. Satterberg, & E.A. Elion (1994) The MAP kinase Fus3 associates with and phosphorylates the upstream signaling component Ste5. *Genes Dev.* **8**: 313–327.

ARTICLE 14

Whiteway, M., K.L. Clark, E. Leberer, D. Dignard, & D.Y. Thomas (1994) Genetic identification of residues involved in association of α and β G-protein subunits. *Mol. Cell. Biol.* **14**: 3223–3229.

1. Discuss the model of the *ste4* haploid-lethal mutant selection depicted in Figure 1A. List the following.
   (a) The genotype of the strain used for the selection. (Include the plasmid genes.)
   (b) The growth conditions used for the identification of the clones carrying haploid-lethal mutations.
   (c) The *STE4* mutagenesis method.

2. Discuss the significance of obtaining mutations only in the region of Ste4p between residues 126 and 150.

3. Describe the method used to isolate suppressors of the *ste4* haploid-lethal mutations. Be specific about the *ste4* mutant allele used for this selection.

4. List the results that indicate that *GPA1-E307K* is an allele-specific suppressor of the *STE4* haploid-lethal mutations. Draw a model of the interaction of Gpa1p and Ste4p based on these results. Indicate the location (within the region of interaction or not) of the various mutant alterations in Gpa1p and Ste4p identified in this study.

5. The results in Figure 2 and Table 1 indicate that the *GPA1* mutation Hls 4.3 is a silent mutation. That is, it exhibits wild-type-like activity.
   (a) Discuss the results that support this conclusion.
   (b) What results would you have expected if the *GPA1* mutation Hls 4.3 had interfered with the activity of Gpa1p?

6. Table 2 uses two-hybrid analysis to explore the direct interaction of the *GPA1* Hls 4.3 mutant protein with wild-type Ste4p protein and with the Hpl 21.3 mutant protein.
   (a) Which results indicate that the interaction between the Gpa1 Hls 4.3 mutant protein and Ste4p or Ste4 Hpl 21.3 mutant protein is comparable? How is this consistent with the phenotype analysis reported in Figure 2 and Table 1?
   (b) Which results indicate that the Ste4 Hpl 21.3 mutant protein interferes with the interaction with Gpa1 protein?
   (c) Which results indicate that the *GPA1* mutation Hls 4.3 re-establishes the interaction with Ste4 Hpl 21.3 mutant protein?
   (d) Which results indicate that the interaction between these mutant proteins is allele specific?

7. Discuss other Gpa1 mutations in the region of residue 307 that strengthen the hypothesis that this portion of the Gα subunit is involved in the interaction with G$\beta\gamma$ and may play an important role in the selectivity of the interaction of different Gα subunits with their specific G$\beta\gamma$ targets.

ARTICLE 15

Valtz, N., M. Peter, & I. Herskowitz (1995) *FAR1* is required for oriented polarization of yeast cells in response to mating pherones. *J. Cell Biol.* **131**: 863–873.

Chenevert *et al.* (1994) devised a screen to identify mutants defective in the ability to undergo the properly oriented morphological changes (schmoo formation) associated with pheromone exposure. They reasoned that such mutants would be capable of mating to a wild-type strain but should exhibit defects when paired with an enfeebled mating partner. Among the several mutant genes identified, Chenevert *et al.* (1994) identified new alleles of *FAR1* that they called *far1s* alleles. Article 15 characterizes these *far1s* alleles.

Chang & Herskowitz (Article 8) identified *FAR1* and characterized its role in cell cycle arrest. They demonstrated that Far1p functions as a negative regulator of the G1 cyclin Cln2p. This inhibitory effect involves the direct binding of Far1p to Cdc28p/Cln2p cylin-dependent kinase but, in contrast to other cyclin kinase inhibitors, Far1p binding does not appear to result in the inhibition of kinase activity and thus the mechanism of action is novel (Peter & Herkowitz, 1994; Gartner *et al.*, 1998). Article 15 focuses on quite a different function of Far1p, namely cell polarization during schmoo formation.

Chang & Herskowitz (Article 8) suggested a role for Far1p in cell polarization during mating based on their initial analysis of different *far1* mutant phenotypes. Additionally, Chang (1991) describes *far1-c*, a C-terminal truncation mutation, capable of cell cycle arrest in response to pheromone but defective in mating, perhaps due to an inability to orient toward the mating partner. These studies illustrate the value of detailed analysis of the pleiotropic phenotypes of multiple mutant alleles.

1. Describe the pheromone confusion assay.

2. Describe the orientation assay.

3. Describe in detail the pleiotropic phenotypes of *far1-s* alleles. Compare the phenotype of strains carrying the four different *far1-s* alleles described in Article 15 with wild-type *FAR1* strains and with the phenotype of strains carrying other *far1* mutant alleles. Where possible, include each of the phenotypes listed below.
   (a) Cell cycle arrest.
   (b) Mating defects with different partners.
   (c) *FUS1* transcription.
   (d) Far1 protein expression.
   (e) Mating confusion.
   (f) Orientation to pheromone gradient.

4. Describe the experiments that demonstrate that Far1p has a function in mating in addition to its role in cell cycle arrest.

5. Describe the experiment and the analysis of the experimental results that allows the authors to conclude that the *far1-s* mutants orient towards the incipient bud site. What does this suggest with regard to the structure of the incipient bud site while the cell undergoes reorientation for schmoo formation?

6. Four *far1-s* alleles were isolated, sequenced, and characterized. Discuss the value of analyzing multiple alleles. How might their conclusions differ if mutant B4 were not available?

7. *far1-60F3* complements *far1s*-D1. Assuming that the Far1 protein does not form homomultimers, propose a mechanism for this intragenic complementation.

REFERENCES

Chang, F. (1991) Regulation of the cell cycle by a negative growth factor in yeast. Ph.D. Thesis, University of California, San Francisco.

Chenevert, J., N. Valtz, & I. Herskowitz (1994) Identification of genes required for normal pheromone-induced cell polarization in *Saccharomyces cerevisiae*. *Genetics* **136**: 1287–1297.

Gartner, A., A. Jovanovic, D.I. Jeoung, S. Bourlat, F.R. Cross, & G. Ammerer (1998) Pheronome-dependent G1 cell cycle arrest requires Far1 phosphorylation, but may not involve inhibition of Cdc28-Cln2 kinase, in vivo. *Mol. Cell. Biol.* **18**: 3681–3691.

Peter, M. & I. Herkowitz (1994) Direct inhibition of the yeast cyclin-dependent kinase Cdc28/Cln2 by Far1. *Science* **265**: 1228–1231.

ARTICLE 16

Butty, A-C., P..M. Pryciak, L.S. Huang, I. Herskowitz, & M. Peter (1998) The role of Far1p in linking the heterotrimeric G protein to polarity establishment proteins during yeast mating. *Science* **282**: 1511–1516.

In addition to identifying *far1-s* mutants, Chenevert *et al.* (1994) isolated the mutant alleles of several other genes involved in polarized morphogenesis during mating (schmoo formation) as well as in vegetative cell division. These included alterations in *CDC24*, a GDP–GTP exchange factor for Cdc42p, referred to as *cdc24-m* mutants because they affect mating at permissive temperatures in addition to having a defect in cell division at higher temperatures. Cdc42p is a small GTPase in the same family of proteins as the mammalian Rho1p and is involved in organizing the actin cytoskeleton (reviewed in Johnson, 1999; Pruyne & Bretscher, 2000; Takai *et al.*, 2001).

Chenevert *et al.* (1994) also identified schmooless alleles of *BEM1*, a gene previously shown to be involved in cell polarization during vegetative budding (Chant *et al.*, 1991; Chenervert *et al.*, 1992). Reports that Bem1 protein binds to actin and interacts with Cdc42p, Cdc24p, Far1p, Ste5p, and Ste4p suggested the possibility that these proteins form a large complex that is essential for directing the recruitment of the actin cytockeleton to the region of the cell surface exposed to the highest concentration of pheromone (Peterson *et al.*, 1994; Leeuw *et al.*, 1995; Lyons *et al.*, 1996; Park *et al.*, 1997; reviewed in Pruyne & Bretscher, 2000). The authors of Article 16 used two-hybrid analysis to demonstrate this proposed complex and to explore specific interactions among the components of the complex.

1. Diagram the *FAR1* 'bait' construction, the *BEM1* 'prey' construction, and the reporter gene used to demonstrate an interaction between Far1p and Bem1p.

2. What experimental data supports the statement, 'Far1p preferentially bound to Cdc42p in its active GTP-bound state, . . .'?

3. What experimental data are presented to support the specificity of the Far1p–Cdc42p interaction?

4. What experimental data indicate that the Far1p interaction with Cdc42p is **not** direct but is mediated via Bem1p?

5. What pairs of constructs would you test to explore the interactions of Ste20p with the components of this complex? Be sure to examine the possibility that the interactions you detect are not direct and propose how you might test this.

6. Comment on the following. Interactions between two heterologous (nonyeast) proteins detected using the yeast two-hybrid system are most probably direct.

7. Figure 2 shows the two-hybrid results obtained with the *far1-s* mutant alleles B4 and H7. Discuss how these results are consistent with the phenotype exhibited by strains containing these mutations.

8. The authors cite unpublished results describing *STE4* mutant alleles that cause defects in mating but not pheromone signaling. Based on the results described in this article, propose a possible mechanism for this class of *ste4* mutation. Outline a series of experiments including two-hybrid analysis and at least one other genetic approach that you might use to investigate this novel class of *STE4* alleles to support your hypothesis.

REFERENCES

Chant, J., K. Corrado, J.R. Pringle, & I. Herskowitz (1991) Yeast *BUD5*, encoding a putative GDP–GTP exhange factor, is necessary for bud site selection and interacts with bud formation gene *BEM1*. *Cell* **65**: 1213–1224.

Chenevert, J., K. Corrado, A. Bender, J. Pringle, & I. Herskowitz (1992) A yeast gene (*BEM1*) required for cell polarization whose product contains two SH# domains. *Nature* **356**: 77–79.

Chenevert, J., N. Valtz, & I. Herskowitz (1994) Identification of genes required for normal pheromone-induced cell polarization in *Saccharomyces cerevisiae*. *Genetics* **136**: 1287–1297.

Elion, E.A. (2000) Pheromone response, mating and cell biology. *Curr. Opin. Microbiol.* **3**: 573–581.

Johnson, D.I. (1999) Cdc42: An essential Rho-type GTPase controlling eukaryotic cell polarity. *Microbiol. Mol. Biol. Rev.* **63**: 54–105.

Leeuw, T., A. Fourest-Lieuvin, C. Wu, J. Chenevert, K. Clark, M. Whiteway, D.Y. Thomas, & E. Leberer (1995) Pheromone response in yeast: association of Bem1p with proteins of the MAP kinase cascade and actin. *Science* **270**: 1210–1203.

Lyons, D.M., S.K. Mahanty, K.Y. Choi, M. Manandhar, & E.A. Elion (1996) The SH3-domain protein Bem1 coordinates mitogen-activated protein kinase cascade activation with cell cycle control in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **16**: 4095–4106.

Park, H.O., E. Bi, J.R. Pringle, & I. Herskowitz (1997) Two active states of the Ras-related Bud1/Rsr1 protein bind to different effectors to determine yeast cell polarity. *Proc. Natl Acad. Sci. USA* **94**: 4463–4468.

Peterson, J., Y. Zheng, L. Bender, A. Myers, R. Cerione, & A. Bender (1994) Interactions between the Bud emergence proteins Bem1p and Bem2p and Rho-type GTPase in yeast. *J. Cell Biol.* **127**: 1395–1406.

Pruyne, D. & A. Bretscher (2000) Polarization of cell growth in yeast. I. Establishment and maintenance of polarized states. *J. Cell Sci.* **113**: 365–375.

Takai, Y., T. Sasaki, & T. Matozaki (2001) Small GTP-binding proteins. *Physiol. Rev.* **81**: 515–524.

ARTICLE 17

Erdman, S., L. Lin, M. Malczynski, & M. Snyder (1998) Pheromone-regulated genes required for yeast mating differentiation. *J. Cell Biol.* **140**: 461–483.

This article uses the genome-wide transposon mutagenesis approach to identify additional genes involved in cellular processes involved in mating such as agglutination, polarized growth for the formation of the mating projection, cell fusion, and nuclear fusion. Searches for mutants exhibiting defects in mating have identified a number of genes involved in these processes. However, it is likely that many genes have been missed possibly because of redundancy in the *Saccharomyces* genome or because alterations in these genes do not cause defects severe enough to produce a sufficiently distinct phenotype. The approach described here uses transposon mutagenesis (see Chapter 12) to produce random *lacZ* fusions to ORFs throughout the genome and to screen these for pheromone regulation.

1. Describe the *lacZ* insertional mutagenesis scheme used in this article to randomly tag genes in the *Saccharomyces* genome. Include:
   (a) The structure of a typical library plasmid carrying a yeast DNA fragment with a Tn insert creating a *lacZ* fusion.
   (b) The genotype of the host strain into which the library was transformed.
   (c) How were transformants selected?
   (d) How were transformants screened to identify fusions to pheromone-regulated genes?

2. For this study:
   (a) Why did the authors use a *bar1*Δ strain?
   (b) Why were both haploid *MAT***a** and homozygous *MAT***a**/*MAT***a** diploid strains used?

3. List the phenotypes used to test the novel pheromone-regulated genes identified in this study for a potential affect on mating. Describe the reasons for selecting *FIG1*, *FIG2*, *FIG3*, and *FIG4* for further study.

4. What is a PRE sequence and what is the significance of finding PRE sequences in the promoter regions of *FIG1, FIG2, FIG3*, and *FIG4?*

5. Describe the method used to demonstrate that cell cycle arrest does not alter the expression of the *FIG* genes.

6. Describe the complete phenotype of the *fig2* mutant in detail. Include results discussed throughout this article.

7. Describe the Fig2 protein. Include sequence motifs and the results of cellular localization studies. What do the authors propose is a likely function of Fig2p?

8. The study identified 54 pheromone-regulated genes but the authors estimate that there are between 67 and 132 such genes in the *Saccharomyces* genome. Describe how they derived this estimate.

9. Only nine previously known pheromone-regulated genes were identified in this screen.
   (a) Name **one** gene that you know to be pheromone regulated that was not identified.
   (b) How do the authors explain the fact that they missed many genes?
   (c) How might the Tn library be improved so as to make the mutagenesis method more random?

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