Regulation of Sugar and Ethanol Metabolism in *Saccharomyces cerevisiae*

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ABSTRACT

This review briefly surveys the literature on the nature, regulation, genetics, and molecular biology of the major energy-yielding pathways in yeasts, with emphasis on *Saccharomyces cerevisiae*. While sugar metabolism has received the lion's share of attention from workers in this field because of its bearing on the production of ethanol and other metabolites, more attention is now being paid to ethanol metabolism and the regulation of aerobic metabolism by fermentable and nonfermentable substrates. The utility of yeast as a highly manipulable organism and the discovery that yeast metabolic pathways are subject to the same types of control as those of higher cells open up many opportunities in such diverse areas as molecular evolution and cancer research.

I. INTRODUCTION

The yeast *Saccharomyces cerevisiae* is one of the simplest eukaryotes, with a genome size only about three times that of *Escherichia coli*.[238] Nonetheless, it has an extremely versatile metabolism, and its ability to respire anaerobically has made it a domesticated microorganism with two important uses. Baker's yeast has been selected over millennia for high rates of CO_2 production, and brewer's yeast may have been selected for high ethanol production for hundreds of thousands of years.[50] This yeast is versatile; it can grow on a great variety of fermentable and nonfermentable substrates, and under some conditions can produce, and survive in the presence of, very high levels of ethanol. Cells capable of efficient *sake*-type fermentation, in which the sugar is added stepwise over long periods of time, can regularly produce 20% ethanol.[140]

The discovery by Buchner in 1893 that cell-free extracts of yeast can break down added sugars not only dealt the final blow to vitalistic theories that made a distinction between living and nonliving matter, but also opened up the possibility of studying these and other living processes *in vitro*. Following this ground-breaking work, glycolysis in yeast was one of the first pathways to be worked out in some detail. Other pathways, such as the glycerophosphate-dihydroxyacetone phosphate shuttle, were first detected in yeast.[233] Also, while the role of mitochondria in aerobic metabolism was largely elucidated through work on mammalian mitochondria,[322] the discovery of mutants ("petites", so-called because they produce small colonies) that lack mitochondrial function[106] opened the way for many important studies of oxidative phosphorylation.

Many important reviews have covered this early work and should be consulted for details. In particular, Sols et al.[286] have surveyed the work up to 1970 on yeast energy-yielding metabolism. Casey and Ingledew[50] have surveyed the immense literature on ethanol tolerance in yeasts up to 1985. Also, Fraenkel[111] has examined the work done on metabolic pathways up until 1981, examining particularly the burgeoning number of mutants affecting these metabolic processes that had been isolated up to that time.

As our understanding of these pathways grows, and as more tools of molecular biology become available, the primary attention of workers in the field has turned away from a mere elucidation of the steps in the various pathways and their relative importance and toward the question of their regulation. Gancedo and Gancedo[121] have reviewed the mutants affecting carbon catabolite repression that have been isolated up to 1985, and Thevelein[308] has reviewed the extensive work that has been done on trehalose metabolism and its regulation up to 1983. This is not an exhaustive list, but it will serve to direct the reader to a number of important reviews.

The present article concentrates primarily on the new information which is emerging from the application of molecular biology to questions of yeast intermediary metabolism, and only lays out the details of the pathways to the extent necessary for the reader to understand the regulatory work that has been done.

The recent impact of molecular biology on this field has been enormous. Wild-type genes can be readily cloned in yeast by complementation of the mutant phenotype by a shuttle plasmid carrying the wild-type allele derived from a yeast DNA library.[337] Because introns are uncommon in yeast genes, it is usually not necessary to construct a cDNA library. Other types of cloning techniques can also be employed, and searches for homologous genes in the small yeast genome can readily be made. The introduction of pulsed-field electrophoresis[273] and orthogonal field alternation gel electrophoresis (OFAGE)[45] allows 15 of the 16 chromosomes of yeast to be separated electrophoretically.[46] Coupled with Southern blotting, this permits the rapid localization of a cloned gene to a chromosome. Genes such as the glucoamylase genes, the effects of which are only detectable during sporulation, can be located by this method.[257]

Cloned genes can be incorporated into the genome, using an integrating plasmid, in the place of a mutant allele. They can be disrupted by the insertion of a marker allele and integrated to produce a strain in which no vestige of the gene

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activity remains.[265] Also, mutant strains can be transformed with high-copy-number plasmids carrying the cloned gene, to determine the effect of increasing the dosage of the gene.[36] Regulatory as well as structural genes can be treated in this way. Regulatory genes can also be mapped functionally through deletion analysis, so that the regions of the proteins they encode that are responsible for various regulatory functions can be located.[17]

Binding of small regulatory compounds to the cloned gene products can be investigated, and DNAse footprinting[118] can be used to determine the points at which regulatory proteins bind to the DNA. Regulatory genes in yeast can now be transferred to other organisms to determine their effects[75] and the growing library of gene sequences can be scanned by computer to determine the relationship of newly cloned yeast regulatory and structural genes to those of other organisms. In some cases, unexpected findings turn up, like the discovery that yeast mating type loci have homology to Drosophila and amphibian homeobox domains,[279] a similarity which extends to their DNA-binding capabilities[127] and their central role in gene expression.[228]

All these and many other techniques have been brought to bear on the question of the regulation of yeast metabolic pathways, and a picture of remarkable complexity has been emerging. Regulatory systems can be positive or negative.[130] That is, positive regulation may be effected by the binding of a protein to a DNA regulatory region, permitting transcription of a downstream gene. Negative regulation may be effected by the removal of a protein bound to a region, again permitting transcription to commence. As will be seen, it appears often to be the case that both positive and negative regulators may interact with each other, or may bind to slightly different parts of a DNA regulatory region.

Many structural genes seem to be subject to more than one regulatory system. When one layer of regulation is removed, other regulatory mechanisms, the effects of which were imperceptible before, appear to be able to take their place. A picture is emerging of a highly regulated, highly homeostatic cell which can withstand not only a great range of environmental insults but also the malfunction or loss of function of many of its regulatory systems.

II. CATABOLIC PATHWAYS

Figure 1 shows in outline some of the major pathways by which yeast derives energy. Fructose-6-phosphate, the starting point for the Embden-Meyerhof pathway of glycolysis, can be derived from external sources of glucose or fructose, or from the mobilization of glycogen and trehalose. The diagram shows in outline the synthetic and breakdown pathways for these two important storage compounds, and the conditions under which they are accumulated. Trehalose accumulates, particularly under conditions of severe stress such as those leading to sporulation, up to as much as 23% of the cell dry weight.[183] Glycogen tends to accumulate under somewhat less stressful conditions.[129] Both of these compounds can be readily broken down to glucose. This, in turn, enters the glycolytic pathway.

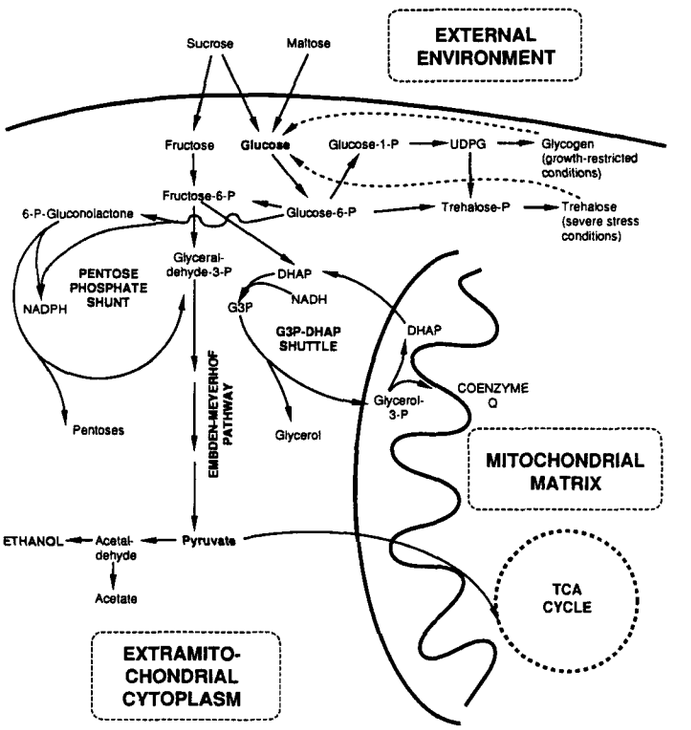

FIGURE 1. An outline of the pathways involved in the utilization of sugars in the environment and of storage compounds in the cell. All these pathways are under strong and complex regulatory control. Although the majority of the enzymes of the Embden-Meyerhof and pentose shunt pathways are synthesized constitutively, the flow of compounds into these pathways is highly regulated. Note the important role played by the glycerol phosphate-dihydroxyacetone phosphate shuttle. The relative flow of the pyruvate produced by glycolysis to ethanol or alternatively into the TCA cycle and oxidative phosphorylation is ultimately governed by the availability of oxygen. A number of mutants, discussed in the text, can alter this flow.

The diagram also shows the relative relationships of the pentose phosphate shunt and the glycerol-3-phosphate dihydroxyacetone phosphate shuttle to the Embden-Meyerhof pathway. The shunt is the only source of pentoses and is the primary source of reduced NADP in the cytoplasm. It is, therefore, always operating during glycolysis, though it tends to receive a larger fraction of the carbon flow during aerobic than during anaerobic growth. Under anaerobic conditions, the majority of metabolized glucose travels down the Embden-Meyerhof pathway to pyruvate. A variable amount, up to 30% during aerobic growth,[58] is diverted from glucose-6-phosphate into the pentose phosphate shunt.

Recently, Aguilera[2] isolated suppressors of a deletion mutation at the locus coding for phosphoglucose isomerase, *PGI1*. These suppressors were able to grow on glucose. He found that mutations falling into one complementation group, *SPG1*,

were all recessive and were only able to grow if mitochondrial function was unimpaired. No ethanol was produced, and the respiration level was high. Aguilera suggests that these very interesting mutants are involved in the diversion of carbon flow into the pentose phosphate shunt. They will repay further thorough study.

The G3P-DHAP shuttle, as seen next, is very important in yeast and leads to the production of appreciable quantities of glycerol during fermentation.

The major monosaccharides that can be fermented to *S. cerevisiae* are the D-stereoisomers of glucose, fructose, and mannose, and, after a period of adaptation, galactose. Among the disaccharides, sucrose is broken down at a very high rate by invertase, located on the cell surface,[175] and absorbed by carrier-mediated facilitated diffusion as a mixture of glucose and fructose. Maltose is apparently taken up by an inducible active transport system[277] since it can reach a higher concentration in the cell than in the surrounding medium and its transport can be interrupted by antimetabolites such as DNP and CCCP. Once in the cell, it is hydrolyzed to glucose by maltase,[229] which can also hydrolyze sucrose but is apparently prevented from doing so *in vivo* because only small amounts of sucrose can enter the cell directly. Secretion of invertase is necessary in order to hydrolyze external sucrose.

A complex relationship exists between maltose utilization and the accumulation of trehalose and glycogen. The *MAL4* gene, involved in the regulation of the maltose fermentation pathway, also confers an ability to accumulate trehalose rapidly during logarithmic growth,[244] suggesting that it may be involved in the regulation of both pathways.

The early work on lactose metabolism has been thoroughly reviewed by Oshima.[239] Also diagrammed are the two major alternative routes open to the further metabolism of pyruvate, leading either to ethanol under anaerobic conditions or into the TCA cycle when oxygen is present. The diversion of some acetaldehyde to acetate is the probable source of the acetate produced during fermentation.

Under aerobic growth, pyruvate can enter the mitochondrion. There, two of its carbons are used to acetylate Coenzyme A (acetyl CoA) through the action of the pyruvate dehydrogenase complex, with the release of the carboxyl carbon as CO_2. Under anaerobic conditions, or if the activity of the mitochondria has been interrupted, the same two carbons are converted to the intermediate storage compound ethanol. This occurs in two steps: the conversion of pyruvate to acetaldehyde by pyruvate decarboxylase, with the release of CO_2, and the subsequent conversion of acetaldehyde to ethanol through the action of isoenzyme I of alcohol dehydrogenase.

Some pyruvate can also be converted to oxaloacetate through the activity of the cytoplasmic pyruvate carboxylase,[136] and this may enter gluconeogenesis. A mutant found in our laboratory, unable to grow on ethanol on minimal medium, was thought to be defective in this enzyme,[345] but this was found not to be the case using other assays.[184] The structural gene for this enzyme has been cloned but not yet disrupted;[223] it will be instructive to determine the phenotype of a defect for this enzyme since this will determine the relative importance of this part of the gluconeogenic pathway.

The amount of pyruvate entering these three pathways is governed by the physiological state of the cell. Ethanol can be produced, particularly if fermentable substrates in the medium are abundant, even under aerobic conditions. There is also some evidence that both glycolysis and gluconeogenesis can occur simultaneously, even though this might lead to futile cycling.[147] The danger of such cycling may be overestimated; Bañuelos and Fraenkel[13] found little indication of futile cycling in wild-type or mutant cells in which fructose-bisphosphatase and phosphofructokinase were both present and active.

The mutants these authors tested for evidence of futile cycling, from both *S. cerevisiae* and *S. carlsbergensis,* were unable to inactivate fructose-bisphosphatase normally in the presence of glucose, though their primary lesions have not been determined. It may be that they are constitutive mutants of the type that has been found to affect the inducible alcohol dehydrogenase.

Glycerol and acetate are minor products of anaerobic fermentation. The former is produced through the action of the glycerol-3-phosphate-dihydroxyacetone phosphate shuttle, which is an important generator of reducing power in some animal cells such as those of insect muscle. The amount of glycerol produced is greatly enhanced in cells lacking alcohol dehydrogenase activity, in which all the cytoplasmic NADH must be produced by this pathway.[346]

Under alkaline conditions, the amount of acetate accumulated in the medium increases. Whether this involves a "third form of fermentation",[286] or whether it is simply due to the inability to utilize ethanol completely, resulting in a partial diversion of acetaldehyde to acetate, remains to be determined.

Glycerol can be used as a substrate by *S. cerevisiae* to a limited extent. YEPG, a complete medium with 2% (v/v) glycerol, is commonly used to distinguish grande from petite yeast, the latter being unable to grow on this medium. In fact, however, only about 10% of the glycerol is used by the commonly employed genetically defined strains of yeast, even on this complete medium.[341] Much less is used on minimal medium with glycerol as a sole carbon source (MVG), on which most laboratory yeast strains will hardly grow at all. Mutants able to utilize glycerol at higher levels have been isolated, and this enhanced growth can be prevented by the transaminase inhibitors aminooxyacetate and cycloserine.[341] This suggests that their growth phenotype may depend on the malate-aspartate shuttle or some other transaminase-dependent process, but the precise mechanism remains to be determined.

Lactate is utilized poorly by *S. cerevisiae,* although there are two D-lactate dehydrogenases and one L-lactate dehydrogenase in the cell.[72,267] The D-lactate dehydrogenases, though

FAD dependent, do not appear to be connected directly with the electron transport chain. The L-lactate dehydrogenase is, however, intimately connected with it since this activity is one function of cytochrome b_2 located in the mitochondrial intermembrane space.[153] The reduced cytochrome b_2 directly reduces cytochrome c, so that reducing equivalents from lactate enter near the end of the electron transport chain. As a result, this permits the phosphorylation of only one ATP per lactate.

The pyruvate produced by the activity of this enzyme presumably enters the TCA cycle. The reason that lactate is such a poor substrate may be that there is no immediate source of cytoplasmic NADH when the cell is growing on this compound, but this possibility has not been investigated. Mutants lacking cytochrome b_2 show no obvious phenotype beyond an inability to grow on L-lactate.[134,267]

III. PATHWAYS OF GLUCONEOGENESIS

Certainly, the commonest substrate for gluconeogenic growth in yeast in the wild is ethanol. Because there is no active transport system for ethanol in yeast, this compound, once it is produced, leaves or enters the cell solely by passive diffusion.[191] Thus, all the internal components of the yeast cell must be adapted to be able to function or at least survive in the presence of high concentrations of ethanol. This was recently vividly illustrated by the isolation by Aguilera and Benitez[3] of a large number of mutants that conferred ethanol sensitivity. The 21 mutants investigated fell into 20 complementation groups, indicating that many essential cellular components have been selected over the course of evolutionary time for high ethanol tolerance.

It is striking that this substrate provides energy resources to the cell in such a variety of ways that it is difficult to prevent growth on it.

The major pathways of ethanol utilization are shown in Figure 2. Ethanol is converted first to acetaldehyde by alcohol dehydrogenase (chiefly isozyme II[340]) and then to acetate, probably primarily by the K^+-activated aldehyde dehydrogenase,[82] which seems to be responsible for most of this conversion. These two steps result in the concomitant reduction of two NAD^+. The acetate is then converted to acetyl CoA by the cytoplasmic ATP-dependent acetyl CoA synthetase.

The activated acetyl groups can follow two major routes. First, they can enter the cytoplasmic glyoxylate cycle,[90,136] in the course of which two acetyl groups are linked together to form succinate. This can, in turn, enter the mitochondrial matrix through the malate-succinate shuttle.[343] Alternatively, the acetyl groups can enter the mitochondrion directly through the acetyl carnitine shuttle. In this shuttle, acetyl groups are transferred from CoA to carnitine in the cytoplasm through the action of carnitine acetyl transferase. They are then carried to the mitochondrial matrix where they are transferred back to CoA. The existence of this shuttle in yeast can be demonstrated

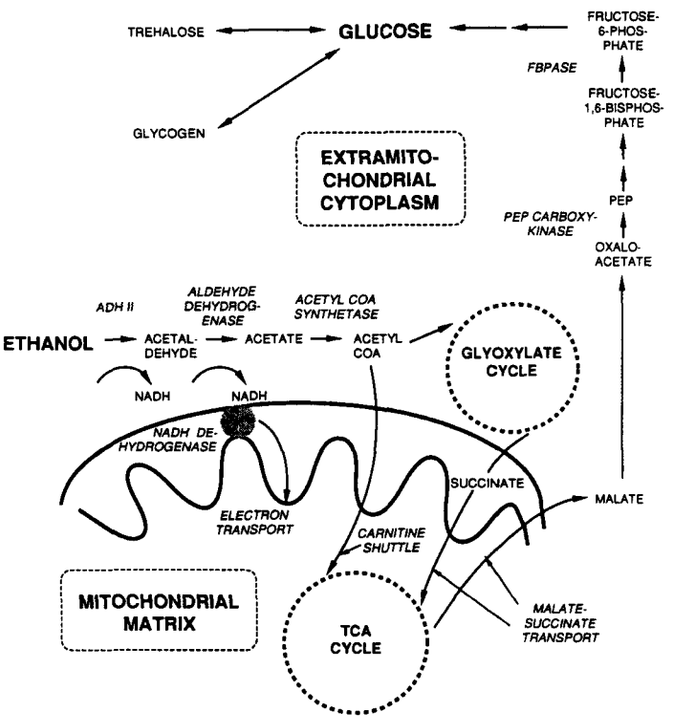

FIGURE 2. An outline of the pathways involved in the synthesis of sugars from simpler nonfermentable compounds, particularly ethanol. Note that there are at least three routes by which energy from ethanol can generate ATP through mitochondrial activity. These are directly by the production of cytoplasmic NADH, and indirectly through the production of cytoplasmic acetyl CoA or succinate.

by supplying isolated yeast mitochondria with acetyl carnitine and following the subsequent phosphorylation of ADP.[343] Carnitine acetyl transferase is an active enzyme in yeast.[358]

Once the TCA cycle has been primed, excess malate exported from the mitochondrion by the entry of succinate can be converted to oxaloacetate and enter the cytoplasmic gluconeogenesis pathway. It may be that certain parts of this pathway operate only when glycolysis is not functional. Wilson and Bhattacharjee[348] found that pyruvate kinase and phosphoenolpyruvate carboxykinase tend not to be present in the cell simultaneously, which would reduce the likelihood of futile cycling. Further investigation of this regulation will prove rewarding.

The gluconeogenesis pathway based on ethanol is highly redundant, and even mutants that would be expected to disrupt it completely do not do so. Subik et al.[294] isolated what appeared to be a mutant of the α-ketoglutarate dehydrogenase complex which could still grow slowly on ethanol. The same growth pattern was seen in more extensive recent work by Dickinson et al.,[83] who isolated a mutant apparently defective in lipoamide dehydrogenase. In the isolation of this strain, they used Subik's approach and screened mutants that were able to grow on ethanol but not on glycerol.

Lipoamide dehydrogenase, which makes up part of both the pyruvate dehydrogenase and the α-ketoglutarate dehydrogenase complexes of the TCA cycle, has been investigated extensively in *E. coli*, and mutants in which the gene is disrupted have been found to prevent the TCA cycle from operating in that organism.[292] The gene has recently been cloned and sequenced, and upstream regulatory sites similar to those found in other yeast genes were discovered.

In the yeast mutant isolated by Dickinson et al., all three enzyme activities were undetectable, and a complementing presumptive clone of the lipoamide dehydrogenase gene on a high copy number plasmid resulted in an overproduction of that enzyme. This could be visualized after affinity chromatography on lipoate-Sepharose.

The phenotype of the mutant is interesting for a variety of reasons. Homozygotes for the mutant cannot sporulate on acetate medium, but heterozygotes sporulate slightly more readily than the wild type. The authors suggest that the α-ketoglutarate dehydrogenase complex may be essential for sporulation. The question of why this mutant is able to grow on ethanol, though not on acetate or glycerol, remains unresolved, but one possibility is that the reducing power of cytoplasmic NADH, produced by the first steps of ethanol metabolism in the cytoplasm and perhaps by the action of the glyoxylate cycle, may be transferred directly to the electron transport chain. This might occur through the membrane-bound NADH dehydrogenase, which yeast mitochondria possess in common with the mitochondria of plants.[71,343]

Yeast has other means of escaping what would appear at first sight to be metabolic blocks that should completely prevent ethanol metabolism. For example, the cytoplasm may supply metabolic steps that are normally thought of as the province of the mitochondria. This is vividly illustrated by the effects that are seen when mutants are blocked in citrate synthase.

Such a block, in an essential step of the TCA cycle, would be expected to convert the blocked cells into petites. The first mutants isolated[39] were glutamate auxotrophs, but were still able to grow on nonfermentable substrates. Rickey and Lewin[260] isolated mutants defective in the mitochondrial enzyme, and found that there was residual activity in the cytoplasm, which they attributed to the cytoplasmic enzyme that forms part of the glyoxylate cycle. These mutants were also able to grow on nonfermentable substrates. More recently, Rosenkranz et al.[264] disrupted both the mitochondrial and the cytoplasmic genes, and at last succeeded in producing a strain that behaved as if it were petite. Later, they found that strains disrupted in the mitochondrial enzyme were unable to grow on acetate, though they could grow on other nonfermentable substrates, and had roughly twice the concentration of malate found in wild-type cells.[168]

The most likely explanation for this phenomenon of continued growth on most nonfermentable substrates on the part of the mutant blocked in the mitochondrial citrate synthase is that the citrate synthase in the cytoplasmic glyoxylate cycle can take over the function of its mitochondrial counterpart. The sequence of events required to complete the TCA cycle would be as follows. First, intramitochondrial malate accumulates because of the metabolic block. It is then shuttled out of the matrix against extramitochondrial citrate and converted to citrate in the cytoplasm through the action of the cytoplasmic MDH and citrate synthase. This citrate enters the mitochondrion in exchange for more malate. It will be interesting to determine whether this is the correct explanation.

McAlister-Henn and Thompson[216] have succeeded in disrupting a nuclear malate dehydrogenase gene by using its homology with the mitochondrial gene. The gene-disrupted strain was still capable of growth on glycerol and lactate on complete medium, though it was not capable of growth on acetate. It is possible that the gene these workers disrupted was the one found in the glyoxylate cycle, leaving a second cytoplasmic MDH, presumably responsible for the conversion of mitochondrion-produced malate to oxaloacetate, still intact.

A subunit of the succinate dehydrogenase gene has recently been disrupted and shows a remarkably small phenotypic effect even though once again this block would be expected to interrupt the TCA cycle.[189] It grows more slowly than the wild type on glycerol, but grows readily on ethanol and pyruvate.[358]

These metabolic pathways in yeast thus show a great deal of redundancy. In the case of ethanol metabolism, this reflects the many different routes, in both the extramitochondrial cytoplasm and the mitochondrion, through which the products of ethanol can and do travel in the course of anabolic activity. What appears to be a highly redundant system must actually reflect the differing anabolic needs of the cell as the amounts of oxygen and of nutrients vary.

It becomes apparent during the course of this article that yeast regulatory systems are similarly redundant. This redundancy has the effect of providing the cell with the ability to take rapid advantage of changing conditions by directing the flow of metabolites into alternative pathways.

IV. MUTANTS OF THE GLYCOLYSIS PATHWAY

Many mutants in this centrally important pathway have been isolated, both in structural genes and more recently in regulatory genes. The characteristics of mutants isolated up to 1981 have been summarized by Fraenkel.[111] A number of interesting aspects of this earlier work are worth reexamining here.

A great variety of mutants affecting specific steps in the glycolytic pathway itself have been isolated since the first probable mutants were found by Skoog and Lindegren.[284] A number of workers have, for example, used specific selective procedures to screen for mutants defective in various parts of the pathway. Maitra, for example, examined mutants unable to

grow on glucose and isolated a subclass that was still capable of utilizing fructose. One of these was a phosphoglucose isomerase mutant.[200] A valuable selective tool was 2-deoxyglucose, which inhibits glycolysis but only when it is phosphorylated. Resistance to 2-deoxyglucose was used to screen for hexokinase mutants.[197]

Up to 1979, most of the mutants isolated were first characterized simply by an inability to grow on glucose. This was true of the best-studied of these mutants, those deficient in hexokinases,[187] phosphoglucose isomerase,[198] and pyruvate kinase.[202,289] In that year, Ciriacy and Brietenbach[65] reported the isolation of mutants at seven different loci that were deficient in the rate of flux through the glycolytic pathway.

One problem with the isolation of mutants in glycolysis is that many of the steps of glycolysis are shared by gluconeogenesis, so that a mutant blocked at such a step would be unable to grow on either fermentable or nonfermentable substrates. Ciriacy and Breitenbach circumvented much of this problem by mutagenizing cells and plating them on medium containing both glycerol and ethanol. They then screened for mutants that could ferment neither glucose nor maltose. Those that could ferment neither were considered likely to be mutants in glycolysis rather than transport mutants since glucose and maltose are taken up by different pathways.[295]

The reasoning behind the use by Ciriacy and Breitenbach of both glycerol and ethanol in their permissive medium is as follows. Since glycerol enters the glycolytic pathway at the level of glyceraldehyde-3-phosphate, the only steps of the Embden-Meyerhof pathway needed to complete gluconeogenesis starting with glycerol would be aldolase, fructose-bisphosphatase, and phosphoglucose isomerase. Fructose-bisphosphatase is not part of the glycolytic pathway, so only the steps catalyzed by aldolase and PGI would be inaccessible to their mutant hunt.

Mutants were found that were deficient in phosphofructokinase, triose phosphate isomerase, phosphoglycerate kinase, phosphoglucomutase, and pyruvate kinase. Surprisingly, mutants were also found that were deficient in phosphoglucose isomerase. The fact that they were found at all suggests that they were probably slightly leaky. All the mutants were inhibited by glucose when first isolated, in most cases apparently because the introduction of glucose resulted in a buildup of phosphorylated intermediates.

Different mutants in the set showed an accumulation of various glycolytic intermediates, not always obviously correlated with the particular block. The only common denominator among all the mutants was a rapid depletion of ATP when grown on glucose. There was a very interesting lack of correlation between the levels of fructose-1,6-bisphosphate in the mutant cells and the degree of inactivation of fructose bisphosphatase, suggesting that these levels may not be connected to catabolite inactivation *in vivo* (see the following).

Since that time, a number of these genes have been cloned, sequenced, and disrupted, and this type of investigation is continuing. As a consequence of these more detailed studies, our understanding of the enzymatic basis of the steps of glycolysis continues to grow.

The continuing story of the phosphofructokinase genes provides a vivid illustration of this. It now appears to be a case of an enzyme in which function can be provided by more than one subunit.

There are two subunits of phosphofructokinase, α and β, and a functioning enzyme consists of four subunits of each type, $\alpha_4\beta_4$.[170] Two loci corresponding to these two subunits have been found to affect levels of this enzyme in yeast.[66,33]

The single phosphofructokinase mutant isolated by Ciriacy and Breitenbach turned out to be leaky in its growth on glucose, and, in fact, to be a double mutant. One of these showed low levels of phosphofructokinase but was, when placed in an otherwise wild-type background, still able to grow on glucose. The other, of unknown nature, was inhibited by glucose but showed normal levels of all the glycolytic enzymes.

Clifton et al.[68] used this observation to isolate a mutant, *pfk1-1*, as a double mutant in a *pyk* (pyruvate kinase deficient) mutant. Their reasoning was that, because the step catalyzed by phosphofructokinase came before that catalyzed by pyruvate kinase, a *pfk* mutant might be epistatic to a *pyk* mutant and permit glucose growth. They screened a number of revertants, slightly resistant to this growth inhibition, for PFK activity. Segregants carrying the *pfk* mutation alone were able to grow more readily on glucose than the double mutant.

Clifton and Fraenkel[66] isolated another nonallelic mutant, *pfk2-1*, using the same technique and found that it also could grow on glucose while the double mutant could not. They tentatively identified *PFK1* as specifying the α subunit, on the basis of overproduction of this subunit by a clone of the gene. They also found that their *PFK2* mutant, presumably responsible for the β subunit, had an increased K_m value for fructose-6-phosphate but still showed some activity. They concluded that the β subunit might be the catalytic one (which is in accord with information from the enzyme's biochemistry[312]) and that this subunit might be able to carry out catalytic activity alone. Their assumption was that the *PFK2* mutant was leaky, and they also considered the possibility that both subunits had catalytic activity.

There were alternative possibilities, however. Lobo and Maitra[188] had earlier found evidence for a particulate PFK and suggested that it might be coded by a third gene that was able to provide catalytic activity by sharing α or β subunits with the cytoplasmic enzyme.

Breitenbach-Schmitt et al.[34] suggested another explanation. They hypothesized that there might be a bypass pathway involving the pentose phosphate shunt, allowing the *pfk* blocks to be circumvented. They found some mutants that might have been blocked in such a pathway.

These alternative explanations have been rendered less likely

by the results obtained from cloning of both genes[142] and their disruption by insertion of *LEU2*.[141] In spite of the disruptions, the single mutants were still able to grow on glucose, though they accumulated both fructose- and glucose-6-phosphate. As with the conventionally isolated mutants, the double mutant was unable to ferment glucose. This provided strong *prima facie* evidence that both subunits have catalytic function.

The most parsimonious explanation at the moment seems to be that both the α and β subunits are capable of carrying out catalytic function, and indeed there is some evidence that both subunits are very similar.[144]

The power of the techniques of molecular biology to unscramble formerly confused stories is also shown by the analysis of the phosphoglucose isomerase structural gene. The enzyme is a dimer composed of identical subunits.[166] Three isoenzymes had been detected by physicochemical means,[167] but only one complementation group was found.[201] The likelihood of there being only a single catalytic structural gene was reinforced by molecular investigations. Aguilera and Zimmermann[4] were able to demonstrate that strains carrying the cloned *PGI1* gene on a multicopy plasmid showed increased activity and that there was only one copy of the gene in the genome. This does not rule out the possibility of protein subunits modifying the activity that is coded by other genes and perhaps lost during purification, but it would appear that *PGI1* codes for the only peptide with catalytic activity and that the isoenzymes that were observed earlier are produced by post-translational modification.

Disruption of this gene, a hunt for integrants in a diploid, and subsequent genetic analysis should be instructive since a strain carrying a totally disrupted gene should not be able to carry out either glycolysis or gluconeogenesis.

There is some evidence for an interaction of the temperature-sensitive cell division cycle mutant *cdc30* with *PGI1*. This remarkable division cycle mutant is blocked in cell division at high temperatures on glucose, but is not blocked when it is grown on nonfermentable substrates. Dickinson and Williams[84] found that it carries an altered PGI isozyme, suggesting that there might be an interaction between the two unlinked genes, perhaps involving posttranscriptional modification of the enzyme in the absence of the wild-type *CDC30* activity.

V. THE REGULATION OF GLYCOLYSIS

There is some disagreement in the literature on the degree of constitutivity or inducibility of the enzymes in the glycolytic pathway. Maitra and Lobo[199,200] found that the synthesis of the enzymes in this pathway appears to be influenced by products of hexokinase. Key enzymes in the pathway showed substantial levels of induction when cells were transferred to glucose medium after growth on gluconeogenic media. On the other hand, Clifton and Fraenkel[67] and McAlister and Holland[215] found that the levels of gluconeogenic enzymes did not change greatly on either glucose-containing or gluconeogenic media over a variety of conditions. This difference in results may be due to strain-specific effects, and the *gcr* glycolysis regulatory mutant found by Clifton and Fraenkel (see the following) might also be strain-specific in its effects on glycolytic enzyme synthesis, though this possibility has not been explored.

Because most mutants that repress several enzymes of glycolysis are likely to prevent gluconeogenesis as well, entirely prohibiting cell growth, it has been difficult to isolate mutants with a strong repressive effect on this central pathway. Similarly, it has been difficult to find mutants in which glycolysis is permanently derepressed, perhaps because such mutants are likely to fall victim to futile cycling when grown under conditions leading to the simultaneous activity of gluconeogenic enzymes. Thus, workers have been confined to the study of leaky mutants. The most thoroughly investigated of these has been named *gcr* (for glycolysis regulation).

A. The *gcr* Mutation

Clifton et al.[68] initially selected a number of mutants unable to grow on glucose but able to grow on pyruvate. They found that a subclass of these were able to grow on pyruvate alone but were not able to grow on this same medium when glucose was added. One of these latter was deficient in pyruvate kinase.[65] Its inability to grow in the presence of glucose is presumably due to the accumulation of PEP.

Clifton et al. reasoned that a search for suppressors of this mutation would pick up mutants blocked earlier in glycolysis. It might also pick up mutants in which some or all of the steps in glycolysis were conditionally repressed. This property could be used to select for mutants defective in the regulation of at least part of the glycolytic pathway.

Suppressors of the *pyk* phenotype, selected for their ability to grow slowly on glucose-pyruvate medium, were investigated. These mutants proved to have markedly reduced levels of several glycolytic enzymes when grown on nonfermentable substrates, but were still able to grow moderately on glucose. The gene was named *gcr* (for glycolysis regulation).

Clifton and Fraenkel[67] showed that in the *gcr* mutant the majority of glycolytic enzymes were synthesized to less than 5% of wild-type levels on gluconeogenic compounds, but were synthesized to 20 to 50% of wild-type levels on sugars. (Enzymes that were relatively unaffected were hexokinase, glucose-6-phosphate dehydrogenase, and phosphofructokinase.) This reduced level of activity of some of the glycolytic enzymes on sugars was apparently enough to reduce the concentration of inhibitory compounds accumulating in the cell. This, in turn, permitted slow growth of the *pyk gcr* double mutant.

The *gcr* phenotype was connected with lowered mRNA levels of the affected glycolytic enzymes. It appears that its inability to grow on gluconeogenic compounds can be explained by this reduction since it would be unable to synthesize the required levels of those glycolytic enzymes which are involved in both glycolysis and gluconeogenesis.

These workers also made the interesting observation that on minimal medium the growth pattern of the mutant was reversed. On this medium it was unable to grow appreciably on lactate but was able to grow at least as well on glucose and maltose as the wild type. It will be instructive to investigate the relative roles of the glycolytic and gluconeogenic pathways in this mutant when it is grown on a variety of media. When wild-type cells are grown on minimal medium supplied with nonfermentable substrates such as ethanol or lactate, the gluconeogenic pathway is normally highly derepressed.[342] The steps shared by glycolysis and gluconeogenesis that are affected by *gcr* might be limiting in this case, which would explain this mutant's slow growth on such a medium. The increased growth of the mutant on minimal medium with glucose as a carbon source, compared with its growth on complete medium with the same substrate, however, remains a mystery. It may be that the slow growth on complete medium with glucose is not the result of the somewhat repressed levels of glycolytic enzymes (which are present in high abundance in the cell in any case), but rather the result of some other pathway which *gcr* affects.

The wild-type *GCR1* gene was cloned by complementation of its growth phenotype on glucose[163] and later sequenced and disrupted. The disruption was thorough, involving an insertion of *LEU2* in the place of a 4-kbp deletion spanning the entire *GCR1* reading frame,[9] or an insertion of *URA3* in place of a deletion spanning 90% of the reading frame.[146] In both these studies, it was found that the phenotype was the same as in the original *gcr1* mutation.

Survival of the integrant cells indicated that *GCR1* is not essential to the cell. The activity profiles of 12 enzymes in 9 different strains showed no significant difference between the original *gcr1* mutant and disrupted or disrupted-deleted strains.[9] The *GCR1* gene is a large ORF of 844 codons, showing very little bias in codon usage. This suggests (in view of the strong bias found in glycolytic and other highly expressed genes)[18] that this regulatory protein is expressed at sufficiently low levels that tRNA availability is not a factor in its translation.

Both normal and overproduced levels of glyceraldehyde-3-phosphatase and enolase were reduced about 50-fold in strains carrying the original or the disrupted *gcr* mutation, indicating that it codes for a positive transcriptional regulator.[146]

There is some indication that the GCR product may bind to DNA since it shows an alpha-turn-alpha region near the carboxy terminus of the polypeptide. The sheer size of the molecule, and its variety of phenotypic effects, suggests that like other regulatory proteins in yeast it may be involved in a number of regulatory processes. Because it does not completely repress the affected glycolytic enzymes, it appears to be required only for high-level expression.

The complexity of the interaction of GCR and other regulatory proteins with the regulatory regions of the glycolytic genes that they affect is illustrated by the story of the enolase loci. Enolase, which interconverts 2-phosphoglycerate and phosphoenolpyruvate, can take part in both glycolysis and gluconeogenesis, and perhaps because of this there are two unlinked genes in yeast, *ENO1* and *ENO2*, coding for distinctly different isozymes. These share, as do the major isozymes of alcohol dehydrogenase, 95% of their amino acids.[145] In contrast to the alcohol dehydrogenase situation, in which one isozyme is constitutive while the other is derepressed only in the absence of glucose, both enolase isozymes are synthesized in approximately equal amounts on nonfermentable substrates. When the cells are grown on glucose, however, enolase 2 is increased 20-fold. Both isozymes can serve in glycolysis. There seems, at least on the media tried, no effect on growth rate when the *ENO1* gene is deleted.[214]

Uemura et al.[323,325] located an 87 bp upstream activation site (UAS) 300 bp from the ENO1 transcription initiation site. UASs are specific regions of the DNA, upstream from the structural genes, that bind to regulatory proteins. The functionality of this 87 bp segment was demonstrated by replacing the UAS of the *PHO5* gene with it, resulting in enhanced transcription of *PHO5*.[324]

Cohen et al.[69] found that the situation at the *ENO2* locus was somewhat different. They located two upstream UASs flanking a point 461 bp upstream of the transcription initiation site. Deletion analysis showed that each of these UASs was sufficient for glucose-dependent induction. This region, like many other UASs, could function in reverse orientation, and it was found to be homologous with a similar site in the UAS of *pgk*.[291] When both these *ENO2* UASs were deleted, the gene was not expressed. Cohen et al.[70] later investigated the regulatory region of *ENO1* further and showed that in addition to the UAS there was a repressor site between it and the structural gene that was responsible for negative regulation. This negative regulatory element appears to be more important than the UASs in regulating the differential expression of the two enolase genes since either *ENO1* or *ENO2* UASs, placed upstream of *ENO1*, resulted in similar regulatory patterns.

In would appear at first sight that the *ENO* genes should be a prime target for regulation by *GCR1*, but it has proved very difficult to see what relationship the genes have to each other. A small increase in the transcriptional level of *ENO1* in an *ENO1-lacZ* fused gene was observed in a multi-copy plasmid carrying the *GCR1* gene.[324] Machida et al.[195] used gel retardation assays to demonstrate that three different protein species could bind to the *ENO1* UAS. DNA protection analysis showed that all three of these factors bound to one half of each of two dyad-symmetrical regions in the UAS.[195] Such dyad-symmetrical regions have been found in most of the UASs of yeast so far investigated.[32,148]

One of these species, S1, was increased relative to the others when the cells were grown on glycerol plus lactate. When cells were transformed with a multi-copy plasmid carrying *GCR1*, there was no effect on any of the three species when they were grown on glucose. There was, however, a slight increase in

the sum of S2 and S3 when the transformed cells were grown on glycerol plus lactate.

These observations suggest that *GCR1* is not the only regulator of this gene, and indeed its effects appear to be relatively minor. They reinforce the point made earlier, that mutants with a dramatic effect on expression of centrally important glycolytic genes such as the *ENO* genes will prove difficult to find and study.

B. Heat Shock Elements and UASs

Heat shock proteins in a great variety of organisms including yeast are induced to high levels by a sudden increase in temperature from 25 to 38 or 42°C.[185] Heat shock elements, or HSEs, are found upstream from the genes coding for these proteins and are characterized by a short imperfect palindromic sequence.[220] They are the sites for binding of heat shock transcription factor. The mode of action of this factor is complex. Once it is bound, it probably binds in turn to another protein transcription factor already associated with the TATAA box, in order to initiate the remarkably high levels of transcription seen in this system.[352]

This latter factor is required for normal transcription initiation and has recently been cloned and sequenced.[93,137] It turns out to be identical to a factor already known, SPT15, which suppresses Ty element insertions. In its guise as a TATAA-binding factor, it has been named TFIID, and forms the nucleus of an active transcription complex with other transcription factors, TFIIA, B, and E.[40] The characterization of this protein is an important advance and will allow its interaction with heat shock transcription factors and many other regulatory proteins to be analyzed in detail.

Evidence for a negative regulator acting on the hsp70 HSE has also been found,[285] so that the HSE region and the regions downstream from it seem to have much in common with UASs.

In yeast, unlike other organisms investigated, it appears that the heat shock transcription factor must be phosphorylated before it can bind to the HSE.[287] The promoters of some yeast glycolytic genes have also been found to be activated by heat, showing kinetics similar but not identical to those of the heat shock proteins. Phosphoglycerate kinase and one of the three glyceraldehyde-3-phosphate dehydrogenase enzymes, designated *hsp35*, have been found to be made as abundantly as heat shock proteins after a jump from 25 to 38°C,[252] though their synthesis drops markedly relative to HSPs if the cells are subject to a more extreme jump to 42°C.[251]

A very diagrammatic representation of how all this might work is shown in Figure 3.

Piper et al.[250] deleted the presumptive HSE upstream from the phosphoglycerate kinase gene and found that it abolished the heat shock response. These authors suggest that the incorporation of an HSE into the promoter region of certain metabolic genes may be an adaptation to ensure that glycolysis and other essential processes continue even after a strong environmental insult.

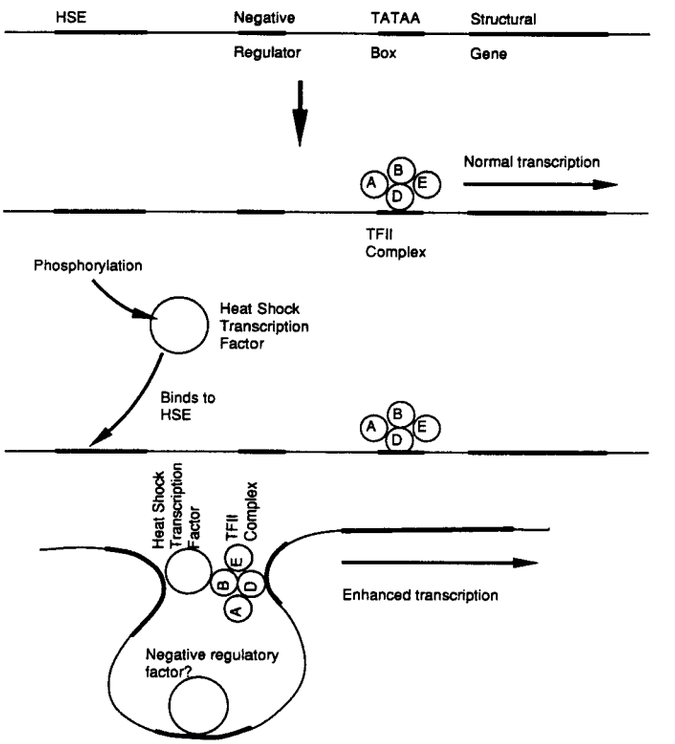

FIGURE 3. A very diagrammatic view of the interactions of the upstream heat shock element, and the transcription factor that binds to it, with the TATAA box and the TFII transcription element family that bind to that region. The binding of TFIID directly to the TATAA box, followed by the binding of TFIIA, B, and E, permit the initiation of normal levels of transcription. When the temperature is raised, the heat shock transcription factor is phosphorylated (in yeast, though this is not apparently necessary in all organisms), permitting it to bind to the heat shock element (HSE). The TATAA box and HSE are then brought into proximity, permitting greatly enhanced transcription of the heat shock protein or glycolytic enzyme protein. A negative regulator which also modulates transcription in at least some yeast systems is also shown. Presumably this is mediated by yet another regulatory protein, though no details are yet available.

VI. THE REGULATION OF GLUCONEOGENESIS AND OTHER HIGHLY REPRESSIBLE PATHWAYS

It is much easier to find and study mutants that affect the regulation of anabolic enzymes, or enzymes that are induced by growth on substrates other than glucose or fructose, than it is to find mutants with a dramatic effect on glycolysis.

In *S. cerevisiae*, glucose appears to be the substrate of choice — given a mixture of glucose and mannose or glucose and fructose, the glucose will be metabolized first.[295] This preference is mediated through carbon catabolite repression, in which glucose present in the medium has the effect of repressing a great variety of both catabolic and anabolic enzymes not directly concerned with its metabolism.

The term "carbon catabolite repression" was coined by

Magasanik[196] and was carefully chosen to convey the idea that it may not be the sugar itself that is directly acting as a repressor, but a catabolite of it.

Gancedo and Gancedo[121] have expertly summarized the bewildering array of mutants, discovered by many different groups, that are involved in carbon catabolite repression in yeast. Their Table 2 lists eight mutants, two of which are alleles, that do not permit the derepression of enzymes once the fermentable carbon source has been removed from the medium. Their Table 3 lists 26 mutants that permit enzymes normally repressed to be synthesized. Some of the mutants affect only one enzyme; some affect several. The multienzyme effects are difficult to compare directly since different workers tested different lists of enzymes. However, even this partial determination of their properties reveals an interlocking palimpsest of regulatory levels quite daunting to anyone used to working with prokaryotes. An exhaustive survey of the properties of all these mutants is beyond the scope of this article. We will explore some of those that have been well-studied and have particularly interesting properties, with an eye to searching for regulatory characteristics that they might have in common. We begin by examining some of the features of the regulation of transcription.

A. The Nature of Yeast Upstream Activation Sites (UASs)

The UASs of yeast have properties that distinguish them from operator and repressor loci found in bacteria.

To begin with, there do not appear to be single operator or repressor loci in yeast or other higher organisms that are directly analogous to those that are found in bacteria. However, a variety of regulatory regions, groupable into positive and negative control sites,[130] have been discovered. Positive regulators not associated with TATAA boxes have been designated UASs[125] and may sometimes be hundreds of bases upstream from the initiation site. They can exert their regulation independently of the TATAA boxes since these boxes have been deleted from the *CYC1* gene[132] and the *HIS3* gene[293] without affecting the regulation exerted by their still-intact UASs. The UASs examined so far are sometimes but not always highly conserved and may or may not show rotational symmetry. For example, the HAP1 activator protein binds to the UASs of the cytochrome *c* genes *CYC1* and *CYC7*, but, aside from the fact that these UASs both have lengths of about 23 bp, they show no obvious resemblance to each other.[247]

Furthermore, these activation sites provide another level of control over and above that seen in bacterial systems. If bacterial genes are controlled by more than one regulatory pathway, this is accomplished by two or more sites at which transcription is initiated, and these sites may be at some distance from each other. This results in mRNAs of markedly different length, depending on which initiation site is utilized. However, in yeast and higher eukaryotes, several different UASs may interact with a single initiation site. This results in a very uniform family of transcripts even from genes under multiple forms of regulation, which may yield some advantage to the cell.

UASs exhibit yet another level of complexity, in that they can bind to more than one protein. This can make a specific UAS the target of more than one regulatory system. Such systems are not unique to yeast, but have been found in some viruses. The thymidine kinase promoter of herpes simplex virus binds more than one regulatory protein.[156] In lambda phage, the lambda repressor binds to the promoter of the repressor gene, but is replaced at exactly the same site by the product of the *Cro* gene during lytic infection.[258] It seems likely that similar situations will be found in many other organisms.

In yeast, the best investigated system involves the interactions of *UAS1*, a UAS found upstream about 250 bases from the cytochrome *c1 (cyc1)* gene. There are two regions of *UAS1*, A and B, both of which are required for activation.[174] Furthermore, the UAS is not activated beyond very low basal levels in heme-deficient cells, but can be activated strongly on addition of heme.[132] A protein RC2 which binds to region B was found to be present in heme-deficient cells grown in the presence of heme, but not in its absence.[6]

Pfeiffer et al.[248] showed that another protein, the product of a gene *HAP1*, could bind to region B, and that binding of HAP1 prevents the binding of RC2 and vice versa. Footprint analysis showed that while RC2 and HAP1 bound to essentially the same short region, the two binding patterns were slightly different. Evidence was found for another protein, RAF, which can form a complex with HAP1 and bind to the A region. Since RC2 binding would prevent the binding of this HAP1-RAF complex and the activation of the UAS, it may be that RC2 is a negative regulator of the locus.

This system has been mentioned here because it may form a paradigm for the complex and multivalent controlling elements that appear to be involved, as we will see, in both glycolytic and gluconeogenic regulation. The reader will note that it has strong similarities with the ways in which regulatory proteins bind to the UAS of *ENO1*. Multiple sites within UASs, each capable of binding to more than one regulatory protein, appear to be the rule rather than the exception.

Most recently, it has been shown that yeast UASs are very similar in their action to mammalian enhancer sequences.[12] The strongest evidence that their actions are similar was obtained by Kakidani and Ptashne[158] and by Webster et al.,[334] who showed that, when the yeast *GAL4* gene was expressed in mammalian cells, it could activate a number of different mammalian promoters into which the UAS_g binding site which binds the GAL4 product had previously been inserted.

Guarente[131] has recently summarized models of UAS action, in which it is assumed that the DNA between the TATAA box and the UAS loops back on itself. This would have the effect of putting the UAS activator and the TATAA box factor into close proximity, as we saw with TATAA and the heat shock

element, so that they can in turn interact with the RNA polymerase to initiate transcription. Because more than one factor can often bind to the UAS, it seems likely that the preferred model is one in which the activator protein interacts directly with the TATAA box factors, and the resulting complex binds to the RNA polymerase at the TATAA site. This seems more likely than a model in which each factor interacts separately with the polymerase. In the latter case, there would have to be many specific binding sites for the activator proteins on the surface of the RNA polymerase.

B. The Hexokinases

Carbon catabolite repression of a variety of gluconeogenic and other enzymes has been well studied.[255] However, glucose and other carbon sources may also act as inducers — glucose induction —[100] and this fact led to the uncovering of a complex story of competing regulatory systems that has been repeated with variations in other parts of the yeast genome.

Carbon catabolite repression in yeast is apparently mediated by mechanisms different from the cAMP-dependent system seen in *E. coli*.[357] The first step in the investigation of the mechanisms of this repression lay in the isolation of mutants, affecting both single enzymes and entire pathways, that were insensitive to it.

The story of hexokinase regulation begins with the processes by which hexoses are obtained from the medium. Invertase catalyzes the hydrolysis, chiefly extracellular in yeast, of sucrose into glucose and fructose. Monetenecourt et al.[222] isolated mutants with constitutive invertase production, by means of an overlay procedure in which mutagenized cells were first plated on a high-fructose medium, which normally represses invertase. They were then overlaid with reagents of a commercial system for the chromogenic detection of glucose. Invertase-positive colonies turned red.

Zimmermann and Scheel[356] obtained further mutants having a clearcut phenotype by an ingenious selective procedure. They utilized the fact that invertase, located on the outside of the cell membrane, can slowly break down raffinose to yield fructose. This surrounds the cell with a fructose "cushion". They coupled this with the observation that 2-deoxyglucose exerts carbon catabolite repression,[351] but that this repression can be overcome by the presence of sufficient fructose to swamp the effects of the phosphorylated 2-deoxyglucose intermediate. Cells producing large amounts of fructose, and therefore carrying a derepressed invertase, should be able to grow on raffinose-2-deoxyglucose medium.

The mutants they isolated in this way could be attributed to two genes. The first, *hex1*, was no longer repressible for a variety of glycolytic enzymes and for some mitochondrial enzymes such as cytochrome *c* oxidase.[218,100] The *hex1* mutant was discovered to be missing one of the isoenzymes of hexokinase, designated PII.[103] This was in fact a defect in the structural gene, for its allelism to the structural gene *hxk2* obtained earlier by Lobo and Maitra[187] was demonstrated by Entian.[98]

The second mutant, *hex2*, did not appear to be a defect in a structural gene. It showed elevated rather than depressed levels of hexokinase PII when grown on fermentable substrates and was sensitive to maltose. The mechanism of this maltose sensitivity, which proved to be very useful in isolating subsequent mutants, was shown to be due to uncontrolled maltose uptake and a consequent high intracellular concentration of glucose.[97]

These were exciting observations since both the absence of PII and its overproduction had pronounced pleiotropic effects on the cell. These findings suggested the possibility that it is the levels of PII that determine the level of glucose repression since hexokinases catalyze the first steps of glycolysis.[99] It seems equally possible, of course, that the levels of PII seen in the *hex2* mutant are a response to the increased glucose levels in the cell and are unconnected to the effects of *hex2*. (It may be feasible to determine the effects of *hex2* on the cell with more precision since the gene has now been cloned.[234]) Certainly, the *hex1* mutant, by whatever mechanism, appeared to reduce the amounts of glucose repression markedly.

A third mutant, *cat80*, was isolated on galactose-2-deoxyglucose medium.[355] It was found that the presence of the wild-type gene was necessary for the production of elevated hexokinase in *hex2* mutants.[105] This mutant, however, unlike *hex2*, was not found to be sensitive to maltose.

Yet another layer of regulatory mutants could be produced, starting with a *hex2* strain and screening for mutants resistant to maltose.[96] Two of these, *cat1* and *cat3*, had a variety of pleiotropic effects. The most notable of these were the prevention of derepression of maltose-metabolizing enzymes, along with similar effects on a number of enzymes of the glyoxylate shunt and gluconeogenesis. The *cat1* and *cat3* mutants had, however, no effect by themselves on invertase regulation.[231] Because these mutants are epistatic to *hex2*, this suggests that they affect an earlier regulatory step.

Because of its clearcut effect in preventing glucose repression, the *hxk2* (né *hex1*) mutation, which you will recall affects the structural gene for isoenzyme PII of hexokinase, has undergone intensive investigation. The catalytic function of the enzyme is not involved in its regulatory capabilities since mutants retaining this function have been found that still prevent glucose repression.[100]

Posttranscriptional modification of PII appears to be ruled out as a triggering signal for carbon catabolite repression. This is because of the finding that PII did not appear to break down in the course of carbon catabolite repression, even when a search for such breakdown products was carried out using a strain transformed with a multicopy plasmid carrying the PII gene.[108] Phosphorylation of the protein may, by producing a conformational change, provide an alternative mechanism for its regulatory abilities; the kinetics of phosphorylation and

inactivation of the enzyme are the same, though no connection has yet been found with its effects on carbon catabolite repression.[109]

An encouraging avenue of research has been opened up by the discovery of potential alternative initiation patterns for this gene. An additional hexokinase PII isoenzyme, PIIM, is found in wild-type yeast,[169] but not in one of five mutants in the gene with altered regulatory but unaltered catalytic properties.[101] Sequencing of *HXK2* revealed two minor initiation sites inside the coding region, suggesting that an alternative transcription start might provide a possible mechanism for the production of this modified isoenzyme.

Further studies of this transcriptional modification may provide clues to the difference between the wild type and the *hxk2* mutants. In view of the web of interacting regulatory mutants that have been discovered to contribute to carbon catabolite repression, this gene may not have quite the primacy its discoverers suggest,[102] but it obviously plays an important role.

C. Other Genes Regulating Carbon Catabolite Repression

The *hxk2* mutant is not unique in its effects. In addition to the somewhat similar *cat80* mutation, a nonallelic mutation *grr1* has been isolated through galactose-2-deoxyglucose selection that results in constitutive synthesis of invertase, maltase, galactokinase, and cytochrome *c* oxidase, and that shows elevated hexokinase levels.[8] A similar recessive mutant, *glr1*, was found by Michels and Romanowski,[219] who isolated it by its ability to grow on maltose and glucosamine.

Dominant mutants with similar effects have been isolated by a variety of methods. Ciriacy[63] utilized the fact that a strain lacking the constitutive alcohol dehydrogenase, ADHI, grows slowly on high glucose, conditions under which mitochondrial activity is repressed. This is because the mutant cell has difficulty producing ethanol and thus completing the fermentative pathway. Mutants of an *adh1* strain that grew well on high glucose were found to carry a mutation, *CCR80*, which, in addition to derepressed mitochondrial functions, was also found to be derepressed for malate dehydrogenase and several enzymes of the glyoxylate cycle. Ciriacy suggested that this mutant provided evidence that all these functions, both cytoplasmic and mitochondrial, might be under common control.

Once again, however, it was possible to find mutants that affected the regulation of specific parts of these pathways, independently of the others. Böker-Schmitt et al.[29] overlaid cells grown on high glucose with triphenyl tetrazolium chloride (TTC). Cells able to respire aerobically reduce the TTC to the insoluble red pigment 2,3,5-triphenylformazan, so this screen allowed them to select for cells with high levels of mitochondrial activity even in the presence of inhibiting levels of glucose. Two apparent alleles at a locus permitting aerobic respiration under these conditions, *CCR91* and *CCR96*, were found to have high levels of mitochondrial protein synthesis and enzyme activity, but maltase and malate synthase (the latter a glyoxylate cycle enzyme) showed normal levels of repression. Thus, these mutants were clearly distinct from *CCR80*.

Interestingly, these workers reported that this selection scheme produced a number of other mutants with similar phenotype, but a cytoplasmic mode of inheritance. This methodology should provide an opportunity for investigation of the regulation of nuclear activity by the level of activity of the mitochondrial genome, a phenomenon that had previously been detected by the patterns of gene translation in grande and petite cells.[246]

These mutants have an effect indistinguishable from that of *CCR80*, but exert this effect on only some of the enzymes that *CCR80* influences. Further study of these mutants will almost certainly reveal a story of UASs which can bind more than one transacting regulatory protein. A detailed study of the dynamics of these binding processes will eventually elucidate how some enzymes and pathways appear to be under more than one co-equal system of control. It will also eventually explain the puzzling fact that disruption of any of these binding processes appears to disrupt the entire regulation of that gene.

D. The GAL System

One of the best understood examples of this multiple regulation is provided by the control of three genes involved in galactose metabolism, *GAL1*, *GAL7*, and *GAL10*. These catalyze steps in the production of UDP-galactose, a precursor for lactose formation and the production of polysaccharide side-chains in glycoproteins.[87] All three of these genes are repressed in the absence of galactose.[290]

The expression of the *GAL* genes is affected by *cis*-acting UASs upstream from the initiation of transcription of the *GAL1-GAL10* pair and of *GAL7*. In the case of *GAL1* and *GAL7*, the genes are in opposite orientation and the UAS lies between them.[353] This permits divergent transcription.[133]

The GAL UASs have been characterized in detail.[335] They have been found to interact with at least two *trans*-acting elements, the effect of which is to communicate alterations in the cell environment to the genetic system.

The first of the major *trans*-acting elements is a positive regulator, coded for by the *GAL4* gene.[88] This regulatory protein is required to initiate transcription.[94] The second is a negative regulator, *GAL80*.[207] This is the type of regulator familiar to workers on *lac* and similar operons in *E. coli*. Its product normally binds to the regulatory region of the DNA, and must be released before transcription can be initiated.

GAL4 transcription seems to be constitutive, but the quantity of *GAL80* transcripts can be increased five- to tenfold by a shift in the medium from glycerol or glucose to galactose.[280] The gene products of the positive and negative regulators (determined to be 9.9 kDa for GAL4[139,180] and 4.2 kDa for GAL80[236]) were also found to interact with each other in a remarkable way.

When the GAL4 protein was bound to the *GAL1-GAL10* and the *GAL7* promoters, DNA footprinting experiments initially identified two protected regions at each promoter.[31,301,125] Later experiments suggested there are at least four[276] — though deletion analysis showed that not all may contribute equally to promotion.[335]

The amount of GAL4 product present in the cell is normally high and not limiting. If multiple copies of the *GAL4* gene are introduced, there is only a small increase in the galactose-induced synthesis of GAL1, 7, and 10.[154,155,178] However, in a strain carrying more than 200 copies of *GAL7* and one copy of *GAL4*, the amount of GAL7 transcript was increased 13-fold, indicating that GAL4 product is in fact limiting in this extreme situation.[11]

When the low-affinity binding sites for GAL4 at the *GAL1-GAL10* UAS were examined singly and in combination, it was found that they acted synergistically in stimulating transcription, indicating that binding can be cooperative.[124]

The binding sites of GAL4 were examined in more detail. There are two highly acidic activating regions in the GAL4 molecule, but aside from this enrichment for acidic residues they show no detectable homology with each other.[194] When random *E. coli* DNA fragments were hybridized to the segment of the *GAL4* gene that specifies DNA binding, these fragments were also found to code for peptides rich in acidic residues.[193] The result of these experiments suggests that the binding of these acidic protein regions to a TATAA factor or to the RNA polymerase itself can be rather nonspecific, though exactly how much latitude can be allowed in this interaction remains to be determined.

A model proposed by Oshima[239] suggested that, in the absence of galactose, the GAL4 and GAL80 gene products bind together and are rendered inactive. When galactose is introduced, the complex breaks up and GAL4 product is free to become a positive regulator.

This basic model has undergone some modifications as the complexity of the system has been revealed. Baker et al.[10] constructed strains with high numbers of the *GAL4* gene. They found that, while there was no great effect on transcription of a single *GAL7* gene as a result of increasing the number of copies of *GAL4*, this increase could produce a 50-fold increase in transcription if the strain also carried many copies of *GAL7*. Thus under normal circumstances, the amount of GAL4 product is saturating if there is only one target gene present, but it would not be enough to exert control if there were many copies of the target gene and only one *GAL4* gene. This reinforces the conclusions reached earlier by the same authors[11] from experiments in which only the dosage of *GAL4* was altered.

The amount of *GAL80* can also be limiting if enough target sequences are present. Disruption of the *GAL80* gene had rather little effect in strains with multiple copies of *GAL4*, indicating that the negative regulation exerted by the GAL80 product when only one *GAL4* gene was present could be overcome if there were sufficiently large amounts of *GAL4* transcript. If a GAL4-GAL80 complex is indeed formed, its effects can apparently be overcome by increasing the amount of GAL4 present.

Another level of interaction was observed. Baker et al.[11] had earlier found that if the copy number of *GAL7* was increased, the amount of transcription of *GAL10* was reduced. The most reasonable explanation was that GAL4 product was in short supply in such a strain, or perhaps that released *GAL80* product became bound to *GAL7* UAS regions. However, even when high-copy-number plasmids carrying *GAL4*, or plasmids carrying a disrupted *GAL80* gene, were introduced, the transcription of *GAL10* remained low.[10] It therefore seems likely that yet another transcription factor is present and necessary for transcription of the structural genes and that when there are large numbers of copies of *GAL7* they compete with *GAL10* for this factor. Reciprocal experiments in which *GAL10* copy number is varied while *GAL7* number is held constant may cast some light on this.

The amount of *GAL4* transcript does not appear to be affected by the medium on which the cells are grown.[179] However, this is not the case with *GAL80*. Shimada and Fukasawa[280] found that the transcription of *GAL80* increased tenfold on transfer of the cells from glucose to galactose. It was then found that this regulatory gene is actually regulated (at least in part) in a very similar way to the structural genes it affects.

This became apparent as the regulation of *GAL80* began to be investigated. Nogi and Fukosawa[236] found a UAS-like sequence upstream of *GAL80*. This sequence appears to be functional and to be very similar to the UASs of the structural genes. Remarkably, it turned out that GAL4 protein binds at this UAS.[32]

Igarishi et al.[152] then fused *GAL80* to *lacZ* and to structural genes that were in turn fused to *lacZ*, which could be used as a marker for induction. They were then able to show directly that *GAL80* is regulated by galactose in a fashion very similar to the genes that it regulates in the galactose pathway. They also found that the *GAL80* UAS by itself could regulate a *GAL7-lacZ* fusion. The results of these experiments force these authors to suggest that the uninduced levels of *GAL80* must be sufficient, in combination with *GAL4*, to repress the genes of the galactose pathway. However, it would seem counterproductive on the part of the cell to have the levels of the negative regulator *GAL80* increase with the introduction of galactose, unless the further unraveling of this complex of genes shows that the role of *GAL80* is not that of a simple negative regulator. It seems likely that the *GAL80* product interacts, on galactose induction, with yet another regulatory molecule. Hidden complexities abound in this system as well.

E. Regulation of Alcohol Dehydrogenases

The genes regulating the alcohol dehydrogenases have turned out to be similarly complex and multivalent in their interactions.

The regulatory interactions between mutants discussed in this section are illustrated in Figure 4, which attempts to illustrate the relationship between those that act directly on the inducible ADH and those that affect the entire gluconeogenesis pathway.

Mutants in yeast alcohol dehydrogenases were first selected in *Schizosaccharomyces pombe* by Megnet[217] using allyl alcohol. This compound, harmless to the cell, is oxidized to the poisonous aldehyde acrolein. There are three major isozymes. ADHI and ADHII are found in the cytoplasm,[192,347] and the closely related ADHIII is found in the mitochondrial matrix.[59,346]

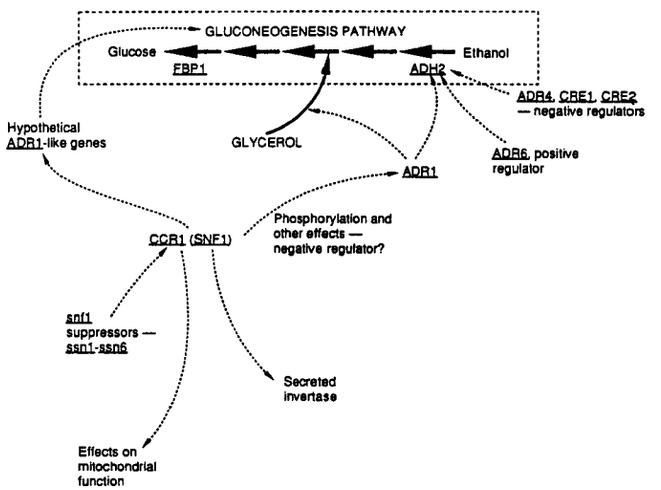

FIGURE 4. An outline of the effects of the regulatory genes discussed in the review that affect the gluconeogenesis pathway. Solid lines indicate metabolic pathways and dotted lines indicate regulatory effects. It is suggested that the *CCR1* gene may act on a set of *trans*-acting regulatory genes like *ADR1* that, in turn, regulate individual steps in the gluconeogenesis pathway. However, these have not yet been found. Note the polyvalent regulatory activities that have so far been discovered for *CCR1* and *ADR1*.

It was shown that cells lacking the cytoplasmic isozymes cannot survive when they are converted to petites by ethidium bromide[346] since they are unable to generate NAD^+ from either glycolysis or mitochondrial activity. Ciriacy later took advantage of this observation by utilizing Antimycin A, a specific inhibitor at the level of cytochrome *b* in the respiratory chain, to produce reversible petite phenocopies.[65] This enabled Williamson et al.[337] to clone the gene for ADHI by complementation, the first time cloning by this method had been carried out in yeast. Transformants of an ADH-negative strain by plasmids carrying a wild-type library were screened for their ability to grow in the presence of antimycin. Cloning of all three of the ADH genes enabled the coding and flanking sequences to be determined for *ADH1*,[19] *ADH2*,[268] and *ADH3*.[354] An amino-terminal extension of 28 amino acids is involved in targeting the ADHIII product to the mitochondrial matrix. Pilgram and Young have recently shown that only about half of this presequence is actually required for the targeting. They also showed that the targeting is unaffected by the introduction of various point mutations into the sequence and that targeting cannot take place if the mitochondria are uncoupled.[249]

A very interesting ADH, expressed normally at low levels and therefore missed by all the workers in the field for many years, was recently found by Paquin and Williamson[245] through a screen for revertants of ADH-negative mutants that resulted from insertion of a Ty element. These insertions permitted growth on antimycin, and some of them were found to make the "revertant" strains constitutive for this new ADH. This enzyme is very distinct from the other ADHs of yeast and shows strong homology with an iron-activated ADH from the fermentative bacterium *Zymomonas mobilis*.[339] However, unlike the bacterial enzyme, it appears to be zinc activated.[89] The enzyme appears to have only limited utility in the cell, but the gene is functional and has not become a pseudogene. This enzyme appears to form an excellent candidate for horizontal transfer between bacteria and yeast in the course of evolution. An alternative possibility, however, is that enzymes of this type are widespread but as yet undetected in eukaryotic lineages, just as another candidate for horizontal transfer, the hemoglobin gene in the legumes, has turned out to be more widespread in the plant kingdom than previously thought.

Ciriacy isolated a number of regulatory mutants affecting the ADH structural genes. These have undergone some name changes in order to make the nomenclature more logical.[298] The newer names are used here.

Ciriacy found two genes that altered the regulation of ADHII.[60] One of these, *ADR3*, was tightly linked to the *ADH2* gene, and a variety of mutants were isolated in it. These mutants, and this region, are now designated as part of the *ADH2* gene itself. Several *cis*-dominant constitutive mutants were isolated by screening for mutants of the strain *adh1 ADH2 adh3* that were able to grow rapidly on high-glucose medium.[61] These were found not to affect enzymes of the glyoxylate cycle, so their effect appears to be confined to the *ADH2* gene itself. The *cis*-dominance was demonstrated by the fact that their effects were confined, in a diploid heterozygous for two allelic forms of ADHII, to the allelic form to which they were linked.

Further mutants at this locus were isolated from high-glucose medium containing antimycin.[64] The mutants fell into two classes, distinguishable by the degree of derepression of ADHII. The "promoter-up" mutants *ADR3-4*^{c} and *ADR3-5*^{c} produced high levels of enzyme, while a second class of mutants resulted in lower levels and seemed to interfere with the polypeptide itself, reducing the degree of subunit association. The molecular lesions of these mutants have been determined. The "promoter-up" mutants have been found to have a lengthened poly-A tract 5′ to the *ADH2* coding sequence. This is normally 20 residues long, but has been lengthened in the mutants to over 50.[269] Complete deletion of this region had no detectable phenotype,[282] leading the group doing the deletion work to conclude that this region is not important in ADHII regulation.

Their conclusion does not take into account, however, the dramatic effect of lengthening this poly-A tract, suggesting that certain classes of modification of this region can have a pronounced effect.

The second class of mutants resulted in constitutive ADHII production, though not to derepressed wild-type levels. They were all found to be due to the insertion of mobile Ty1 or Ty2 elements at various positions in the 5′ region.[338] It remains to be determined why these mutants, although they are outside the structural gene, seem to affect the association of the polypeptide subunits themselves, as Ciriacy had earlier observed.

The mechanisms by which these upstream mutations affect the translation of the ADH2 gene are beginning to be investigated at the level of the DNA structure itself, as well as from the standpoint of the interaction of the DNA with various proteins that bind to it. Della Seta et al.[76] have examined the topological changes found in the region upstream from this gene when torsional stress is applied to the DNA in the process of supercoiling and were able to predict that the pattern of this torsional stress ought to be greatly altered in up-promoter mutants.

In addition to the mutants in the regulatory region upstream of *ADH2*, the effects of a variety of unlinked loci on the expression of the structural gene *ADH2* have been investigated. One such gene of particular interest has been designated *ADR1*.[62] Mutants at this locus were found in two forms: recessive mutants *adr1* which prevented the synthesis of ADHII[62] and *trans*-dominant constitutive mutants *ADR1*^{c} which affected both alleles in a diploid.[64] The *ADR1* gene has been cloned,[80] sequenced,[138] and found to code for a protein of 151 kDa with a zinc finger region and some homology to the transcription factor TFIIIA from *Xenopus*. Furthermore, some *adr1* mutants were found to be located in the zinc finger region, indicating that this region is essential for function.[27]

The homology of this gene with the *Xenopus* transcription factor gives some hope that what appears at the moment to be a bewildering variety of transcription factors will turn out to be a relatively small number of related protein families.

The ADR1 product interacts with an inverted repeat region upstream from *ADH2*, as shown by deletion analysis.[282] These workers found that mutants in which this region had been deleted failed to respond to the presence of constitutive *ADR1* mutants.

Most recently, the ADR1 protein has been made in large quantity as a hybrid *ADR1*-β-galactoside fusion protein in *E. coli*. This hybrid protein was then bound to the two upstream activation sequences of ADH2. The strongest binding was to a palindromic sequence, and the binding was zinc dependent.[92]

Both of the zinc finger regions have been found to be necessary for sequence-specific binding.[311]

ADR1 is expressed constitutively. Its transcription is not affected by the conditions under which the cells are grown, or by the presence of alleles of the more broadly regulatory locus *CCR1*, or even by whether the ADR1 allele is wild type or a mutant conferring constitutivity on *ADH2*.[79] However, Blumberg et al.[28] showed that the levels of expression of *ADR1* are dependent on the yeast strain carrying it, with some strains showing only a 1- to 2-fold increase in *ADR1* mRNA after glucose depletion, and others showing a 10- to 20-fold increase. These authors showed that the amount of expression of *ADR1* had no effect on the level of derepression of the *ADH2* gene and obtained some evidence from experiments with an *ADR1* gene fused to *lacZ* that regulation of *ADR1* activity was not controlled at the translational level either.

This leaves posttranslational control, of which there could be a number of types. Perhaps the most interesting possibility, and one that is consistent with the *GAL4* story, is that of regulation by interaction of the ADR1 protein with a negative regulator.

Such a possibility is suggested by the structure of the ADR1 protein. Denis et al.[79] characterized one constitutive mutant at this locus, *ADR1-5*^{c}, as being due to an arg to lys substitution. They found that this substitution occurred in a region of the protein that is a probable recognition sequence for phosphorylation by cAMP-dependent protein kinase. It may, therefore, be that the phosphorylation state of this protein affects its regulatory functions. *CCR1*, which has been shown to have protein kinase properties (see the following), is a likely candidate for such an interaction and may play the role of the negative regulator.

Detailed deletion mapping of the *ADR1* gene has also demonstrated that it has more than one regulatory function. Analysis of a series of carboxy-terminal deletions of the *ADR1* gene[17] revealed that the amino-terminal 220 amino acids are absolutely required for transcriptional activation of *ADH2*. The analysis also provided evidence that much of the rest of the gene is involved to a lesser extent in activation of *ADH2* since shorter deletions also have an effect — though not so pronounced — on transcription levels.

When the putative phosphorylation region was deleted, the deletion mutant had no effect on the glucose sensitivity of *ADH2* expression. This shows that the constitutive mutants that had previously been located in this region, while integral to *ADR1* function, appear not to be involved in carbon catabolite repression per se. The *CCR1* gene (see the following) was shown to affect *ADR1* even when the protein kinase region was removed, indicating that, whatever the effect of *CCR1*, it is not entirely dependent on phosphorylation of this specific part of the ADR1 peptide.

Finally, the N-terminal half of the ADR1 molecule was found to be required for growth on glycerol, a quite unexpected observation that vividly demonstrates the polyvalent properties of this regulatory molecule.

Unsurprisingly, it turns out that *ADR1* is not the only gene involved in regulation of the ADHII isozyme's expression. Three negative regulators, *ADR4*,[62] *CRE1*, and *CRE2*,[77] have been described. A recessive mutant with positive regulatory

activity, *adr6*, was isolated by screening for mutants that had reduced expression of ADHII in a strain carrying the *ADH2-6*^{c} mutation.[300] (This latter mutation is due to the insertion of Ty1 upstream from the structural gene *ADH2*, so that it comes under the control of Ty1.[298])

The *cdc6* chromosome loss technique,[162] in which chromosomes are randomly lost in a *cdc6* homozygote at 34°C, was used to map *adr6* to chromosome XVI.

The *adr6* mutant was also found to repress the wild-type *ADH2* allele; however, it had no effect on isocitrate lyase or malate dehydrogenase, both of which are under carbon catabolite control.[16] Thus, since the regulatory element or elements affected by this mutant are not involved in carbon catabolite repression, it was suggested that *ADR6* may be involved in the production or stabilization of *ADH2* mRNA.[299]

The *adr6* mutant resembles the pleiotropic regulatory mutants *ccr1*, *2*, and *3* to the extent that when it is homozygous it is unable to sporulate — incidentally showing that it does, like most regulatory mutants in yeast, have pleiotropic effects. It does not, however, affect the same set of enzymes as the *ccr*, *cat3*, or *CCR80* mutants.

F. A Mutant with Effects on More Than One Enzyme — the *CCR1* Locus

Another mutant with much broader effects on carbon catabolite repression, but with equally polyvalent activities, had been isolated earlier, and this mutant turns out to interact with *ADR1* in a complex fashion. This is *ccr1* (ccr standing for carbon catabolite repression), reported by Ciriacy in 1977, along with two similar but less-studied mutants, *ccr2* and *ccr3*.[62] These mutants were isolated by starting with a strain deficient in ADHI, which cannot survive as a petite. Mutagenized cells of this strain were grown on ethanol medium with a small amount of glucose, transferred to glucose plates, and then checked for growth on ethanol, acetate, and glycerol. Those unable to utilize nonfermentable carbon sources were examined further, on the assumption that they were not petite since they lacked ADHI. Many of these mutants were found to be deficient in single enzymes of the gluconeogenic pathway, but some were found to be deficient in several. These were the *ccr* mutants.

When grown on dextrose to the point where dextrose is exhausted from the medium, wild-type cells derepress gluconeogenic enzymes. However, *ccr1* mutants fail to derepress a number of these enzymes: ADHII, isocitrate lyase, fructose-bisphosphatase, and the cytoplasmic malate dehydrogenase. The syntheses of TCA cycle enzymes or mitochondrial electron transport components were not affected. The inability to derepress these enzymes also prevents the *ccr1* strain from growing on nonfermentable substrates and gives it the phenotype of a petite. In fact, however, the inability to derepress these enzymes is not complete. When *ccr1* is grown on yeast extract and peptone in the absence of other carbon sources, it will go through about ten cell divisions and during that time derepress the enzymes of the gluconeogenic pathway quite normally.[343] Furthermore, the *ccr1* mutant appears to have abnormal mitochondrial metabolism. Isolated *CCR1* mitochondria are unable to utilize dicarboxylic acids to phosphorylate ADP, although the remainder of their metabolic functions appears to be unaffected.[343] It is unclear what relationship this defect in mitochondrial metabolism may have to the derepression process, but it seems very likely that they are related.

G. Relationship of *CCR1* to Other Regulatory Genes

The interactions of *CCR1* with other regulatory genes affecting individual steps in the same pathway have generated a good deal of interest. As with other systems we have examined, the interaction of these regulators has turned out to be highly complex. I have tried to diagram some of these interactions in Figure 4, which the reader might wish to look at as we delve into the complexities of this regulation.

The *ccr1* mutation was found to reduce the amount of ADHII made in the presence of the constitutive mutation *ADR1-5*^{c}.[64,77] Whatever the mechanism of this interaction, it would not appear to be at the level of transcription. Denis and Gallo[79] concluded that they were unable to affect the levels of *ADR1* mRNA in either the presence or absence of *CCR1* by manipulation of the environment. A translational or posttranslational mechanism, most likely the latter, would appear to be implicated.

It was, therefore, a reasonable supposition that the CCR1 product would be found to interact with the ADR1 product, and perhaps to be the negative regulator of *ADR1* that was mentioned earlier. Denis and Gallo cited work showing that a mammalian protein kinase had reduced ability to phosphorylate a synthetic peptide similar to that found in the *ADR1-5*^{c} mutation.[164] Thus, if the *ADR1* protein must be phosphorylated in order to repress, its relationship to *CCR1* with its phosphorylating activity would appear to be straightforward. However, if it must be phosphorylated in order to *derepress*, then the *ADR1-5*^{c} mutant product would be less phosphorylated than the wild type and would require the activity of a phosphatase. This phosphatase, in turn, would presumably have to be activated by phosphorylation, perhaps by *CCR1*, in what appears to be an unnecessarily elaborate set of regulatory interactions.

Denis[78] investigated in detail the effects of varying gene dosages of both *CCR1* and *ADR1*. Under conditions in which *ADH2* was repressed, increasing the number of *ADR1* genes had the effect of increasing the amount of *ADHII* mRNA linearly. When *ADH2* was derepressed, a similar increase in the number of *ADR1* genes produced only a three- to fourfold increase at maximum. This indicated that *ADR2* levels governed the regulation. He then showed that, while an increase in *ADR1* dosage could partly mask the *ccr1* defect, an increase in the number of *CCR1* genes had no effect on *ADH2* expression. Thus, it appears that the regulatory order is *CCR1-ADR1-ADH2*.

One particularly interesting observation was that the presence of a large number of *ADR1* copies was toxic to the cell, perhaps because of nonspecific binding of the ADR1 protein to various regulatory regions in the DNA.

Because of the large amount of information which has been accumulated about the regulation of *ADH2*, we may have a biased view of the pattern of regulation of the gluconeogenesis pathway as a whole. As I have suggested in Figure 4, it is possible that other steps in the gluconeogenesis pathway may be similarly regulated, with *CCR1* affecting *ADR1*-like genes that in turn affect the expression of individual steps in the pathway. Examples of such mutants may be the mutants that were investigated by Bañuelos and Fraenkel that either prevent the breakdown of fructose-bisphosphatase or allow it to be synthesized constitutively.[13]

If this should prove to be the case, it still remains to be determined why there should be such complex regulatory systems at each step in a metabolic pathway when it might be expected that one regulatory system should suffice. It is possible that yeast faces an environment of such diversity that to maximize its growth potential it must have the capacity for continuous fine tuning of the levels of each component of the pathway.

H. Regulation of Sucrose Fermentation

The polyvalent nature of the *CCR1* gene was revealed by the results of an investigation of quite another regulated system. A few years after Ciriacy's investigations, an allele of *ccr1* was isolated by quite a different procedure, and it turned out to have quite different effects. Marian Carlson and co-workers[47] isolated the first of a series of mutants that were unable to utilize sucrose, *snf1* (snf stands for sucrose nonfermentor). This mutant cannot derepress the production of the RNA that codes for the secreted form of invertase, which it will be recalled is present in the cell membrane and splits sucrose into glucose and fructose. However, the *snf1* mutation has no effect on the levels of the alternatively processed RNA, with a different 5′ end, that produces an intracellular form of the enzyme from the same gene.[48]

This mutant also had pleiotropic effects on the enzymes affected by *ccr1*, and it was eventually discovered to be allelic to it.[77] It is, however, considerably different from *ccr1* in its effects. The *ccr1* mutant, unlike *snf1*, is readily able to utilize extracellular sucrose (unpublished observation). Also, the *snf1* mutant appears to be generally more severe in its effects than *ccr1*. It will not grow on yeast extract and peptone alone, and although it will survive as an ADH negative and, therefore, has intact mitochondrial activity, it has not been possible to isolate coupled mitochondria from it to determine how their physiology has been altered.[343]

Because of its clearcut phenotype, the genetics of *snf1* have been intensively investigated. Carlson and co-workers[49] isolated suppressors of *snf1* that fell into eight complementation groups. Only one of these, *ssn6* (*s*uppressor of *snf*), made wild-type levels of enzyme, and it was also found to confer constitutivity for the secreted form of invertase even in a wild-type background. It caused the cells to clump. This mutant appears to be an allele of *cyc8*, a mutant that causes overproduction of iso-2-cytochrome c[266] and also causes the cells carrying it to clump.

The *snf1* gene has been cloned by complementation[54] and found to be located on the right arm of chromosome IV. The levels of its mRNA were found to be uninfluenced by either the presence or the absence of glucose. Disruption of the gene did not produce any additional phenotype.[55] Sequencing of the gene revealed that it has homology to three mammalian protein kinases (cAMP-dependent kinases from bovine cardiac and rabbit skeletal muscle, and pp60$^{v\text{-}src}$ from Rous sarcoma virus). Indeed, the homology is very high, approximately as strong as the three mammalian kinases exhibit among themselves. Furthermore, the wild-type *SNF1* allele appears to be able to phosphorylate itself at serine and threonine residues.[53]

While the function of *SNF1* remains unknown, another sucrose nonfermentor isolated by Carlson and her group,[231] *snf3*, is better understood. The defect has been traced to the high-affinity glucose transport system; the low-affinity system appears to be unaffected.[25] Immunofluorescence studies show clearly that the SNF3 gene product is localized in the cell membrane.[56]

The *SNF3* gene has been cloned and disrupted,[232] and its sequence has recently been determined.[56] The gene codes for a large protein of 884 amino acids, and residues 86 to 581 show 28% identity with the HepG2 human glucose transporter. The homology is even more strongly supported by the fact that both the human protein and the yeast protein have 12 putative membrane-spanning regions.

Two other mutants in the series, *snf2* and *snf5*, have been investigated.[1] These genes are involved in high level expression of secreted invertase since when they are disrupted they still permit low levels of expression of this secreted enzyme. Like *snf1*, they are unable to utilize galactose, maltose, or nonfermentable substrates. Production of their mRNAs is not subject to glucose repression. Abrams et al.[1] found that mutants carrying these genes were unable to derepress acid phosphatase under low phosphate conditions, suggesting to these authors that these genes are not involved in glucose regulation per se, but rather in a general regulation of high level gene expression for a great range of target genes, perhaps through effects on transcription or mRNA stability.

Mbonyi and Thevelein[213] found that the cAMP pulse induced in the cell by glucose (see the following) is still present in a *snf3* mutant, so that while a functional kinase is required to induce the pulse the transport protein is apparently not necessary. Sugars were added at 100 m*M*, however, which is a high concentration. It would be most instructive to see whether the cAMP pulse can be produced in *snf3* mutants in the presence

of levels of glucose that are low enough to ensure that very little will enter the cell through the low-affinity pathway.

Very recently, a fascinating synergism of the GAL and SNF systems has been used to demonstrate that SNF1 and SNF4 interact in the cell. Fields and Song[110] have constructed genes specifying a pair of hybrid proteins, consisting of SNF1 protein fused to the DNA-binding region of GAL4 and SNF4 protein fused to the transcription activating region of GAL4. In a cell containing both of these genes, SNF1 product associates with SNF4 product and the entire GAL4 activity is reconstituted. This will prove to be a valuable new tool for investigation of protein-protein interactions in yeast.

VII. THE ROLE OF cAMP IN CARBON CATABOLITE ACTIVATION AND INACTIVATION

A. Glucose as a First Messenger

There are both similarities to and differences between yeast and mammalian cells, so far as regulation of their metabolic pathways by external influences are concerned. In both types of cell, signals from the outside are conveyed though the cell membrane by a first messenger. In mammalian cells, the first messengers are hormones. A very important first messenger in yeast cells may be glucose. Yeast cells grown on 0.02% glucose will arrest in G_0/G_1, and when more glucose is introduced this stimulates inositolphospholipid turnover just as growth factors do in mammalian cells.[157] Furthermore, glucose appears to be involved in Ca^{2+} mobilization since addition of extra glucose after cell arrest increases both the influx and efflux of $^{45}Ca^{2+}$.[157] It is, however, unclear what the glucose sensor might be, and whether the receptor is in the membrane. Glucose can produce immediate effects such as a pulse of cAMP, or its effects can be delayed, and range through a gamut from modification of transcription rates to the initiation of processes that covalently modify proteins.

B. cAMP as a Second Messenger

The intracellular compound that has received the most attention in yeast for its role as a second messenger is cAMP, though it seems likely that there are other second messengers yet to be discovered. In Figure 5, I have sketched some of the pathways leading from glucose through cAMP to the cAMP-dependent protein kinase and shown some of the effects on enzymes and cellular processes that are influenced by the kinase. As will be seen, there is much left out of this figure, in particular alternative pathways. The most remarkable result that has emerged in recent years is that the cell is able to carry out most or all of these regulations in the absence of detectable levels of cAMP.

Glucose triggers an increase in cAMP levels. It is, however, only a transient increase, lasting 1 or 2 min, during which cAMP levels rise about fivefold in wild-type cells.[259] The pulse is comparable in size and duration to that seen on hormone stimulation in *Dictyostelium*.[203] It is assumed that this brief pulse is sufficient to begin a cascade effect, leading to a number of protein phosphorylations, which, in turn, result in activation or inactivation of enzymes of several different metabolic pathways.

This brief signal was studied in detail by Beullens et al.,[20] who used mutants in the early stages of the glycolytic pathway to show that the pulse was apparently triggered by events very early in gluconeogenesis. Mutants blocked in phosphoglucoisomerase exhibited the pulse, but mutants blocked in the first phosphorylation step catalyzed by the hexokinases or by glucokinase did not. If the mutant lacked the hexokinases but still had glucokinase, then addition of fructose produced no pulse while addition of glucose resulted in a pulse. Glucose-6-phosphate and fructose-6-phosphate, the products of these kinases, peaked immediately after glucose addition, while fructose-1,6-bisphosphate peaked somewhat later. It is not yet possible to tell which if any of these molecules might be the trigger for the cAMP pulse.

Very interesting evidence was obtained that the triggering mechanism itself involves a glucose-repressible step, mutants in which can be isolated. Wild-type cells grown on glucose do not show a pulse when more glucose is added, strongly suggesting glucose repression. However, an *hxk2* mutant, defective in glucose repression, does show a pulse when grown on glucose, suggesting that in this mutant the gene product or products involved in the production of the cAMP pulse are synthesized constitutively.

VIII. CARBON CATABOLITE (SUBSTRATE-INDUCED) ACTIVATION AND INACTIVATION

Once the enzymes of a pathway, such as that of gluconeogenesis, have been synthesized to high levels, it may be inappropriate for the cell to retain them if a fermentable substrate has been reintroduced. Mechanisms have evolved for their rapid destruction within the cell. The early work on this phenomenon was reviewed by Holzer,[147] who coined the term catabolite inactivation. At the same time and in the same cells, catabolite *activation* can occur,[308] and in some cases the mechanisms involved are very similar.

Thevelein points out[308] that catabolite activation and inactivation may not be very accurate terms since it is certainly not the catabolite itself that is the primary agent. He suggests substrate-induced activation or inactivation as more meaningful terms.

Many examples of such activation and inactivation have been studied, some in considerable depth. These studies have led to a greater understanding both of these processes and of the regulatory systems that lie behind them. As with the other systems we have surveyed in this review, redundancy and a complex interlocking web of regulators are the rule rather than the exception.

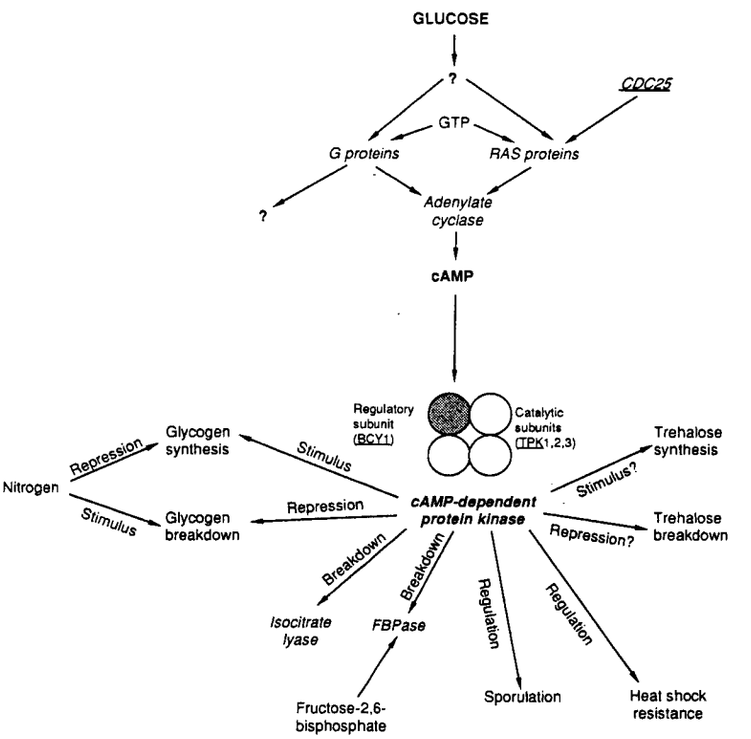

FIGURE 5. The pathways leading to the regulation of the cAMP-dependent protein kinase, and the various cellular functions that it has been found to regulate in turn. Included in the diagram are some of the other factors that are known to interact with this regulation, such as nitrogen and fructose-2,6-bisphosphate. It should be noted that these processes continue to be regulated fairly normally even in the absence of cAMP, so long as the kinase has been permanently activated through the disabling of its regulatory subunit. This suggests the existence of another type of regulation of the kinase, or even a completely separate regulatory system.

Substrate-induced inactivation has been most studied in, but is not confined to, the enzymes of the gluconeogenic pathway. After growth on a gluconeogenic substrate and the rapid addition of glucose, many unneeded enzymes are rapidly inactivated rather than simply diluted out by further cell growth. These include, among others, the major cytoplasmic form of malate dehydrogenase[91,107,230] and isocitrate lyase.[57,190] Both of these enzymes are required for the operation of the glyoxylate cycle, which as we saw earlier is central to the metabolism of ethanol. Other gene products reported to be inactivated in this fashion include a secreted proteolytic enzyme, aminopeptidase I,[116] and the proteins that are involved in the maltose uptake and utilization system[126] and the galactose uptake system.[206]

A. Substrate-Induced Inactivation of Glucose Transport

Gene products other than enzymes are also subject to carbon catabolite repression. The regulation of glucose transport forms an excellent example. The early work of Serrano and de la Fuente[278] came to the conclusion that there was a single system for glucose transport into the cell, with different degrees of affinity depending on the presence or absence of oxygen. It was shown more recently that there are in fact two systems. The first is a system with high affinity (low K_m) for glucose that is subject to catabolite repression. The second system is constitutive and has a low affinity (high K_m) for glucose.[24] The low-affinity system can continue to operate even under nonmetabolizing conditions.[332] Both the high- and low-affinity systems appear to operate by carrier-mediated facilitated diffusion.

Both the low-K_m glucose transport and the transport of fructose are affected by the presence or absence of glucokinase and the two hexokinases.[22,23] Analysis of triple mutants lacking all three kinase activities confirms this observation.[177] It may be that the high-affinity glucose and fructose transport systems are the same.

The activities of the high- and low-affinity glucose uptake systems appear to be differentially affected by ATP levels in the cell. The high-affinity system is more pronouncedly affected by the degree of phosphorylation of the sugar substrates mediated by the hexokinases and glucokinase. Some evidence that the low-affinity system is not so affected was obtained by

Schuddemat et al.,[272] who found that the rate of uptake of the nonphosphorylatable sugar analog 6-deoxyglucose was not affected by regimes that altered phosphorylated sugar uptake and ATP levels in the cell.

The transport systems, normally stable, are inhibited rapidly when protein synthesis is interfered with,[5] providing an explanation for the rapid loss of the ability to take up sugars when cells are starved for nitrogen.[173] Busturia et al.[41] found that inactivation of both the high- and low-affinity systems occurred with a half-life of about 6 h when protein synthesis was inhibited by nitrogen starvation or by addition of cycloheximide. This inactivation was substrate dependent; it occurred when the cells were grown on glucose, galactose, or maltose, but not when they were grown on ethanol.

Nine different complementation groups of mutants unable to grow at low glucose concentration were recently investigated by Bisson,[21] who found that all had pleiotropic effects indicative of a constitutive derepression of glucose-repressible functions and that the *snf1,4*, and *6* mutants were also derepressed for high-affinity glucose uptake. This provided strong evidence that catabolite inactivation is involved, and incidentally provides investigators with armies of new mutants defective in glucose-repressible functions.

B. Substrate-Induced Inactivation of Gluconeogenesis Enzymes

Enzymes directly involved in gluconeogenesis that are subject to glucose inactivation include those catalyzing the two major steps in the reversal of the glycolytic pathway: phosphoenolpyruvate carboxykinase[120,135] and fructose-1,6-bisphosphatase.

The mechanism of inactivation of the latter enzyme is of particular interest because of the possibility that, were it to be present and operating at the same time as phosphofructokinase, a futile cycle would ensue with the useless expenditure of ATP. This enzyme is known to be strongly inhibited *in vitro* by AMP, and the addition of glucose to the medium results in a rapid inactivation *in vivo*.[117]

The inactivation process itself is complex. Lenz and Holzer[181] showed that, after addition of glucose to the medium of cells grown on nonfermentable substrates, there is a reduction of activity of the enzyme by about 50% in 3 min. This inactivation can be reversed by rapid washing of the cells and resuspension in the presence of acetate and cycloheximide, provided that these operations are carried out immediately. The reversal thus appears to be dependent on protein synthesis as well as the removal of glucose. This reversible part of the inactivation is also accompanied by an incorporation of phosphate into the protein.[211,212,224]

The amino acid sequence of the phosphorylation site has been determined.[261] The sequence is one of multiple basic residues, and is characteristic of the regions of other loci known to be acted on by cAMP-dependent protein kinase.[164] A search for such sequences in a number of genes has been used as a diagnostic tool for determining likely sites of kinasing activity.[79]

A slower, irreversible inactivation is accompanied by proteolysis.[117] The kinetics are such that 90% of the remaining activity of the enzyme is irreversibly destroyed in 1 h. Added glucose increases the rate of degradation of the enzyme about 200-fold over the rate seen on gluconeogenic media.

Two intracellular compounds, cAMP and fructose-2,6-bisphosphate, have been implicated in the regulation of inactivation of fructose bisphosphatase. Mazòn et al.[212] noted that the rate of inactivation could be increased by the use of proton ionophores such as 2,4-dinitrophenol and CCCP, which are known to increase cAMP levels in yeast.[320] Then two groups found, through *in vitro* experiments on crude extracts of cells, that the rate of cAMP-induced inactivation can be greatly accelerated by the addition of fructose-2,6-bisphosphate.[122,254]

Fructose-2,6-bisphosphate is a molecule with important regulatory properties and has been implicated in the regulation of carbohydrate metabolism in a number of eukaryotic systems.[330] In animal and higher plant systems, the enzymatic activities involved in the synthesis and breakdown of this compound are both located in a bifunctional protein, but the synthetic and breakdown activities can be separated in yeast.[114,171]

An *in vitro* preparation of the phosphorylated form of fructose bisphosphatase, phosphorylated at ser[11], was found to have increased sensitivity to AMP and fructose-2-phosphate inhibition, although the increase was only about threefold. Mutant proteins in which the phosphorylatable ser[11] was replaced by an alanine or an aspartate were purified, and it was found that the alanine-substituted enzyme had the same behavior as the dephosphorylated enzyme, while the asparate-substituted enzyme had the characteristics of the phosphorylated enzyme.[204] It will be interesting to see whether the phosphorylation plays a role in the degradation of the enzyme, as well as in its sensitivity to AMP.

(It will not surprise the assiduous reader of this review to learn that cAMP-triggered protein phosphorylation is not the only factor involved in the inactivation of this enzyme. Lamponi et al.[176] examined a strain deficient in adenylate cyclase which is auxotrophic for cAMP[210] and found that while extracellular cAMP resulted in normal phosphorylation of FBPase, the actual proteolytic breakdown also required the addition of glucose.)

This inactivation pattern, of a reversible followed by a further irreversible inactivation, has not been seen in some other enzymes. Malate dehydrogenase[120] and phosphoenolpyruvate carboxykinase[95] cannot be reactivated after substrate-induced inactivation. However, a very similar inactivation pattern to that for FBPase has been seen for isocitrate lyase.[190] These authors built on observations that acidification of the medium greatly increases the amount of intracellular cAMP produced by both glucose and ionophores.[51] They observed further that

addition of proton ionophores or the polyenic antibiotic nystatin to cells grown at pH 5.5 produced rapid inactivation of both fructose bisphosphatase and isocitrate lyase, but that they had no effect at pH 7.5, where little change in cAMP level is seen even upon addition of glucose.[309] Nonetheless, inactivation did occur at the higher pH, though at a lower rate, suggesting that with this enzyme as well as with FBPase there may be more than one inactivation pathway and that the second pathway may not be directly mediated by cAMP levels.

The yeast FBPase gene has been cloned and sequenced[104,263] and has been found to have very strong — almost 50% — homology with the mammalian enzyme. There is an indication of a UAS upstream from the enzyme.[104] When the cloned gene was expressed using a *PHO5* or *GAL1* promoter, the cell accumulated high levels of FBPase even when grown on glucose, suggesting that the inactivation mechanisms do not function correctly when the gene is expressed using a heterologous promoter.[263] Dissection of the region upstream of the gene will undoubtedly reveal a degree of regulatory complexity comparable to that of other genes discussed in this article.

cAMP levels have also been implicated in the inactivation of a number of other enzymes of gluconeogenesis subject to carbon catabolite inactivation,[316,317] though none have progressed beyond a preliminary stage of investigation.

C. Catabolite-Mediated Inactivation of Trehalase

Trehalose, a disaccharide of D-glucose, is a major component of insect hemolymph and an important storage compound in fungi, particularly in spores. In yeast, it has been proposed as a regulator of glycolytic flux.[241] High trehalose levels are essential if the yeast cell is to survive starvation.[183] Trehalose also appears to protect against the effects of freezing[73] and preserves membranes during desiccation[237] and during the sudden changes in temperature[149] that also induce heat shock proteins.[7]

Thus, there appear to be a number of reasons why accumulation of trehalose rather than glycogen will be favored during sporulation since spores are required to survive long periods of environmental stress. The necessity to survive these periods of stress explains why trehalose appears to be preferred over glycogen as a storage compound when cells are grown on media that trigger sporulation.[308] Interestingly, the immediate mobilization of trehalose appears not to be necessary for the germination of spores since a careful study has shown that none of the trehalose present in spores is mobilized during their germination.[86] It is, of course, possible that trehalose may supply energy very slowly over long periods during spore metabolism, and of course it is available once the spores have germinated.

The synthesis of this compound involves a number of steps[240,296] (see Figure 1) and involves the formation of trehalose phosphate by the enzyme trehalose phosphate synthase. However, its breakdown into glucose is simpler, catalyzed in one step by the enzyme trehalase (EC 3.2.1.28). The regulation of this enzyme has proved to be a more manageable problem than the regulation of the synthetic pathway, but once again it is found to be under a number of types of regulatory control.

In *Saccharomyces* and *Pichia*, though not in some other yeasts, the mobilization of trehalose has proved to be controlled by several regulatory systems. In some of these there is strong evidence of a role for cAMP. As a result, the levels of this storage compound are, as we will see later, often found to be useful as indicators of the degree of involvement of cAMP and its metabolism in several important cellular pathways.

Literature on the regulation of trehalose metabolism has been ably reviewed up until 1984 by Thevelein.[308] Early results indicated that mobilization was governed by both glucose and the availability of nitrogen,[318] but it now appears that the physiological state of the cell, as well as the availability of glucose and nitrogen, also plays a role. Under some circumstances, it appears that the regulation of trehalose levels is at the level of hydrolysis rather than accumulation and that this hydrolysis can be stimulated by nitrogen.[310]

Genetic as well as physiological information about this breakdown is now available. Panek et al.[244] isolated mutants unable to accumulate trehalose when grown on nonfermentable substrates and found that this inability was apparently due to stimulation of the breakdown pathway by residual nitrogen. The interaction of the carbon source with nitrogen was vividly illustrated by the fact that, when maltose rather than glucose was used, nitrogen showed no stimulatory effect.

A great deal of evidence has accumulated that glucose-induced changes in cAMP levels are involved in trehalose mobilization. Conversion of low- to high-activity trehalase could be accomplished in cell extracts with the addition of cAMP,[329] though efforts to purify the proteins responsible for this activation resulted instead in the loss of cAMP stimulation.[331] Furthermore, *in vitro* experiments showed that there was a correlation between activation and phosphorylation of trehalase and even suggested that movement of the enzyme from the cytosol to the vacuole could be mediated by this phosphorylation.[336]

More recently, attention has focused on the regulation of the trehalose synthesis pathway. The trehalose-phosphate synthase complex, which may be activated by heat shock, also appeared at first to be regulated by cAMP-dependent protein phosphorylation.[242] This has turned out not to be the case on further investigation; no effect of cAMP levels could be found on the levels of trehalose-6-phosphate synthase or trehalose-6-phosphate phosphatase, both of which appear to be part of the same enzyme complex.[333]

Heat shock can actually produce a kind of futile cycling between glucose and trehalose since it stimulates both the synthetic and the breakdown pathways.[150] This co-stimulation may be due to the fact that both functions may be found on the same protein.

D. The Regulation of Glycogen Accumulation and Breakdown

As noted earlier, the precise connection between the cAMP pulse and trehalase activation still remains obscure. It is likely that it takes place in a number of steps and probably involves interactions with other regulatory pathways. Similar interactions have been found in the course of the study of glycogen metabolism.

The polysaccharide glycogen accumulates through the activity of glycogen synthase and is broken down through the activity of glycogen phosphorylase. Glycogen appears to accumulate in the cell primarily when growth is restricted, not only as a result of nitrogen starvation,[319] but also under conditions of carbon, phosphorus, or sulfur starvation[183] and even when the cells are grown on maltose, a less easily utilized substrate than glucose.[243] The involvement of cAMP was strongly indicated when it was discovered that the addition of uncoupling agents increased glycogen degradation.[14] Remarkably, this increase occurred even when the cells subject to the uncoupling agents were being grown on glucose. This is not what would be expected if the only triggering mechanism was a cAMP pulse induced by the sudden introduction of glucose and suggests that glucose and uncoupling agents may actually be working antagonistically and through different mechanisms.

François et al.[115] investigated this interaction and found that indeed the addition of glucose with its attendant cAMP pulse resulted in a relatively small increase of glycogen synthase and a decrease of glycogen phosphorylase by the same order of magnitude. This should increase the rate of glycogen formation, as expected. Galactose and xylose, which can use the glucose transport system but are not phosphorylated,[143] had no effect on the levels of these two enzymes.

The increases and decreases in activity of both these enzymes occur through complex sets of interconversions, different for each enzyme.[113] Glycogen phosphorylase interconverts between an active and an inactive form, while glycogen synthase interconverts between two active forms, one of which is strongly inhibited by glucose-6-phosphate.

The addition of nitrogen or the uncoupling agent dinitrophenol, which as noted previously also results in a cAMP pulse, produces an effect opposite to glucose on the levels of the two enzymes. Interestingly, the magnitudes of the effects were about the same as those seen on glucose, though as noted the directions were opposite. That is, nitrogen or DNP lowered glycogen synthase levels by about the same amount as glycogen phosphorylase was decreased by a glucose pulse and increased glycogen phosphorylase levels by about the same amount as glycogen synthase was increased by glucose. The changes in level were four- to fivefold in all cases.

The nature of the nitrogen source used was important: peptones had the greatest effect, and a much smaller effect could be produced by the addition of ammonium sulfate, glutamate, aspartate, glutamine, or asparagine. We had observed a similar ranking of the effects of nitrogen-rich substrates on the induction of alcohol dehydrogenase II and fructose bisphosphatase, with peptones again having the largest effect.[342,344]

It is striking that the addition of nitrogen-rich compounds stimulates the synthesis of gluconeogenic enzymes and, at the same time, increases the rate of glycogen mobilization. In this case, the same external stimulus appears to have different and indeed opposite effects at opposite ends of the gluconeogenic pathway. This makes sense in physiological terms since cells growing in the absence of glucose but in a nitrogen-rich medium would be able by utilizing this regulation to generate glucose both by synthesis from nonfermentable substrates and by mobilization from storage compounds.

François et al.[115] also found that a temperature-sensitive mutant deficient in adenylate cyclase, *cdc35*,[30] showed a greater increase of synthase levels and reduction of phosphorylase levels than did the wild-type cell at the restrictive temperature. This finding suggested that there had been a release of control as a result of the lowering of cAMP levels. Unfortunately, the effects of nitrogen and uncouplers were not examined with this mutant. The *cyr1* mutant, which is also deficient in adenylate cyclase but is able to utilize exogenous cAMP,[210] behaved like the wild type in the presence of glucose, and like the wild type it showed a decrease of the synthase and an increase of the phosphorylase upon the addition of nitrogen or uncoupling agents. This effect could also be produced by the addition of exogenous cAMP. François et al. suggest two possibilities for these results. Either the opposing effects of glucose and of dinitrophenol and nitrogen are due to subtly different kinetics in the release of cAMP triggered by the introduction of these compounds, or alternatively the dinitrophenol and nitrogen effect is due to a quite different process, perhaps the regulation of Ca^{2+}. If the latter, then the pulse of cAMP seen with these agents does not contribute to their primary effects and indeed is overwhelmed by them.

It is unlikely in any case that a cAMP-dependent protein kinase is solely responsible for the regulation of these two proteins. First, their regulation is and must be complex, with the relative amounts of the two enzymes highly dependent on a variety of external conditions such as the type and availability of carbon sources, nitrogen sources, pH, and so on. Second, *in vitro* experiments have shown that the proteins are phosphorylated by both cAMP-dependent and -independent kinases.[253,349,350]

Strains carrying a disrupted copy or multiple copies of glygocen phosphorylase have recently been constructed.[151] No effect on survival, sporulation, or ability to accumulate treholase was found.

The likely existence of cAMP-dependent and -independent regulatory mechanisms that interact in the same regulatory pathway should be borne in mind as we examine how adenylate cyclase is itself regulated in yeast.

IX. THE RELATIONSHIP OF cAMP METABOLISM TO PROTEIN PHOSPHORYLATION AND EXTERNAL ENVIRONMENTAL TRIGGERS

Several lines of investigation have been drawn together to show the importance of second messengers in the control of gluconeogenesis and a number of other centrally important metabolic processes in yeast. They also show that cAMP is certainly not the only second messenger involved. The story begins with the discovery of homologs of vertebrate oncogenes in yeast. In particular, the discovery that homologs of the *ras* genes in vertebrates are found in yeast[74,81,256] opened up the exciting possibility of investigating the function of these genes in an organism more amenable to experimental manipulation than mammalian cells.

Three yeast *ras*-like genes have been found. One, originally called *YP2* and renamed *YPT1*, has 40% amino acid homology to the 160 amino-terminal amino acids of mammalian *ras*,[119] while two others, *RAS1* and *RAS2*, have greater than 60% homology in the same region. But the carboxy-terminal regions of *RAS1* and *RAS2* have no homology to the mammalian gene, and aside from the fact that the two regions are roughly comparable in size, these regions have no homology to each other. The similarities between the yeast and mammalian RAS proteins are great enough that the yeast proteins can precipitate antibodies raised against the mammalian product.[160] The similarities also extend to function. Most remarkably, both yeast-mammalian hybrid genes and a mutant yeast *RAS1* gene were found to be able to transform NIH 3T3 cells.[75,297]

The RAS proteins of yeast, like the mammalian proteins RAS, G, elongation factor Tu, and α-transducin, bind both GTP and GDP and have a small amount of GTPase activity.[274,303,306] Mutant RAS proteins in mammalian cells that lead to transformation have been found to have an even lower GTPase activity.[38,123,307]

Complex interactions of GTP and GDP with yeast RAS mutant proteins have been observed. Temperature-sensitive mutants were found that lowered the affinity of the protein for these nucleotides, and others were found that altered the exchange rate between GTP and GDP. The former class of mutant killed the cell at the nonpermissive temperature, but the cell could be rescued by the addition of copies of a mutant (ala[18] val[19]) that increased the rate of exchange.[283] Since it is presumed, by analogy to the G proteins, that the ras-GTP complex is more active than the RAS-GDP complex, it may be that this rapid-turnover mutant generates enough RAS-GTP complex to rescue the cell even in the presence of protein with low affinity. Co-transformation in these experiments with the wild-type *RAS2* gene did not rescue the cells.

It quickly became apparent that the *RAS* genes in yeast have a number of profound physiological effects on the cell. If both genes are disrupted, the cell fails to grow.[161,304] Tatchell et al.[305] and Fraenkel[112] independently found that *ras2* mutants were unable to grow on gluconeogenic substrates. This was later found, however, not to be because of any defect in gluconeogenic enzymes or regulation, but rather because the production of *RAS1* mRNA is repressed, even in the wild type, when cells are grown on gluconeogenic substrates. This repression has the effect of converting a *ras2* mutant grown on such substrates into a phenocopy of a *ras1 ras2* double mutant.[35]

Tatchell et al.[305] also found that *ras2* cells were unable to accumulate normal amounts of the storage compounds glycogen and trehalose. Furthermore, homozygous *ras2* diploids had the remarkable property of being able to sporulate after growth on either glucose or ethanol, in contrast to wild type, which requires a period of growth on glucose before being transferred to sporulation medium.[305,315] In general, a number of regulatory systems in these cells appeared to be disturbed, with the result that some metabolic pathways functioned and others were repressed under conditions which produced very different effects in wild-type cells.

These RAS proteins, like the mammalian G proteins, may act as a link between extracellular environmental stimuli and the posttranslational modification of enzymes and regulatory proteins in the cell. In yeast, unlike mammalian cells, these physiological effects appear to be mediated through interactions, not only with GTP, but also with the proteins responsible for cAMP metabolism.

The story in mammalian cells remains rather unclear, and it is too early to rule out completely a role for mammalian RAS proteins in cAMP metabolism. The p21 proteins that are coded by mammalian *ras* genes appear to have no effect on mammalian adenylate cyclase activity either *in vivo*[182] or in a reconstituted *in vitro* system.[15] However, a human H-*ras* protein can activate yeast adenylate cyclase in an *in vitro* system.[38] Furthermore, an increased adenylate cyclase activity and cAMP content were found in untransformed thyroid epithelial cells infected with *ras*-carrying viruses.[288] Also, the high levels of cAMP induced by forskolin treatment of both normal and neoplastic B cells were recently found to be associated with a transient reduction in the amounts of both *myc* and *ras* proteins.[26] The question of the degree to which the yeast and mammalian *RAS* genes resemble each other in their effects on adenylate cyclase continues to be an open one.

A. The Role of Protein Kinases in Regulation

There are a large number of protein kinases in yeast,[53] most of which have been poorly characterized. The one that has been most thoroughly investigated is activated by cAMP and is thought to mediate a series of alterations in key enzymes and regulatory proteins (see Figure 5).

It has been possible to investigate the interaction of the RAS proteins with this kinase and with other proteins involved in cAMP metabolism, thanks to the availability of mutants that are defective in adenylate cyclase (*cyr1*) and in a regulatory

subunit of the cAMP-dependent protein kinase (*bcy1*). These were isolated by Matsumoto et al.[210]

The *cyr1* mutant was isolated by a search for mutants dependent on exogenous cAMP. The *bcy1* mutation was isolated as a suppressor of *cyr1*. Temperature-conditional *cyr1* mutants, defective in adenylate cyclase activity, have been isolated which arrest in the G_0/G_1 phase of mitosis at the nonpermissive temperature.[208]

The cAMP-dependent protein kinases of yeast and of mammals differ both structurally and kinetically. There are two mammalian enzymes, types I and II, which each consist of two catalytic and two regulatory subunits. They share their catalytic but not their regulatory subunits,[302,313] as can be seen from the fact that identical catalytic subunits are released from both type I and type II isoenzymes after treatment with cAMP. When the regulatory subunits are bound to the catalytic subunits, they inhibit their kinase activity. Addition of cAMP decreases the affinity of the regulatory and catalytic subunits by over 1000-fold,[128] resulting in a large increase in kinase activity from the freed catalytic subunits.

In the yeast enzyme, by contrast, there are at least three separate genes that encode catalytic subunits and only one for the regulatory subunit.[44,314] Each functioning enzyme molecule is made up of one of each of the four subunits. Kuret et al.[172] found that addition of cAMP to the purified enzyme decreased the affinity of the regulatory and catalytic subunits by only 20-fold, a markedly smaller effect than the change observed for the mammalian enzyme. This can be traced to the fact that the regions of the mammalian and yeast regulatory subunits that interact with their catalytic subunits have very different sequences.

In yeast, the regulatory subunit inhibits the catalytic subunits because it binds directly to their active sites. There is a phosphorylatable region of the regulatory subunit that acts as a "pseudosubstrate" binding site.[165] When nonphosphorylatable amino acids are substituted for the serines that are normally phosphorylated by the enzyme, this decreases the affinity of the regulatory subunit for the rest of the molecule five- to tenfold.[172]

B. Effects of RAS on Protein Kinases

A direct connection of the *RAS* genes to protein kinase activity became apparent when it was discovered that *bcy1* allows *ras1 ras2* cells, normally lethal, to grow. Toda et al.[315] looked for an effect of various combinations of these mutants on cAMP levels and found a complex relationship.

Mutants at one of the two *RAS* loci had relatively little effect. A *ras1* mutant decreased cAMP levels only slightly, while *ras2* decreased them about twofold. A more pronounced effect was seen with a $ras2^{val19}$ mutant, in which the wild-type product had been modified to increase its similarity to that of a mammalian oncogene, by means of the substitution of a valine for a glycine at position 19.[161] This mutant was found to increase cAMP levels about fourfold.

More recently, it has been found that a far greater increase in cAMP levels can be achieved by placing the $ras2^{val19}$ mutant in a strain lacking cAMP phosophodiesterase activity, so that cAMP cannot be broken down. This results in a 1000-fold increase in cAMP levels, but remarkably this immense boost does not result in a noticeable change in the levels of the effects already due to the $ras2^{val19}$ mutant (see the following).[235]

The *byc1* mutant by itself had no effect on cAMP levels. This is not unexpected since this mutant only affects the activity of cAMP-dependent protein kinase. This would not be expected to have a direct effect on cAMP levels themselves unless the kinase, in addition to its other functions, also governs cAMP production by phosphorylating enzymes in the cAMP pathway.

The most striking effect on cAMP levels was in the *bcy1 ras1 ras2* strain, in which there was no detectable cAMP. There were two possibilities to explain this dramatic reduction in cAMP levels. First, while *bcy1* by itself has no effect on these levels, it might have a strong effect in combination with the two *ras* mutants. Alternatively, the double *ras1 ras2* mutant might simply prevent the formation of cAMP. This second possibility is made more likely by the finding that multiple copies of the *RAS* genes in yeast increase the amounts of membrane-bound adenylate cyclase.[38]

The survival of this triple mutant appears to show that the cell can survive drastically lowered levels of cAMP so long as the protein kinase is permanently activated and, therefore, that it does not need regulation of the kinase to exhibit normal metabolism. An alternative possibility, as seen next, is that the kinase continues to be regulated, but that this regulation is under the control of other agents, the effects of which are only fully revealed in the absence of cAMP. Indeed, this regulation may be just as important as cAMP-mediated regulation, though the latter has received the most attention from investigators.

C. Effect of RAS on Adenylate Cyclase

After the original isolation in yeast of adenylate cyclase mutants, defective in the gene *CYR1*,[210] the investigation of this gene and its interaction with the protein kinase and the RAS genes could proceed. *CYR1* was cloned by complementation.[52,205] The gene was sequenced and found to encode for a large protein of 2026 amino acids. The catalytic domain, however, was found to be coded by only about a fifth of the gene, at the 3′ terminal end.[159]

It was assumed that the sites of interaction with regulatory proteins must be located somewhere in the remaining four fifths of the gene. Uno et al.[328] investigated the interaction of yeast RAS proteins, expressed in both yeast and *E. coli*, with *RAS2* and with *CYR1* genes that carried a range of deletions.

In *E. coli*, possession of the full-sized *CYR1* gene resulted in no cAMP production, but cells carrying a region encompassing the catalytic site could produce cAMP constitutively. Intermediate-sized pieces of gene could only produce cAMP in association with the RAS2 protein, indicating that a region adjacent to the catalytic region was necessary in

order for an interaction with the RAS2 product to take place. Addition of a biologically active GTP analog produced the expected result; adenylate cyclase activity was stimulated when the regulatory region of *CYR1* as well as *RAS2* were present, but was not stimulated when only the catalytic region of *CYR1* was present. Furthermore, GTP stimulation was not observed when the *RAS2* gene was replaced with *RAS2*^{val19}. This can be explained by the observation that the RAS2^{val19} product has reduced GTPase activity.

Parallel results were observed in yeast transformants, which could be studied because of the ability of *RAS1* and *RAS2* to rescue *cyr1* cells. The only major difference between yeast and *E. coli* was that *cyr1*-deleted yeast transformed with the entire *CYR1* gene were able to produce normal levels of cAMP and exhibited normal regulation. The inability of *E. coli* cells to perform similarly may simply be a peculiarity of the *E. coli* system.

The results obtained by using deletions of different sizes in these experiments delineated a region of about 0.9 kb located 5′ to the catalytic region which is primarily responsible for interaction with *RAS2*. The role of the 5′ terminal 600 amino acids of the adenylate kinase, which primarily consist of tandem repeats of a 23 amino acid unit, is still unresolved, as is any role of *RAS1*.[159]

Further dissection of the regulatory properties of *RAS* genes was possible with this system. It had been observed that wild-type cells starved for sulfur synthesized high levels of four proteins characteristic of the resting G_0 state of the cell. A similar response was seen in mutant cells carrying a temperature-conditional *cyr1* allele that had been transferred to the restrictive temperature.[281] In the experiments of Uno et al.,[328] it was found that yeast cells carrying only the catalytic region of *CYR1* were unable to respond to sulfur starvation by synthesizing the G_0 proteins, but those that carried the catalytic and flanking regulatory region were able to do so. It therefore appears that the RAS proteins act to mediate this, and probably also to mediate other environmental signals.

Toda et al.[315] performed some further experiments bearing on the control of adenylate cyclase by *RAS*. They built on the observation that *ras* mutations produce a set of defects remarkably like those seen in *bcy1*.

You will recall that the *ras* mutants appeared to be under less stringent control than wild type. *ras2/ras2* diploids sporulate on rich medium, which does not normally support sporulation, and accumulate higher levels of trehalose and glycogen than the wild type.[305] However, Toda et al. found that cells homozygous for the mutant *ras2*^{val19}, which has a substitution homologous to one that produces transformation in mammalian cells, had quite the opposite phenotype. They did not sporulate under any of the conditions tried, and *ras2*^{val19} mutants accumulated far less trehalose and glycogen than wild-type cells. The depressed trehalose accumulation was due to elevated levels of trehalase activity.

Furthermore, they found that cells left on plates at room temperature or at 4°C lost viability far more quickly than wild-type cells, and indeed were able to show that long-term viability of cells requires some *ras* function.[161]

All these properties turned out to be remarkably like those that had been observed in *bcy* mutants, which are defective in the regulatory subunit of cAMP-dependent protein kinase. Homozygotes for this defect sporulate poorly[209] and *bcy* mutants quickly become inviable.[208] They are defective in trehalose[326] and glycogen accumulation. Yet, *RAS2* is not allelic to *BCY*, despite their similarity.

Addition of cAMP to the medium stimulated production of trehalase in wild-type cells to the levels seen in *bcy* and in another derepressed mutant, *IAC*. However, addition of cAMP to *bcy* mutants[326] or *ras2*^{val19} mutants[315] had no further effect on their already high trehalase levels.

Toda et al.[315] then investigated the effects of the *ras* genes on the regulation of adenylate cyclase. When membranes from an otherwise wild-type strain lacking adenylate cyclase (*cyr1*) were mixed with membranes from a strain of genotype *CYR1 ras1 ras2 bcy1*, normal amounts of adenylate cyclase were produced, indicating that one or both of the *RAS* genes is responsible for factors that regulate the enzyme.

Because *ras2* has the larger effect on cAMP levels,[37] it would appear that *RAS2* is primarily responsible for the regulation of adenylate cyclase.[315] *RAS1*, on the other hand, has been shown to be more responsible for the initiation of inositolphospholipid turnover than *RAS2*, although *RAS2* has some effect.[157]

It should be noted that the levels of cAMP in the cell can have pronounced and important effects in pathways other than the ones that concern us here. When sufficiently high levels of cAMP are supplied to wild-type cells, this can actually overcome the cell arrest in low glucose medium that these cells exhibit. Also, the mutant that produces the largest amount of endogenous cAMP, *RAS2*^{val19}, does not arrest at the low glucose levels that produce G_0/G_1 arrest in wild-type cells, but keeps on growing.[157] The parallel between this mutant and similar mutants in mammalian oncogenes that lead to uncontrolled growth is very suggestive.

As noted earlier, there is another mutant which can, in the absence of wild-type, cAMP-dependent protein kinase, have large effects on cAMP levels without pronounced phenotypic effect. This is *cdc25*, which appears to regulate the levels of RAS proteins.[37,262] The gene, coding for a protein of about 1600 amino acids, has been sequenced and shows no detectable homology to other genes.[37,43]

The functional domains of this gene have been dissected by deletion analysis.[225] So far as the arrangement of the functional domains is concerned, there is a notable resemblance to other regulatory genes in yeast, even though there is no sequence homology. The carboxy terminal half of the protein is necessary for the survival of the cell (unless, as noted previously the cell carries *bcy1* or *ras* mutations). The 38 carboxy terminal residues

are required for sporulation. Also, the amino terminal half is required for growth on gluconeogenic substrates, just as the amino terminal portion of *ADR1* is required for growth on glycerol. It will be most informative to see what properties these apparently functionally homologous (analogous?) regions of these two genes have in common, and in particular whether mutants can be isolated that suppress the effects of double *cdc25 adr1* mutants with deletions in homologous regions. This may cast important light on the regulation of the gluconeogenic pathway.

Tripp and Piñon[321] have found four 31 kDa proteins, using two-dimensional electrophoresis, the phosphorylation of two of which is controlled negatively by *CDC25*. A suppressor of the *cdc25* mutant alters the phosphorylation pattern and appears to act upstream of it and therefore of the *RAS* genes in the regulation of the cAMP-dependent protein kinases.[359]

D. Other Second Messengers?

The primacy of cAMP as a second messenger has recently become less clear with the discovery that none of this elaborate machinery is necessary for the proper regulation of the processes that from earlier work appeared to be highly dependent on cAMP levels. These recent investigations were triggered by the observation that the *bcy1 ras1 ras2* triple mutant is relatively normal in its growth in spite of the fact that its cAMP has been reduced to undetectable levels.

In yeast, three catalytic subunits of cAMP-dependent protein kinase are coded by three structural genes, *TPK1*, *TPK2*, and *TPK3*.[44,186,314] Once the regulatory subunit has been inactivated, the structural subunits are released to phosphorylate proteins to a much greater extent than in the wild-type cell. This, in turn, appears to lead to the deregulation of sporulation, the suppression of the pathways leading to trehalose and glycogen accumulation, and the inability of the cell to survive heat shock or nutrient starvation.

The three catalytic subunits that have been investigated are not equivalent.[314] They were cloned by complementation into the temperature-sensitive cell division cycle mutant, *cdc25*. While the mutants all complemented each other, and all were found capable of blocking heat-shock resistance, the three mutants had different nutritional requirements. Furthermore, spores in which all three of the genes had been disrupted were still capable of germination and growth, although they grew slowly.

As was found with the *ras2*^{val19} mutant when it is coupled to phosphodiesterase mutants, cells with mutant TPK proteins can be produced that exhibit grossly elevated levels of cAMP without pronounced phenotypic effects. Strains in which *TPK2* and *TPK3* have been disrupted and a revertant *TPK1* allele is expressed show a 1000-fold increase in cAMP levels, even when the phosphodiesterase genes are active.[235] This indicates that the wild-type cAMP-dependent protein kinase exerts a very strong positive feedback control on cAMP levels. When mutant *ras* or *cdc25* genes were placed in this background, the cells survived (though wild-type cells would not have done), and cAMP was reduced to undetectable levels.

The availability of these mutants permitted a new question to be asked. What happens if the structural subunits have been damaged in such a way that they can no longer carry out their phosphorylation function as efficiently as in the wild type? Such mutants should presumably still be dependent on cAMP levels, provided that these levels are the only factor regulating the various cellular processes that are apparently under cAMP control, such as heat shock resistance and trehalose and glycogen accumulation.

To answer this question, Cameron et al.[42] looked for revertants that would suppress the *bcy1* phenotype. They found that strains carrying *bcy1* and only one of the three *TPK* structural genes did not grow on acetate, and grew to varying degrees on galactose, with *bcy1 TPK2* showing the least growth. They therefore looked for suppressors of *bcy1 TPK1*, *2*, or *3* that could grow on acetate and found that in each case the suppression was due to a mutation in the active *TPK* structural gene that lowered its activity.

They also found, to their surprise, that these mutant cells were now normally regulated in storage compound accumulation, sporulation, and heat-shock resistance. Also, they remained normally regulated even when mutant adenylate cyclase and *ras* alleles replaced the wild-type alleles in these strains. These workers were able to show that removal of the entire cAMP regulatory system had had no effect on the restored regulatory stability of the multiple mutant cells. These mutant cells behaved in a way indistinguishable from wild type. It would seem that another level of regulation had been revealed, perfectly capable of carrying out all the processes which up to that point had been attributed to alterations in the level of cAMP. It remains to be seen whether this regulation acts through a different kind of regulation of the levels of cAMP-dependent protein kinase, or whether a simple lowering of the kinase levels reveals this new regulation. In the latter case, the regulation may not be dependent on the activity of this particular kinase, and may only be revealed when the kinasing is held to a low and constant level.

One possible candidate for a gene involved in this new level of regulation is *YPT1*.[275] This *ras* homolog has been found to bind GTP and is essential for cell survival and spore production.[271] Its location between the actin and β-tubulin genes and its effect on the distribution of these proteins suggests that it is involved in cellular organization. Segev and Botstein[275] showed that the cells die rather than entering G_1 arrest upon nitrogen starvation. Also, the *bcy1* mutant does not compensate for the *ypt1* defect, suggesting that cAMP metabolism is not involved.

These remarkable results reveal a pattern similar to those we have seen repeatedly in the course of this article. This pattern is one of highly redundant regulatory systems, and it

must have the effect of ensuring that the cell maintains metabolic homeostasis. The regulation of major metabolic pathways in yeast is not only a function of polyvalent regulatory molecules that interact with each other in complex ways as they bind to UASs, but may also be rendered more complex by the existence of more than one second message carrying signals from the external environment to different regulatory pathways or even to different receptors on the proteins of the same pathway. Each regulatory system becomes a palimpsest which must be peeled away layer by layer.

E. G Proteins in Yeast

Two membrane proteins, G_s and G_i, have been implicated in the regulation of adenylate cyclase in mammalian cells (see Reference 104 for review). These have sequences that show a distant relationship to the mammalian and yeast *ras* proteins and share with them the ability to bind GTP. G_s stimulates and G_i inhibits the activity of adenylate cyclase. Recently, two genes homologous to G_s and G_i have been isolated in yeast. The first of these, *GPA1*,[226] codes for a protein of 472 amino acids that is 110 amino acids longer than the α subunit of mammalian G_i but that has 60% amino acid homology in the alignable region. Deletion of this gene in yeast destroyed mating-factor-mediated signal transduction.[85,221] Another gene, *GPA2*, was found later,[227] and its product showed a remarkable pattern of homology both with the GPA1 product and with $G_{i\alpha}$ and $G_{s\alpha}$ proteins from rat brain. It has an insertion of about 80 amino acids, and the insertion occurs at a different point from that of the 110-aa insertion found in the GPA1 protein. If these insertions are left out of the alignment of the yeast and mammalian α subunits, 60% of the amino acids are found to be identical or functionally conserved in all four genes, without exception. The other 40% vary widely across the four genes, indicating that 60% of the gene has been very strongly conserved and the other 40% has been completely free to vary, not only during the divergence of yeast and mammals, but also during the divergence of the *RAS* family within each group.

Disruption of *GPA2* did not affect viability. It also did not affect the size and duration of the transient pulse of cAMP formation after the introduction of glucose. However, insertion of the wild-type gene on a high-copy-number plasmid doubled the amount of cAMP produced and lengthened the duration of the pulse from about 1 min to greater than 30 min. One possible explanation for this effect, which would also explain the fact that a *GPA2*-null mutant is nonlethal, is that the effect of a *gpa2* mutant is to suppress the formation of the phosphodiesterase that breaks down cAMP. This possibility is reinforced by the observation that mutants that lack the phosphodiesterase activity are also unaffected in their viability.[270,327]

The effects of this gene on other genes involved in cAMP metabolism were investigated.[227] The introduction of the gene in high copy number did not complement the inability to grow of a *ras1 ras2* double mutant or a *cyr1* mutant, but did restore the ability of a $ras2^{ts}$ mutant to make cAMP after glucose stimulation at the restrictive temperature.

It will be of the greatest importance to investigate the relationship of these G-like genes to the RAS genes and to other genes with polyvalent regulatory capabilities.

X. CONCLUSIONS

A small part of the web of effects produced by the activity of the cAMP-dependent protein kinase has been sketched out in Figure 5. As if this set of interactions was not complicated enough, it now appears that there is a kind of fallback regulatory system available to the cell that can operate in the absence of cAMP. So far as can be seen at the moment, this system can do everything that the cAMP-mediated system can do, though it is certain that differences in the two systems will become apparent on further study.

It was suggested earlier, in the discussion of the regulation of the gluconeogenesis pathway, that the cell must require a kind of fine tuning of each step of its metabolic pathways through separate *trans*-acting regulatory genes that are themselves under control of more than one regulatory system. We can see now that this is clearly the case for the cAMP regulatory pathways.

It is encouraging to note that mutants are now beginning to be found, like *snf1-ccr1*, that in spite of their very different phenotypic effects turn out to be alleles of polyvalent regulatory molecules. It appears that there are not an unlimited number of such genes in yeast and that with persistence it should be possible to reach the bottom layer of the regulatory palimpsest at some point!

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