CONTROL OF CELL GROWTH AND DIVISION IN *SACCHAROMYCES CEREVISIAE*

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I. INTRODUCTION

The survival of any eukaryotic microorganism or higher eukaryotic cell depends upon accurate duplication of its genetic material and cellular organelles for partitioning into progeny. The success of the cell, however, depends not only on the fidelity of these processes, but also on its ability to selectively initiate mitotic division or enter a quiescent state. In complex multicellular organisms, cells comprising specialized tissues respond to a variety of intercellular mediators: hormones, growth factors, electrical impulses, and perhaps physical contacts. These dictate responses not necessarily beneficial to the individual cell, but rather, to the organism as a whole. The microbial organism, instead, assesses a spectrum of nutritional signals in the environment and responds in ways consistent with its own viability. In view of the overriding concerns of unicellular eukaryotes for their own survival, it is not surprising they are able to synthesize most essential vitamins, cofactors, and macromolecular precursors in a highly regulated fashion and in a manner highly adaptable to environmental change.

In this section, we will review these broad issues of cellular regulation in the yeast *Saccharomyces cerevisiae*. We will describe the major facets of energy-yielding metabolism in yeast and discuss recent contributions to understanding the intricate regulatory circuits responsible for preferential metabolite utilization. We will then discuss the basic features of the yeast cell cycle and the cell cycle-dependent, nutritional-sensing mechanisms which determine entry into proliferative or quiescent states. Finally, we will discuss aspects of the regulation of developmental pathways leading from mitotic division to diploidization and sporulation.

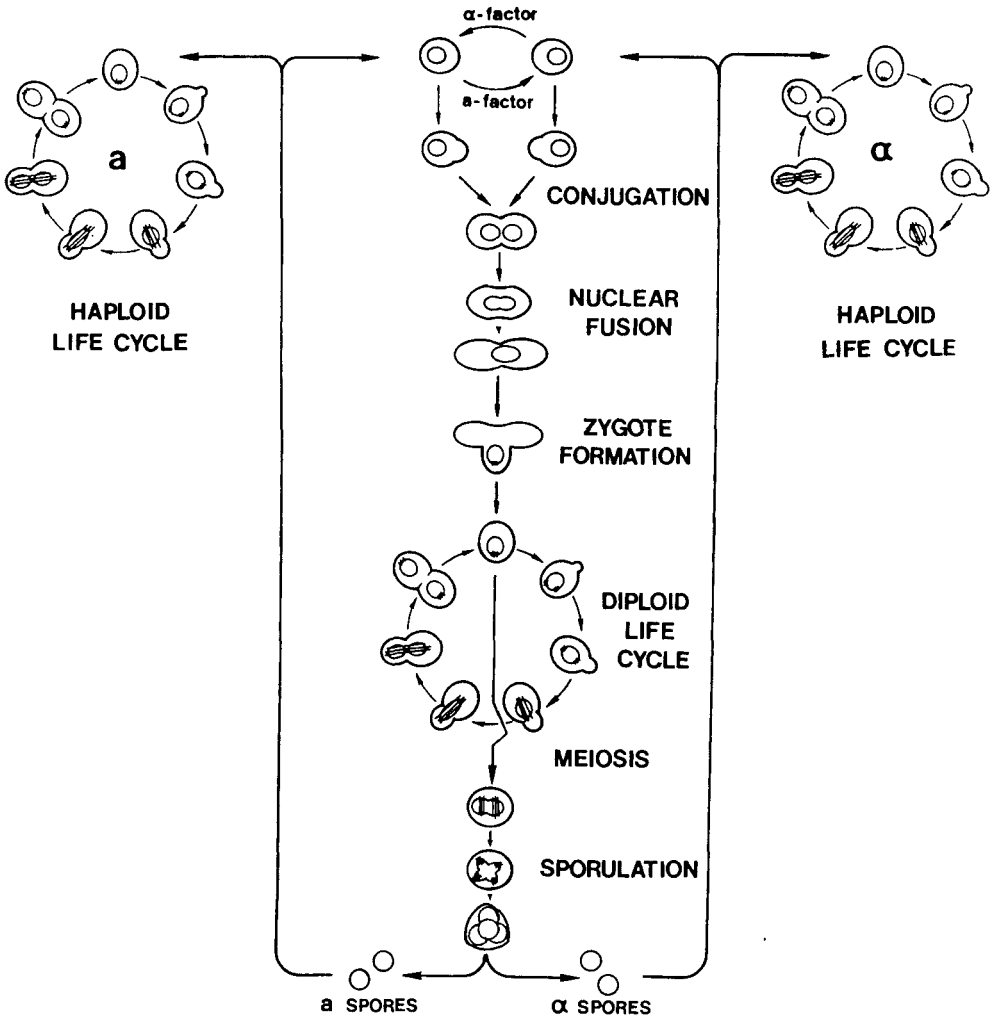

FIGURE 1. Life cycles of heterothallic strains of *S. cerevisiae*.

A. Life Cycle

S. cerevisiae grows vegetatively in one of three distinct developmental states, as a-haploids, alpha-haploids, or a/alpha diploid cells (Figure 1). The a and alpha designations refer to sexual mating types and specialized properties of the haploid cell. Only cells of the opposite mating type undergo conjugation and diploidization under normal circumstances. The resulting a/alpha cell then grows vegetatively, while maintaining a diploid state. This stable diploid exhibits properties not found in either of the haploid parental cells, such as the ability to sporulate or show resistance to effects of mating pheromones. Meiotic reduction leads to the production of four haploid ascospores, two of each mating type. The scheme is complicated, however, by the fact that most wild strains of yeast are able to switch their haploid mating type as often as once per cell generation (not depicted in Figure 1).

Each cell type represents a distinct developmental state and expresses a unique set of gene products. Ultimate controls over these pleiotropic effects are derived from the genetic information contained at a single chromosomal locus, MAT. These subjects have been reviewed comprehensively[1-7] and are discussed later (Section IV).

Haploid cells respond to mating pheromones produced by cells of the opposite mat-

ing type in a programed manner very much like hormone responses in higher cells. The a cells produce an undecapeptide which halts cell division in alpha cells at start, but which allows continued cell growth.[6] Alpha cells secrete alpha factor, a tridecapeptide which has analogous effects on a cells.[6] Both pheromones are encoded by nuclear genes as prepropheromones and are processed post-translationally into mature peptides[8-11] in a manner similar to processing of mammalian neuropeptide enkephalins[12] (for reviews that detail the physiological responses of cells to mating pheromones, see References 1 and 6). Mating factors bind to high affinity cell surface receptors,[13] and the signals for division arrest are transmitted to nuclear genomic sites by unknown intracellular mechanisms, perhaps via cyclic AMP (cAMP)[14] or perhaps by other pathways.[15,16] It appears that both a and alpha cells utilize similar mechanisms for signal transduction in response to mating pheromones. This is evidenced by experiments that demonstrate to some extent that cell surface receptors in a and alpha cells are interchangeable.[17]

The first level of regulation of cell-type specific genes may occur at the cell membrane itself. In a response similar to "down regulation" of cell-surface receptors in mammalian tissues, alpha-factor binding is reduced after prolonged exposure without successful conjugation.[18] Cells become desensitized to mating factor effects, perhaps as a result of decreased receptor number or affinity. In addition, proteolytic degradation of the pheromones occurs by the products of the BAR1 and SST2 genes.[19,20]

B. Cell Division Cycle

Once committed to a mitotic division cycle, a yeast cell replicates about 1.4×10^7 base pairs (bps)[21] or 4.7 mm of DNA, distributed among its 17 or so chromosomes. Extrachromosomal genetic elements, i.e., the endogenous 2 micron plasmid, the cytoplasmic killer double-stranded RNAs, and the mitochondrial genomes, are also replicated. During each cell doubling, histone proteins and nonhistone chromosomal proteins must be produced in sufficient quantity to maintain both the structural integrity and the biological state of activity of the DNA in the progeny cells. The continued synthesis of total RNA, protein, phospholipid, and cell wall carbohydrate is reflected in the rise in total cell mass throughout the cell cycle. Ribosome content increases continuously by a highly coordinated process coupling the synthesis of ribosomal proteins and rRNA. Levels of metabolic enzymes appear to increase steadily or in a stepwise fashion during the growth of the cell as it approaches mitosis. Some structural components are similarly duplicated during each round of cell division, while other cell structures must be synthesized *de novo*. Spindle pole body (SPB) duplication is an example of the former, while the microtubular networks connecting the SPB with chromosomal elements must be polymerized each cell cycle.

Observation of morphological or biochemical events during the course of cell division reveals the process to be highly ordered and reproducible. Two fundamental models have been proposed to explain how the coordination of these processes occurs: the "dependent-growth" mechanism or the "independent-timer" mechanism. Both have valid features (see Section III.A), but neither can predict the behavior of yeast cells under all experimental conditions. Regardless of the actual control mechanisms employed, there is a necessity for periodicity in expression of genetic information. This may take place at the level of the synthesis of gene products or at the level of regulation of their activities. Presumably, the timing of expression of specific gene products relative to known cell cycle events will be of importance in understanding their biochemical roles and the regulatory circuits controlling both their synthesis and activity.

The cytology of the yeast cell has been elegantly reviewed by Byers.[22] Several major morphological changes serve as stage-specific markers in cell cycle progression. These allow for monitoring of cell synchrony under experimental conditions, tentative assign-

ment of the execution point for cell division cycle mutants, and discrimination among cell cycle-dependent or independent events.

The most predominant feature of the *S. cerevisiae* cell is its asymmetric growth by budding of a daughter cell from a mother cell, resulting in two cells of different size. By contrast, cell division in higher eukaryotes almost always results in progeny cells of equal size. Initiation of bud formation in yeast occurs only when the mother cell reaches a critical size, determined by both genetic and nutritional considerations.

The yeast cell division cycle can be divided into G_1, S, G_2, and M phases by analogy with higher cells. Towards the end of G_1, duplication of the SPB occurs. This cytoplasmic structure appears to be analogous to the microtubule organizing center (MTOC) of higher eukaryotes[23] and serves as a nucleation site for microtubule polymerization. The SPBs begin divergent migration and eventually form the spindle apparatus for mitotic or meiotic chromosome segregation. Shortly after SPB duplication, the cell begins DNA synthesis characteristic of S phase. Coincident with the initiation of chromosome replication is the process of bud emergence. The appearance of the cell wall component chitin, a homopolymer of *N*-acetyl-glucosamine, marks the site of subsequent bud growth and can be visualized by cytochemical staining during and after bud formation. Light microscopy shows that bud growth occupies most of the remaining cell cycle, S, G_2, and M, eventually leading to cytokinesis. Often, however, the daughter does not physically separate from the mother cell for several generations.

Completion of DNA synthesis marks the end of S phase, during which time the nucleus has migrated towards the neck separating mother from bud. During G_2 and M, the microtubule networks of the mitotic spindle elongate and effect the intricate process of chromosome segregation. At no time during mitosis does the nuclear membrane dissolve nor do yeast chromosomes visibly condense. This is in sharp contrast to well-studied higher eukaryotic cells, indicating that neither process is obligatory for eukaryotic cell division. Mitochondrial DNA (mtDNA) replication appears to occur throughout the cell cycle.[24] Partitioning of mitochondria into the bud seems to be a random process,[25] the dynamic state of mitochondria ensuring the daughter cell will receive a sufficient portion of total "chondriome". Nuclear division marks the end of the cell division cycle since cytokinesis may or may not have been completed before a new round of division begins.

The asymmetric nature of the budding process results in daughter cells which must substantially increase in mass to attain the minimum size required to initiate another cycle. This is shown schematically in Figure 2. The size difference between mother and daughter cells at cell separation is more dramatic in slow-growing cultures[26] and can be a factor in evaluating cell synchrony experiments. This results in an extended cycling time for the daughter cells due exclusively to elongation of G_1, while the lengths of S, G_2, and M phases are unaffected.[28]

II. CONTROLS OVER CELL GROWTH

We will focus our discussion in this section on the enzymes of energy metabolism, outlined in Figure 3 (for reviews on carbohydrate metabolism in yeast that detail the biochemical pathways and describe available mutants, see References 29 and 30). Recent reviews have succinctly covered other aspects of yeast metabolism and its regulation, e.g., nitrogen metabolism,[31] amino acid,[32,33] nucleotide,[32] and phospholipid[33] biosynthesis. A review of general amino acid control by Hinnebusch will appear shortly in this series.

A. General Considerations

Vegetatively growing yeast cells utilize a wide variety of carbon and nitrogen sources

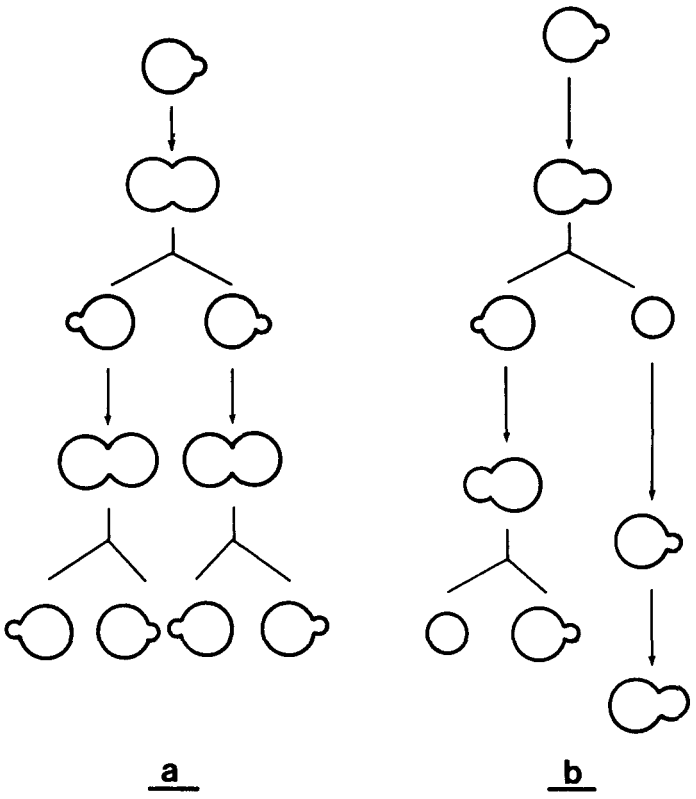

FIGURE 2. Size differences in *S. cerevisiae* at cell division. (a) Cells grown in nutrient-rich medium (fast growth rates). Mother and daughter cells are similar in size at mitosis and maintain synchronous growth. (b) Cells grown in nutrient-poor medium (slow growth rates). Mother and daughter cell sizes are disparate at mitosis and cultures rapidly become asynchronous. (Modified from Carter, B. L. A., Piggott, J. R., and Walton, E. F., *Yeast Genetics*, Spencer, J. F. T., Spencer, D. M., and Smith, A. R. W., Eds., Springer-Verlag, New York, 1983, 1.)

for growth and have evolved intricate regulatory circuits designed to preferentially utilize the most effectively metabolized nutrients. Glucose and certain other sugars are readily fermented as a major source of energy, and eventually as carbon; this is illustrated in Figure 3. Nonfermentable carbon sources are pyruvate, lactate, acetate, and ethanol. *S. cerevisiae* is constitutive for the ability to metabolize glucose, mannose, and fructose and is inducible for the metabolism of many glycosides, e.g., sucrose, galactose, maltose.

In glucose-rich medium in batch culture, yeast grow aerobically or anaerobically by fermentation, a very inefficient process in the rate of energy conversion per mole of carbon. However, under these conditions, cells achieve maximum growth rates, doubling within 2 hr. Energy is derived exclusively by substrate-level phosphorylations during glycolysis, while enzymes of the tricarboxylic acid (TCA) cycle, electron transport system, and oxidative phosphorylation are in a repressed state. Glucose does not appear to directly inhibit respiration, but rather, repression occurs in part through common networks of positively and negatively acting modulators of gene activity. For this reason, this phenomenon is referred to as carbon catabolite repression. Mitochondria are found to comprise only about 3% of the cell volume as opposed to upwards of 12% in actively respiring cells.[25] Thus, mitochondrial components, which can com-

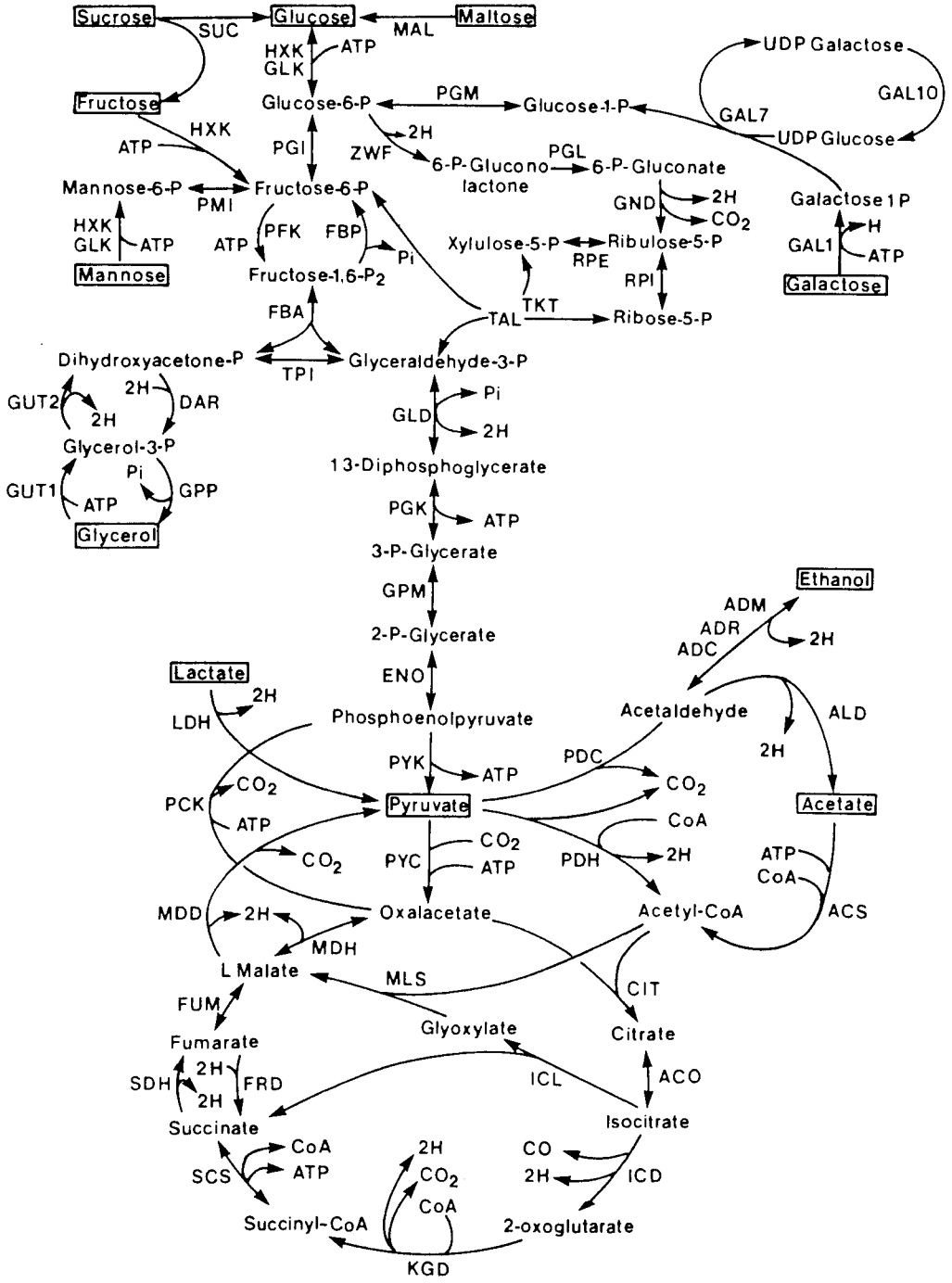

FIGURE 3. Pathways of yeast intermediary metabolism and sugar utilization. Substances commonly used as carbon sources for growth are boxed. Many of the reactions are incompletely described. For example, for kinases, ATP is indicated as a reactant, but ADP is not shown as a product. The existence, function, or direction of several of the indicated reactions is speculative. Gene designations are referred to in the text. Pi indicates inorganic phosphate and 2H indicates a dehydrogenase reaction. (Adapted from Fraenkel, D. G., *The Molecular Biology of the Yeast Saccharomyces: Metabolism and Gene Expression,* Strathern, J. N., Jones, E. W., and Broach, J. R., Eds., Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1982, 1.)

prise a substantial portion of the total cell mass, need not be synthesized or assembled above a low basal level. In respiring cells grown on nonfermentable carbon sources (lactate and ethanol), the role of oxygen must be considered as superimposing another level of control. Complete derepression of mitochondrial function can only be achieved in the presence of adequate oxygen concentrations. Catabolite repression extends beyond the mitochondrial boundary, affecting many of the sugar degradative pathways.[34] When glucose is abundant, enzymes for utilization of other sugars are repressed.

Much is known about the regulation of glycolytic enzymes and those of the TCA cycle at the level of control of enzyme activity, most of which involve allosteric feedback control and effects of the energy charge of the cell (ATP/ADP ratios). In higher eukaryotes, hormonal signals are relayed via cAMP and calcium-dependent protein kinases, and the resulting phosphorylations and dephosphorylations dictate energy production and utilization patterns. Similar alterations in reversible phosphorylation states of several yeast enzymes have been shown to influence their activity. In addition, increasing evidence is accumulating for the regulation of several enzymes by alterations in their rates of synthesis. These effects can be easily measured by quantitating mRNA by hybridization with cloned DNA probes, which are now available for many enzymes of energy metabolism.

B. Glycolysis

During fermentation, glycolysis forms the backbone of yeast intermediate metabolism (Figure 3). The breakdown of a variety of sugars provides the carbon skeletons and energy essential for biosynthetic pathways. Table 1 shows the enzymes and reactions involved in glycolysis. Most of the structural genes for these enzymes have recently been cloned by complementation. Their level of expression varies, depending upon growth conditions, but is always moderately high, consisting of about 0.5 to 5% of the total cellular protein (for general reviews on yeast glycolysis, see References 29 and 30).

Biochemical data[29] indicate that the rate of glycolysis is regulated by a number of cell intermediates: ATP, ADP, acetyl-CoA, citrate, glucose-6-phosphate, 6-phosphogluconate, fructose diphosphate, phosphopyruvate, aspartate, and ammonium. These compounds act as activators or inhibitors to influence key glycolytic enzymes involved in irreversible reactions. These include hexokinase (HXK), phosphofructokinase (PFK), and pyruvate kinase (PYK). All three reactions are believed to be critical control steps in glycolysis.

Phosphorylation of glucose, the first step in glycolysis, is catalyzed by three isozymes; HXK P-1 (HXK1), P-II (HXK2), and glucokinase (GLK).[30] These enzymes differ by their substrate specificity. The HXKs phosphorylate fructose, glucose, and mannose, whereas GLK is active only upon the latter two substrates. The individual roles of these three isozymes in vivo are not understood. They are all insensitive to allosteric inhibition by the end product glucose-6-P, in contrast to the situation in higher eukaryotes.[29,47] HXK2 has recently been shown to consist of a catalytic subunit and a regulatory subunit which contains a recognition site for metabolite(s) of carbon catabolite repression (see Section II.D). Evidence implicating HXKs in glucose and fructose uptake has also been presented,[48] suggesting that uptake could be an initial step in regulation.

The enzyme PFK shows positive and negative cooperative kinetics. Its activity is affected by a variety of intermediates of the TCA cycle, the electron transport system, and gluconeogenesis.[29,30] These effectors, fructose 2,6-diphosphate (activator), ATP (inhibitor as well as substrate), AMP (activator), and citrate (inhibitor), in conjunction or singly, exert an effect on the intracellular ATP/ADP ratio and hence play an im-

Table 1
ENZYMES OF GLYCOLYSIS AND FERMENTATION

Enzyme	Gene	Reaction catalyzed	Molecular weight	Subunits	Cloned	Ref.
Hexokinase	HXK	Glucose + ATP → glucose 6-P + ADP	96,000	4	+	35—37
Phosphoglucose isomerase	PGI	Glucose 6-P ⇌ fructose 6-P	145,000		−	
Phosphofructo-kinase	PFK	Fructose 6-P + ATP → fructose 1,6-diP + ADP	580,000	13	+	38
Fructose-biphosphate aldolase	FBA	Fructose 1,6-diP ⇌ glyceraldehyde 3-P + dihydroxyacetone	70,000	2	−	
Triosephophate isomerase	TPI	Dihydroxyacetone-P ⇌ glyceraldehyde 3-P	54,000	2	+	39
Glyceraldehyde-3-P dehydrogenase	GLD	Glyceraldehyde 3-P + P_i + NAD ⇌ 1,3-diP-glycerate + NADH	120,000	2	+	40
Phosphoglycerate kinase	PGK	1,3-diP-glycerate + ADP ⇌ 3-P-glycerate + ATP	44,500		+	41,42
Phosphoglycerate mutase	GPM	3-P-glycerate ⇌ 2-P-glycerate	112,000	4	−	
Enolase	ENO	2-P-glycerate ⇌ P-enolpyruvate + H_2O	67,000	1	+	43
Pyruvate kinase	PYK	P-enolpyruvate + ADP → pyruvate + ATP	150,000	2	+	44
Pyruvate decarboxylase	PDC	Pyruvate ⇌ acetaldehyde + CO_2	175,000	2	+	45
Alcohol dehydrogenase	ADC	Acetaldehyde + $NADH_2$ ⇌ ethanol + NAD_2	150,000	4	+	46,65
	ADR		150,000	4	+	67
	ADM		—	—	—	

portant role in the Pasteur effect.[49,50] The Pasteur effect describes the observation that from a given quantity of glucose a higher cell yield is obtained when cells are grown aerobically rather than anaerobically. The physiological explanation is that only in the presence of oxygen is yeast capable of reutilizing ethanol, the end product of fermentation. Changes in the concentrations of the effectors listed above mediate this transition from glycolytic to respiratory growth.

The inhibition of PFK increases the concentration of glucose-6-phosphate, thus favoring the formation of polysaccharide reserves and enhancing feedback inhibition of hexose transport across the plasma membrane. This results in effective inhibition of glucose phosphorylation to glucose-6-phosphate. In addition to these allosteric effects, the concentration of PFK has been shown to vary under different conditions of growth.[51] Recently,[52] a temperature-sensitive mutant lacking PFK activity has been found which causes a depletion in energy charge and has pleiotropic effects on transport. Genes affecting PFK activity, PFK1 and PFK2, have also been identified and cloned.[38] They appear to be alpha and beta subunits, respectively, of the enzyme. It has been suggested that the regulatory and catalytic domains reside on different subunits, with beta possessing catalytic activity.[38] Recently, the PFK1 and PFK2 genes were subcloned on multicopy plasmids and transformed into yeast.[53] Northern blot analysis showed high levels of PFK1 and PFK2 mRNA, which correlated with a 3.5-fold increase in enzyme activity. However, the transformants did not show an increase in ethanol production, indicating that this enzyme is not the only limiting step in glycolysis.

An example of regulation governing the major pathways of glycolysis and gluconeogenesis is the antagonistic reactions catalyzed by fructose 1,6 biphosphatase (FBP) and PFK. When glucose is depleted from the medium or when cells are grown on a nonsugar carbon source, FBP is derepressed.[54,55] In order to eliminate futile cycling and depletion of ATP, the enzymes must be regulated reciprocally. Allosteric effectors of PFK have been described and discussed above.[30] In the case of FBP, a rapid reversible inactivation is obtained by phosphorylation of the enzyme.[56] Irreversible inactivation of this enzyme was considered to be due to proteolytic degradation.[57] It has been suggested therefore that derepression of the enzyme occurs by *de novo* synthesis.

The third irreversible reaction, catalyzed by PYK, results in the production of pyruvate and one molecule of ATP. Under anaerobic conditions, respiratory enzymes are repressed and pyruvate cannot be metabolized via the TCA cycle, and $NADH_2$ generated during glycolysis cannot be oxidized via the cytochrome system. Instead, pyruvate is decarboxylated to produce acetaldehyde, which in turn is reduced to ethanol via oxidation of $NADH_2$. The activity of PYK is modulated by many effectors.[58] These include the positive allosteric effector fructose 1,6 diphosphate, a glycolytic intermediate, and negative effectors such as ATP and citrate. Although PYK is synthesized constitutively at a high level, changes in the carbon source from a gluconeogenic to a glycolytic medium induce a 6- to 20-fold increase in activity.[59,60]

Although most of the glycolytic enzymes are synthesized constitutively in cells grown under glycolytic and gluconeogenic conditions, there are reports of variations for individual enzymes.[61] The only mutant isolated which affects a broad spectrum of glycolytic enzymes is the gcr mutant described by Clifton et al.[62] When gcr mutants are grown on various nonfermentable carbon sources, a decrease in enzyme activity to 5% or less of the wild-type levels is observed. When the mutant is grown in the presence of fermentable sugars, the decrease in enzyme activities is only 20 to 50% relative to wild-type cells. The lowest relative values were found for phosphoglycerate mutase and enolase. The protein profiles of cell extracts and in vitro translation products of total cellular RNA from mutant and wild-type cells were compared. While the pattern of polypeptides were similar qualitatively and mRNA levels reflected protein levels in

vivo, quantitative differences were observed. Both in vivo protein concentrations and in vitro translatable mRNAs decreased in the mutant. Thus, although the exact lesion is unknown, it is likely to somehow affect mRNA half-lives or synthesis for a discrete number of enzymes.[63] The identification of the gcr mutant infers that global mechanisms controlling the synthesis of the glycolytic enzymes exist. Further investigation of this mutant using the cloned glycolytic structural genes will extend our understanding of the regulation of this pathway.

C. TCA Cycle, Glyoxylate Cycle, and Gluconeogenesis

The essential role of the TCA cycle (Figure 3) in energy metabolism is the oxidation of acetate in the form of acetyl-CoA to carbon dioxide, with the concomitant reduction of NAD^+ to NADH.[64] The energy equivalents of the reduced nicotinamide adenine dinucleotide are then passed on to the electron transport proteins, where a series of oxido-reductions, coupled to the phosphorylation of ADP, end with the reduction of molecular oxygen to water. The carbon skeletons of the TCA cycle intermediates undergo a series of isomerizations, decarboxylations, and energy-yielding oxidations. The oxidations (dehydrogenations) of isocitrate, 2-oxoglutarate (formerly known as alpha-ketoglutarate), succinate, and malate are, of course, coupled to the reduction (hydrogenation) of coenzymes NAD^+ or FAD^+, thereby capturing energy in a usable form. Several catabolic pathways provide breakdown products which are channeled via acetyl-CoA into the TCA cycle for energy production. These include glycolysis, oxidation of fatty acids, degradation of certain amino acids, and utilization of alternate carbon sources such as ethanol and acetate. The reactions of the TCA cycle take place in the inner mitochondrial matrix, a soluble fraction within the inner mitochondrial membrane. The genes encoding these enzymes are, however, of nuclear origin and their mRNAs are translated on cytoplasmic ribosomes.

Several intermediates of the TCA cycle are also members of biosynthetic pathways, especially as nucleotide and amino acid precursors.[64] Removal of these intermediates, e.g., 2-oxoglutarate for glutamate synthesis or oxaloacetate for purine and pyrimidine synthesis, would cripple the energy-yielding cycle. The purpose of "anaplerotic" reactions is to maintain the levels of these key TCA cycle intermediates. An example is the carboxylation of pyruvate to oxaloacetate, occurring in cells grown on glucose and catalyzed by pyruvate carboxylase (PYC). These reactions replace the lost carbon skeletons from sources outside the cycle and allow balanced cycling to continue.

1. Glyoxylate Cycle

A source of TCA cycle intermediates unique to yeast and other microorganisms grown on ethanol or acetate is provided by the glyoxylate cycle. Under these conditions, respiratory activity is at a maximum and the TCA cycle is the only energy-yielding pathway since glycolysis does not occur. The net effect of the glyoxylate cycle is the production of succinate from acetyl-CoA (Figure 3). The pathway diverts isocitrate (6-carbon) from its usual decarboxylation to 2-oxyglutarate (5-carbon) and CO_2, producing instead succinate (4-carbon) and glyoxylate (2-carbon). This reaction is catalyzed by isocitrate lyase (ICL). Glyoxylate is then condensed by malate synthetase (MLS) with another molecule of acetyl-CoA to form malate (4-carbon). The overall effect is to avoid the loss of carbon atoms as CO_2, to elevate the level of 4-carbon TCA cycle intermediates, and to promote the energy-yielding metabolism of the TCA cycle. Functioning of the glyoxylate cycle is also linked to gluconeogenic growth.

The need for 6-carbon sugars for biosynthetic purposes necessitates the production of glucose-6-phosphate from simple two-carbon nutrients like ethanol and acetate.[64] The process requires the synthesis of malate and oxaloacetate as intermediates of gluconeogenesis. These are provided as a result of the glyoxylate cycle. Malate is con-

verted to pyruvate by malate dehydrogenase (MDH), and oxaloacetate is converted to phosphoenopyruvate (PEP) by PEP carboxykinase (PCK). Both reactions begin a reversal of the normal glycolytic pathway. Subsequent reactions are reversible, except for the interconversion of fructose-1,6-biophosphate and fructose-6-phosphate. The forward reaction in glycolysis is catalyzed by the allosterically regulated PFK (Section II.B), whereas the gluconeogenic enzyme is FBP. The latter enzyme is subject to regulation by covalent modification (phosphorylation) as discussed below.

2. Bypass of the TCA Cycle: Fermentation

The final byproduct of glucose oxidation during fermentation is ethanol. In growth on high levels of glucose, pyruvate is diverted from entry into the TCA cycle, converted to acetaldehyde, and subsequently reduced to ethanol at the expense of $NADH_2$ (Figure 3). The enzymes involved in these reactions have recently been shown to respond to glucose concentrations at the mRNA level. The yeast gene encoding pyruvate decarboxylase (PDC1) was isolated by genetic complementation and used to detect mRNAs under various growth conditions.[45] A rapid 20-fold increase in the amount of hybridizable mRNA occurred within 30 min after glucose was added to cells growing in ethanol-containing medium. PDC1 mRNA was also detected in cells utilizing galactose, glycerol, or succinate as carbon sources. This implies that glucose is not the sole inducer of enzyme synthesis. One might contend that the increase in PDC1 mRNA results from a release of repression caused by ethanol. However, when cells were grown with the nonmetabolizable glucose analog 2-deoxy-glucose in the absence of ethanol, no induction was observed, suggesting the latter may not be true. In either case, it is clear that enzyme activity correlates with mRNA levels under various conditions of growth.

The second enzyme required for conversion of pyruvate to ethanol is alcohol dehydrogenase I (ADHI), which reduces acetaldehyde to ethanol. The gene for this enzyme (ADC1) has also been cloned,[46] sequenced,[65] and used as a probe to study its expression.[66] Alcohol dehydrogenase II (ADHII), an isozyme of ADHI, is encoded by a very closely related gene (ADR2).[67] It is altered in its catalytic activity such that it favors the reverse reaction, i.e., from ethanol to acetaldehyde, thus initiating the entry of two carbon compounds into the TCA cycle. One would expect reciprocal regulation of the genes encoding these isozymes. In fact, the steady-state levels of ADC1 mRNA are high in glucose-containing medium, while ADR2 transcripts are repressed. These results are based on the hybridization of mRNA to cloned DNA probes (despite a high degree of cross-hybridization) and their translation in vitro.[66] Sequences upstream of the ADR2 gene act in *cis* to mediate glucose repressibility.[68] When ADR2 5′ regions were excised and placed upstream of the ADC1 gene, ADHI, normally active in fermentable carbon sources, was repressed by glucose. It appears therefore that the two isozymes of ADH are synthesized in response to the appropriate nutritional stimuli, in a reciprocal manner. This would make sense physiologically, avoiding futile cycling between acetaldehyde and ethanol. In fact, as alluded to by Denis et al.,[66] the regulation of ADHI and ADHII may represent a general feature of metabolic enzymes which catalyze reversible (equilibrium) reactions. That is, several metabolic enzymes are represented by multiple forms, and cells may respond to various physiological demands by synthesizing the appropriate form of the enzyme, depending on which direction the pathway is taking. This may also be the case for the two enolase genes.[69] ENO2 is highly induced in glucose grown cells while ENO1 is not. The latter may be of greater importance during growth on gluconeogenic carbon sources. It will be of interest if biochemical studies can distinguish substrate preferences for the two ENO gene products. According to the model, one would expect ENO2 to have greater specificity for 2-phosphoglycerate and ENO1 to prefer PEP. If dual roles for isozymes are a general

phenomenon of reversible enzyme reactions, it suggests an evolutionary "driving force" for the creation of multiple (nonidentical) genes which are often found for a particular enzyme. A similar situation may also exist for isozymes catalyzing irreversible reactions, such as the duplicated and reciprocally regulated acid phosphatase genes PHO5 and PHO3, whose products may have opposing or different metabolic roles.

3. TCA Cycle Enzymes

Several key reactions in the TCA cycle are catalyzed by the enzymes citrate synthase (CIT), isocitrate dehydrogenase (ICD), and the oxoglutarate (alpha-ketoglutarate) dehydrogenase (KGD) complex.[64] These reactions are highly favored energetically, as indicated by their large changes in free energy. As is true for most biochemical pathways involving coupled reactions in series, the nonequilibrium nature of the reactions catalyzed by these enzymes implicates their role as "flux"-generating steps.[64] These steps effectively pull the preceding reactions along, making the pathway as a whole energetically more favorable. Regulating the rate of flux-generating steps is an economical way of controlling the activity of the entire pathway. It is of no surprise then that these enzymes are regulated at the level of activity by numerous effectors. In cells of higher organisms, CIT is regulated in a positive manner by its cosubstrate oxaloacetate and allosterically inhibited by citrate. ICD is activated by Ca^{2+}, ADP, and NAD^+, while KGD is activated by Ca^{2+}, NAD^+, and coenzyme A, and is negatively affected by ATP. These alterations are all consistent with a low energy status, causing induction of TCA cycle activity for production of reducing equivalents. The Ca^{2+} effects, especially prevalent in muscle tissue, are a means of elevating total respiratory activity in the mitochondria. Its relevance to yeast energy metabolism, however, is uncertain. Although reports suggest the existence of Ca^{2+}-binding proteins (calmodulin) in yeast[70] and mutants (cal1) which arrest as cell division cycle (cdc) mutants in Ca^{2+}-poor medium have been isolated,[71] the role of Ca^{2+} in yeast cell physiology is largely unknown. A gene encoding a calmodulin-like protein in yeast has recently been cloned and shown to be single copy in the genome and essential for growth.[72]

In studying the activities of TCA cycle enzymes, it is important to keep in mind a fundamental difference between yeast and higher organisms. In mammalian systems, glycolysis and respiration are intimately coupled, and low cellular energy charge ratios (high ADP/ATP) induce both pathways coordinately. Yeasts, however, are able to uncouple the two pathways during fermentative growth. Hence, despite a high cellular energy content, yeast cells growing in glucose-rich media will not induce the levels of the critical TCA cycle enzymes discussed above.

It is clear that these flux-generating enzymes of the TCA cycle are critical control points and constitute good candidates for investigating patterns of enzyme synthesis. Making use of available antibodies to CIT, Hoosein and Lewin[73] studied the biosynthesis of CIT in *S. cerevisiae*. They showed it was synthesized as a cytoplasmic precursor protein processed during import across the mitochondrial membrane. Cells grown in glucose-rich medium had insignificant amounts of CIT activity. As cells entered mid-logarithmic-phase growth (past the point of inflection on the growth curve), a marked induction of the enzyme occurred. This induction was monitored by measuring enzyme activity and by immunoprecipitation of both in vivo labeled proteins and in vitro translation products of the yeast mRNA. The latter is taken to represent the relative levels of CIT mRNA, assuming no change in its translatability in vitro. Assuming there are no alterations in post-transcriptional events, it is reasonable to suspect that glucose repression of this TCA cycle enzyme occurs in part by a transcriptional mechanism.[73] It is interesting that cells begin this induction long before growth rates taper off, suggesting that mechanisms exist to monitor rates of utilization of nutrients long before they are fully depleted. In this regard, regulation of CIT levels in chemostat

cultures would be informative. That is, do cells trigger derepression of citrate synthase (and other TCA cycle enzymes) when glucose concentrations fall below a critical point or are they sensitive to relative changes in glucose levels or growth rates?

To our knowledge, transcriptional effects on production of ICD or KGD have not been studied. Both seem likely candidates for this type of regulation. A few other enzymes which may reveal interesting transcriptional regulation and deserve comment here are PYC, PCK, and FBP. These enzymes function to route carbon skeletons out of the TCA cycle and reverse the glycolytic pathway for biosynthesis of glucose (or glucose-6-P).

4. Gluconeogenesis

The gluconeogenic pathway is active in cells grown in glycerol, lactate, ethanol, acetate, and other nonfermentable carbon sources which do not yield 6-carbon sugars as intermediates. The pathway consists principally of reversals of the normal glycolytic reactions; the exceptions are the nonequilibrium steps catalyzed in glycolysis by PFK and PYK. PYC catalyses an anaplerotic reaction as mentioned previously, feeding 4-carbon skeletons into the TCA cycle in the form of oxaloacetate, under conditions of high respiratory activity. Conditions which favor gluconeogenic growth require high rates of respiration. Thus, oxaloacetate can serve the dual role of increasing the level of TCA cycle intermediates and participating as a substrate for conversion to PEP in gluconeogenesis. This reaction is catalyzed by PCK, which has recently been demonstrated to show transcriptional regulation in mammalian cells.[74,75] Using an isolated nuclei transcription "runoff" assay, these authors showed a rapid cAMP and glucocorticoid stimulation of transcription, whereas insulin inhibited the synthesis of PCK mRNA. It is likely that the yeast enzyme will show a similar transcriptional control, although the role of cAMP is more difficult to predict.

One of the final steps of gluconeogenesis is the dephosphorylation of fructose-1,6-biophosphate to fructose-6-phosphate, a reaction which bypasses the normal glycolytic step. Some detailed biochemical studies on the enzyme FBP by Mazon et al.[54] and Noda et al.[55] have demonstrated its regulation by covalent modification. It is inhibited by AMP, glucose, and other sugars and is activated in cells grown in ethanol or acetate. The enzyme is catabolite repressed and therefore is purified from cells undergoing gluconeogenic growth (on acetate) when levels are greatly induced. A model based on in vitro data suggests that the accumulation of the substrate fructose-1,6-biophosphate during growth on glucose stimulates a cAMP-dependent kinase activity which phosphorylates the enzyme. This covalent modification of FBP is believed to "tag" the enzyme for proteolytic degradation, thus inactivating it. This type of active destruction of glucose-repressible enzymes is a form of catabolite inactivation and may also occur for other gluconeogenic enzymes (for review, see Reference 76). It has not yet been established if FBP is regulated by changes in synthesis at the level of RNA transcription, although this seems to be a reasonable expectation.

D. Electron Transport Proteins

More is known about mitochondrial function in yeast than in any other organism. This is primarily because yeast are facultative aerobes and can grow in the absence of respiratory activity, allowing mutational analyses that would be lethal in other cell types. The ease with which genetic crosses are made has also facilitated analysis. Thus, many mitochondrial mutations of both nuclear and mitochondrial origin have been isolated and studied. The field of mitochondrial genetics is vast and many excellent reviews[77-79] and books[80] on the subject exist.

The role of nuclear-mitochondrial interactions are best exemplified by ATPase and some of the electron transport proteins, which are large complexes whose subunits are

encoded by both nuclear and mitochondrial genes. Most mitochondrial proteins (about 95%) are coded for in the nucleus and must be imported into mitochondria through the outer membrane and into or through the inner membrane to the matrix (for reviews on protein import, see References 81 and 82). As mentioned previously, TCA cycle enzymes function in the soluble matrix fraction. Electron transport proteins (except cytochrome c and ubiquinone) and the mitochondrial ATPase are assembled as membrane-bound complexes whose function is dependent on their association with the inner membrane. There are about 7 protein-coding genes in the mitochondrial genome, in addition to the large 21S and small 15S mitoribosomal RNAs, and 25 tRNA genes.[83] The transcripts produced by mitochondrial RNA polymerase (nuclear encoded) are translated on 74S mitochondrial ribosomes. There are also several unidentified open reading frames (URFs), most of which are located within introns of the structural genes for apocytochrome b and cytochrome c oxidase. Much evidence has accumulated implicating the URFs as coding sequences for RNA maturases involved in the splicing of RNA precursors. One URF is also found in the 21S mt-rRNA, but its product is uncharacterized. As will become clear, in spite of the small size of the yeast mitochondrial genome, there exist intricate control mechanisms to express these gene products.

We will discuss briefly the essential features of regulation of genes encoding three mitochondrial enzymes; apocytochrome b, cytochrome c oxidase, and the nuclear-encoded isocytochrome c. These are perhaps the best-studied components in yeast electron transport. The expression of these genes is under catabolite repression, and their induction is dependent on the presence of oxygen. Baldacci et al.,[84] in a survey of the transcriptional patterns of mitochondrially encoded genes in cells grown under various physiological conditions, concluded that the control of production of mature mRNAs is not under general transcriptional control, but varies individually for the different subunit encoding genes. This implies that the complex RNA processing pathways play a role in controlling the levels of production of the final mature proteins. However, as will be discussed below, many of the yeast mitochondrial gene introns are in fact "optional". Intron presence is strain dependent, suggesting that their processing is not an intrinsic feature of their physiological regulation. This notion is supported by the fact that intron-containing and intron-less strains are apparently equal in their respiratory competence. The physiological regulatory patterns of the mitochondrially encoded protein-coding genes are not understood; reports have indicated apocytochrome b levels do not appear to be under heme control[85] nor are they reduced in anaerobically grown cells.[86] In the case of the nuclear-encoded cytochrome c genes, much is known about the physiological regulation of their expression by glucose, O_2, and heme and will be discussed briefly.

In contrast to the nearly complete absence of introns in the yeast nuclear genome (except certain ribosomal protein genes and actin) yeast mtDNA seems to welcome these intruding sequences. The apocytochrome b, cytochrome oxidase subunit I, and 21S rRNA contain 5, 6 to 9, and 1 introns, respectively.[78] The optional nature of the introns in different strains precludes an absolute requirement for the presence of introns or the maturases they encode. Why the mitochondrial genome is subject to interruption by sequences not characteristic of the nuclear genome is a mystery. Splicing signals common to exon-intron boundaries in yeast nuclear introns and the introns of higher eukaryotic cells are not found in mitochondrial split genes. Although transcription and translation do not appear to be coupled in mitochondria, there is evidence that mitoribosomes may play a role in the splicing of pre-mRNAs.[87] Apocytochrome b gene mutants, unable to process intron 2 by normal RNA splicing mechanisms, "cleanly" remove their intron sequences at high frequency.[88] The mechanisms for this DNA splicing are unknown.

In a recent report, Michel and Lang[89] found sequence similarities between cyto-

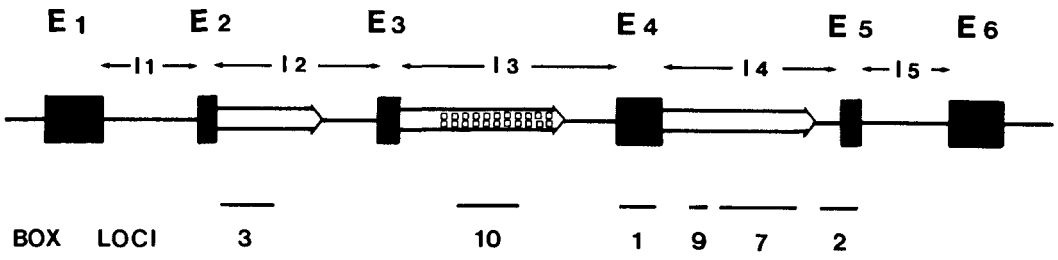

FIGURE 4. Organization of the cytochrome b gene (cob). The long form of cob containing five introns (I1 to I5) is shown. Exons 1 to 6 are indicated by filled bars. Open arrows indicate open reading frames, confirmed by DNA sequencing data. The hatched area represents incomplete sequence determination. Map locations of selected box mutations are indicated. (Adapted from Dujon, B., *The Molecular Biology of the Yeast Saccharomyces: Life Cycle and Inheritance*, Strathern, J. N., Jones, E. W., and Broach, J. R., Eds., Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1981, 505; De La Salle, H., Jacq, C., and Slonimski, P. P., *Cell*, 28, 721, 1982; and Evans, I. H., *Yeast Genetics*, Spencer, J. F. T., Spencer, D. M., and Smith, A. R. W., Eds., Springer-Verlag, New York, 1983, 269.)

chrome oxidase subunit I (oxi3) intron-encoded proteins and retroviral reverse transcriptases. Perhaps reverse transcriptase activity in oxi3 or other intron-encoded maturases could explain the "mobile" nature of mitochondrial introns (for further discussion, see Reference 90).

1. Cytochrome b

The apocytochrome b gene (referred to as cob or cob-box) exists in different strains of *S. cerevisiae* in "long" and "short" forms, based on the number of introns. The complete nucleotide sequence of the short version has been determined.[91] These data, along with that of others (for review, see Reference 78), reveal that the short version lacks introns 1 through 3 (bI1, bI2, and bI3) which are present in the long version of the cob gene. The long version contains 5 introns in all. The organization of the long form of the cytochrome b gene is shown in Figure 4. At least two introns contain URFs encoding RNA maturases. The box mutations have mit$^-$ (respiratory-deficient) phenotypes and are characterized by the absence of normal-sized apocytochrome b polypeptides. Genetic and biochemical analysis of these mutants[92] provided the first evidence for the existence of introns and *trans*-acting factors within them. Analysis of many independent box mutations,[93-95] aided recently by DNA sequencing,[87,96-98] has uncovered novel mechanisms of RNA processing.

The models, based on studies cited above and others, suggest that the first intron, bI1, is excised by a mechanism independent of mitochondrially encoded gene products, since it can be removed in certain petite mutations (which lack mitochondrial translation machinery). Most probably, bI1 is excised by a nuclear-encoded splicing enzyme, but this remains to be demonstrated. The removal of intron 1 joins the coding sequences of exon 1 (E1), exon 2 (E2), and the URF of intron 2. Since the 5′ end of the intron 2 URF is adjacent to and in-frame with exon 2, a fusion protein consisting of E1/E2/bI2-URF results. This fusion protein then acts as a splicing enzyme ("spligase") which removes bI2, thus causing the destruction of its own mRNA species in order to join the next exon-encoded sequence. This represents a novel type of autoregulation in eukaryotic systems. A similar model for the processing of intron 3 has been suggested by genetic studies.[77] *Trans*-recessive box10 mutations have been found to map within intron 3. These mutants have properties similar to the *trans*-recessive box3 mutants of bI2, and box7 and mutants of bI4. *Trans*-recessive intron mutations suggest the presence of intron-encoded, diffusible maturases, as has been strongly implicated for bI2 and bI4.[78] In addition, partial DNA sequencing of bI3 has revealed an open reading frame at the 5′ end, in-frame with exon 3.[99]

Interestingly, mutations in intron 4 (box7) are pleiotropic in that they affect not only the processing of cob-box transcripts, but are also defective in splicing RNAs of the mitochondrial cytochrome oxidase subunit I (oxi3) gene.[93] The dependence of oxi3 RNA processing on the product of the apocytochrome bI4 maturase is another novel finding and will be discussed below. The intron 4 maturase is believed to be proteolytically processed from a 55,000-dalton precursor consisting of E1/E2/E3/E4/bI4-encoded sequences into a final product of 27,000 daltons.[96,97] Several *cis*-dominant bI4 mutations have also been characterized at the DNA sequence level;[87] box1 mutants map at the E4-bI4 junction, just inside the coding sequence of the exon; box2 mutations are in the region beyond the 3′ end of the bI4 URF and therefore not within a protein coding sequence; and box9 mutations are within the bI4 URF, but cannot be complemented in *trans*. These three *cis*-acting mutations therefore define regulatory sequences required for the processing of the cob intron 4.

As mentioned before, the exon-intron junctions of mitochondrial split genes do not follow the strict "GT---AG" consensus rules at the 5′ and 3′ intron borders as observed in split nuclear genes of yeast and higher cells. The *cis*-acting splicing signal TACTAAC found near the 3′ junction of yeast nuclear introns also is not found in mitochondrial introns.[100] Instead, internal sites identified by *cis*-dominant box2 and box9 mutations appear to be involved. These are far removed from the splice junctions.[96] DNA sequence analysis revealed that single base substitutions were responsible for many of the box9 mutants isolated, some of which would not result in amino acid changes. The mutations did not result in frameshifting of the bI4 URF and could not be complemented in *trans*, implying that bases in box9 identify sequence elements required in *cis* as substrates in the splicing reaction(s). In a follow-up study,[98] revertants were isolated and in several cases second-site suppressors (sup) were identified. Through a series of genetic crosses and DNA sequence analyses, the two sup mutants were found to result from single base changes in other sites within the same intron. One model consistent with these findings is that the critical sequence elements act by promoting the formation of a specific RNA secondary structure which serves as a substrate for the splicing reaction. This is supported by the finding[98] that the second-site suppressors were compensatory base substitutions which could, in theory, restore appropriate base pairing to the original box9 mutation. Finally, the excision of the fifth intron of cob apparently proceeds without the involvement of self-encoded maturases, as no open reading frames have been found in bI5.

2. Cytochrome c Oxidase

Cytochrome c oxidase is a complex multisubunit enzyme found in association with the inner mitochondrial membrane. It functions as the ultimate electron carrier, reducing oxygen to water. In yeast it has been thought to consist of seven nonidentical subunits, four of which are encoded in the nucleus and three in the mitochondrial genome.[78] More recent biochemical analysis has shown two additional nuclear-encoded subunits (VIIa, VIII), by comparison of their *N*-terminal sequences to oxidases of higher organisms.[101-103] The three mitochondrial subunits of cytochrome oxidase, COXI, COXII, and COXIII, are encoded by the oxi3, oxi1, oxi2 genes, respectively. The oxi1 and oxi2 are not split genes. However, oxi3 contains between six and nine introns, depending on the strain and criteria used in making exon assignments. Since the yeast protein subunits have not been sequenced, oxi3 exon assignments are based on comparisons of translated DNA sequence data and protein sequences of bovine and human cytochrome subunits. In addition, it is generally assumed that yeast introns and those of higher organisms will not be heavily conserved, as are the cytochrome-coding sequences. Below, we present some features of the regulation of the split gene encoding cytochrome c oxidase subunit I (oxi3).

Although hundreds of oxi3 mutants have been isolated, it has been extremely difficult to find mutations which map within intron regions. This is unlike the case of the cob gene, where elegant genetic studies have revealed numerous complementation groups in intron regions (box mutants). Lack of appropriate mutants has hampered study of oxi3 transcript processing, and while open reading frames have been found in nearly all oxi3 introns except intron 5 (aI5), it has been difficult to demonstrate their role as RNA maturases. A new set of mutants analyzed by Slonimski and colleagues[104] has succeeded in demonstrating an intron 1-encoded maturase using analyses similar to cob-box studies. The data suggest that the maturase is necessary for removal of its own intron 1 and can function in *trans*. Mutations in aI1 accumulate novel polypeptides and cannot synthesize mature COXI subunits. The identification of other intron-encoded maturases in oxi3 must await the isolation of the appropriate mutants, i.e., those which map to the respective introns, are *trans*-recessive, and are independent of cob-box7 mutations.

As discussed earlier, box7 mutations deficient in the apocytochrome b intron 4 maturase are also defective in processing cytochrome oxidase I transcripts. Detailed analysis of two *cis*-dominant oxi3 mutations in aI4 revealed extensive nucleotide homology between the fourth introns of the oxi3 and cob genes.[105] More importantly, base changes in oxi3 aI4 mutants are in positions strictly analogous to those identified in the *cis*-acting box9 mutations in bI4. Thus, it appears as if both genes contain introns with similar signals necessary for processing by the box7 maturase. The oxi3 aI4 does contain a URF in-frame with exon 4 which could potentially code for a protein with homology to the box7 maturase. It is unclear if this protein is synthesized and why it does not function as its own maturase. One can speculate about the evolutionary implications of this apparent intron conservation. Perhaps the aI4 URF-encoded product is a mutant form of maturase which is no longer able to splice its intron, or perhaps it codes for a protein with other unknown functions. It is also interesting to consider the possibility that the synthesis of apocytochrome b and cytochrome c oxidase, which function in concert as respiratory chain proteins, may be coordinated by a rate-limiting intron removal pathway dependent on the common box7 maturase. In addition, it has been observed that the oxi3 primary transcript encompasses the downstream genes encoding ATPase subunits 8 and 6.[106] The functional significance of this multigenic transcription unit in regulating the genes it contains is not understood, however, it may serve to link their timing of expression as energy (ATP) production requires both enzyme complexes.

Several classes of nuclear mutations exist which suppress mitochondrial RNA processing defects. Pleiotropic mutations such as NAM2-1 affect both cob-box and oxi3 genes.[107] Inactivation of NAM2 by gene disruption results in a complete loss of the mitochondrial genome, generating petites at a frequency of 100%.[108] Mutations have also been isolated which effect only oxi3 processing; one example is the nuclear mss mutation.[109] The complex interplay between nuclear and mitocondrial gene products which promote the processing of cob and oxi3 transcripts is only beginning to be unraveled. The nature of the nuclear suppressors is particularly intriguing, since some allow correct excision of certain mitochondrial introns in the absence of their normal maturases. It is likely that these mutations represent defects in nuclear-encoded maturases.

3. Cytochrome c

Cytochrome c is one of the best-studied proteins in any cell. It has been purified from eukaryotic organisms as diverse as humans, bovine, pig, tuna, higher plants, yeast, and *Tetrahymena*, and its crystalline structure has been determined (for review see Reference 110). Evolutionarily, it is well conserved at the amino acid level, but

more remarkable is the extraordinary conservation in its tertiary structure.[111] The structure about the heme-pocket, for example, is nearly identical in all cytochromes examined. The extent to which cytochrome c has been studied is due in part to its small size, its isolation as a soluble protein, and its characteristic spectral properties after heme attachment to the apocytochrome.

The gene(s) encoding cytochrome c has also been the focus of much research. Yeasts have been shown to contain two distinct, but homologous genes, iso-1-cytochrome c (CYC1) and iso-2-cytochrome c (CYC7), comprising, respectively, about 95 and 5% of the total inactively respiring cells.[112] Hundreds of cytochrome c mutations have been isolated by Sherman and Steward,[112] most of which result from translational defects, i.e., nonsense, frame-shifts, or initiator codon mutations in the structural gene for CYC1. More recent approaches have facilitated the isolation of mutants affecting cyc gene transcription (see below). Current models suggest that gene duplication and divergent evolution have resulted in two distinct unlinked genes in yeast, each with characteristic patterns of regulation.

Both CYC1 and CYC7 are subject to carbon catabolite repression. Derepression is achieved only during cell growth on nonfermentable carbon sources under aerobic conditions. The synthesis of CYC1 was shown to parallel the levels of in vitro translatable CYC1 mRNA during glucose derepression,[113] and later these effects were demonstrated to occur by transcriptional mechanisms.[114] It has been difficult to distinguish the effects of heme and of oxygen on cytochrome c synthesis due to the requirement of O_2 in heme formation.[115] However, results from studies using heme analogs and intermediates from the heme biosynthetic pathways indicate primary effects by heme on CYC1 gene transcription and suggest that the effects of O_2 are, in fact, mediated through heme levels.[116] In contrast, expression of CYC7 does not appear to be affected by heme levels since apoiso-2-cytochrome c protein[116] and mRNA levels[117] are unaffected in hem1 mutants defective in delta-aminolevulinic acid (ALA) synthase and thus unable to synthesize heme. In this same study,[117] another interesting mutant, cyc3 (defective in heme attachment) was analyzed for its effects on CYC1 and CYC7 expression. This revealed the existence of post-transcriptional mechanisms regulating the accumulation of apoiso-1-cytochrome c, but not apoiso-2-cytochrome c protein. Clear differences in the kinetics of appearance of CYC1 and CYC7 mRNAs during glucose exhaustion were also demonstrated.[117] Thus, both similarities and differences exist in the regulation of this duplicated gene pair, although the physiological significance of these variations is not yet understood.

Detailed analysis of the molecular basis for glucose and heme control has been carried out primarily on the gene for the more abundant of the two cytochrome c proteins. In a report using a powerful and now common tool for studying gene regulation in yeast, Guarente and Ptashne[118] fused the *Escherichia coli* lacZ gene to CYC1 protein-coding sequences, creating a hybrid protein with functional beta-galactosidase activity in yeast. This construction enabled the isolation of mutations affecting CYC1 transcription by simple screening procedures for the enzymatic activity of the hybrid protein. In a series of elegant studies, Guarente and colleagues[119-121] scrutinized the 5′-flanking region of CYC1 for sequence elements involved in heme control and catabolite derepression. They defined two upstream activation sites, UAS1 and UAS2, centered around −275, and −225 bp, respectively, upstream of the transcription start site. These results have been reviewed briefly.[122] UAS sequences have been found associated with other catabolite repressed genes including those of the galactose system, GAL1/GAL7/GAL10,[123,124] and also with the HIS3,[125] HIS4,[126] and LEU2[127] and other yeast genes. In CYC1, the exact upstream location of UAS1 and UAS2 relative to the transcription start site is not critical for heme or glucose derepression,[119,120] in apparent contrast to results obtained by Lowry et al.[128] Similar studies by Guarente and Hoar[121]

indicate the CYC1 UAS cluster is functional in either orientation with respect to CYC1 gene transcription. In addition, UAS sequences can operate on heterologous promoters,[120,123] reminiscent of the properties of viral enhancer elements[129,130] and binding elements of hormone-sensitive genes in mammalian cells and their viruses.[131,132]

The UAS sequences listed above all operate in a positive manner, but there are examples of upstream modifying sites (UMS) which repress transcription. One instance was described for the ADR2 gene (Section II.B), and may be similar to a "negative enhancer". However, UAS elements do not function when located downstream of the transcription start site[121] or when displaced by several kilobase pairs, and, thus, analogies drawn with enhancer elements have their limitations.

One of the more interesting features of the upstream regulatory region of CYC1 is the separability of UAS1 and UAS2 function. Deletion analysis of hybrid CYC1-lacZ fusions tested in cells grown under aerobic vs. anaerobic conditions or in cells harboring the hem1 mutation suggest that heme activation occurs almost exclusively through UAS1.[119,120] Positive control of CYC1 by heme via UAS1 occurs in the absence or presence of glucose and appears to be independent of UAS2. In contrast, when heme is present, cells derepress CYC1-lacZ gene transcription (in lactate medium) through *both* UAS1 and UAS2. Isolation of *trans*-acting mutations, hap1, which affects only UAS1, and hap2, which affects only UAS2, supports the notion of differential regulation via tandem activation sites.[120] Why neither UAS is activated in the absence of heme is an intriguing question, which, upon clarification, should reveal the role of heme in the release of CYC1 from catabolite repression. The authors suggest that increases in intracellular heme concentration during catabolite derepression (perhaps due to induction of heme biosynthetic enzymes) are responsible for HAP1-dependent UAS1 activation.[120] Heme alone is insufficient to activate UAS2, but its presence is necessary for derepression in lactate medium, a process dependent upon HAP2. Indeed, heme analogs induce UAS1, but not UAS2, driven expression.[120] Determining the role of the HAP1 and HAP2 gene products will be important for further understanding CYC1 expression and catabolite repression in general.

E. Catabolite Repression

Whenever glucose is present in the growth medium, it is utilized as the sole source of carbon. Repression of several metabolic pathways not required for glycolysis occurs, including repression of enzymes of the glyoxylate shunt pathway and gluconeogenesis and enzymes of the TCA cycle and of the respiratory system, some of which are present at very low levels. Enzymes for the metabolism of disaccharides are also under similar regulatory control.

In *E. coli,* carbon catabolite repression is mediated by the product of a single gene, CRP. The CRP protein, when complexed with cAMP, activates transcription of enzymes involved in catabolism of sugars other than glucose.[133-140] In yeast, the system is much more complex and appears to result from active repression of genes involved in metabolizing alternate sugars when glucose is present. Mutations have been isolated which define a variety of genes affecting several different enzymes (Table 2). An exclusive role of glucose in this repression is unresolved, although no clear involvement of glycolytic intermediates has been demonstrated,[141,142] and the precise mechanism of regulation is yet to be revealed. Genetic evidence leads to the conclusion that although catabolite repression appears ultimately to be under the control of relatively few pleiotropic effectors distinct branch points exist in these regulatory pathways.

1. Genes Involved in Catabolite Repression

Mutants in carbon catabolite repression can be divided into two major groups, those that prevent repression (constitutive expression) and those that fail to show derepres-

Table 2
GENES INVOLVED IN CARBON CATABOLITE REPRESSION

Gene	Function controlled	Ref.
HEX1	Repression of maltase, invertase, malate dehydrogenase, and hexokinase PII	143
	Respiratory enzymes	144
HEX2	Repression of maltase, invertase, malate dehydrogenase, and hexokinase PII	143
	Maltose uptake system	144
CAT80	Repression of maltase, invertase, and malate dehydrogenase	142, 144
SNF1	Derepression of invertase; galactose, melibiose, and maltose metabolism; gluconeogenic enzymes; unable to grow on non-fermentable carbon source; ssn mutants are suppressors of snf1	145
REG1	Derepression of galactose utilization enzymes; invertase	146
CAT1 CAT3	Derepression of isocitrate lyase, fructose 1,6 biphosphatase, and maltase; unable to grow on glycerol as sole carbon source	147
MUR1	Derepression of maltose metabolism	147
CCR1	Derepression of isocitrate lyase, fructose 1,6 diphosphatase, and alcohol dehydrogenase	148
CCR2 CCR3	Repression of isocitrate lyase, fructose 1,6 diphosphatase, alcohol dehydrogenase II, and to a lesser extent, malate dehydrogenase and succinate dehydrogenase	148
GLR1	Repression of maltase, galactokinase, alpha-galactosidase, NADH-cytochrome c reductase, cytochrome c oxidase	149
ccr80	Repression of respiratory and gluconeogenic enzymes	150
ccr91 ccr96	Repression of mitochondrial functions: succinate, cytochrome c oxidoreductase	34

sion (see Table 2). These may be further subgrouped into mutants with pleiotropic effects or those that affect only one enzyme. The latter are usually structural gene mutants. The number of genes involved is unclear, as are the relationships among the various mutants. A variety of phenotypes for affected enzymes have been observed, but no systematic comparison of mutants has been made.

One of the earliest mutants isolated, FH4C,[151] is insensitive to glucose repression of invertase and maltase, but is not well characterized because the strain mates poorly and is not amenable to genetic analysis. A similar mutation, flaky,[152] is resistant to glucose repression of maltase, invertase, and succinic dehydrogenase, due to a lesion in a single nuclear gene. The mutant is extremely flocculant and not readily analyzed biochemically. Other indirect affects, such as sporulation deficiency, have been observed with other mutants.

More recently isolated mutants, hex1, hex2, and cat80, show glucose constitutive expression of the enzymes invertase, alpha-glucosidase, and MDH.[143,144,153] Mutants hex1 and hex2 also fail to repress respiratory enzymes, and hex2 has been shown to affect the maltose-uptake system. All three mutants differ with respect to HXK2 activity, one of two identified isozymes of HXK, normally catabolite repressible. Cat80 has wild-type activity.[143] The hex2 mutant has elevated HXK2 activity. The increase in activity is dependent upon *de novo* protein synthesis, as evidenced by nutritional shift experiments.[143] Another allele of this gene, hex2-3, is unable to grow on maltose as a carbon source. The hex1 mutation is allelic to the structural gene of HXK2, and biochemical data show that the mutant does not synthesize the enzyme.[154,155] The hex2 mutants are presumed to possess a mutant allele of a regulatory gene affecting HXK2 synthesis. The epistatic relationship of cat80 to hex2[143] suggests that CAT80 is also involved in HXK1 synthesis. The glr1 mutants of *S. carlsbergensis* described by Michels and Romanowski[149] are similar to the hex1 mutants. The enzymes maltase, galactokinase, alpha-galactosidase, NADH-cytochrome c reductase, and cytochrome c-oxidase are expressed constitutively in glr1 mutants. Although initially GLR1 was believed

to be a new gene, recent work indicates that it is allelic to hex1 (hxk2), the structural gene of HXK2 in *S. cerevisiae*.

Entian and Frohlich[156] have proposed that HXK2 is bifunctional, with catalytic and regulatory subunits, and that the regulatory subunit is required for catabolite repression. This notion is supported by their isolation and characterization of mutants defective in glucose repression (nonrepressible), in which lesions map to HEX1 (hex1^r). In these mutants, HXK2 activity is unaffected. A recent analysis of the catalytic properties of the enzyme isolated from mutant cells revealed no differences in kinetic properties.[448] In one mutant, however, a recently identified in vivo modified form of the enzyme (PIIM) was not present.[448] The authors suggest that this modification of HXK2 may be directly involved in triggering glucose repression.

The second group of mutants fail to derepress catabolite-repressed enzymes. Mutants cat1, cat3, and mur1,[147] isolated by their ability to suppress the maltose sensitivity of hex2-3 mutants, are unable to derepress a variety of enzymes (see Table 2 for details): maltose, ICL, and fructose 1,6 diphosphatase. No regulatory effect on invertase or the respiratory enzymes was observed, and the mutants grow on glycerol as a sole source of carbon.[147] An allele of CAT1, CAT1-2^d, accelerates derepression of the above enzymes after the media has been depleted of glucose.[157] Another group of mutants, ccr1, ccr2, and ccr3, were isolated by Ciriacy.[148] The ccr1 mutation is allelic to cat1-1. Mutations in the two other genes, ccr2 and ccr3, fail to derepress maltase and the enzymes involved in oxidative metabolism, but were still respiratory competent. The mitochondrial TCA cycle enzymes succinate dehydrogenase and MDH were not significantly affected.[148]

Based on the epistasis relationships between cat1 and cat3 and the constitutive mutants hex1 and hex2 and the phenotypes of the above mutants, Entian and Zimmermann[147] have presented a view of how these genes participate in catabolite repression. They suggest that catabolite repression can be separated into two groups of enzymes that are either stringently repressed (gluconeogenesis and glyoxylate shunt pathway enzymes) and under the control of CAT1, CAT2, CCR2, CCR3 (positive), and CCR80 (negative), or those not repressed when sugars such as galactose are in the medium, controlled by HEX1, the structural gene for HXK2, and to varying extents, HEX2 and CAT80. Together, hex1 and hex2 are involved in repression of respiratory function, whereas cat80 mutants have no influence. Mutants hex1, hex2, and cat80 do not release the stringently repressed enzymes from repression.

Carlson et al.[158] have recently isolated another catabolite-repression mutant, based on its sucrose nonfermenting phenotype. This phenotype results from a defect in one of five gene loci, SNF1-SNF5.[159,168] A sixth mutant, snf6, shows reduced sucrose utilization, but not the pleiotropic effects. The snf mutants fail to express glucose-repressible genes required for the catabolism of galactose, maltose, melibiose, and several nonfermentable carbon sources.[159,168] The snf1 mutant is unique in that the gene has been cloned by complementation and genetically mapped to chromosome IV.[145] Interestingly, the amino acid sequence of the SFN1 protein, predicted from the nucleotide sequence of SNF1, shows homology to sequences in the conserved domain of protein kinases.[160]

SNF1 has been studied predominantly with respect to invertase. Invertase exists in two forms, cytoplasmic and secreted, the latter being glycosylated.[161-163] Only the secreted form is glucose repressed, and this occurs at the transcriptional level.[164-166] The snf1 mutations produce only the constitutive cytoplasmic invertase. Suppressors of the snf1 mutation, ssn, have also been isolated. These fall into eight complementation groups. All showed exocellular invertase production under conditions of glucose limitation (at lower levels than in the parent strain), and ssn6 produced the enzyme even in the presence of excess glucose.[167] The double mutant ssn6 SNF1 exhibited constitutive

synthesis of secreted invertase, indicating that the suppressor mutation acts independent of the SNF genotype. When the concentration of glucose is low, the SNF1 gene product appears to act positively to derepress glucose-regulated genes. The pleiotropic nature of the ssn mutants (clumpy growth) and the properties of ssn6 (allelic with cyc8 sporulation deficiency in homozygous diploids, illegitimate mating, and overproduction of cytochrome c) have led Carlson and co-workers[167,168] to propose that ssn6 is a *trans*-acting negative regulatory gene, acting in response to external glucose concentrations as a repressor of SUC2 and the other pleiotropically affected genes. The role of the SNF and SSN genes in expression of invertase, and more generally, in catabolite repression has been more thoroughly detailed by genetic analysis, providing further support for the existence of positive and negative regulators.

In addition to the above mutants, other reports show that certain mitochondrial functions can be released from carbon catabolite repression while cytoplasmic functions remain unaffected. CCR91 and CCR96[34] mutants are unable to repress a spectrum of mitochondrial functions, including cytochrome systems. The observation that these cells have a high growth rate and exhibit normal fermentation suggests this phenotype is not due to a decrease in glucose utilization. Defects in the nuclear genes CCR91 and CCR96 release mitochondrial genes from glucose repression. Since the respiratory chain is fully equipped and functional, this implies that the expression of many nuclear genes involved in mitochondrial function are also released from repression. This concomitant release of mitochondrial and nuclear gene expression, combined with the dominant nature of the mutations, suggest the existence of a common regulatory gene function. Defects in SNF1, CAT1, and CAT3, on the other hand, do not affect mitochondrial function. It remains to be determined if the cytoplasmic-mitochondrial boundary defines separate regulatory domains.

In summary, data reported by a number of laboratories suggest that carbon catabolite repression of many different glucose-repressible enzymes is under the control of numerous interdependent and overlapping genes. This complex network appears to consist of positive and negative regulatory factors which may interact in conjunction or singly through specific regulatory sites to exert catabolite-repression control. It is clear that for a number of these regulated enzymes, catabolite repression occurs primarily at the level of transcription of the structural genes (invertase, maltase, ADH, and galactose utilization enzymes). More detailed molecular and genetic analysis of these systems, such as those described below, should reveal how this repression is achieved in yeast.

2. Regulation of Sugar Utilization Pathways

Yeasts utilize a variety of sugars for growth, including galactose, melibiose, sucrose, and maltose (Figure 3). Specific enzymes catalyze their metabolism to an intermediate stage where they enter the glycolytic pathway. Synthesis of these enzymes is repressed in cells grown in excess glucose, but undergo derepression, under glucose-limited conditions, in the presence of the appropriate sugar(s).[30,169] The enzymes are coded for by structural genes which are regulated at the transcriptional level. This regulation is mediated by regulatory genes specific for each system, in addition to mediators for catabolite control. The precise mechanisms of catabolite control are not well understood. Recent reports emphasize the genetic aspects of the catabolite regulation of these sugar metabolism pathways.[170,146]

Several of the systems responsible for differential sugar utilization by yeast have been documented at the molecular level. These systems exhibit differences in genomic organization of structural and regulatory genes, and although they all show regulation at the transcriptional level, available evidence suggests that distinct mechanisms of regulation exist. In the remainder of this section, we will discuss the molecular basis of

regulation in the most extensively studied of these systems, the galactose utilization pathway.

Recently reviewed genetic evidence combined with molecular-level data provide some understanding of the basic regulatory mechanisms involved in galactose metabolism.[169] The Leloir pathway enzymes, which metabolize galactose to glucose-6-phosphate (Figure 3), are repressed in the presence of glucose even when galactose is present. A 1000-fold induction occurs at the level of transcription when cells are grown in a galactose-based medium. The products of the structural gene cluster comprising GAL1 (galactokinase), GAL7 (galactose-1-phosphate uridyltransferase), GAL10 (uridine diphosphoglucose 4-epimerase), and GAL2 (galactose permease) are controlled by a well-defined group of regulatory genes GAL4, GAL80, and GAL3.[169,171] Based on genetic data (see Reference 169), a model for this regulation has been formulated, wherein galactose-mediated interaction of regulatory protein complexes directs transcriptional expression of the GAL structural genes.

The recent review by Oshima[169] details the genetic and biochemical evidence supporting a regulatory model where both the positive regulator GAL4 and the negative regulator GAL80 are constitutively expressed.[172,173] Recent developments in molecular cloning techniques have provided recombinant clones of the structural GAL gene cluster,[174] as well as the regulatory genes GAL4[175,176] and GAL80.[177] RNA blot hybridization analyses have provided conclusive data that the regulatory genes are constitutively expressed. The GAL4 transcript is maintained at 0.1 copies per cell,[176] and the GAL80 mRNA is fivefold more abundant.[178] Present data suggest that GAL4 and GAL80 function by direct protein-protein interaction. The GAL80 regulatory function can be titrated out in neutral (lactate) and repressing (glucose) media when multiple copies of the GAL4 gene are introduced[175] into the cell on plasmid vectors, supporting the existence of protein-protein interactions between these positive and negative regulators.[177,178]

Matsumoto et al.[170] have suggested that either the GAL4 or GAL80 gene product may be involved in glucose repression. They isolated several catabolite repression mutants affecting the galactose pathway enzymes, based on the synthesis of galactokinase, normally unexpressed in medium containing glucose.[146,170] These mutations define three different complementation groups. Both gal82 and gal83 are recessive mutations, showing greatly enhanced resistance to glucose repression, but only when combined with GAL81 or gal80 constitutive mutations. They are probably specific for the GAL structural genes. The reg1 mutants show additive effects to those of gal82 and gal83 and also show glucose resistance to invertase synthesis, but not to alpha-D-glucosidase. Based on an elaborate biochemical and genetic study of these various mutants, the authors have proposed a regulatory model for catabolite repression of the galactose utilization enzymes, involving transmission by three independent circuits. One circuit, mediated by the REG1 gene product, is pleiotropic for repression of various catabolic enzymes. The second, mediated by the products of GAL82 and GAL83, is specific for the galactose pathway enzymes. Both circuits were suggested to convey glucose signals either to GAL4 (or in the case of REG1, a positive factor) or directly to the structural genes. The third circuit is mediated by a competitive interaction of galactose and glucose for GAL80 or through allosteric inhibition of galactose permease, thereby lowering intracellular inducer concentrations.

Data presented by Johnston and Hopper[175] argued that the GAL80 protein, and not the GAL4 gene product, is involved in transmitting the glucose response. They reported two observations. First, multiple plasmid-borne copies of the GAL4 gene partially overcome the uninducible nature of GAL80^r mutations (*trans*-dominant over wild-type and gal80 alleles). Second, the level of expression of both MEL1 and GAL7 in GAL80^r cells transformed with a high copy number plasmid carrying GAL4, grown

in glucose medium, was higher than in a GAL80 transformant with the same plasmid.[179,175] This observation also applies to untransformed GAL80^r and gal80 strains. The gal80 mutants transformed with the GAL4 plasmid showed wild-type inducible levels of transferase activity when grown in glucose medium. Thus, lesions in the GAL80^r or gal80 alleles were proposed to alter the response of the GAL80 protein not only to galactose, but also to glucose. The mutant proteins were presumed to be less effective in transmitting the catabolite repression signal as the wild-type GAL80 protein.

The construction of a GAL80 null mutant,[177] however, provides strong evidence that the GAL80 protein is purely a negative regulator and not directly involved in catabolite repression. The steady-state levels of galactokinase and alpha-galactosidase were measured in the null mutant grown in various media. Both enzymes were regulated as in wild-type cells; no enzymatic activity was detected in glucose medium, but in neutral glycerol-lactate medium or in galactose medium, enzyme activities were detected. Enzyme levels were higher overall than in the wild-type strain. When the null mutant was grown on glycerol-lactate-glucose medium, no enzymatic activity was detected. RNA blot hybridizations confirmed that the increases or decreases in enzymatic activity were due to changes in the levels of GAL1 and MEL1 transcripts. Independently, in a series of experiments conducted to delimit the regulatory elements of the inducible divergent GAL1-GAL10 promoter (in promoter fusions to the *E. coli* lacZ gene), Yocum et al.[180] have confirmed the latter observation. Full glucose repression of beta-galactosidase activity was observed in gal80 strains. These data eliminate any direct role for GAL80 in catabolite repression.

Reports from a number of laboratories analyzing the 650-bp region separating the GAL1-GAL10 genes showed that a 365-bp DNA fragment[123] from this intergenic region contains all the required DNA sequences for galactose-induced transcription and glucose repression.[181] Subsequent deletion mutation analysis of this region indicated the importance of a 108-bp G + C-rich DNA sequence[181] located approximately halfway between GAL1 and GAL10. The 45 bps proximal to GAL10 are sufficient for normal galactose-induced transcription of GAL10, whereas the 55 bps upstream of this showed only weak induction of the GAL10 gene.[180] These experiments, however, were unable to differentiate between those regulatory sequences responsible for normal galactose induction and for glucose repression of GAL10. The authors suggest that the distance between the GAL sequences (necessary for galactose-mediated high-level transcription) and the transcriptional start site of GAL10 is not critical since a deletion which brings the UAS about 100 bps closer to the initiator codon does not signficantly affect expression. West et al.[182] reported that deletions toward the GAL1 transcriptional start do alter the expression of the GAL1 gene. When the UAS was placed 40 bps closer to the GAL1 TATA box (within 40 bps), the GAL1 promoter was functional in the absence of galactose. Furthermore, cells harboring this construct grown in a glucose medium showed reduced glucose repression (from about 150-fold to about 5- to 10-fold). It is not clear whether the reduced glucose repression is simply a function of the decreased distance between UAS and the TATA box region or whether specific protein-binding inhibitory sequences have been removed.

Giniger et al.[183] developed an in vivo footprinting technique for analysis of GAL4 protein DNA binding domains, based on protection from methylation by dimethyl sulfate (DMS). The data suggest that GAL4 binds to four related 17-bp sequences (each possessing dyad symmetry) located midway between the GAL1 and GAL10 genes and about 250 bps from the transcriptional start site of both. The protection pattern observed in a normal GAL4 strain grown in the presence of galactose was absent when cells were grown in the same medium with glucose, suggesting that one mechanism of glucose repression is inhibition of binding of the GAL4 protein to UAS sequences.

A novel experiment by Brent and Ptashne[184] has further dissected the nature of the

interaction of GAL4 with DNA. First, the GAL UAS of a "tester" plasmid was replaced with a bacterial operator sequence which binds the *E. coli* lexA repressor protein. Yeast cells containing this plasmid were transformed with a second construct encoding a lexA:GAL4 hybrid protein. The *N*-terminus of the hybrid contained the first 87 amino acids of the lexA protein (sufficient for operator binding) fused to about 800 residues of the GAL4 protein (known to interact with GAL80). Cells expressing the lexA:GAL4 hybrid protein were able to activate transcription of the test promoter, while those producing the lexA protein itself could not. However, the lexA:GAL4 construct was unable to activate the wild-type GAL structural genes as inferred by its inability to complement a gal4 mutation. Presumably, this is because the binding domain for the GAL UAS is missing in the hybrid protein. These results imply the following: (1) binding of GAL4 to DNA is required for this protein to activate transcription, and (2) in general, transcriptional activation is not a consequence of protein-DNA binding per se, but rather, the immobilization of a specific activator protein(s) upstream of a promoter.

One inherent problem in using the GAL1/GAL10 cluster to study catabolite control is that the effects of glucose repression are difficult to assess without consideration of GAL4-mediated galactose induction. This problem has been largely avoided by construction and analysis of UAS_{gal}/HIS3 fusions.[447] The 365-bp region from GAL10 was placed upstream of the intact HIS3 transcription unit. HIS3 transcripts and the corresponding enzyme activity (IGP dehydratase) were shown to be repressed in glucose-grown cells containing these constructs integrated at the HIS3 locus. In raffinose-grown cells, IGP dehydratase enzyme levels were comparable to the wild type. These experiments demonstrate the separability of catabolite repression and galactose induction within the GAL UAS element. HIS3 expression was reduced by glucose repression in both normal and amino acid starvation conditions, suggesting that catabolite repression occurs by a mechanism involving negative repression of transcription. This is unlike the situation in *E. coli,* in which a catabolite repressor protein (CRP) is a transcriptional activator.

As evidenced from the above investigations, a comprehensive molecular framework for the regulation of the galactose-metabolizing enzymes is beginning to emerge. Other sugar-metabolizing systems are less well characterized. They usually exist in multigene families of structural and regulatory genes, each family consisting of several loci. Usually, only one functional copy of each gene of a cluster is necessary for metabolism. In the case of maltose metabolism, five unlinked MAL loci have been characterized, MAL1 to MAL4, and MAL6.[185,186] The MAL6 locus has been cloned and shown to consist of a regulatory gene (MAL63) and two structural genes (MAL61 and MAL62).[186-188] The regulatory gene is required for induction of both structural genes, maltase (MAL61) and maltose permease (MAL62). More recently, regulatory genes from two other loci, MAL2 and MAL4, have been cloned.[189,190] Strains carrying the MAL6 and MAL2 loci exhibit inducible expression of maltase subject to catabolite repression, whereas strains carrying the MAL4 locus synthesize maltase constitutively.[189] MAL4 showed strong homology to MAL3 and significant differences to MAL1 and MAL2.[190] Transformation of a maltose nonfermenting strain with a plasmid carrying the cloned MAL2 regulatory gene showed a maltase-inducible and catabolite-repressible phenotype, whereas the same strain transformed with a plasmid carrying the MAL4 regulatory gene showed constitutive maltase expression, suggesting that catabolite repression of maltase is mediated via the MAL regulatory genes.

The maltose gene cluster, like the galactose system, exhibits divergent transcription of coordinately regulated structural genes. Two different sized transcripts were identified from the MAL61 locus. One transcript was detected only in maltose-grown cells and not in glucose- or glycerol-grown cells, whereas the other transcript, although

inducible, was present under all growth conditions.[188] Two transcripts from the MAL63 gene were also detected and were shown to be differentially regulated; one is constitutive and the other is induced by maltose. This mode of regulation, production of mRNAs transcribed from different promoters within a single gene, was reported also for the SUC2 gene. Beyond this, little is known about the detailed mechanisms controlling this complex regulon.

F. Compartmentation and Transport

Compartmentation and transport appear to play central roles in maintaining cellular homeostasis, particularly with regard to degradation, storage, and salvage pathways. They are also important in the starvation response of yeast.[191] Starvation of cells for glucose, nitrogen, sulfate, phosphate, biotin, or K^+ will cause cell division arrest at G_1.[192] Cells outside of G_1 will complete a cycle of division and then arrest prior to execution of start or the cell cycle stages defined by CDC28, CDC24, and CDC7.[193] Mobilization of metabolic reserves and salvage pathway enzymes and the induction of alternative transport systems provide the necessary nutrients to sustain this growth.

The most prominent organelles in these processes are the plasma membrane and the vacuole.[194,195] The vacuole is analogous to mammalian lysozymes, containing a number of salvage pathway enzymes, principally glycoproteins synthesized as proenzymes,[196] and small metabolites, *S*-adenosylmethionine,[197] allantoin and allantoate,[198] and polyphosphate.[199,200] A majority of the amino acid pools, with the exception of leucine, aspartate, and glutamate, are also found in the vacuole.[201,202] In exponential growth, the vacuole consists of small vesicles of 300 to 1000 nm in diameter.[203] In stationary phase, it is the most prominent organelle. The vacuole undergoes distinct morphological changes during the cell cycle. From freeze-thaw fractionation studies, Wiemken et al.[204] have shown that prior to bud initiation the relatively few large vacuoles present begin to shrink and fragment into numerous small vacuoles. Migration of vacuoles to the newly forming bud occurs, followed by expansion and fusion during G_2, in the preparation for cytokinesis. In the transition to G_0, morphological changes occurring in vacuole membrane ultrastructure coincident with lipid body uptake[203,205] suggest these processes may be involved in degradation and storage pathways for storage of carbon and energy.

Several examples exist in which metabolism appears to be regulated by inter- and intracellular metabolic compartmentation. Perhaps the best studied are the enzymes of nitrogen metabolism. Cooper[31,206] has shown that transcriptional regulation of these genes is highly interactive with compartmentation for balancing the acquisition, production, and utilization of nitrogen metabolites. Similarly, it has been suggested that the cycling of polyphosphate accumulation and degradation in the vacuole may serve as a metabolic buffer, by consumption of ATP and by balancing intracellular concentrations of inorganic phosphate.[207,208] All of the enzymes of the polyphosphate cycle are regulated in concert with an inducible inorganic phosphate transport system[209] and with repressible exocellular acid phosphatase.[210]

Inorganic phosphate (Pi) is an essential cellular constituent, and its metabolism has been studied in conjunction with cell cycle events. Yeast stores excess Pi in polyphosphate (polyP) deposits, representing 20% of the dry weight of the cell under certain growth conditions.[211] Two classes are found in the cell: acid-insoluble polyP with an average chain length of 165 phosphate residues and acid soluble polyP containing up to 10 residues.[212] PolyP synthesis and breakdown follow a cyclic pathway, controlled by changes in external Pi concentration.[213] Under nonlimiting conditions, synthesis is strongly inhibited by ADP and is antagonistic to nucleic acid and phospholipid synthesis. During exponential growth, nucleic acid synthesis inhibits polyP deposition and stimulates its degradation. Accordingly, polyP barely accumulates. In cells reaching

stationary phase, the growth rate declines, degradation is inhibited, and polyP levels increase. The polyP cycle therefore appears to balance cellular levels of Pi with the energy charge of the cell.[214]

When external Pi is limiting, a high-affinity scavenging system consisting of polyphosphatase and alkaline phosphatase is derepressed.[207,215] This leads to the consumption of polyP and the formation of Pi.[216] An exocellular acid phosphatase is also derepressed under these conditions, potentially providing an external source of Pi from phospho-ester substrates.[210,217,218] The direct interconversion of polyP and ATP by polykinase does not occur in vivo. Rather, polyP is sequentially hydrolyzed to smaller chain length molecules by polyphosphatase and by vacuolar alkaline phosphatase.[207,212,213] Free Pi resulting from enzyme derepression is then used in biosynthesis of nucleic acids and phospholipids.[207,219] In addition, a decrease in low-density vesicles, sterol esters, and triacyl glycerols is observed, presumably releasing free Pi.[220] Thus, cells growing in medium depleted of Pi generate two pools of Pi. The molecular basis of repression and derepression of these enzymes by Pi is not understood. However, genetic and biochemical data indicate regulation is at the transcriptional level.

Cells in synchronous cultures also exhibit a flux in Pi utilization. In the period when initiation of DNA synthesis occurs, for example, external Pi concentration decreases simultaneously with an increase in polyP. During the S phase, if external Pi is low, polyP is consumed, presumably by acting as a substitute source of Pi for nucleotide biosynthesis.[221]

The yeast plasma membrane has been studied extensively using electron microscopy.[222] Recent morphological studies have revealed a rearrangement of particles on the cytoplasmic side of the membrane into semicrystalline arrays during transition to stationary phase or carbon starvation, although the function of this is unknown.[223,224] Transport has been characterized both genetically and biochemically.[222] Three modes of transport exist in yeast; passive diffusion, facilitated diffusion, and active transport.[206] The distinguishing characteristic of facilitated vs. active transport is the coupling to energy consumption in the latter. This enables extreme concentration gradients across the plasma membrane.

Little, if anything, is known about uptake of acetate, lactate, glycerol, and ethanol. Based on uptake rates and glucose-inhibition studies,[225] it appears that hexose transport involves both a constitutive system of facilitated diffusion (glucose-hexose system) and a number of inducible systems for transport of mono- and disaccharides.[206] Induction of the galactose-uptake system,[226,227] responsible for facilitated diffusion of galactose, fucose, or arabinose, is under control of the GAL4, GAL3, GAL80 system, which transcriptionally regulates the galactose-metabolizing enzymes. Maltose transport appears to be mediated by an active transport system inducible up to 150-fold.[228-230] Four mutants, dsf6, dsf7, dsf17, and dsf21, are defective in maltose uptake but transport glucose, galactose, and sucrose normally.[231] Sucrose is believed to be hydrolyzed to glucose and fructose within the periplasmic space by an inducible exocellular invertase prior to transport across the plasma membrane,[232] although recent kinetic data suggest that multiple uptake systems may be involved.[233] Trehalose, a storage carbohydrate in yeast, is also transported.[234] The lack of efflux of trehalose in trehalose-depleted medium suggests that this sugar is indeed sequestered, unlike other disaccharides.

Major energy reserves in the yeast cell are the branched polysaccharide glycogen and the disaccharide trehalose, each of which are believed to play distinct metabolic roles in carbohydrate storage and mobilization. The total cellular content and ratio between the two can vary greatly, depending on the nutritional status of the cell (for reviews on glycogen and trehalose biosynthesis in yeast and their involvement in energy metabolism, see References 207 and 235). Recently, Lillie and Pringle[237] measured separately

the glycogen and trehalose levels in cells under a wide variety of growth conditions. Several important observations were made regarding the role of each under conditions of starvation for nitrogen, phosphorus, and carbon sources. Based on their findings, these authors suggested that glycogen may be an important energy source during the transition from glycolytic to respiratory growth, while both glycogen and trehalose are accumulated prior to, and utilized during, starvation. The time course of glycogen and trehalose accumulation during diauxic growth in glucose-rich medium is indicative of distinct control mechanisms for the net synthesis of these two carbohydrates. Glycogen accumulation actually preceded depletion of glucose from the medium, as if the cell projected the number of cell generations to glucose exhaustion. Trehalose, however, accumulated significantly only after cells reached a late logarithmic growth phase, when glucose levels had fallen precipitously.[237]

Although trehalose is not a significant storage carbohydrate in mammalian systems and has been studied very little, glycogen synthesis and breakdown has been intensively studied for decades. Much of what we know about regulatory biochemistry resulted from these studies. Covalent modification (phosphorylation/dephosphorylation) of proteins plays a major role in acute hormone responses in tissues of higher organisms. Insulin and glucagon have reciprocal effects on the activity of key enzymes in glycogen synthesis (glycogen synthase) and glycogen breakdown (glycogen phosphorylase). These effects are mediated through a series of kinases (phosphorylase kinase; cAMP-dependent protein kinase) and a generalized protein phosphatase. Energy consumption or storage (i.e., glycogen turnover) is regulated rapidly without invoking nuclear events such as gene transcription. Only recently have phosphoprotein phosphatases been characterized from yeast and their involvement in glycogen production by inactivation of glycogen phosphorylase implicated.[238] Oritz et al.[239] obtained evidence for the activation of yeast trehalase (which catalyzes the breakdown of trehalose) by a cAMP-dependent protein kinase in a glc1 mutant. This mutant fails to accumulate both glycogen and trehalose, thought to be due to a defect in the regulatory subunit of a cAMP-dependent protein kinase. The constitutive activity of the protein kinase in the glc1 mutant, according to the authors,[239] may lead to both activation of trehalase and inactivation of glycogen synthase by phosphorylation of the two enzymes.

Another study using bcy1 (positive regulator of cAMP-dependent protein kinase) and cyr1 (adenylate cyclase structural gene) mutations support a case for the regulation of trehalase by phosphorylation.[240] Further evidence comes from the analysis of cells with a mutation in the yeast gene RAS2, whose function has been implicated in the adenylate cyclase system[241] (Section III.C.1). The $RAS2^{val19}$ mutant, a missense mutant analogous to one resulting in tumor-transforming potential in the mammalian ras gene, fails to accumulate glycogen and trehalose and has elevated trehalase activity.[241] These phenotypes all appear to result from elevated levels of intracellular cAMP. Regulation of the storage carbohydrate metabolic enzymes has not yet been studied at the level of their synthesis and presumably awaits further biochemical characterization of the proteins and cloning of their structural and regulatory genes.

G. Cell Surface Growth

Discussion of growth regulation would be incomplete without reference to cell surface events. In yeast, the cell surface is defined by two interactive structures, the plasma membrane and the cell wall. The former is composed of a lipid bilayer containing both integral and peripherally associated proteins. While the extent of fluidity of membrane components in yeast is uncertain, there are indications that localization of membrane components occurs. The cell division cycle gene CDC24 involved in bud initiation (Section III) may play a role in mediating this restriction process.

The cell wall is composed primarily of structural carbohydrates and mannoproteins.

This structure has recently been reviewed.[242] Glucan, a polysaccharide composed of D-glucopyranose, is synthesized continually throughout the cell cycle. Chitin, a polymer of *N*-acetylglucosamine, is deposited in a localized ring on the cell surface just prior to bud emergence and is most actively synthesized during septation. Mannoproteins appear to be continuously synthesized and are deposited to form the outermost layer of the yeast cell wall. The periplasmic space is the site of localization of numerous secretory proteins, including invertase, acid phosphatase, alpha-galactosidase, and several forms of glucanase. Transport systems for hexoses, amino acids, and other metabolites as well as mating pheromone receptors and agglutinins are comprised of integral membrane proteins.

Growth at the cell surface in *S. cerevisiae* occurs exclusively at the emerging bud and is a cell cycle-regulated process. The problem of how cell wall components are delivered to a localized region of the cell surface for assembly has interested many investigators. Using radio-labeled precursors, it can be shown that newly synthesized cell wall constituents are deposited in the bud wall.[243] Mannoprotein synthesis occurs on membrane-bound ribosomes of the endoplasmic reticulum, which are sorted through the golgi apparatus into secretory vesicles which fuse with the plasma membrane. This was demonstrated by early studies using electron microscopic and autoradiographic techniques and is consistent with more recent studies using conditionally lethal secretory mutants (for a review of yeast secretory pathways, see Reference 244). As in exocrine cells of the mammalian pancreas, the secretion of mannoproteins as well as other yeast glycoproteins is a vectoral process. Studies by Field and Schekman[245] have shown that secretion of invertase and repressible acid phosphatase (PHO5) is also localized to the area of bud growth. Thus, the processes of bud growth and protein secretion are intimately coupled and involve exocytosis of secretory vesicles for delivering components needed for cell surface growth.

Another enzyme transported to the cell surface is chitin synthetase. Work of Cabib[247] and Cabib and Farkas[246] led to the model that this enzyme is a membrane-bound zymogen, activated by proteolysis from the inner side of the plasma membrane. It appears that chitin synthetase activity may be confined to the nascent septum (and prebud ring) by directing the zymogen protease to the bud (or prebud site) via the secretory pathway. Cabib et al.[248] have reviewed these and other related topics. The CDC24 mutant mentioned above fails to bud and grows in an apolar (spherical) manner at the restrictive temperature.[249] Chitin synthesis is not blocked in these mutants, but rather, is distributed evenly over the cell surface.[250] Vectoral secretion of acid phosphatase was also altered in CDC24 mutants at the restrictive temperatures.[245] These data suggest: (1) that the polar mode of yeast cell growth is coupled to secretory processes and (2) that the CDC24 gene product may be involved in maintaining the polarity of membrane constituents. Identification of the mechanisms regulating this cell cycle-dependent localization of cell surface constituents and their temporal mode of synthesis awaits further study.

III. CONTROLS OVER CELL CYCLE

A. cdc Mutations and Start

The absence of any gene product essential for continued cell growth eventually prevents further cell division. However, a lesion which results in arrest at a defined point in the cell cycle more likely represents a gene function involved directly in events required for cell cycle progression. The cdc mutants are conditionally blocked at a particular step in the cell cycle and identified by arrest at a characteristic terminal morphology. Hartwell et al.,[251,252] Klyce and McLaughlin,[253] and Reed[254] have isolated a large number of temperature-sensitive cdc mutants. Cold-sensitive mutants have also

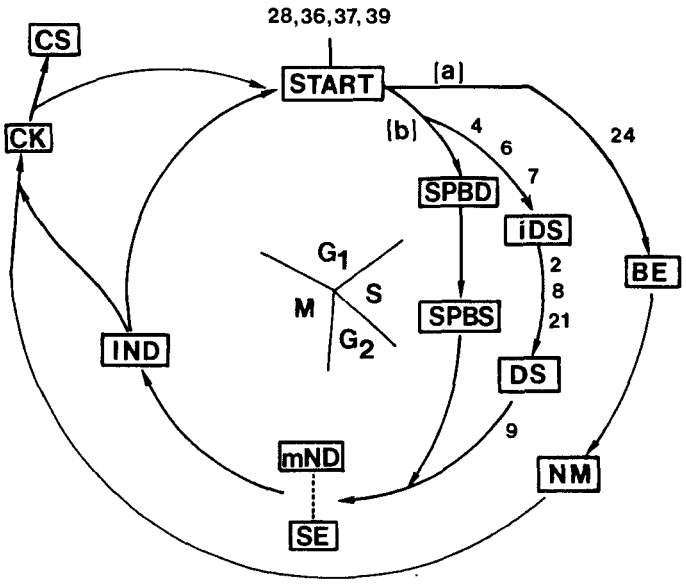

FIGURE 5. Generalized version of landmark events in the yeast cell cycle. There are several parallel pathways; the two major ones are shown here. (a) Bud emergence; (b) DNA synthesis. The DNA synthesis pathway can also be subdivided into two parallel pathways as indicated. The execution points of cdc mutations discussed in the text are indicated. Many others have been ordered in these pathways, but are not shown. Abbreviations: SPBD, spindle-pole-body duplication; SPBS, spindle-pole-body separation and spindle formation; iDS, initiation of DNA synthesis; DS, DNA synthesis; BE, bud emergence; NM, nuclear migration; mND, medial stage of nuclear division; SE, spindle elongation; IND, late nuclear division; CK, cytokinesis; and CS, cell separation.

been identified.[255] In total, about 50 cdc mutants have been isolated, and this number is likely to represent an upper limit using conventional temperature-selection schemes. The point during the cell cycle at which a mutant cdc gene product fails to carry out its normal function(s), resulting in a subsequent terminal phenotype, is termed the "execution point". Traditionally, execution points have been determined by reciprocal shift experiments using a characteristic terminal morphology as a cell cycle marker. More recently, however, Botstein and colleagues[256] have used cell viability rather than terminal morphology to define the execution point of topoisomerase II (TOP2). Studies of this sort may broaden current definitions of cdc mutations. Characterization of cdc mutations has lead to several models of the *S. cerevisiae* cell cycle and has been the subject of numerous reviews.[192,249,257-259,261,262] A summary is shown in Figure 5.

Two basic models have been suggested[249] as ways to control progression through the cell cycle: (1) the dependent control model, whereby an obligatory sequence of events occurs, each step dependent on completion of the previous one; or (2) the independent control model, in which cell cycle events are triggered independent of one another by a molecular timer-clock mechanism which acts once per division cycle on each process. As depicted in Figure 5, it appears that elements of both are probably involved. Analysis of cdc mutations has shown the existence of at least two parallel pathways: DNA synthesis/nuclear division and bud emergence/nuclear migration.[249] Each pathway consists of a series of obligatory events, yet each pathway can, under most circumstances, progress without the other. Both converge at the G_1 event known as "start".

Since the study of cell cycle regulation relies heavily on the use of mutants which are

predominantly recessive in nature, it follows that nearly all experimental approaches involve haploid cells. This avoids masking of the mutant phenotypes by wild-type alleles normally present in diploid strains and facilitates further genetic and molecular biological analysis. This section deals almost exclusively with haploid cells during vegetative growth.

The mutants cdc28, cdc36, cdc37, and cdc39 all arrest at the restrictive temperature within the G_1 phase, just prior to SPB duplication and initiation of DNA synthesis. This is also the point of arrest by mating pheromones (Section I.A) and after nutritional shift down (Section III.C). The properties of this point called start are very much analogous to the "restriction point", G_0, of higher cells.[263] Current models suggest that start is a mechanism by which the cell becomes committed to one of several alternative pathways: reinitiation of mitotic division, entry into a quiescent state, pheromonal arrest in preparation for mating (Section IV), or, in the case of diploid cells, initiation of sporulation (Section V). As a central control point, it is clear that much information must be processed at or just prior to start. A "partial" commitment to several developmental pathways would probably prove lethal.

Reciprocal shift experiments have shown that cdc28, cdc36, cdc37, and cdc39 mutants arrest at a point approximately equivalent to alpha pheromone arrest, and yet they form two distinct categories of cdc mutations.[254] In these experiments, Reed found that at the restrictive temperature all four start mutants continue to grow in size (protein and RNA synthesis continue, initiation of DNA synthesis is blocked) and are conjugation proficient, similar in both respects to pheromone-arrested cells. Two observations, however, distinguish cdc28 and cdc37 from cdc36 and cdc39. First, the conjugation frequencies were much higher in arrested cells containing cdc36 or cdc39 mutations, nearly the same as wild-type cells.[254] Second, in a/alpha diploid backgrounds, these two mutants do not continue growth at the restrictive temperature and do not arrest synchronously. Instead, they arrest at random points in the cell cycle.[254] Thus, cdc36 and cdc39 appear to be under control of the mating-type locus (MAT), and the suggestion is that they are involved in pheromone arrest. These proposals received support in experiments by Shuster,[264] who showed that cdc36 and cdc39 were able to suppress two mating-defective mutations, ste4 and ste5, while cdc28 and cdc37 could not. It may be suggested therefore that cdc28 and cdc37 act in nutritional/growth control by initiating the mitotic cycle, while cdc36 and cdc39 function in the pheromone arrest pathway in preparation for conjugation. It appears that various start mutations arresting at the same point in G_1 may, in fact, be part of distinct, but converging, pathways. Further study of these four start mutants will be facilitated by their isolation on recombinant plasmids by genetic complementation.[265,266]

The length of the G_1 phase is highly variable, depending on the growth medium of the cells.[27] Studies in other species of yeast and in mammalian tissue culture systems have indicated there is, in fact, no absolute requirement for a measurable G_1 phase. Several lines of "G_1-less" cells exist in mammalian systems, most of them having undergone tumorigenic transformation. Analysis of cdc start mutants in the fission yeast *Schizosaccharomyces pombe* suggests their actions are during G_2 or M phase.[267] Recently, mutations in the *S. pombe* cdc2 start gene were shown to be complemented by the *S. cerevisiae* CDC28 gene,[268] thus functioning across both species and cell cycle phase boundaries. These observations suggest that events necessary for the completion of start (or in the mammalian case, the G_0 to G_1 transition) may not be intrinsically bound by a time restriction in G_1, but instead simply represent a culmination of a particular series of molecular events. That start events are unlinked to G_1 per se is also supported by experiments in which the duration of S-phase was greatly extended in *S. cerevisiae* by treatment with low doses of hydroxyurea, a known inhibitor of DNA synthesis.[269] The duration of G_1 was either reduced or eliminated, demonstrating that completion of start must have taken place prior to G_1.

B. DNA and Histone Synthesis

The structure, organization, and replication of the yeast genome has been reviewed by others.[270] Here, some aspects covered in these reviews will be highlighted and the regulation of histone gene expression and its coordination with DNA synthesis during the cell cycle will be discussed. Mitochondrial genomes have also been covered in great detail in recent reviews[77,78] and will only be briefly mentioned here.

Chromosomal DNA synthesis, by definition, is limited to S phase, occupying about one fourth to one half of the yeast cell cycle time. In rapidly growing cultures (doubling time of 1.5 to 2 hr), the DNA of diploid cells can be replicated in about half the time required by haploid cells.[271,272] Under certain conditions of nitrogen starvation, S phase may be lengthened along with G_1 and G_2.[272] However, under most circumstances, the duration of S phase is relatively constant, with most variations occurring in G_1.[26] The number of chromosomal origins of replication, estimated by electron microscopic observation of DNA from S-phase cells, is approximately 400 per haploid genome.[273] The timing of activation of these replication origins appears to occur in a temporal fashion, as evidenced by differential sensitivity of specific genes to the mutagenic effects of nitrosoguanidine during S phase.[274-276] Rivin and Fangman[272] have shown that cells grown under conditions which prolong S phase exhibit a continuous activation of chromosomal origins throughout S phase. This is contrary to results obtained by Petes and Williamson,[277] which suggest (in cells grown in rich media) that all chromosomal origins are initiated simultaneously upon entry into S phase. More recent studies[278] have used recombinant plasmids containing chromosomal autonomous replication sequences (ARS), presumed to represent true replication origins. Using hybridization probes derived from these plasmids, the authors demonstrated the sequential activation of different chromosomal ARS elements (ARS1, ARS2, and 10Z), supporting the notion that there is a temporal order of replication of yeast chromosomal genes. Some evidence also exists for sequential activation in higher cells, based on the timing of replication of mouse alpha-globin genes, immunoglobulin genes before and after rearrangement, and normal and translocated copies of c-myc genes.[279] The timing of replication of genes may have implications for their transcriptional activation.

The *S. cerevisiae* mitochondrial genome is about 76 kb in length. Detailed physical and genetic maps have been compiled.[280] The mtDNA contains at least 7 protein-coding genes, about 25 tRNA genes, and single copies of both the large (21S) and small (15S) mt-rRNA gene. Replication is not limited to S phase, but occurs throughout the cell cycle.[281,282] The number of copies of mtDNA depends on growth conditions, paralleling the respiratory activity of the cells. The mtDNA synthesis is presumed to depend on a nuclear-encoded mtDNA polymerase activity which is subject to glucose repression. Although no histones have been found in mitochondria, a basic histone-like protein is found associated with mtDNA.[283]

Several cdc mutants defective in DNA synthesis have been isolated by selection of cells that arrest just prior to or during S phase after shifting to the nonpermissive temperature. The properties of these mutants have been reviewed elsewhere.[192,259] Of these, cdc4, cdc6, and cdc7 are defective in initiation of DNA synthesis; cdc2, cdc8, and cdc21 are mutants in DNA replication; and cdc9 and cdc40 are deficient in joining of replicated fragments. The biochemical identity of only a few CDC genes has been determined. Among these are: CDC8, thymidylate kinase;[284-286] CDC9, DNA ligase;[287] and cdc21, defective in thymidine monophosphate (dTMP) synthesis.[288] More recent analysis of cdc2[289] indicates that this mutant initiates replication randomly, leaving about one third of the genome unreplicated, due to failure to activate all origins. Given the number of gene products involved in bacterial DNA replication, it is likely that many more yeast genes are required for replication than have been identified by current isolation schemes. Kuo et al.[290] screened more directly for mutants in the DNA repli-

cation process by identifying nitrosoguanidine-mutagenized cells which, after detergent permeabilization, were incapable of incorporating ^{32}P-dTTP at restrictive temperatures (36°C). Out of about 400 mutants, they found 14 new complementation groups, whose analysis may shed light on new genes and their products required for DNA replication.

Although yeast chromosomes do not visibly condense during mitosis, yeast chromatin is very similar by physical and biochemical criteria to that of higher eukaryotic cells. Electrophoretic patterns of yeast chromosomal proteins show remarkable similarities[291] to those of higher cells, as do ultrastructural features.[292] On a molecular weight basis, histone to DNA ratios are 1:1, while total chromosomal protein is 1.5:1.[293,294] These amounts are comparable to those determined for a wide variety of organisms.[295] Histones H2A, H2B, H3, and H4 have been identified in yeast by Wintersberger et al.[291] and others, but H1 does not appear to be present (for review and additional references, see Fangman and Zakian[270]). The core histones are arranged in a nucleosome structure[296] with a repeat unit of about 165 bps.[292] Employing nuclease sensitivity as a probe of chromatin structure has proved useful for detection of nucleosome phasing[297] and for correlations with gene transcription.[298]

The absence of histone H1 is the most conspicuous difference between yeast chromatin and that of higher eukaryotes and may explain the lack of chromosome condensation. It is known that H1 serves as a molecular link between nucleosome subunits.[299] Nuclease digestions of bulk yeast DNA have not distinguished actively vs. nonactively transcribed genes,[300] as demonstrated in avian and other systems.[301] This may be a consequence of the unusually high percentage (>40%) of the yeast genome which is actively transcribed. Despite these differences, yeasts offer several advantages in studying both histone and nonhistone chromosomal proteins. First, of course, is the facile genetics and sophisticated molecular biological capabilities, i.e., ability to transform and selectively integrate cloned and mutagenized genes. In the case of histone genes, low gene copy number allows detailed analysis of their expression and the in vivo function of reintroduced copies.

The most detailed analysis of histone gene expression in yeast has concerned H2A and H2B; both have been made available by molecular cloning.[302] Analysis of recombinant clones showed the genes for H2A and H2B to be separated by 700 bps and divergently transcribed. Of special interest was the identification of only two copies of each H2A and H2B in the yeast genome. This compares with 300 to 600 copies in sea urchins, 100 in *Drosophila*, 40 to 100 in *Xenopus*, and 10 to 40 copies in most mammalian genomes.[303] Thus, yeasts provide a unique opportunity to explore regulation of these highly conserved genes. Also unusual is the polyadenylation of yeast histone mRNAs.

DNA sequence analysis of both copies of H2B reveals only 4 amino acid changes out of 130, most nucleotide changes (41/49) being silent.[304] It is therefore unlikely that one of the copies is a pseudogene. The time of H2B divergence in this report was estimated at 190 million years ago. Cells containing a disruption of one or the other, but not both, copies of H2B are viable, demonstrating that both genes (HTB1 and HTB2) are functional.[305] The function of H2B is therefore not impaired by the amino acid changes, despite the fact that three out of four are not conservative substitutions. These three substitutions all lie near the basic *N*-terminus at positions 2, 3, and 27. This region is not believed to be a central structural component in the nucleosome particle, as suggested by its susceptibility to trypsin digestion. Wallis et al.,[305] in very elegant experiments, created haploid strains with null mutants of both HTB1 and HTB2. These strains are viable because they contain plasmids encoding various forms of HTB2. They demonstrated that deletions of 18 or 20 amino acids near the *N*-terminus of the plasmid-encoded H2B gene are capable of functioning in vivo with no detectable changes in cell growth or morphology. Whether or not these sequences func-

tion in some subtle way in histone assembly into higher order chromatin structures is unknown. These findings are especially intriguing since there is considerable conservation of both charge and amino acid sequence of this region across a great range of species.

Histone synthesis and the presence of translationally active histone mRNA occurs only during S phase in both mammalian systems and in yeast.[306,307] The coupling of histone and DNA synthesis has been observed in numerous experimental systems. Most of the initial studies have been performed in mammalian tissue culture and sea urchins. Evidence has accumulated implicating post-transcriptional and perhaps post-translational controls in histone regulation (for recent reviews on histone genes and their expression, see References 303, 308, and 309). As a way to investigate these possibilities in yeast, Osley and Hereford[310] introduced multiple copies of the H2A/H2B transcription unit into various chromosomal loci by integrative transformation. Their results indicate that although the rate of synthesis of histone mRNA was dosage dependent the steady-state levels did not change. Pulse-chase experiments demonstrated that the degradation rate of histone H2A/H2B mRNA had increased to compensate for the excess copy number. This provides evidence for post-transcriptional regulation, but does not directly address the issue of cell cycle-dependent synthesis. One interesting model suggested by these authors involves autogenous regulatory mechanisms. In the absence of DNA synthesis, free histones could act as regulators of their own synthesis, perhaps by binding to nascent mRNAs, thereby promoting an increased rate of turnover. Similar mechanisms exist for the synthesis of *E. coli* ribosomal proteins. In the absence of available precursor rRNA, free proteins bind to their respective mRNAs, inhibiting translation.[311] Integrated multiple copies of various H2A and H2B-lacZ fusion genes are currently being used to delimit the sequences in H2A and H2B responsible for the dosage compensation phenomenon.

There exist various experimental approaches to the study of periodic expression of gene products during the cell cycle, but all in some way involve the use of cell cycle stage-specific populations. Several methods presently and formerly employed to study the cell cycle dependence of enzyme synthesis are discussed in Section III.D. One particularly good method makes use of the increase in size of budding yeast during cell cycle progression, allowing "age" fractionation by centrifugal elutriation. Ludwig and McLaughlin[312] examined cell cycle expression of histone proteins by comparing their instantaneous rates of synthesis over the course of one cycle time. Cultures of yeast received long-term ^{14}C-amino acid labeling, followed by a short pulse of ^{3}H-amino acids. Cells were then fractionated by centrifugal elutriation. Total protein extracts of three fractions representing individual cell cycle stages, were subject to two-dimensional gel analysis. The instantaneous rates of synthesis of H2A, H2B, and H3, determined by the ^{3}H/^{14}C ratios in spots corresponding to histone proteins from each "age" fraction, were determined. Results indicated a sharp peak in the rate of histone synthesis just prior to the peak of DNA synthesis. Histone H4 was not amenable to this sort of analysis due to its aberrant migration in the gel system used. These patterns were specific to histone synthesis, as ribosomal proteins showed a pattern of continuous synthesis.

A sequence of about 1.3 kb was shown to be responsible for the periodic synthesis of H2A mRNA.[313] This element is found at the 3′ end of the adjacent, divergently transcribed H2B gene and acts independent of orientation. A similar sequence element was found by other researchers near the 3′ end of a H3-H4 gene pair, perhaps constituting a histone-specific enhancer-like element. Another interesting feature of this element is its ability to function as an ARS,[313] which has lead to speculation about the role of DNA replication in cell cycle-specific activation of histone gene transcription.

C. Nutritional Shifts and G_1

We wish to highlight here some aspects of the nutritional status of cells which influence commitment to mitotic division. We will pay special attention to recent findings which suggest a role of cAMP and protein kinases in the ability to traverse start. The recent discoveries of components of the adenylate cyclase system in yeast and the possible involvement of the RAS gene products are of special interest.

In animal systems, the mitogenic activity of single cells is dictated by exocellular signals received in the form of hormones or growth factors. In yeast, the signals are the availability of essential nutrients in the medium and in the absence of mating pheromones. Higher cells and yeast starved of essential nutrients arrest in G_1 regardless of the deficiency.[314] Tumor cells, in general, have lost the normal growth mechanisms which allow uniform arrest at G_0 in the absence of serum growth factors. Yeast mutants which also have an altered ability to sense nutrient deficiency and fail to arrest at an unbudded state (i.e., at start) have been isolated. These whi mutants[315] display two characteristic phenotypes. One is most noticeable under conditions of slow growth and results in cells which are able to bud at a size much smaller than the "critical size" that must be attained in wild-type cells. Both whi1 and whi2 mutants show this phenotype, whereas the latter is also deficient in synchronous arrest by nutrient limitation. Arrested cells containing the whi2 mutation are not in the same physiological state characteristic of wild-type cells in stationary phase; the mutant cells are sensitive to heat shock and cell wall degrading enzymes, lack the appropriate levels of storage carbohydrates, and are generally reduced in viability.[27] Interestingly, whi2 is defective in monitoring only carbon source limitation, since, under conditions of nitrogen starvation, it does arrest synchronously in G_1.

The coordination of growth with cell division has been reviewed by others.[27,192] The stage-specific events of bud emergence and nuclear division are not rate limiting in successive rounds of cell division. Rather, overall growth, as characterized by accumulation of macromolecular components, seems to determine the "critical size" needed for cell division. The correlation of absolute cell mass with completion of start under various growth conditions is not strict. Thus, it is unlikely that total cell size is being measured, but rather, the cell is monitoring for the presence of some unidentified metabolic indicator. Whether the cell monitors the synthesis or degradation of specific proteins is not known. However, models implicating a "labile protein(s)" can be correlated with cell behavior in both yeast[316] and higher cells[263] exposed to protein synthesis inhibitors (cycloheximide). Nutritional assessment mechanisms may be independent for each type of nutrient limitation, but the final signals are ultimately channeled through start (see Section III.A and Figure 6). The general control mechanism for regulation of amino acid biosynthetic pathways (GCN genes) may also function in control at start.[319] Mutants in the GCD1 gene (formerly TRA3), known to be a negative regulatory element for general control structural genes,[317,318,321] are also temperature sensitive for growth. At elevated temperatures, cells with this mutation arrest synchronously at the CDC28 step. The GCD1 gene product may thus represent a link between amino acid availability and cell cycle regulation at start.

1. Adenylate Cyclase System

The role of cyclic adenosine 3′,5′-monophosphate has been well studied in bacteria[136,322] and clearly acts as an intracellular signal for carbon source starvation.[323] Intracellular levels of cAMP are depressed when *E. coli* are grown in the presence of glucose. When grown on an alternate carbon source, levels of the cyclic nucleotide are elevated and, upon binding to the CRP (CAP) protein, stimulate transcription of several operons for utilization of alternative carbon sources. Many attempts have been made to identify a similar role for cAMP in catabolite repression in yeast, but no

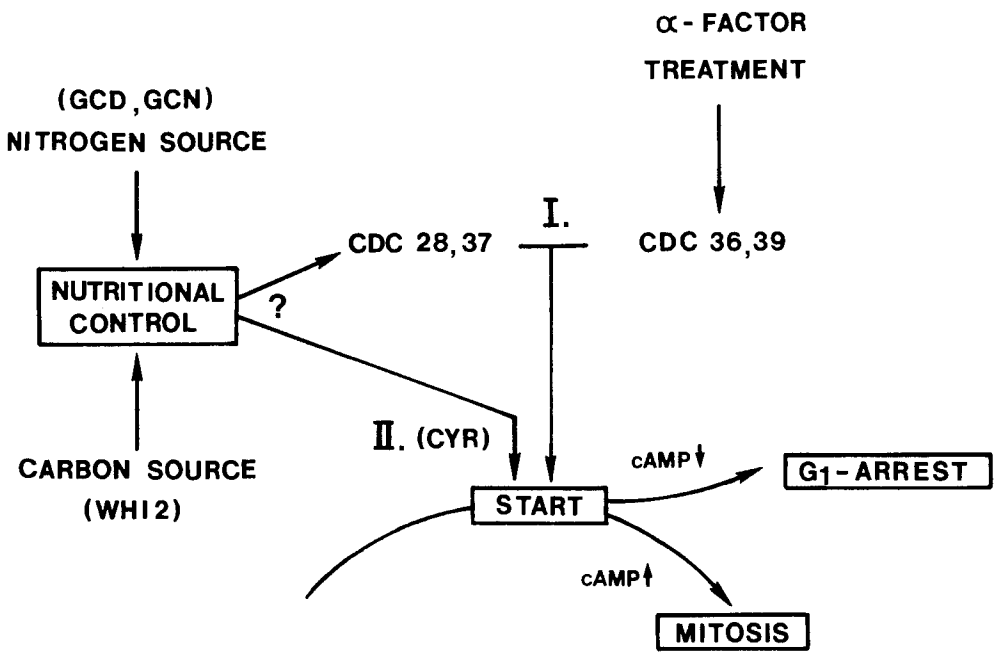

FIGURE 6. A model for possible control pathways operating at start in haploid cells. WHI2,[315] GCD,[317,318] and GCN[319] genes are involved in monitoring essential nutrients and influencing the developmental switch at start. Their precise roles in this process have not been defined (see text). Mutations in CDC28, CDC36, CDC37, and CDC39 are all class I start mutations.[254] The cyr1 is a class II start mutation.[320,15] High levels of cAMP are thought to be required for cell division.[18]

definitive evidence for such a role has emerged. Some evidence to the contrary has been presented by Matsumoto et al.[324] and will be discussed below. It appears that in yeast cAMP may influence the decision between mitotic growth and G_1 arrest or meiosis. A model presented in Figure 6 depicts the genes and metabolic signals which may influence decisions made at start.

The signal transducing system regulating adenylate cyclase activity in higher cells is a subject of great interest.[325] Binding of the appropriate hormones to beta-adrenergic receptors in specialized tissues (liver, for example) results in a stimulation of adenylate cyclase activity and a subsequent rise in intracellular cAMP. This coupling is accomplished in part by two regulatory proteins with stimulatory and inhibitory effects on the catalytic activity of adenylate cyclase. These proteins are referred to as G_s and G_i, respectively, and may also be involved in transduction of signals by mechanisms *not* involving cyclic nucleotides[326] (for reviews on G-proteins in higher cells, see Reference 327). G-protein-mediated effects are stimulated by guanine nucleotides and both G_s and G_i possess GTPase activities, hence their names. The biological effects of altered cAMP levels are propagated by cAMP-dependent protein kinases, part of a network of protein kinases whose activities are controlled by their phosphorylation state.[328]

These types of regulatory circuits are becoming increasingly popular subjects of inquiry for yeast biologists. Studies by Liao and Thorner[329] have demonstrated that the alpha factor mating pheromone inhibits adenylate cyclase activity in yeast membrane fractions. Membranes were also prepared from a temperature-sensitive sterile mutant (ste5) which is unable to mate at 34°C. No inhibition of adenylate cyclase activity was observed in membranes from ste5 cells assayed at the restrictive temperature, whereas at 23°C alpha factor caused a decrease in adenylate cyclase activity similar to that seen in wild-type cells. These observations led to the model that alpha pheromone inhibits

Table 3
GENES INVOLVED IN THE ADENYLATE CYCLASE SYSTEM

Mutation	Enzyme	Comments	Ref.
cyr1	Adenylate cyclase	ts Mutant; arrests in G_1 if not supplemented with exogenous cAMP	320
cyr2	Catalytic subunit cAMP-dependent protein kinase	ts Mutant; arrests in G_1 if not supplemented with exogenous cAMP; lowered affinity for ATP; cAMP-induced kinase activity reduced 100-fold	332
CYR3	Structural gene for regulatory subunit of cAMP-dependent protein kinase	ts-Dominant mutant; partially blocked at G_1 if not supplemented with exogenous cAMP; affinity of CYR3 for cAMP reduced tenfold at restrictive temperature	333
bcy1*	Regulatory protein necessary for production of regulatory subunit of cAMP-dependent protein kinase (CYR3)	Suppresses cyr1, CYR3, as expected; also suppresses cyr2, suggesting that critical lesion in cyr2 is defective (enhanced) in binding to regulatory subunit; suppresses ras1/ras2 double mutant	331, 333
glc1	cAMP-dependent protein kinase	High level cAMP-dependent protein kinase activity; fails to accumulate storage carbohydrate glycogen and trehalose; elevated trehalose activity, thought to result from increased kinase activity	239
$RAS2^{val19}$	Membrane-bound GTPase	Dominant point mutation (created in vitro) analogous to activated mammalian ras oncogene; fails to accumulate glycogen and trehalose; elevated trehalase activity; high levels of intracellular cAMP; elevated levels of GTPase activity uninducible by GTP; diploid RAS2/$RAS2^{val19}$ unable to sporulate	241
pde1	Phosphodiesterase I	Suppressor of CYR3; accumulates intracellular cAMP; not essential for growth	334
ppd1	Phosphoprotein phosphatase	Suppressor of cyr2; dispensible for growth	335

* In contrast to the results of others,[331,333] Toda and Wigler[236] suggest that bcy1 encodes the structural gene for the regulatory subunit of cAMP-dependent protein kinase. This result follows molecular cloning and sequence analysis of BCY1 and comparison to the bovine regulatory subunit and the bacterial CRP (CAP) protein.

cAMP production which, in turn, potentiates responses in the mating pathway for cell cycle arrest at start. Results of others,[15,330] however, are not entirely consistent with these findings; it seems likely that as in higher organisms the exact role of cAMP as an intracellular signal will be difficult to establish. Fortunately, several important mutants have been identified and characterized genetically and biochemically.

Several cAMP-requiring temperature-sensitive mutants (cyr) have been isolated,[331] and their characterization has yielded a wealth of information, which is summarized in Table 3 and Figure 7. The cyr1 defect was shown to be a mutation in the adenylate cyclase structural gene itself,[320] while cyr2 is defective in the catalytic subunit of cAMP-dependent protein kinase.[332] CYR3 is defective in the structural gene for the regulatory subunit of cAMP-dependent protein kinase.[333] These three genes do not complement each other genetically, and the first two are recessive while the latter has a dominant phenotype. All three require cAMP-supplemented medium for growth at restrictive temperatures and all can be suppressed by the mutation bcy. This mutation is likely to be in a gene which encodes a positive regulatory protein controlling expression of the regulatory subunit of cAMP-dependent protein kinase (cyr3). Cells harboring the bcy mutation have constitutively high levels of cAMP kinase activity.[331,333] Study of these

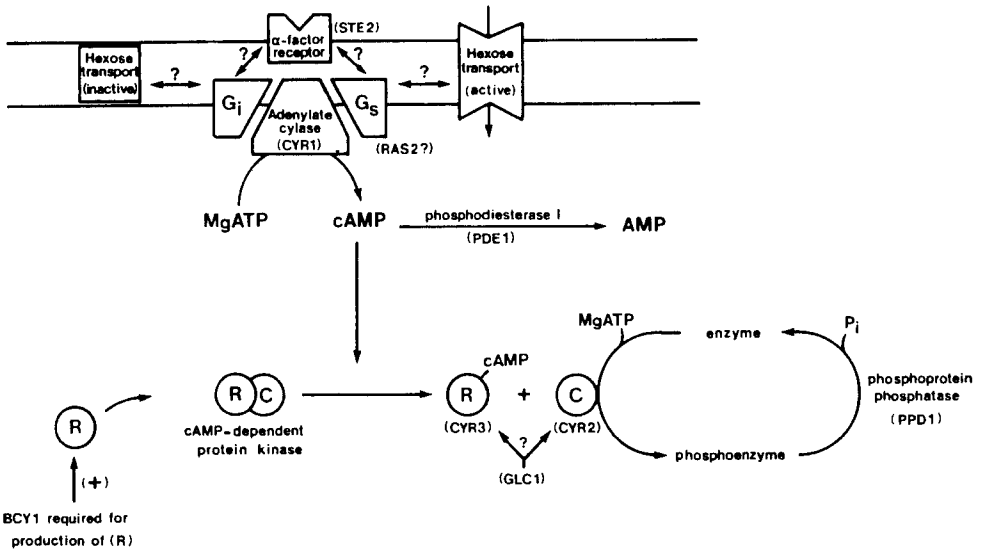

FIGURE 7. An heuristic model for the relationship between components of the adenyl cyclase signal transducing system in yeast. Assignments for CYR1,[320] CYR2,[332] CYR3,[333] BCY1,[331,333] PDE1,[334] and STE2[13] have been documented. RAS2 has been shown to have GTPase activty and to affect cAMP levels,[241] but its role as a G-protein is not established. Studies with glc1 mutations[239] have not distinguished if the lesion is in the regulatory or catalytic subunit of cAMP-dependent protein kinase. The cAMP-dependent protein kinase holoenzyme (in higher systems) exists as a tetramer, composed of regulatory and catalytic subunits, R_2C_2.[336] Tandem cAMP-binding domains are found in each regulatory subunit. These domains share primary sequence homology with the bacterial CAP protein cAMP-binding region.[337]

mutants in conjunction with alpha pheromone arrest and nutrient limitation have suggested a role of cAMP-dependent protein kinase in control of the yeast cell cycle.

Using a strain carrying a temperature-sensitive allele of the adenylate cyclase gene (cyr1-2) and standard reciprocal shift experiments,[16] Matsumoto et al.[15] determined the point within start that CYR functions. The timing of action occurs just prior to the point of alpha pheromone arrest and prior to the arrest point arrived at by nitrogen starvation. Their data also demonstrated that cyr behaves like a class II start mutation;[254] at the restrictive temperature, it no longer continues growth or retains the ability to conjugate. Thus, the mutant acts in a manner reminiscent of cells which are undergoing nutrient limitation. Class I start mutations (e.g., cdc28) are unable to reinitiate bud emergence or DNA synthesis, but do continue to grow in size and obtain an oblong "shmoo" morphology, which is characteristic of pheromone-treated cells preparing for conjugation. These results suggest that contrary to the model proposed by Liao and Thorner,[329] adenylate cyclase appears not to be involved in the pheromone arrest pathway directly, but instead acts at a distinct (earlier) step. The involvement of this step in nutritional control of cell division is suggested by studies using a mutation which suppresses cyr. This mutant, bcy1,[333] is able to suppress cyr1 by allowing cells to grow in the absence of exogenous cAMP. More interestingly, it also allows nutrient-deprived cells to reinitiate the bud emergence/nuclear migration pathway, thus partially suppressing nutrient-deficient arrest pathways. However, cells with the bcy1 mutation retain their sensitivity to mating pheromone, further supporting the notion that cAMP levels are not affected by alpha factor arrest, or at least not through mechanisms involving cAMP-dependent protein kinase.

A clear picture emerging from these studies is the importance of the membrane-associated cAMP system to regulation of yeast cell physiology and the need for more

research in this area. Recent studies show remarkable similarities between the yeast system and those well characterized in higher eukaryotes. In addition to identifying genes for adenylate cyclase, the cAMP-dependent protein kinase regulatory and catalytic domains, and a gene which regulates cAMP-dependent protein kinase, biochemical evidence has been obtained for the existence of G-proteins in yeast.[338] In addition, mutants defective in cAMP phosphodiesterase (pde1) have been isolated by their ability to suppress CYR3 mutants.[334] The involvement of phosphoprotein phosphatases in the cAMP regulatory network is supported by recent studies with the mutant (ppd1).[335] This mutation is deficient in phosphoprotein phosphatase activity and suppresses the cyr2 defect, consistent with the model presented in Figure 7.

The RAS1 and RAS2 genes, recently cloned and characterized,[339-341] have also been implicated in the adenylate cyclase system.[241] These genes were identified by homology in the viral Harvey murine sarcoma ras oncogene, known in higher cells to encode proteins with membrane-bound GTPase activities,[342] stimulated by epidermal growth factor.[343] A third yeast gene more distantly related to ras, YP2, is essential for cell growth, is unable to complement ras1 and ras2, and probably has a separate cellular function.[344,345] Ras oncogene proteins share significant homology with bovine-derived G-protein sequences[346] and with the retinal signal protein transducin.[347] A lethal phenotype is observed only when both RAS1 and RAS2 are disrupted.[348,349] However, this can be overcome by the bcy mutation.[241] That is, ras1/ras2/bcy1 cells are viable (Table 3). Therefore it appears that constitutively high levels of cAMP-dependent protein kinase activity can overcome ras1 and ras2 defects. Intracellular levels of cAMP were also correlated with the presence of functional RAS genes, RAS2 being the major determinant. Finally,[241] it was demonstrated that membrane preparations from ras1/ras2/bcy1 cells do not show GTP stimulation of adenylate cyclase activity as do membranes from RAS1/RAS2 cells. Hence, it is likely that RAS function in yeast involves control of cAMP levels and perhaps that RAS proteins are in fact G-proteins. Another interesting observation is a single amino acid substitution in RAS2 resulting in cells unable to undergo synchronous G_1 arrest during nutrient deprivation.[241] This amino acid substitution is analogous to one (glycine to valine) which in mammalian systems transforms the normal ras protein into a tumorigenic variant. In addition, membrane preparations from these $RAS2^{val19}$ cells show constitutively high levels of adenylate cyclase activity, uninducible by GTP. These results together suggest that yeast RAS genes may function in nutritional control of cell growth through mechanisms involving cAMP and protein kinases.

The role of protein kinases in control of cell division at start is further substantiated by recent analysis of the CDC28 gene. Significant homology has been found between the amino acid sequence of CDC28 and the protein kinases encoded by several vertebrate oncogenes.[350] Biochemical evidence has been obtained showing that the product of CDC28 is, in fact, a protein kinase.[351] This identification should provide impetus for further studies aimed at elucidating the molecular events occurring at start.

A limited homology has also been reported among the predicted amino acid sequences of CDC4, CDC36, and the ets oncogene of avian erythroblastosis virus.[352] The function of the ets protein is unknown. In our own laboratory, we have recently identified and isolated a gene, ESS1, from *S. cerevisiae*[353] by virtue of cross-hybridization with the simian sarcoma virus oncogene, v-sis.[354] The cellular (c-sis) gene encodes platelet-derived growth factor, a potent mitogen in higher systems.[355,356] We have shown ESS1 to be essential for growth.[353] Although there is very little amino acid sequence homology between ESS1 and v-sis, limited homology at the level of secondary structure was observed.[353] It will be interesting to determine whether the product encoded by ESS1 is also involved in control of cell proliferation.

It should be mentioned that other intracellular second messengers may also have

important functions in yeast. Hubbard et al.[70] have presented evidence for the existence of the Ca^{2+}-binding protein calmodulin in yeast, and Ohya et al.[71] (Section II.C.3) have suggested, based on genetic studies, that Ca^{2+} and calmodulin may influence cell division at bud emergence and nuclear division.

2. Stationary Phase Transition

Cells committed to exiting exponential growth and entering a quiescent state undergo a distinct set of metabolic changes. In cells deprived of essential amino acids, these changes include inhibition of DNA, RNA, and protein synthesis, decreased glucose uptake, and increased protein degradation. In mammalian cells, these phenomena have been well studied and are termed the "negative pleiotypic response".[357] In yeast, as in bacteria, these responses are known as the "stringent response".[358] During the transition from rapid growth to stationary phase, the total cellular ribosome content falls as a result of a rapid, coordinate depression of ribosomal RNA and ribosomal protein synthesis.[358,359] The initial drop in rRNA and mRNA specific for ribosomal proteins precedes the decrease in tRNA and bulk mRNA synthesis.[360] Decreases are then observed in total RNA synthesis, followed by G_1 arrest and cessation of DNA synthesis.

In a recent study, the synthesis of 400 major *S. cerevisiae* proteins was monitored by two-dimensional gel analysis after pulse-labeling of cells undergoing glucose exhaustion.[361] As cells entered the stationary phase, protein synthesis rates dropped to 10% of previous levels and RNA synthesis became undetectable. Of the 400 proteins detectable in their gel system (out of an estimated total of 4000 in yeast[260]), the synthesis of all but 20 ceased upon entry into the stationary phase. Several of these proteins were identified: glyceraldehyde-3-phosphate dehydrogenase, enolase, HXK, aldehyde dehydrogenase, actin, and five heat shock proteins. The first two of these may become active in gluconeogenesis, but the reason for continued synthesis of HXK, aldehyde dehydrogenase, and actin is not readily apparent. Heat shock proteins are known to act in situations of environmental stress, and their continued synthesis is not unexpected, especially since stationary cells have been found to be thermo-tolerant.[363] These studies, however, do not reveal information regarding the nature of the RNA populations within stationary phase cells. Although the proportion of mRNA with discrete size classes of poly(A) tracts was shown to change in cells entering the stationary phase,[362] not much is known about the presence or stabilities of specific mRNAs in nongrowing cells. Existing evidence suggests that during the transition from rapid growth to stationary phase there is an accumulation of the storage carbohydrates glycogen and trehalose.[237] The cellular content of both is gradually diminished in stationary cells, indicating their utilization as sources of carbon.

D. Enzymes Whose Expression is Cell Cycle Regulated

The cell cycle-dependent synthesis of histone proteins is well established in both higher systems and in yeast. However, the picture is not so clear for most enzymes examined, despite nearly 20 years of study. Oddly enough, the models favored by cell cycle investigators seem to follow a cyclical pattern. In 1964, Gorman et al.[364] published a report demonstrating the cell cycle periodicity of several different enzymes in *S. cerevisiae* or, as commonly referred to, "step" enzymes. Others reported similar findings in *S. pombe*.[365] In all, about 30 enzymes were shown to display a step pattern of synthesis in *S. cerevisiae*,[364,366] the assumption being that enzyme activity reflected the rate of enzyme synthesis. Since total RNA and protein synthesis continued in a linear fashion, it was suggested[364] that transcription of individual genes was ordered according to timing of replication. However, these studies relied on cells synchronized by induction techniques which have since come into question. Induction synchrony can be accomplished by reversal of stage-specific arrest brought about by a variety of met-

abolic inhibitors, including DNA synthesis inhibitors, mating pheromones, nutrient limitation, heat shock, or by using temperature-sensitive cdc mutants. Many of the studies mentioned above achieved synchrony by the releasing of cells from starvation arrest by replacement in rich medium. The metabolic perturbations caused were very difficult to assess since cells were in transition from an inactive state to one of rapid growth.

Mitchison[368] and Mitchison and Carter,[367] upon reexamining step enzymes in *S. pombe*, found that pregrowth conditions used in earlier studies could result in step-like patterns in both synchronous and asynchronous cells. In fact, 18 out of 19 enzymes reexamined failed to show step synthesis when synchronous cultures were obtained by size fractionation using centrifugal elutriation (see below), the only exception being TMP kinase.[368] Studies using both induction synchrony and direct analysis of cells age fractionated by zonal rotor centrifugation indicated step synthesis for alpha-glucosidase in *S. cerevisiae*[364,369,370] and beta-galactosidase in *Kluyveromyces lactis*.[369,370] Experiments on single cells of *K. lactis* also indicated a sharp increase in beta-galactosidase activity during a restricted portion of the cell cycle.[371] However, others failed to detect evidence for step synthesis of these same enzymes in the same organisms using centrifugal elutriation to obtain small unbudded cells for outgrowth of synchronized cultures.[372] Elliott and McLaughlin[373] combined centrifugal elutriation of *S. cerevisiae* cultures and the technique of O'Farrell[374] to monitor potential cell cycle changes in 550 major polypeptide "spots" on two-dimensional gels. Their results indicated no step changes in the patterns of synthesis of 550 major polypeptides. For 111 of these further examined by double-labeling techniques, they demonstrated an exponential increase in their instantaneous rates of synthesis. In agreement with earlier studies,[369,375] they also showed periodic increases in DNA synthesis, while total RNA and protein synthesis increased exponentially. A follow-up study using similar methodologies with the addition of pulse-chase labeling of cell proteins ruled out the possibility of altered degradation patterns for 108 out of 110 of the proteins examined.[376]

In contrast, a more recent study by Geiduschek and colleagues[377] revealed the existence of at least 17 cell cycle regulated proteins. Technical improvements such as computer quantitation of two-dimensional gel patterns allowed them to monitor over 900 *S. cerevisiae* proteins. Extraordinary measures were taken to eliminate artifacts which had plagued earlier studies. Three independent methods were used to obtain cells: (1) zonal rotor centrifugation to isolate synchronous subpopulations for subsequent outgrowth and pulse-labeling, (2) age fractionation by zonal rotor centrifugation of pulse-labeled asynchronously grown cultures, and (3) induction synchrony following alpha factor arrest. In addition, stress-related induction of proteins (heat shock proteins, etc.) due to cell culture manipulations was taken into account. The sharp rise in the labeling patterns of most cell cycle modulated proteins began in late G_1, as was observed for histones H4, H2A, and H2B.

It became clear that methodologies were of particular concern in this area of research. There is no thoroughly acceptable method of examining synchronized populations of cells, as each method is subject to limitations. Lloyd et al.[261] have reviewed methodologies used in cell cycle experiments in yeast and other organisms, detailing the principles and drawbacks of each. In our own laboratory, we have employed zonal rotor centrifugation of asynchronously growing cultures to fractionate cells by age for direct analysis of both enzyme activities and functional specific mRNAs measured by in vitro translation.[378,379] This method obviates the need for cells to reinitiate exponential growth after the disruptive manipulation of centrifugation. Fractions off the gradients are then subject to a variety of control measurements, including remixing of fractions, as suggested by Mitchison,[449] to determine the effect of the centrifugation process itself. In these experiments, we sought to reexamine in detail the patterns of

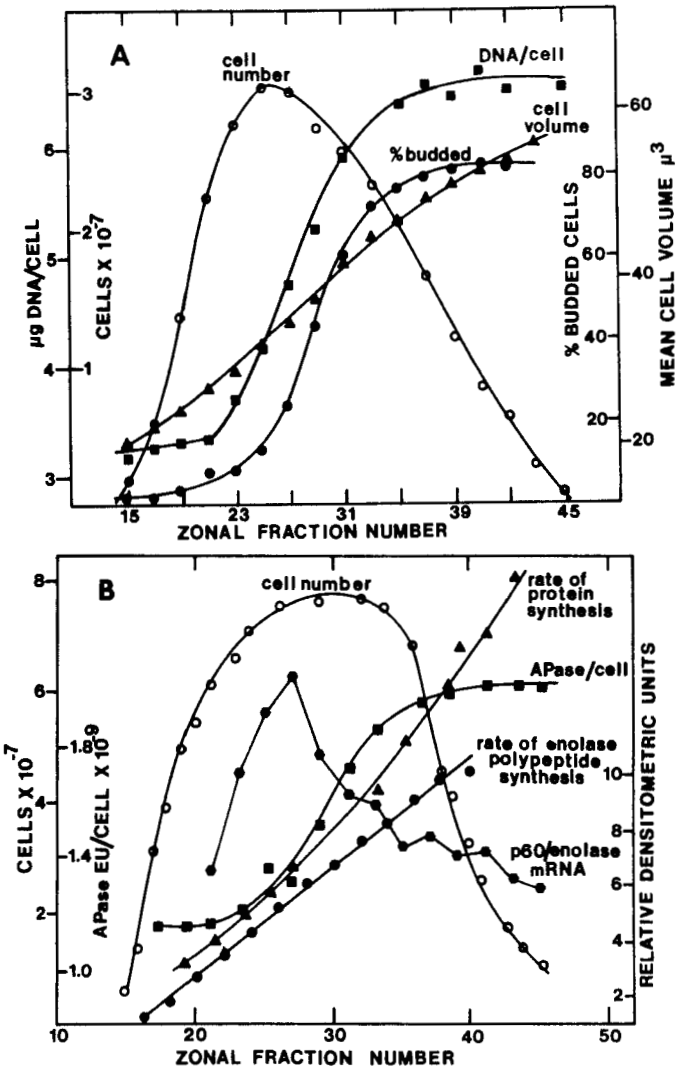

FIGURE 8. Variations in the cell cycle. Strain Y185 was grown asynchronously to mid-log phase in low-Pi medium and the harvested cells fractionated on a zonal rotor. Zonal rotor fractions, representing different stages of the cell cycle were analyzed for: (A) ○, cell number; ▲, cell volume; ●, % budded cells; and ■, DNA/cell. (B) ○, cell number; ▲, rate of protein synthesis; ●, rate of enolase polypeptide synthesis; ■, APase activity per cell; and ●, the ratio of translatable APase (p60)/enolase mRNA.

synthesis of acid phosphatase (PHO5) and GAL1.[378,379] Previous results of others implicated GAL1 as a step enzyme in *S. cerevisiae*[380] and acid phosphatase (APase) as a step enzyme in both *S. cerevisiae*[380] and *S. pombe*.[381] Functional assays for APase and GAL1 mRNAs were developed and molecular clones were isolated for hybridization analysis. As shown in Figure 8A, zonal fractions can be analyzed by a number of biological parameters: percentage of unbudded cells, DNA content, mean cell volume, and overall rates of protein synthesis. The constitutively synthesized enzyme enolase was used as an internal control in these experiments. As can be seen in Figure 8B, the ratio of functional APase mRNA to enolase mRNA reveals a sharp peak characteristic

of step synthesis, which is followed shortly by a rapid increase in APase enzyme activity. These results indicate that the step increase in APase activity during the cell cycle occurs in part by periodic transcription of APase mRNA. These results have been corroborated by two-dimensional gel analysis as described previously.[450] The reason for temporal regulation of APase is unclear, but possibly relates to its participation in a transcriptional regulatory network which may also regulate polyP cycling preceding S phase.

GAL1 enzyme activity in these same reports[379] was again seen to occur in a stepwise pattern, however, this did not result from a simple step increase in GAL1 mRNA levels. Instead, a slow twofold oscillation of hybridizable mRNA levels occurred, which was closely paralleled by a similar oscillation in the rate of GAL1 polypeptide synthesis. The timing of these oscillations was inconsistent with the sharp and dramatic increase in enzyme activity. The possibility of changes in GAL1 mRNA functional capacity was ruled out. These data and those of another study[382] indicate that GAL1 activity is regulated by enzyme turnover or stabilization.

An important point, which has not been mentioned thus far, concerns the specific growth conditions under which these and previous studies have been performed. For example, nutrient-poor medium is often used for metabolic-labeling studies. This results in an artificial elongation of G_1, which may have profound effects on observed patterns of enzyme synthesis or accumulation.[379] In the studies mentioned above, GAL1 enzyme levels were measured under conditions of steady-state growth, while APase accumulation was analyzed in a transition phase (high P_i to low P_i) during a single round of cell division. Thus, growth conditions were tailored to reflect the optimal conditions for induction of each enzyme and may account for the ability to detect step changes in their expression. Therefore, these studies on GAL1 and APase should convey the message that mechanisms of regulation of any enzyme in the context of the cell cycle must be analyzed separately, and such data should be interpreted only after the physiological mechanisms for regulating individual enzymes are well documented.

Experiments with zonal rotors have not escaped criticism. The overriding concern is whether size selection of cells truly represents age fractionation. The concern stems from the asymmetric nature of the budding process as discussed previously (Figure 2) in which daughter cells are smaller in size and thus have slower sedimentation rates than the same "aged" mother cell. This problem is largely avoided by growing cells in sufficiently rich medium, since at higher growth rates the mother/daughter size differential is greatly diminished.[26] Measurement of the total mass increase per cell doubling allows estimates of differences in size between mothers and daughters at cytokinesis.[26,379] In addition, by measuring the DNA content per cell and, more importantly, by determining the percent of unbudded cells across the gradient fractions, one can assess the nature of the separation. Centrifugal elutriator rotors are, by contrast, used to isolate subpopulations of cells for establishing synchronous cultures. One shortcoming is that only a fraction of the original culture is used, usually consisting of the smallest unbudded cells whose physiological state is assumed to be representative of the original culture. The demand is then made to resume exponential growth after the trauma of centrifugation, which may cause exposure to brief periods of starvation or anaerobisis as a result of high cell densities in the rotor assembly.[261]

The cell cycle expression of only a few additional genes has been investigated in detail. These studies depended very much on the availability of cloned genes. While periodic synthesis of many step enzymes is still disputed in the literature, it is generally assumed that enzymes involved in DNA metabolism, particularly in the replication process, will show periodic synthesis or activation. The identification of the CDC9 gene product as DNA ligase has led investigators to determine its pattern of expression during cell division. Peterson et al.,[383] using cloned CDC9 as a hybridization probe and

synchronous cells obtained by both induction and selection techniques, demonstrated a four- to tenfold increase in the relative amount of CDC9 mRNA at the G_1/S boundary, a result that was not unexpected. Another observation in these studies was the rapid induction of CDC9 mRNA following DNA-damaging UV-irradiation and an apparent modification of the ligase protein itself by poly ADP-ribosylation. It will be of interest to determine if UV induction and periodic activation of CDC9 gene transcription are mediated through common mechanisms, or if unique modes of regulation have evolved making use of different effector molecules. Cell cycle regulation has also been shown for CDC21, the gene for thymidylate synthase.[384]

Another gene recently shown to have periodic transcription patterns is SUC2, which codes for both secreted and cytoplasmic invertase. Total invertase activity peaks near the time of bud emergence in cells released from alpha-factor arrest.[385] Levels of functional SUC2 mRNA for both forms of the enzyme reach a maximum midway between peaks in histone mRNA synthesis in two successive rounds of cell division examined. In the three studies described above, the question of transcriptional activation vs. an alteration in processing or stability of the mRNAs was not directly addressed. However, no evidence for alterations in these properties has been found.

The HO endonuclease-mediating mating-type recombinational events will be discussed in some detail in Section IV.B. For now, however, it should be mentioned that recent studies by Nasmyth[386] have shown HO enzymatic activity to peak early in G_1, between the execution points of CDC28 and CDC4. S_1 analysis also reveals that HO mRNA is synthesized within this window of events. Expression of both the HO mRNA and HO endonuclease activity are dependent on bypass of the alpha factor arrest point and completion of the start events of CDC28. Since double-stranded chromosome breaks can be lethal in yeast,[387] appropriate temporal expression of this mating-type switching gene is probably tightly regulated. Another interesting finding is that the HO gene is closely linked to an ARS element,[386,388] as are histone genes. Whether DNA replication promotes transcription of specific genes, perhaps by a restructuring of local chromosomal proteins, is an area of great interest despite some indirect evidence to the contrary.[389] The latter studies used metabolic inhibitors of DNA synthesis, cdc mutations in DNA synthesis, or conditions of restricted cell growth which allow cell division. These showed a dependence of step increases for APase,[381] arginase, and invertase[389] activities on growth parameters, rather than DNA synthesis.

It appears that as techniques used to study cell cycle-specific expression of genes become more sensitive, more examples of periodic regulation will be found. The mechanisms controlling periodic expression remain almost a complete mystery. Studies of the sort carried out by Lorincz et al.[377] should help in the identification of new cell cycle-regulated proteins, and molecular cloning of their respective genomic sequences should facilitate their detailed analysis. The use of gene fusions as well as more traditional deletion and mutation analysis may identify elements in DNA which confer cell cycle regulation of individual genes, gene families, or functionally related sets of genes. Post-transcriptional and post-translational regulation, of which there already are examples, should not be overlooked.

IV. CONTROLS OVER CELL TYPE

The mating system in yeast serves as a premier model for how regulatory genes control the expression of a variety of unlinked genetic loci and how this in turn specifies a developmental pathway. The importance and interest in this system is evidenced by the number of times the subject has been reviewed in recent years.[2,5-7,390] A wide variety of gene control mechanisms have been found, and the interaction between cell cycle and mating pathways (Section V) is becoming increasingly better understood.

A. a/alpha Hypothesis

Alleles at the MAT locus on chromosome III, MATa, or MATalpha determine the mating type of haploid cells. Haploid cells containing MATa information mate as a cells, while those with the MATalpha allele mate as alpha cells. Diploid cells heterozygous at the mating-type loci, MATa/MATalpha, exhibit a third set of properties. The phenotypes associated with each of the three cell types are thought to result from expression of distinct sets of unlinked genes controlled by regulatory activities encoded by the MAT locus.[391,392] These unlinked genes were first identified as mating deficient ste (sterile) mutations.[393] Ste mutations can specifically affect mating competence in either alpha or a cells or nonspecifically by causing sterile phenotypes when present in both cell types. That is, ste3, ste13, tup1,[394] and kex2[395] mutations only result in sterile phenotypes in alpha cells ("alpha-specific") and ste2, ste6, and ste14 only affect mating properties of a cells ("a-specific"). Lesions in nonspecific genes STE4, STE5, STE7, STE11, STE12, and STE15 result in sterility of both cell types. As will be described, several of these nonspecific STE genes are required for the expression of both alpha- and a-specific functions. The properties of ste mutants are summarized in References 2, 5, and 6, and most recently in Reference 7. One important note is that STE genes, whose mating phenotypes are specific to one cell type, may in fact be expressed in cells of both mating types. STE13, for example, is alpha-specific (required for maturation of alpha factor, but not a factor), but its mRNA is expressed in diploids and in both haploid cell types.[6]

The "alpha1/alpha2 hypothesis", first proposed by Strathern et al.,[392] suggests a way in which regulatory proteins encoded by the two transcription units found at MATalpha and one at MATa control mating phenotypes in the three cell types, a, alpha, and a/alpha. The major features of the proposal, reviewed in References 2 and 7, are summarized in Figure 9, including a recent modification incorporating STE12.[402] According to the original proposal, in MATalpha cells, the alpha1 transcript encodes a positive regulator of alpha-specific genes and alpha2 encodes a negative regulator of a-specific genes. In MATa cells, a-specific genes are constitutively expressed. In MATa/MATalpha diploids, the products of a1 and alpha2 form a complex which negatively regulates alpha1 and several unlinked nonspecific genes. Although genetic evidence has so far confirmed nearly all the predictions made by the model, it is only recently that direct effects of MAT alleles on the expression of unlinked genes has been demonstrated. These studies are made possible by molecular cloning of MAT regulated genes, and a few examples are described below.

Sprague et al.[396] have isolated a plasmid containing the STE3 (alpha-specific) gene by complementation of ste3 mutants with a yeast genomic library. This clone was used as a molecular probe for STE3 transcripts in alpha, a, and alpha/a cells, the results indicating their presence only in alpha cells. This is consistent with the proposal that STE3 is positively regulated by the product of the MATalpha1 transcript. Expression of STE3 was also possible in MATa cells transformed with a plasmid which produced alpha1 transcripts from a heterologous promoter.[396] This observation further supports the notion that alpha1 encodes a positive regulator of STE3 rather than the alternative hypothesis that a1 codes for a negative regulator of STE3. It is assumed that STE3 mRNA production is controlled through alteration of the rates of transcription, although this has not been rigorously tested. In addition, a physical interaction of the alpha1 gene product with the STE3 structural gene remains to be demonstrated.

In a similar study, the effects of alpha2 on the repression of the a-specific STE6 gene was examined using cloned STE6 sequence probes.[397] Results demonstrated that STE6 mRNA was expressed only in a cells or cells containing mutations in MATalpha2. They also tested the repression by alpha2 of STE6: lacZ hybrid genes integrated at the STE6 locus in MATalpha, MATalpha/MATa, and MATa cells. Only MATa cells or strains

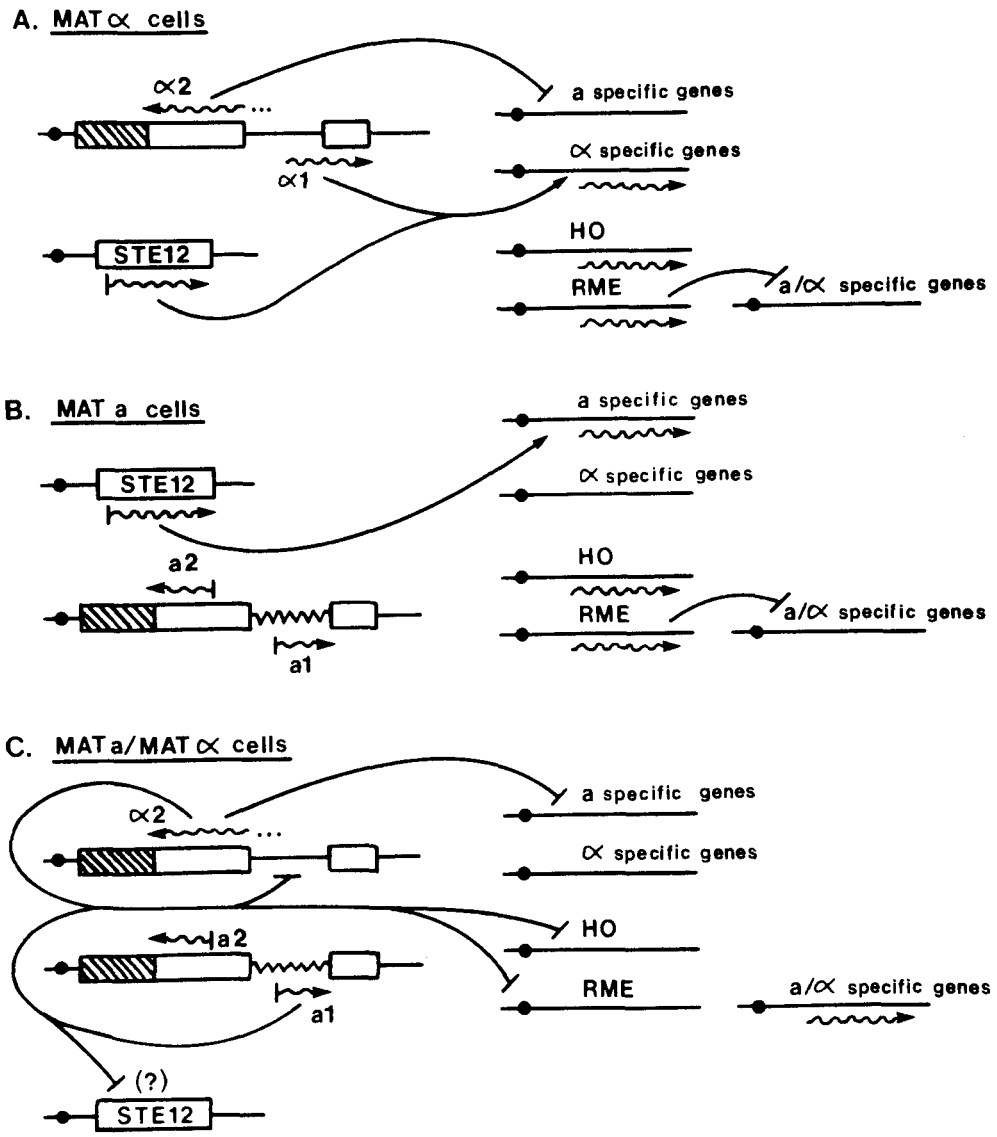

FIGURE 9. The *alpha1/alpha2* hypothesis. Transcription patterns (wavy arrows) of the MAT locus in each of the three cell types: (A) The alpha haploid; (B), a haploid; and (C), a/alpha diploid. The roles assigned to the MAT-gene products and their effects on unlinked genes are denoted by arrows (positive control) and flat lines (negative control). (Adapted from Klar, A. J. S., Strathern, J. N., and Hicks, J. B., *Microbial Development*, Losick, R. and Shapiro, L., Eds., Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1984, 151.)

carrying a matalpha2 mutation expressed beta-galactosidase activity. These results suggest that in alpha cells negative regulation of genes required for the a cell mating type, by MATalpha2, occurs at the level of mRNA production.

Expression of other mating type-specific genes has also been shown to occur by controlling gene activity at the level of RNA production: MFalpha1, the alpha factor structural gene, requires alpha1; the HO endonuclease and STE5 are repressed by a1-alpha2;[398-400] and BAR1 is repressed by the alpha2.[401] These observations are consistent with the original alpha1/alpha2 hypothesis.[392] An additional level of regulation has been introduced into the model based on recent results of Fields and Herskowitz.[402] It

now appears that the product of STE12 is required for expression of both alpha- and a-specific genes. Haploid strains containing mutations in STE12 have greatly reduced or undetectable levels of mRNA from three alpha-specific genes, MFalpha1, MFalpha2 (a second structural gene for alpha-factor), and STE3. Since MATalpha1 levels were unchanged in ste12 mutants, STE12 control of alpha-specific genes is more likely a direct effect, i.e., not mediated via control of the MAT locus.

Surprisingly, several a-specific gene transcripts were also reduced or absent in ste12 mutants. These include MFa1 and MFa2, which encode the structural genes for a-factor, and STE2,[13] which encodes a structural element of the a cell surface receptor for alpha-factor. The transcription of two haploid-specific genes whose expression is not dependent on the MAT locus, HO and STE5, are not affected by STE12. Thus, STE12 appears to be required for expression of genes involved in cell type determination (alpha vs. a) which are also under control of the MAT locus in haploid cells.

To summarize, these new results do not contradict established models of the alpha1/alpha2 hypothesis, but rather, extend its complexity. Simply stated, alpha-specific gene expression requires positive activation by both MATalpha1 and STE12. The a-specific gene expression, previously thought of as constitutive, also requires STE12 function. This will soon be further complicated, however, by the observation that STE7 and STE11 are also required for expression of all alpha- and a-specific genes, while STE4 and STE5 are required for some alpha- and a-specific genes.[402] Thus, although the information contained at the MAT locus (MATalpha vs. MATa) ultimately determines the haploid cell mating type, its control over unlinked loci is exerted through concerted mechanisms involving STE12 and perhaps other regulatory genes. Regulation of STE12 itself, following its isolation on recombinant plasmids, will be of interest. Is STE12 a haploid-specific gene and, if so, how is it turned off in a/alpha diploids? Repression of STE12 by the a1-alpha2 complex would be one obvious possi-Repression of STE12 by the a1-alpha2 complex would be one obvious possibility.

It seems likely that transcriptional activation or repression is involved in the majority of mating-type gene regulatory circuits. However, until the protein products are identified and characterized, other forms of regulation should not be dismissed.

To uncover the molecular interactions between the MAT regulatory proteins and the structural genes they control, investigators have taken several approaches. Herskowitz and colleagues[403] have created alpha2-lacZ hybrid genes. Studies of the hybrid proteins have demonstrated that the alpha2 gene product is localized in the nucleus, consistent with its action as an effector of gene activity.[403] Interestingly, only a short stretch of 13 amino acids near the *N*-terminus of alpha2 was sufficient for nuclear targeting. Other hybrid constructions which contain nearly all of the alpha2 coding region have been used to purify the alpha2 protein.[404] Isolation of the fusion gene product is greatly facilitated by immunoselection with anti-beta-galactosidase antibodies (e.g., by affinity chromatography) from yeast cell extracts. The protein purified by this strategy shows sequence-specific DNA binding properties, having high affinity for sites in cloned STE6 gene fragments in vitro.

Others have used more traditional approaches by creating deletion mutations and identifying DNA sequence elements which confer mating-type regulation. Siliciano and Tatchell[405] deleted sequences in and around the region between the transcriptional start sites of MATalpha1 and MATalpha2. In addition to finding a functional promoter element common to both divergent transcription units, including TATA box regions for alpha1 and alpha2, they identified a region which is necessary for diploid repression of alpha1 RNA production. This site may therefore represent a target site for interaction with the a1-alpha2 complex which shuts off alpha1 in diploid MATa/MATalpha cells. Deletion of this region of 14 bp results in constitutive expression of alpha1 and alpha2 transcripts in diploid cells. A surprising finding in this study was that no alpha-

specific MFalpha1 mRNAs were detected in the diploid cells containing the 14-bp deletion. Since alpha1 is a positive regulator of alpha-specific genes, one would have expected to find transcripts encoding alpha factor (MFalpha1) to be present in these alpha1-producing diploids. It was evident from these studies that alpha1 is necessary, but not sufficient, for expression of at least one alpha-specific gene in diploid cells. In retrospect, it is likely that inability to detect MFalpha1 mRNA is due to the absence (or inactivation) of the STE12 product in these diploids.

The above mutations were created in vitro in order to localize the regions conferring a1-alpha2 regulation. Results from analysis of a seemingly unrelated class of mutations which have arisen in vivo appear to have converged on and extended these findings.[406] Certain Ty insertion mutations, designated ROAM, bring a variety of unrelated genes under control of the mating-type locus.[406] One such mutation (CYC7-H2) results from the insertion of Ty1 in the 5′-flanking sequences adjacent to the CYC7 gene. This insertional mutation results in a 20-fold overproduction of CYC7 in haploid cells, but this effect is abolished in several ste mutants. In addition, levels of the enzyme are about tenfold higher in a or alpha haploids than in a/alpha diploid cells. It appears that overlapping modes of regulation are occurring; a positive effect requiring wild-type haploid functions (e.g., STE7, STE11, and STE12) which results in overproduction of CYC7, and a negative effect on this overproduction in diploids presumably resulting from a1-alpha2 repression. DNA sequencing of the region within Ty1, which is required for this cell type regulation, revealed the presence of two enhancer-like elements.[407] These were identified by homology to the core sequence of the SV40 72-bp repeat, and, in addition, show homology to the "diploid regulation" control sequence of Siliciano and Tatchell discussed above. Deletion of both enhancer-like elements results in a loss of cell-type specific expression of CYC7-H2. The presence of one of these elements is sufficient for both haploid overproduction and for MATa/MATalpha repressibility. As these authors suggested, the a1-alpha2 repressor in diploid cells may be interfering with the positive enhancing potential of this sequence element (or with its associated proteins), perhaps resulting in an obstruction of transcription initiation. A detailed functional study of this region by deletion or mutation analysis should reveal if the positive and negative enhancement effectors compete for the same target sites in the DNA sequence.

Another element in yeast characterized by Brand et al.[408] has properties analogous to an enhancer sequence except that it acts negatively on transcription. Dubbed a "silencer" sequence, this *cis*-acting element represses transcription of the silent mating-type locus, HMR, independent of orientation, at great distances from the transcriptional start site (tested up to 2600 bps away), when positioned at both the 5′ and 3′ ends of HMR. This silencing activity is silent information regulator (SIR) dependent and can repress more than one promoter concurrently.[408]

B. Homothallism and Regulation of the HO Gene Product

Homothallic strains (HO) of yeast have the ability to switch mating type almost once per cell generation.[409,410] The molecular basis of this switching process is well documented[411,413] and occurs by a transposition event which results in a replacement of the genetic information present at the MAT locus with an alternate allele from elsewhere in the genome. The donor loci which contain silent copies of the alpha and a alleles are termed HMLalpha and HMRa. Extensive physical structure analysis of cloned MAT, HML, and HMR regions has revealed much information and has led to elegant experiments confirming the model for mating-type interconversion.[298,405,414,415]

The function of the HO gene product must be essential for this process, since heterothallic (ho) strains show greatly reduced frequencies of switching. The HO gene product has recently been shown to encode a site-specific endonuclease which cleaves a

defined DNA sequence at the MAT locus.[416] This double-stranded cut is believed to initiate the switching event. The products of the SWI genes,[417] the DNA recombination/repair genes RAD51, RAD52, and RAD54,[387,418] and CSM[419] genes are also required for switching. Recent studies have shown that the HO gene transcripts are present in a or alpha haploids, MATa/MATa, MATalpha/MATalpha diploids, but not in heterozygous MATa/MATalpha diploid cells.[398] HO RNA accumulation is therefore controlled by constituents of the MAT locus and is presumed to be negatively regulated by a1-alpha2. Thus, HO expression and cell-type switching is turned off in cells which have diploidized by conjugation and no longer require cell-type diversification for optimizing mating success. The regulation of HO is a complex process as indicated by the following observations: (1) HO RNA production is cell-cycle dependent,[386] (2) pedigree analysis has indicated that only "experienced" cells switch mating type,[410] and (3) HO expression is dependent on SWI genes.[420]

As mentioned previously, the HO endonuclease activity and its mRNA levels were found to peak sharply in early G_1 between the CDC28 and CDC4 steps in the cell cycle.[386] This is consistent with the observation that switching always occurs prior to DNA replication. As in the case of the cell cycle-regulated histone genes, the HO structural gene is closely associated with an ARS element.[388] It was thought, perhaps, that the structural changes which accompany preparation of ARS sequences for initiation of DNA synthesis also promote transcriptional activation. This, however, appears not to be the case for the HO gene, as deletion of the (3′) ARS element did not alter HO transcription.[421] Using a variety of HO deletion constructs integrated at the corresponding genomic locus, Nasmyth[421] identified sequences in the 5′-flanking region of HO which affect cell cycle regulation. Further analysis demonstrated that multiple copies of a repeated element (Pu-N-N-Py-C-A-C-G-A_4) are necessary and sufficient to confer start-dependent cell cycle control of HO.[422] This element does not appear to be conserved in the 5′-flanking regions of histone H2A or CDC9, genes whose expression is also cell cycle dependent.

Nasmyth[386] addressed the question of why it is that only cells which have previously budded ("experienced" cells) are able to switch mating type. Mother cells were separated from daughter cells by centrifugation through osmotic gradients and each was allowed to resume growth in rich medium. Mother cells displayed the characteristic peak of HO mRNA and endonuclease activity during the ensuing cell cycle period. Daughter cells, however, were lacking in both. These results demonstrate that one reason newly budded cells do not switch is the failure to produce the specific endonuclease activity needed to initiate HM to MAT transposition. One can speculate as to the mechanism by which a mother cell retains the potential for immediate expression of HO while the daughter cell inherits an inactivated gene which cannot be expressed during its first round of cell division. *Trans*-acting positive or negative regulators may segregate preferentially with the mother or daughter cells, respectively, or perhaps a distinct chromatin conformation is inherited by each cell.

Worcel and colleagues have shown in vitro and in vivo chromatin assembly in *Xenopus lavis* may occur in such a manner that once assembled a gene-chromatin structure may be "locked" into a state of inactivity, and only when assembly occurs in the presence of the required factors can the gene be activated.[423,424] One can envision a mechanism whereby *trans*-acting factors required for active chromatin assembly segregate with only mother cells, similar to the model proposed by Nasmyth. Alternatively, these factors are present in all cells, but in the course of DNA-strand replication which requires redistribution of chromatin constituents (histone and nonhistone proteins) at the growing fork, only one nascent duplex is competent to reassemble in the active state. Perhaps the basis for a conservative reassembly of chromatin complexes (i.e., one active and one inactive per round of replication) could be dictated by the

intrinsic differences between continuous vs. discontinuous synthesis during DNA chain elongation. The most difficult problem in either model, however, is the means by which cell components, whether they are *trans*-acting factors or *cis*-acting chromatin complexes, are segregated exclusively to only one of the progeny cells. Murray and Szostak[425] have provided evidence that mechanisms do exist for unequal segregation of nuclear components. These observations were based on pedigree analysis of the inheritance of autonomously replicating plasmids which demonstrate a high degree of segregation bias towards the mother cell. Elucidation of the mechanism by which yeasts unequally distribute the potential for mating-type interconversion should also help formulate models concerning the molecular basis of higher cell differentiation.

Insight into the regulation of HO activity during the cell cycle, by mating-type status and by mother/daughter lineages, may come from the study of a recently characterized set of switching mutants, swi1 to swi5.[420] These five nonallelic genes were shown to be required for HO expression as determined by Northern analysis and by measuring the beta-galactosidase activity of HO-lacZ hybrid genes.[420] Four of these mutations are thought to act indirectly on HO expression. SWI5 is postulated to act more directly on HO expression, although RNA transcription rate changes vs. RNA processing or stabilization has not formerly been addressed for any of the swi mutations. Of interest will be whether HO repression in MATa/MATalpha diploids results from negative control of SWI gene function by a1-alpha2.

V. CONTROLS OVER SPORULATION

A. Initiation of Sporulation

Meiosis and sporulation represent a developmental pathway for diploid yeast heterozygous at the MAT locus. The choice between mitotic and meiotic division is very complex, initiated by certain nutrient deficiencies and presumably related to nutritional sensing at start. Cells grown in a nonfermentable carbon source and deprived of a nitrogen source rapidly undergo sporulation. Standard sporulation medium consists solely of potassium acetate, although many complex media will induce sporulation to varying extents. Genetic aspects of sporulation have been studied extensively through the use of sporulation-deficient mutants, many of which are cdc mutants as well. Identification of sporulation-specific genes and the study of their regulation has, however, proved very difficult. The transition of vegetatively growing cells to ascospore production is not easily monitored biochemically for various technical reasons, such as fluctuations in intracellular precursor pools and increased rates of macromolecular degradation. In addition, many of the physiological changes which occur during meiosis and sporulation are not specific to a/alpha diploids and are primarily a consequence of nutrient starvation, which also occurs in haploids and homozygous diploids (for a comprehensive review and numerous references on meiosis and ascospore formation, see Reference 3).

The initiation of meiotic reduction and sporulation illustrates a very interesting and interdisciplinary developmental problem. In haploid cells (or diploids homozygous at the MAT locus), nutritional signals are channeled through start to effect a choice between mitotic cycling and G_1 arrest.[4] Similar nutritional signals in diploid cells heterozygous (a/alpha) at the MAT locus now affect the choice between mitosis and meiosis/sporulation. Two lines of evidence indicate that replacement of the G_1 arrest pathway (as a developmental alternative to mitosis) by a program of ascospore production is a consequence of the alleles present at MAT. First, of course, is that diploidy itself is insufficient to allow meiosis and sporulation since MATalpha/MATalpha or MATa/MATa diploid cells can do neither. Second, haploid cells, which allow expression of

both a and alpha information due to defects in repression of HML and HMR loci (sir⁻ mutants) are able to initiate a meiotic program, although they cannot complete it.

Several mutations exist which allow sporulation of homozygous MATa/MATa or MATalpha/MATalpha diploids, one of which is rme1.[426] Rine et al.[427] demonstrated that the rme1 mutation was not due to cryptic expression of mating-type information (as in the case of sir⁻), but was refractory to MAT, HML, and HMR expression. Their model suggests that RME1 acts as a negative repressor of sporulation-specific genes and is constitutively synthesized in alpha, a, or homozygous diploids, but is turned off in a/alpha cells by the a1/alpha2 regulatory complex.

Several of these predictions have been borne out. Mitchell and Herskowitz[428] cloned the RME1 gene and demonstrated the following: (1) RME1 transcripts are repressed in a/alpha diploids, a process which is dependent on the presence of a1 and alpha2; (2) overexpression of RME1 from plasmid constructs prevents sporulation of a/alpha diploids, consistent with its role as a negative regulator of sporulation-specific genes; and (3) finally, a null mutation in RME1 allows sporulation of cells which are not a/alpha diploids.

Other than defects in mating-type loci, three major classes of mutants altered in initiation of sporulation have been identified. One class involves the cdc genes which operate at start. The cdc28 and tra3 (gcd1) are unable to sporulate at nonpermissive temperatures, suggesting their involvement in initiation of meiosis as well as mitosis. However, two other start mutants, cdc25 and cdc35, show very different phenotypes. Diploids homozygous for either cdc mutation will sporulate at the nonpermissive temperature even in nutrient-rich medium. As concluded by Shilo et al.[429] the functions performed by the cdc28 and tra3 (gcd1) gene products are required to initiate both mitotic and meiotic pathways, while cdc25 and cdc35 products affect the choice between these two developmental alternatives. The second class of mutations, spd, is derepressed for sporulation. Studies by Vezinhet et al.[430] suggest that spd gene products may also influence the decision between cell division and meiotic reduction. These mutations may be defective in nutritional-sensing mechanisms, as they sporulate in media which do not promote sporulation of wild-type cells. The spd1 has been shown to act at or prior to the execution point of CDC28.[430] The third class of sporulation initiation mutants, spo50, spo51, and spo53,[431] can be isolated as revertants of cells derepressed for sporulation (spd). As proposed by Dawes,[4] these genes may promote sporulation at a stage following the branch point (at start) between mitotic growth and meiosis/sporulation (see Figure 10). It will be interesting to determine if spo50, spo51, and spo53 are targets of the proposed negative regulator of sporulation-specific genes, RME1.

As outlined above, the products of several genes have been implicated in "nutritional-sensing" mechanisms involved in initiating sporulation of alpha/a diploid cells. However, it is not known what intracellular metabolite(s) are being monitored, if any. It has been shown that a partial deprivation of any one of the three major media components required for yeast cell growth can trigger a sporulation response.[432] That is, starvation for a nitrogen, carbon, or phosphorus source (in the presence of all other nutrients) can result in the appropriate signals for diversion of a/alpha diploids from vegetative growth to a meiotic program. It is reasonable to postulate the existence of a common intracellular compound(s) whose levels of activity are subject to influence from all three major metabolic systems (N, C, P). Whether adenylate charge, guanine nucleotides, NAD, or cAMP are involved is not yet certain, but substantial evidence implicating the latter compound is emerging (see below). The involvement of guanine nucleotides (GTP) in the initiation of sporulation has also been postulated.[433,434] In addition, there appears to be an element of size control involved in initiation of sporulation, similar to that seen in controls governing the cell division cycle.[435]

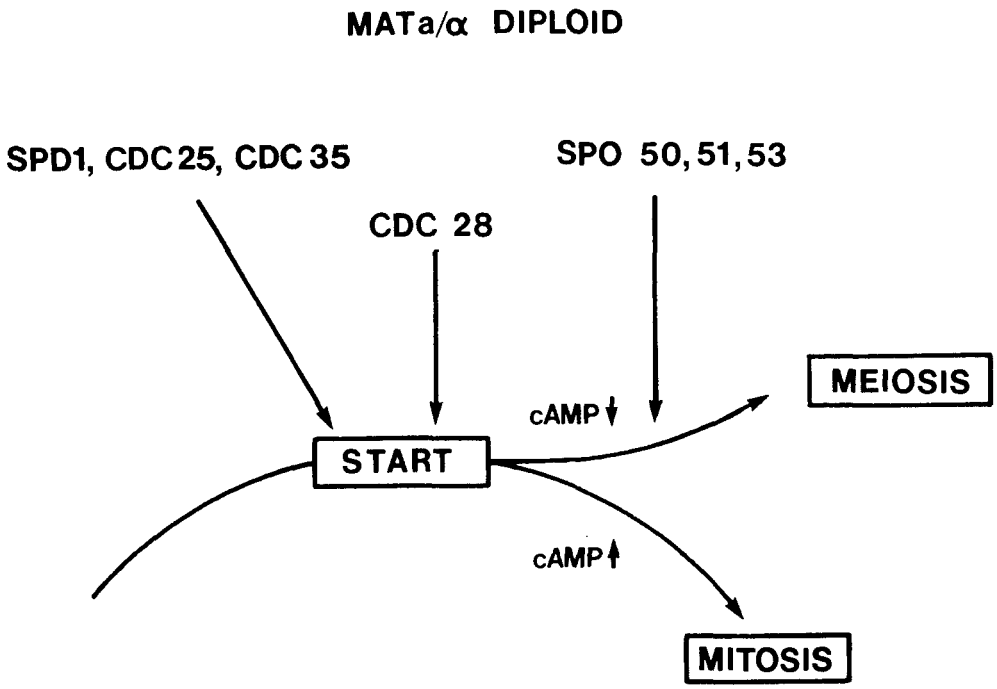

FIGURE 10. A simplified model of genes involved in the developmental switch in a/alpha diploids. SPD1, CDC25, and CDC35 are believed to influence the choice between mitotic and meiotic pathways, perhaps by monitoring nutrient status (mutations in these genes are derepressed for sporulation). CDC28 is necessary for both sporulation and mitosis, but acts after the "switch" has been initiated. SPO50, SPO51, and SPO53 are required for sporulation after the branchpoint as indicated. Low cAMP levels correlate with initiation of meiosis.

The role of cAMP in induction of the meiotic program was investigated using several mutants of the adenylate cyclase system listed in Figure 7. In these studies, Matsumoto et al.[330] tested the ability of adenylate cyclase mutants (cyr1) and cAMP-dependent protein kinase mutants CYR3 to produce ascospores in various media. Diploid cells homozygous for either mutation were able to sporulate at the restrictive temperature in potassium acetate medium, but, in addition, both mutants initiated meiosis in nutrient-rich medium, suggesting the involvement of these genes in the decision between the two pathways. The cyr1 mutant is defective in adenylate cyclase activity.[320] CYR3 mutants produce a cAMP-dependent protein kinase with a lowered affinity for the cyclic nucleotide due to an alteration in the structural gene of the regulatory subunit.[333] Another mutation, bcy1,[331] which leads to high levels of catalytic activity of cAMP-dependent kinase was found to be unable to initiate the meiotic process or to produce spores.[330]

Taken together, these observations suggest that cAMP levels influence the choice between the two developmental pathways; low cAMP is required for meiosis and sporulation while high cAMP levels (or constitutive activity of cAMP-dependent protein kinase) signal mitosis and cell division. It is interesting to note that the dominant mutant $RAS2^{val19}$ has constitutively high levels of membrane-associated adenylate cyclase activity and is unable to sporulate under any conditions.[241] However, a homozygous ras2 mutant will sporulate even in rich medium similar to the cyr1 mutation deficient in adenylate cyclase activity. A most challenging problem will be to establish the links between cAMP metabolism and the "nutritional-sensing" mechanisms involving the CDC25, CDC35, GCD1, SPD gene products.

B. Gene Expression During Sporulation

Although much is known about the requirements for sporulation from a genetic standpoint,[3] virtually nothing is known about the biochemical events involved. Most genes required for sporulation also function during vegetative growth or nutrient deprivation. Cells transferred to sporulation medium continue at first to synthesize protein and RNA. The subsequent breakdown of polysomes to monosomes indicates a block in translation initiation.[436] However, this is not specific to a/alpha diploids, suggesting it is a general starvation response, as in higher cells.[437] mRNA continues to be synthesized despite the increased RNA degradation rate. Only in rare cases have sporulation-specific enzymes been characterized at a biochemical level.[438]

Several recent approaches have been taken for the isolation of sporulation-specific genes and gene products. Using strategies similar to those previously described for cell cycle studies, Wright et al.[439] prelabeled cells with ^{35}S, transferred them to sporulation medium, and fractionated protein extracts on two-dimensional electrophoretic gels. A total of 21 a/alpha diploid cell-specific changes in the protein profile were observed, due either to *de novo* protein synthesis or to modifications of existing proteins. Subsequent two-dimensional gel electrophoretic studies of the in vitro translation products of mRNA isolated from cells undergoing sporulation eliminated the latter possibility. Weir-Thompson and Dawes,[440] using a reticulocyte lysate translation system, identified among 750 identifiable translation products 43 species alterations that were sporulation specific. Surprisingly, only four were presumed to be due to *de novo* mRNA synthesis (accumulation), the remainder resulting from increases or decreases in levels of existing translatable mRNAs. Thus, among species whose abundance and mobility on these gel systems allow detection, most alterations do not involve the synthesis of new proteins, but rather, changes in the level of existing proteins. These findings were extended by Kurtz and Lindquist,[441] who identified ten newly synthesized polypeptide species using a wheat germ translation system. Some of the alterations detected included a subset of the heat shock proteins, while some may be involved in spore wall synthesis and deposition.[442] In addition, they examined the stability of mRNA in ascospores and found essentially no turnover over extended periods of up to 21 days. This compares to normal vegetative cell RNA half-lives of about 15 to 20 min. The mechanism of mRNA stabilization is unknown.

Efforts to clone sporulation-specific genes by techniques repeatedly proven successful in yeasts and higher cells have been used by Clancy et al.[443] and Percival-Smith and Segall.[444] Differential colony hybridizations were used to screen duplicate filters for the presence of sequences preferentially expressed in sporulating cells. In the former study, 16 sporulation-specific clones were identified and in the latter, 14 were found. There are indications that some of these genes are clustered since several of the clones hybridized to multiple sporulation-specific transcripts. The function and regulation of these genes are unknown at present.

It must be noted, however, that genes expressed preferentially during sporulation may not necessarily be required for the sporulation process itself. A study by Kaback and Feldberg[445] demonstrated sporulation-specific patterns of transcript accumulation for several genes (CDC10, GAL10, and HO) believed to be dispensable for sporulation. Thus, the identification of mRNA or polypeptide products showing elevated expression during ascospore formation is not sufficient to implicate their role in this differentiation process. The availability of molecular clones of sporulation-specific genes may allow functional tests (i.e., gene disruption) for the requirement of individual gene products. A recent example of this approach has been carried out by Yamashita and Fukui.[446] They have isolated a sporulation-specific glucoamylase gene (SGA) and shown it to be positively regulated in MATa/MATalpha cells. However, although the expression of SGA is specific to cells undergoing meiosis and sporulation, it is not

essential for these processes. Diploid cells containing double disruptions of SGA have no glucoamylase activity, but are able to sporulate normally and give rise to four viable spores. Unfortunately, genes which have dual roles in mitotic growth as well as in meiosis and sporulation will not be amenable to this type of analysis if double mutations in diploid cells prove lethal.

VI. CONCLUDING REMARKS

We began this section by contrasting the roles of eukaryotic microorganisms like *S. cerevisiae* to those of higher eukaryotes. An emerging picture, documented by the wealth of insight into regulation in *S. cerevisiae,* is a widespread similarity in their basic molecular regulatory mechanisms, despite important differences in cellular function.

In the preceding pages, we presented some of the key features of yeast cell growth and provided examples of how yeasts respond to environmental changes by altering these growth patterns. Although we chose to discuss issues primarily related to carbon metabolism, modes of regulation of this pathway are probably conserved among all the major metabolic systems. Combined genetic and molecular approaches have been highly successful in elucidating detailed mechanisms of regulation of a few select genes (e.g., GAL, CYC, and cob). Understanding how the regulation of these genes is integrated with others in each particular metabolic pathway and in more global cellular circuits remains an exciting and formidable challenge. The identification and characterization of genes involved in general control over related sets of genes (e.g., GCN, CCR, SNF1, and GCR) uncovers only the tip of the metabolic-regulatory iceberg.

Models have been presented concerning nutritional inputs at start that influence the developmental pathways taken by haploid or diploid yeasts. The pleiotropic effects of the well-studied MAT locus establish the developmental options available for cells in response to nutritional challenges. The mechanisms by which this single genetic locus determines cell fate serves as a premier model for developmental biologists.

As in higher cells, membrane-associated signaling systems appear to be in place. The role of cAMP and (Ca^{2+}?) may be essential in coordinating a wide variety of nutritional signals in order to affect a single cellular response, e.g., entry into another round of mitotic division. The biochemical identification of the components of the adenylate cyclase system will, no doubt, stimulate interest in yeast as a model system for further studies on signal transduction. Moreover, the discovery of oncogene homologs in yeast has attracted the attention of many investigators who might otherwise regard studies of this simple eukaryote as inappropriate for extrapolation to higher organismal systems. As more data accumulate about molecular mechanisms operating in yeast and higher cells to control basic cellular functions, i.e., growth and division, the similarities may become even more apparent.

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