Instituto Juan March
de Estudios e Investigaciones

9

CENTRO DE REUNIONES
INTERNACIONALES SOBRE BIOLOGÍA

Workshop on

Control of Gene Expression in Yeast

Organized by

C. Gancedo and J. M. Gancedo

T. G. Cooper
T. F. Donahue
K.-D. Entian
J. M. Gancedo
C. P. Hollenberg
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INTRODUCTION

J. M. Gancedo
C. Gancedo

The idea of this meeting originated about two years ago in discussions with Andrés González after an Advanced course on Biochemistry and Genetics of yeast cosponsored by FEBS and the Fundación Juan March. We thought that it would be interesting to gather a number of specialists on an active field of yeast biology and we choose "Control of gene expression" as a topic on which rapid progresses were being made taking advantage of the experimental possibilities that yeast offers as a model organism.

That an understanding of the mechanisms of control of gene expression is of capital importance to understand how an organism will function in a certain moment is not new . Some thirty years ago a meeting in Cold Spring Harbor with the title of "Cellular regulatory mechanisms" addressed the same question we are aiming presently at: to know how the expression of a gene is controlled. At that symposium Jacob and Monod were presenting their model of control of gene expression in *Escherichia coli*, the existence of mRNA was just being considered and the only paper dealing with yeast was one by Helmut Holzer on "Regulation of carbohydrate metabolism by enzyme competititon" . A great leap since that time has been done and today we are dealing with more complicated eukaryotic organisms among which yeast has been extremely rewarding.

Gene expression is the process of going from DNA to protein and therefore has two distinct steps: transcription and translation. There has been a tendency to identify control of gene expression with control of transcription but this needs not to be so. In some cases control of translation could play an important role and even changes in mRNA stability could regulate gene expression. However, presently the better studied field is that of the control of transcription mechanisms and this was reflected in the content of the communications.

The topic of control of gene expression in yeast is nowadays so vast that it was not possible to cover all its aspects in the time available. The workshop was initiated with presentations on RNA polymerase II and on some of the general factors required for transcription and was followed by others on the role of the chromatin structure in regulating transcription . A number of communications were centered around a regulatory system which affects a great

variety of yeast genes: carbon catabolite repression. Finally cis- and trans-acting elements which control the expression of anabolic and catabolic genes involved in nitrogen metabolism were considered, and some attention was dedicated to post-transcriptional regulatory mechanisms.

A topic which was not addressed is that of the possible control of the rate of transcription. That is a reflect of our lack of information about the following questions: once transcription is initiated does it proceed at the same speed in every gene? Are there factors that could modulate the progress of the transcription machinery? Another question that received perhaps less attention than deserved was that of "who control the controllers?" or put in another form, is the expression of genes that encode transcriptional activators itself regulated?

In addition to the presentations by the invited speakers some oral reports from selected posters among those exhibited by the participants were scheduled. Three round tables around the topics of "Factors affecting transcription", "Carbon catabolite repression" and "Regulation of nitrogen metabolism" were also organized.

The communication between speakers and participants was intense and was fostered by the magnificent organization provided by the Fundación Juan March. It was an unanimous feeling among the people who attended the workshop that the Fundación has done a wonderful job not only in bringing together prominent speakers but in caring for all the participants in the way it did. Therefore it is a pleasure for us to express our sincere thanks to the Fundación Juan March for its generous support of this meeting. We would like also to thank all the invisible persons who helped to the success of the meeting and specially Andrés González who coordinated all the efforts.

Juana María and Carlos Gancedo
Madrid, December 1992

First session

YEAST RNA POLYMERASE SUBUNITS AND CLASS III TRANSCRIPTION FACTORS

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In the past decade, studies with reconstituted RNA polymerase-dependent systems, combined with genetic evidence obtained mainly in yeast, have established that RNA polymerase I (or A) synthesizes ribosomal RNA precursors, RNA polymerase II (or B) transcribes protein-encoding genes, and RNA polymerase III (or C) produces 5S rRNA and tRNAs. Most of the small nuclear RNAs are transcribed by enzyme II or, in some cases, by enzyme III. Each enzyme is directed for promoter selection and correct initiation by a set of general and gene-specific transcription factors. The characterization of yeast RNA polymerases, transcription factors and regulatory proteins have greatly contributed to our understanding of eukaryotic transcription. In this lecture, we give an overview of the molecular structure of the three yeast nuclear RNA polymerases as derived from biochemical and immunological studies, present the recent progress made in their genetic characterization and finally discuss transcription complex formation, illustrated with the pol III system.

Yeast RNA polymerases A(I), B(II), and C(III) are organized around a common core of subunits related to the bacterial core enzyme ($\beta'\beta\alpha_2$) and share a set of five small subunits (ABC27, ABC23, ABC14.5, ABC10α, and ABC10β). All these subunits are essential for growth. In addition, each enzyme contains a variable number of enzyme-specific subunits, some of which are not strictly required for growth. Most subunit genes have been cloned, sequenced, and mutagenized to produce null alleles and, for several of them, conditional mutants. A functional map of RNA polymerase active site, taken in a broad sense, has begun to emerge from a combined genetic and biochemical analysis of the large subunits. Progress is made on subunits that interact with the basal transcription factors.

Two general class III transcription factors have been identified in yeast. TFIIIC which recognizes the A block and B block promoter elements has been highly purified as a large multi subunit protein. TFIIIC acts as an assembly factor to recruit TFIIIB on the DNA, to an upstream position. The initiation factor TFIIIB is less well characterized. It can be separated into two essential components of 70 kDa and 90 kDa. In addition, the involvement of TBP (the TATA box binding protein) has been recently recognized. All these factors participate to the transcription complex allowing RNA polymerase III to initiate correctly. The cloning of TFIIIC and its role in promotor recognition, transcription complex assembly and chromatin derepression will be presented.

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RNA polymerase III (C) and its transcription factors. Trends Biochem. Sci. 16, 412-416.

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RNA polymerase B (II) and general transcription factors. Annu. Rev. Biochem. 59, 711-754.

TFIID AND THE MECHANISM OF TRANSCRIPTIONAL ACTIVATION

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It has become clear over the past decade that there are common molecular mechanisms of transcriptional regulation in eukaryotic organisms ranging from humans to yeasts. The structures of chromatin, RNA polymerases, mRNAs, and promoters are highly conserved throughout the eukaryotic kingdom. Yeast and higher eukaryotes contain structurally similar and functionally analogous transcription factors that recognize essentially identical sequences and can often function across a wide variety of species. Such functional interchangeability not only emphasizes the remarkable degree of evolutionary conservation, but also makes it possible to study molecular mechanisms *in vivo* and *in vitro* by using mixtures of yeast and mammalian components.

Most RNA polymerase II promoters contain a TATA element upstream of the mRNA start site that binds the basic initiation factor TFIID. Binding of TFIID to the TATA element is the essential first step in the ordered assembly of the active transcription complex, and it appears to potentiate the promoter for transcription in the context of chromatin. Besides RNA polymerase II and TFIID, the transcription complex is composed of multiple initiation factors (TFIIA, TFIIB, TFIIE, TFIIF, TFIIH). TFIID interacts with TFIIA and alters the interaction with the TATA element. Next, TFIIB joins the complex and appears to bridge the region between the TATA element and initiation site. Then, TFIIF recruits RNA polymerase II to the complex and finally TFIIE comes on board to form a preinitiation complex capable of synthesizing mRNA. TFIIH is a kinase that phosphorylates the C-terminal tail of the largest subunit of RNA polymerase II, an essential step in initiation. This basic RNA polymerase II machinery supports TATA-element-dependent transcription *in vitro*, but requires gene-specific activator proteins for efficient transcription *in vivo*.

TFIID is highly conserved throughout the eukaryotic kingdom. Yeast and human TFIIDs are functionally interchangeable in basal transcription reactions reconstituted with yeast or human components and have nearly identical TATA-element specificities. The C-terminal 180 residues of TFIIDs from a variety of eukaryotic organisms are at least 80% identical in amino acid sequence. This C-terminal core domain is necessary and sufficient for TATA-element binding and basal transcription *in vitro* and for the essential functions of TFIID in yeast cells. Despite these striking similarities, human TFIID can not carry out the essential functions of yeast TFIID *in vivo*, with differences between the highly conserved core domains being responsible for the phenotypic distinction. However, our *in vivo* and *in vitro* experiments strongly suggest that human TFIID can support basal transcription and respond to acidic activator proteins in combination with yeast initiation factors.

Interactions between activator proteins and TFIID have been observed both genetically and biochemically. However, the question of whether upstream activator proteins stimulate

transcription by directly contacting TFIID or by interacting with a distinct protein that serves as an adaptor to the basic transcription machinery remains unanswered and controversial. In this regard, it is important to stress that mammalian and *Drosophila* TFIIDs are tightly associated with a number of additional factors in multiprotein TFIID complexes, and there exists a family of TFIIA-like proteins that interact with TFIID and regulate promoter activity. Two distinct TFIID complexes have been observed in human cells, only one of which can support transcriptional activation. Yeast TFIID behaves as a simple monomeric protein in cell-free extracts, but it is very likely to associate with other factors *in vivo*.

Gene-specific transcriptional activators generally contain distinct DNA-binding and transcriptional activation functions that are usually located in separate regions of the proteins. Activation domains are often defined by short acidic regions that function autonomously when fused to heterologous DNA-binding domains. Although many different acidic sequences can serve as transcriptional activation regions and negative charge is clearly important, the level of transcriptional stimulation is influenced by other structural features such as the length of the region and possibly α-helical character. It is believed that the DNA-binding domain serves merely to bring the protein to the DNA target, whereupon the acidic activation region interacts with a component(s) of the basic transcription machinery. Since acidic regions function across a diverse range of eukaryotic species, they are likely to contact some part of the basic transcription machinery that is conserved functionally throughout the eukaryotic kingdom. In contrast, the glutamine-rich and proline-rich activation regions found in some mammalian proteins do not function in yeast cells, presumably because they function by different mechanisms.

The mechanism by which acidic activation domains stimulate transcription is a fundamental but unsolved issue. Acidic activation domains might directly interact with a component(s) of the basic transcription machinery or alternatively indirectly affect the function of the basic machinery by associating with distinct proteins termed adaptors, coactivators, or mediators. Finally, the target could be a component of the chromatin template such as histones or associated proteins. Many experiments have been devoted to this question and evidence has been gathered in support of TFIID, TFIIB, the C-terminal tail of the largest subunit of RNA polymerase II, adaptors. There are many opinions, but the situation remains to be clarified.

Characterization of yeast genes involved in transcriptional activation.

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The mechanism by which activators stimulate transcription is largely unknown. Biochemical and genetic evidence indicates that it may be mediated by intervening factors, termed adaptors or co-activators. These factors would bridge the interactions between activators and the basal transcription machinery, and in principle they should be required for transactivation but not needed for basal transcription. We have devised a genetic strategy to isolate the corresponding genes in yeast, based on the property of powerful activators to inhibit yeast growth when over-expressed. This toxic effect requires both the activation and the DNA binding domains of the activator protein, suggesting a close relationship between toxicity and activation capability. The assumption is that a mutation reducing the toxicity potential of a given activator would be also impeding its ability to activate transcription. Our selection scheme uses the chimeric activator Gal4-VP16 expressed from the ADH1 promoter on a high copy plasmid. Whereas this combination completely inhibits growth of a wild type yeast strain, we isolated about 30 different mutants able to grow in the presence of this toxic construct. The isolated mutations fell into three complementation groups, *ADA1*, *ADA2* and *ADA3*, characterized by a very poor growth in minimal media, although no specific auxotrophy has been found associated to them.

The poor growth of the *ada* mutants in minimal media was utilized to clone the corresponding wild type alleles by complementation. Sequence analysis of the three cloned *ADA* genes shows that their are novel genes, with no clear homology to any other gene cloned so far. Different regions of homology have however been found. Specifically, *ADA2* shows Cys-rich region that may encompass a DNA binding domain, although no direct proof have been obtained.

Analysis of the phenotypes of the three isolated *ADA* genes demonstrated that *ada1* mutants escaped Gal4-VP16 toxicity by reducing its expression levels. *ada1* mutations seem to affect the levels of different activators, by a mechanism still unknown. Mutations in

ADA2 and *ADA3* genes do not affect the levels of expression of Gal4-VP16, but reduce its ability to activate transcription. In addition, they have wide pleiotropic effects, showing that they affect multiple genes, although their complete deletions are not lethal.

The *ADA2* gene appears to be required for full expression of a discrete set of activable genes. Genes regulated by Gcn4 seem to be the most severely affected by *ada2* mutations, suggesting that the activation domains of Gcn4, VP16, and, presumably, other activators not characterized yet, do not work in these mutants. Conversely, activation through other UAS (like *CYC1*, driven by both Hap1 and Hap4) and transcription of several house-keeping genes are not affected in *ada2* mutations. The effect of *ada2* mutations is also very selective *in vitro*: response to the activators Gcn4 and Gal4-VP16 is reduced in *ada2* mutant extracts, but not activation by Gal4-Hap4. Basal transcription is also not affected in *ada2* extracts. Given these characteristics, we propose *ADA2* as a transcriptional adaptor.

Mutations in the *ADA3* gene affect transcription of a greater variety of reporters than *ada2* mutations, albeit in very different degrees. Transcription of house-keeping genes like *TBP*, *ACT1* or *GCN4* is not affected in *ada3* mutants. *CYC1* transcription is moderately affected by *ada3* mutations, whereas VP16- and Gcn4-driven activation is severely impeded. In general, transcription of genes that are affected by *ada2* mutations appear to be the most affected in *ada3* mutants. *In vitro*, *ada3* mutant extracts show two different phenotypes. First, activation by any of the above indicated chimeric activators, as well as basal transcription appear to be significantly reduced in *ada3* mutant extracts. Second, the choice of start sites is altered in *ada3* mutants, being the UAS-proximal ones preferred over the distal ones. These characteristics suggest that *ADA3* is a non-essential factor in the basal machinery that receives the activation signal from transcriptional activators through *ADA2* or related adaptors.

Second session

Histones versus transcription factors: chromatin structure and gene regulation in yeast

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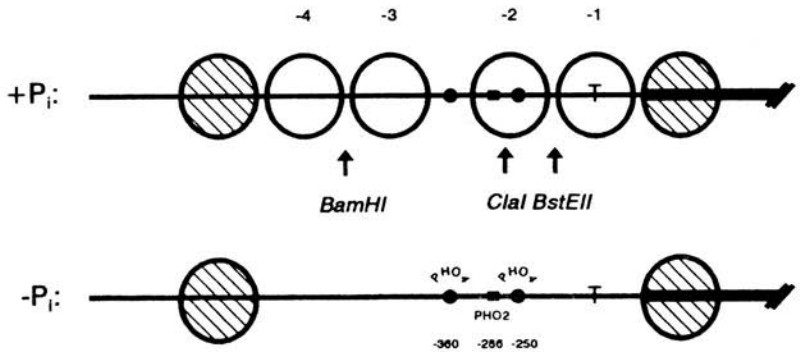
We are investigating the role of histone DNA interactions in gene regulation and have chosen the *PHO5* gene from *Saccharomyces cerevisiae* as a model system. The *PHO5* gene encodes a strongly regulated acid phosphatase which is repressed in high phosphate media. It is induced about 100-fold when cells are starved for phosphate. Two positive regulatory proteins, PHO2 and PHO4, with binding sites at the *PHO5* promoter (Vogel et al., 1989) are required for this to occur. At high phophate conditions, the PHO4 protein is functionally inactivated by a negative factor, the product of the *PHO80* gene, probably by protein protein interactions.

The chromatin structure at the *PHO5* promoter undergoes a massive transition upon induction of the gene. In high phosphate media, there is a short hypersensitive region located about 370 bp upstream of the gene. This hypersensitive region which contains a PHO4 binding site is flanked by specifically positioned nucleosomes (Almer and Hörz, 1986). Upon induction of the gene by starving the cells for phosphate, two nucleosomes upstream and two nucleosomes downstream of this hypersensitive site are selectively removed (Almer et al., 1986).



We have investigated the mechanism of this chromatin transition and asked specifically if DNA replication is required for nucleosome disruption to occur. This question cannot be answered simply by starving cells for phosphate and at the same time preventing cell division because phosphate starvation conditions are not achieved under these conditions due to endogenous phosphate pools.

We have overcome this problem by inducing *PHO5* activation through a temperature shift rather than by phosphate starvation, thus circumventing all the complications inherent in the phosphate signaling pathway. This was made possible by introducing a targeted mutation into the *PHO80* gene which converted this gene to a temperature sensitive allele.

When the chromatin structure at the *PHO5* promoter was analyzed it was indeed found to open up after a temperature shift to 37° in the same way as previously found for wild type cells activated by phosphate starvation. When we prevent DNA replication by starving the cells or taking growth arrested cells we found that chromatin still opened up after raising the temperature to 37°. We even found that the closed chromatin configuration could be regenerated by shifting the cells back to 24° after they had been at 37°, again in the absence of DNA replication (Schmid et al., 1992).

We could show that the cells need glucose for the nucleosome disruption, which might mean that this is an energy dependent process. The reverse reaction, i.e. the reformation of nucleosomes after a temperature downshift, occurs in the absence of glucose.

These results imply that transcription factors, in this case PHO2 and PHO4 (Fascher et al., 1990), must be able to trigger the disruption of a preexisting nucleosomal template. We have analyzed the in vivo binding of PHO4 to its target sites by in vivo footprinting techniques using dimethylsulphate. Characteristic changes in the reactivities of G-residues at both binding sites were observed at low phosphate conditions. That we were really observing interactions of the PHO4 protein could be confirmed by analyzing *pho4* strains and also strains that overexpressed *PHO4*. We conclude from these results that PHO4 binds to both target sites in chromatin after *PHO5* induction.

Based on these results a model will be presented that describes the interplay between histones and transcription factors in the activation of the *PHO5* promoter.

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Gene modulation by the Centromere and Promoter Factor 1 and SPT proteins.

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The centromere and promoter factor 1 (CPF1) is a basic helix-loop-helix -ZIP protein that is required for optimal centromere function and transcription of several genes. The protein is encoded for by the *CPF1* gene (also known as *CEP1* or *CBP1*).

CPF1 binds specifically *in vivo* and *in vitro* to an octanucleotide motif RTCACRTG found at the centromere determining element (CDE) I motif in yeast centromeres and at many other sites in the yeast genome including gene promoters. DNA bound CPF1 bends the DNA and induces marked changes in the chromatin structure around its target site. Deletion of the *CPF1* gene or mutations in the CDEI region result in a ten to twenty fold reduction in the stability of chromosomes and centromere based plasmids indicating that DNA bound CPF1 is required for optimal centromere function. The function of CPF1 at the centromere may be to bend/distort the DNA to aid the formation of other DNA:protein and protein:protein interactions, particularly those formed at CDEIII and with the microtubule.

Marked changes in the position of nucleosomes are observed around CDEI sites in promoters in wild-type strains and *cpf1* strains. Using a *cpf1* strain and an inducible plasmid expressing *CPF1*, these changes in chromatin structure can be shown to be a direct consequence of the presence or absence of the CPF1 protein in the cell. These chromatin changes are evident at the CDEI-like elements in the promoters of two genes, *MET25* and *MET16*, whose products are required for the biosynthesis of methionine in the cell. Deletion of the *CPF1* gene results in an absolute requirement for methionine for growth suggesting that CPF1 might be required for the expression of methionine biosynthetic genes. However, these is no correlation between the nature of chromatin structure at these promoters (reflecting the presence or absence of CPF1 in the cell) and the dependence of the cell on methionine for growth. Cells expressing versions of the CPF1 protein that are unable to bind to DNA *in vitro* show a chromatin pattern expected of a *cpf1* mutant strain yet these strains are methionine prototrophs.

In support of the idea that CPF1 functions in one way to maintain optimal centromere function and in a different way to ensure transcription of genes we have constructed a number of strains containing point mutations in the basic domain and helix 1 region of CPF1 that have distinctive and different effects on centromere function or the ability of strains to grow without methionine. We are currently using genetic and biochemical approaches to unravel the role of CPF1 in the transcription of genes.

We have demonstrated that for optimal transcription of Ty elements and for maintaining methionine independent growth CPF1 functions to antagonise the repressing effects of a related family of proteins including SPT21, RPD1(SIN3), RPD3 and CCR4. In a strain expressing a version of CPF1 with a point mutation that results in methionine auxotrophy, the introduction of a mutation in any one of this family of genes is sufficient to allow growth in the absence of methionine and to increase Ty RNA levels to above normal. Thus *cpf1*, *spt21* strains are methionine prototrophs. We have isolated a number of other genes that are able, at high copy number, to complement the methionine auxotrophy of strains containing a complete deletion of the *CPF1* gene. One of these genes encodes the HLH protein PHO4. I will discuss the role of CPF1 in the transcription of genes and the possible mechanisms for complementation of CPF1 function in different genetic backgrounds.

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Third session

Regulation of glucose repression in *Saccharomyces cerevisiae*

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Glucose repression refers to the absence of certain enzymes if glucose is available in the medium. The many enzymes subject to glucose repression include glycoside hydrolases and those of galactose catabolism, gluconeogenesis, the tricarboxylic acid cycle, the glyoxylate cycle and the respiratory chain. Genetic analysis of glucose repression has revealed two kinds of mutant. First glucose repression mutants in which enzymes that are glucose repressible have high activities, even when glucose is the carbon source. Second glucose derepression mutants which stay in the glucose repressed state even after exhaustion of glucose.

Four groups of mutants can be distinguished phenotypically: (i) *hxk2* (=*hex1, glr1*), *hex2* (=*reg1*), *cat80* (=*grr1*) and *cid1*, which affect glucose repression, (ii) *cat1* (=*snf1*) and *cat3* (=*snf4*), which affect glucose derepression, (iii) *cat2* and *cat4* (=*mig1*) which are epistatic over glucose derepression mutants, and (iv) glucose repression and derepression mutants that have additional regulatory disorders, *tup1* (=*flk1, umr7, cyc9, amm1, aar1, slf2, aer2*), *cyc8* (=*ssn6*) and *rgl1*.

Hexokinase PII appears to have a major function in triggering glucose repression. This function can be complemented by over-expression of isoenzyme PI, but not glucokinase. No further glycolytic steps beyond glucose phosphorylation are necessary for glucose repression and the actual signal for glucose repression is still unknown. The Hex2 protein is located in the nucleus and contains strongly acidic regions, which may compete with transcriptional

factors. The Cat80 protein has leucine-rich motifs, which is indicative of protein-protein interactions, genetic analysis suggests that Cat80 functions early in the pathway of glucose repression.

Genes *cat1* and *cat3* are necessary for glucose derepression. The Cat1 protein appears to be a protein kinase and Cat3 corresponds to a subunit necessary for its function.

Deletion analysis of the glucose repressible promoters identified DNA sequences at which gene repressors (URS elements) or gene activators (UAS elements) can bind. The invertase URS element was used to characterize its gene repressor Cat4 (=Mig1). For fructose-1,6-bisphosphatase acitvator proteins DapI and DapII were identified by gel retardation experiments. DapI binding occurs when cells are grown on ethanol.

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Entian, K.-D. and J.A. Barnett (1992) Regulation of sugar utilization by *Saccharomyces cerevisiae*. TIBS (in press).

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CONTROL OF *HXK2* GENE EXPRESSION. THE ROLE OF PROTEIN PHOSPHORYLATION ON THE GLUCOSE REPRESSION SIGNALING PROCESS OF Saccharomyces cerevisiae.

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The yeast Saccharomyces cerevisiae produces three different enzymes that can phosphorylate glucose and thus initiate glycolysis. Two of these (hexokinase 1 and 2) phosphorylate fructose and mannose as well, allowing growth when either of these sugars is used as the sole source of carbon and energy; the third (glucokinase) phosphorylate only glucose and mannose. The two hexokinases are thus both dispensable for growth on glucose, but the cell requires one or the other for growth on fructose.

The two hexokinases are not functionally interchangeable, as there are differences in the phenotypes of null mutations in the two genes, in the growth rate on glucose and the degree of glucose repression. On the other hand, the high affinity glucose uptake system depends on the activity of any one of the three kinases.

Yeast glucose phosphorylating enzymes have been the subject of a considerable number of biochemical studies but their individual roles are unclear. Mechanisms of their involvement in high affinity glucose uptake, or, for hexokinase 2 and 1, in glucose repression, are also to be explained. There is, therefore, much to be learned about these enzymes. The present paper is a study of the role of hexokinase 2 phosphorylation on the glucose repression signaling process. We will also present preliminary data about the control of hexokinase 2 gene expression.

Hexokinase 2 undergo phosphorylation "in vivo" when the yeasts are grown in medium with a low concentration of glucose and the phosphate incorporation was stimulated by certain pentoses, as xylose. Xylose-induced phosphorylation of hexokinase 2, shows a direct correlation with invertase and α-galactosidase activities, suggesting that the phosphorylation of hexokinase 2 is closely associated with the signaling process of glucose repression.

In order to characterize the hexokinase 2 phosphorylation we have carried out experiments about its reversibility and the effect of competitor sugars on the protein phosphorylation. One conclusion of these experiments is that the phosphate incorporated to

hexokinase 2 is not implicated in the catalytic mechanism of glucose phosphorylation.

In order to identify the phosphorylated site of hexokinase 2, the ^{32}P-labeled protein was digested with TPCK-treated trypsin. The tryptic peptides were resolved on a C-18 reverse phase HPLC column and sequence data were obtained for the purified phosphopeptide. These results lead to the identification of a peptide between Gly_{60} and Arg_{93} as the target for protein phosphorylation.

Site directed mutagenesis and deletion experiments identified Ser_{79} as the phosphate aceptor residue of hexokinase 2. A truncated hexokinase 2 lacking 14 aminoacids, including Ser_{79}, is not phosphorylated and the yeast cells lacks glucose repression. these results strongly support the hypothesis that the phosphorylation of hexokinase 2 is implicated with the glucose repression signaling process.

To study *HXK2* expression yeast RNA isolated from cells growing logarithmically in several media containing distinct non-fermentable (glycerol, ethanol) and fermentable (glucose, fructose, mannose,galactose) carbon sources, was hybridized against *HXK2*. The actin gene, which is constitutively expressed was used as standard. A preliminary analysis of the *HXK2* upstream control region was carried out by using different *HXK2-lacZ* fusions. Our results shows that hexokinase 2 transcription need the presence of glucose in the culture medium.

GLUCOSE REPRESSION OF *GAL* GENE EXPRESSION and ANALYSIS OF A WEAK YEAST PROMOTER.

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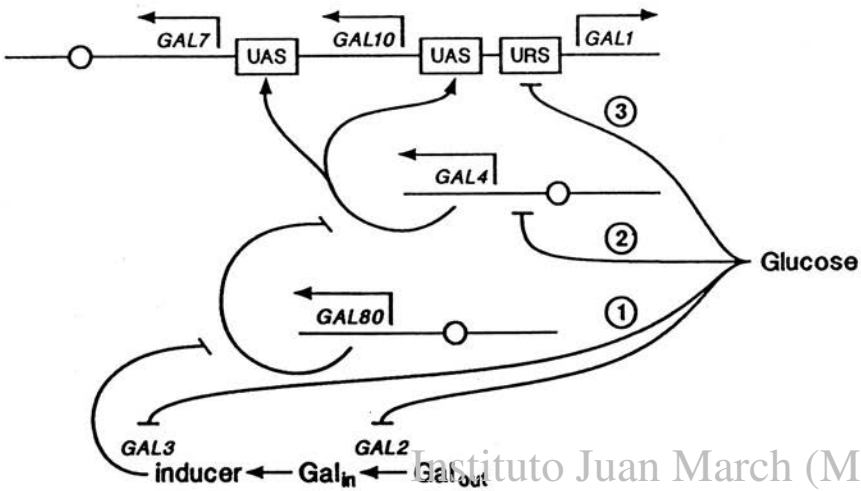
Expression of the *GAL* genes of *S. cerevisiae* is stringently regulated in two ways: galactose *induces* and glucose *represses* their expression. The basis for induction by galactose has long been well-understood: Gal80p inhibits the ability of Gal4p to activate transcription by binding to the transcriptional activation domain of Gal4p; growth on galactose produces an inducer that inhibits Gal80p function.

The basis for glucose repression has more recently become clear. Glucose interferes with Gal4p function in three distinct ways (diagrammed below). **First**, glucose lowers the level of functional inducer in the cell, resulting in increased Gal80p activity, which causes about a 10-fold reduction in *GAL1* expression. This is due to reduced levels of the *GAL2*-encoded galactose permease caused by glucose repression of *GAL2* transcription and glucose-induced inactivation of the permease, and also glucose repression of *GAL3* expression, which encodes a protein necessary for inducer function. **Second**, glucose represses expression of *GAL4*, leading to lower levels of Gal4p, which reduces *GAL* gene expression approximately 40-fold. This is due to binding of the glucose-activated Mig1p repressor to two sites in the *GAL4* promoter. **Third**, glucose repression works directly on the *GAL1* promoter through a URS element that consists of two Mig1p binding sites to reduce *GAL1* expression about 5-fold. These three mechanisms together seem to account for *all* of the approximately 1000-fold repression of *GAL1* expression.

These multiple mechanisms ensure that glucose repression of *GAL* gene expression is rapid and stringent. The mechanism responsible for most of the repression of *GAL1* expression (#2, reduction in Gal4p levels due to repression of *GAL4* transcription) acts slowly: it cannot take effect until the preexisting Gal4p is diluted out (Gal4p is very stable). Indeed, when this is the only repression mechanism operating on *GAL1*, several hours are required to establish repression. The third mechanism (binding of Mig1p to the *GAL1* promoter) achieves rapid repression of *GAL1* expression by inhibiting the function of the preexisting Gal4p bound at the *GAL1* promoter: this mechanism represses *GAL1* expression within 20 minutes after glucose addition. Thus, the role of the third mechanism appears to be to rapidly repress *GAL1* expression until the other two slower mechanisms become established.



We are studying the promoter of the *GAL4* gene for several reasons. First, we want to know why it is among the weakest of all yeast promoters, because we are interested in understanding the basis for promoter strength. Second, it appears to contain no TATA box, and we wish to understand how such promoters function. Third, we want to understand the mechanism of glucose repression of *GAL4* expression. Finally, we want to identify the transcriptional activators of a gene that itself encodes a transcriptional activator. We believe such proteins may be important "global" regulators in yeast.

Our analysis of the *GAL4* promoter reveals three types of regulatory elements: **UAS**, **URS**, and a new type of element we term **UES**. The most upstream element appears to be a **UAS**: deletion of this element reduces but does not abolish *GAL4* expression. This element is likely the binding site(s) for the transcriptional activator of the *GAL4* gene. Between the UAS and the RNA start site is an A+T-rich element termed the **UES** (Upstream Essential Sequence) that may fulfill the role of the TATA box: it is in a location expected of a TATA box, and deletion of this element *abolishes GAL4* expression. However, the UES is clearly functionally distinct from a TATA box: it does not function in place of a TATA box in another promoter, nor does a consensus TATA box function in place of the UES in the *GAL4* promoter. Furthermore, the UAS of the *GAL4* promoter does not function when placed in another promoter containing a TATA box. These results lead us to the conclusion that the weak *GAL4* promoter contains novel elements that appear to be functionally co-adapted. The interaction between these elements may set the low level of *GAL4* expression. We are eager to identify the proteins that bind to these elements. The **URS** consists of two binding sites for the Mig1p repressor. It is located between the UES and the RNA start site of *GAL4*. and is responsible for glucose repression of *GAL4* expression.

Galactokinase, a regulatory protein of lactose/galactose metabolism

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The yeast species Saccharomyces cerevisiae and Kluyveromyces lactis are related and have been shown to share a number of structural and regulatory properties. The comparison of the sequence of equivalent regulatory genes from both organisms has become an effective start to the identification of conserved and therefore potentially important regions in the gene products.
The genes for lactose and galactose metabolism in K. lactis are organized and regulated in an analogous fashion to the galactose system in S. cerevisiae (Dickson and Martin, 1980; Riley and Dickson, 1984). The similarities between both systems include the high degree of homology between the upstream activating sequences mediating galactose induction (Salmeron and Johnston, 1986) and the positive regulatory proteins LAC9 and GAL4, which can function in both organisms (Salmeron and Johnston, 1986; Breunig and Kuger, 1987; Riley et al. 1987; Salmeron et al. 1989).
The genes in S. cerevisiae encoding enzymes of galactose and melibiose metabolism are induced shortly after a cell grown on a nonfermentable carbon source receives galactose (see review Johnston, 1987). The induction mechanism is dependent on the function of GAL4, GAL80 and GAL3. The genes GAL4 and GAL80 encode proteins which are directly involved in transcription activation and repression of galactose governed promotors. The GAL3 gene has been analyzed in more detail, but its function still remains unknown (Bajwa et al. 1988; Torchia and Hopper, 1986; Bhat et al. 1990). Most noticeably, gal3 mutants exhibit an increased induction time of several days, which led to the hypothesis that GAL3 is responsible for the synthesis of the inducer molecule. No K. lactis mutations with a phenotype similar to that of S. cerevisiae gal3 have been described.
In a study on the regulation of lactose metabolism in K. lactis, we have isolated a number of new regulatory mutants. One of these mutants carries a recessive mutation which leads to loss of induction of ß-galactosidase, galactokinase and lactose permease. This phenotype was due to a mutation in the K lactis GAL1 gene (kl-GAL1), which until now was only known to encode the enzyme galactokinase.
We have analyzed the two phenotypically distinct types of gal1 mutants. The gal1-r type of mutant lacks on galactose the induction of ß-galactosidase and the enzymes of the Leloir pathway, whereas the gal1-209 type only lacks galactokinase activity. Our data implicate an additional function for the GAL1 gene product that is required for the induction of the lactose/galactose system (Meyer et al. 1991). This regulatory function is not dependent on galactokinase activity, as it is still present in the galactokinase negative mutant gal1-209. Complementation studies in S. cerevisiae show that K. lactis GAL1 and gal1-209, but not gal1-r can complement the gal3 mutation. We conclude therefore that the regulatory function of GAL1 in K. lactis directly after induction, is similar to the function of GAL3 in S. cerevisiae. Based on these data, we have proposed a new model for galactose induction (Meyer et al. 1991). Recently, Bhat and Hopper (1992) have confirmed this function of GAL1p .
Overexpression of the positive regulator LAC9 or deletion of the negative regulator kl-GAL80 can suppress the uninducibility of gal1-r mutants, which places the site of action of GAL1p upstream of the LAC9 function. Overexpression of kl-GAL1 leads to a partial

derepression of the lactose/galactose genes, which suggests it to act as a regulator downstream of the inducer.
The mutations in the alleles gal1-r and gal1-209 have been localized. A more detailed analysis of functional domaines will be discussed.

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Fourth session

Heme Regulation of Gene Expression in Yeast

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The yeast *Saccharomyces cerevisiae* is a facultative aerobe. It regulates the expression of a large number of genes according to the availability of oxygen. Two sets of such genes are regulated by heme, the biosynthesis of which requires molecular oxygen at two steps. Transcription of the first set is activated by heme and includes nuclear genes that encode mitochondrial functions, such as components of the electron transport chain, and functions involved in controlling oxidative damage, such as superoxide dismutase and catalase. Transcription of the second set is repressed when heme is present in cells and encodes hypoxic functions such as alternate cytochrome subunits and oxidases involved heme and membrane syntheses.

Heme activation of transcription for a given gene is achieved through one (in some cases both) of two possible activators: the heme-dependent Hap1 protein or the heme-dependent, catabolite-repressible Hap2/3/4 complex. These activators bind to specific DNA sequences upstream of the target genes. Heme repression is mediated by the Rox1 repressor which binds to a specific operator sequence upstream from is target genes. Transcription of *ROX1* is heme-dependent, activated by Hap1, while the function of the Rox1 protein is heme-independent. Additionally, the Rox1 protein has a short half-life in yeast cells which permits a rapid induction of hypoxic functions. Thus heme repression results from the regulation of repressor synthesis and not repressor function.

The function of these DNA binding proteins is controlled through other cellular factors that modulate their activity. One such factor is the Tup1/Ssn6 heterodimer. Mutations in either the *TUP1* or *SSN6* gene encoding the proteins of this complex cause constitutive expression of heme-repressed genes, loss of catabolite repression, low level expression of heme-activated genes in the absence of heme, α-sterility (loss of repression of a-specific genes in a α-cell), and flocculence. This pleiotropy can in part be explained by a model in which this complex serves as a negative adapter between a a number of specific DNA binding proteins and the transcriptional machinery; when this complex is anchored to the DNA, repression occurs. In support of this model, the Rox1 repressor binds to its DNA operator site in extracts prepared from *tup1* or *ssn6* deletion mutants, but does not repress transcription *in vivo*. Alternatively, artificially anchoring the Tup1 or Ssn6 protein to DNA causes repression in the absence of a specific DNA binding protein.

Another factor involved in mediating repression is Rox3, an essential, nuclear protein. Cells carrying carboxyl-terminal deletions of Rox3 are viable, but show altered transcription patterns. Possible roles of Rox3 in transcription will be discussed.

CONTROL OF EXPRESSION OF THE GENES FOR THE GLUCONEOGENIC ENZYMES IN *SACCHAROMYCES CEREVISIAE*

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The gluconeogenic enzymes fructose-1,6-bisphosphatase and phosphoenolpyruvate carboxykinase are strongly repressed by glucose. The mechanisms underlying glucose repression of the corresponding genes, *FBP1* and *PCK1* , have not been yet elucidated but we have established that glucose not only decreases the rate of transcription of the genes but also affects the stability of the corresponding mRNAs.

To assess the role of different regions of the respective promoters on the transcription of the *FBP1* and *PCK1* genes we have constructed *FBP1-lacZ* and *PCK1-lacZ* fusion genes and studied the effect of deletions in the promoters in the expression of *lacZ* , both in glucose-grown cells and in derepressed yeast. Several UAS and URS regions have been identified in both promoters, as well as sites implicated in catabolite repression. Both genes harbour consensus sequences for the binding of the same regulatory proteins such as yAP1, MIG1 and the complex HAP2/HAP3/HAP4. In addition the lack of expression of β-galactosidase from fusions not including sequences from the coding regions of *FBP1* or *PCK1* suggests the existence of downstream activating elements.

In the *FBP1* promoter we detected by footprinting assays two sequences able to bind nuclear proteins. They are very similar to the consensus sequence for the binding of the regulatory protein MIG1, thus suggesting a role for MIG1 in the regulation of *FBP1* transcription. However, neither deletion nor overexpression of the *MIG1* gene affected the regulated expression of the *FBP1* or *PCK1* genes. A possible explanation for this observation would be the existence of some other regulatory protein with a Zn-finger sequence similar to that of MIG1 and which could therefore bind to sequences related to those recognized by MIG1.

To investigate the physiological relevance of catabolite repression of the gluconeogenic enzymes we have put the corresponding genes under the control of a promoter which is not repressed by glucose. The expression of the gluconeogenic enzymes under glycolytic conditions has no striking consequences on yeast survival but causes appreciable changes in the metabolism of yeast.

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Fifth session

CIS-ACTING ELEMENTS AND TRANS-ACTING FACTORS REQUIRED FOR TRANSCRIPTIONAL ACTIVATION OF THE ALLANTOIN SYSTEM GENES, AND THEIR RESPONSE TO INDUCER, AND NITROGEN CATABOLITE REPRESSION

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Transcriptional activation of all allantoin pathway genes in *S. cerevisiae* is mediated by two or more copies of the upstream activation sequence, UAS_{NTR} (MCB9:602-608). Saturation mutagenesis of UAS_{NTR} demonstrated it to be a dodecanucleotide containing the sequence GATAA at its core (J. Bacteriol. 173:4977-4982). A **DAL5** UAS_{NTR} has been shown to footprint indicating that protein(s) bind to it. Expression of all allantoin pathway genes is highly sensitive to nitrogen catabolite repression (NCR). This physiological response was explained by the observation that operation of UAS_{NTR} is highly NCR-sensitive (MCB 9:5440-5444). We previously demonstrated that transcriptional activation mediated by UAS_{NTR} also requires a functional **GLN3** gene product (J. Bacteriol. 172:1014-1018). Minehart and Magasanik reported that the **GLN3** product contained a sequence homologous to the GATA protein family of DNA binding proteins which also bind DNA fragments containing the sequence GATAA (MCB 11:6216-6228).

In addition to the negative effects of nitrogen catabolite repression, allantoin system genes are negatively regulated by the **DAL80** product; steady state levels of **DAL** gene-specific mRNAs are markedly elevated in **dal80** mutants. **DAL80** control is not, however, confined to the allantoin pathway. **UGA1** expression occurs at high levels in the absence of inducer in **dal80** mutants. We cloned the **DAL80** gene, determined the response of its expression to induction and nitrogen catabolite repression (NCR), its nucleotide sequence, and generated a chromosomal **dal80** null mutant. **DAL80** gene expression does not respond to induction by the allantoin pathway gratuitous inducer, oxalurate. However, in contrast to the positively-acting allantoin pathway regulatory proteins, **DAL80** expression is reduced to undetectable levels when cells are provided with readily used nitrogen sources. Not surprisingly, the DAL80 promoter contains multiple copies of the **DAL** UAS_{NTR} (MCB 9:5440-5444). One of the most intriguing characteristics of the **DAL80** protein sequence is the presence of a stretch of amino acids sharing high homology with the yeast **GLN3** protein, a **Neurospora** transcription factor (NIT2), and a vertebrate transcription factor (GATA-1) both of which bind to a sequence that is homologous to the **DAL** UAS_{NTR} (MCB 9:602-608 & 9:5440-5444).

To ascertain whether or not **DAL80** protein binds to UAS_{NTR} sequences of the allantoin pathway genes as suspected from its structural homology to mammalian GATA binding proteins, EMSA and foorprinting assays employing **DAL80** protein produced in **E. coli** were used to demonstrate that **DAL80** protein specifically binds to UAS_{NTR}-homologous sequences.

The structural relationships between **DAL80** and **GLN3**, participation of UAS_{NTR} in positive and negative regulation mediated by **DAL80**, and UAS_{NTR} mediation of nitrogen catabolite repression of allantoin pathway

genes prompted us to ask whether or not these correlations extended broadly to other nitrogen catabolic genes. Therefore, we tested 14 genes with multiple copies of UAS_{NTR} in their upstream regions for: sensitivity to NCR and **DAL80** regulation, requirement of a functional **GLN3** product for their expression. Every catabolic gene we tested that contained multiple copies of UAS_{NTR} upstream of its TATA sequences was sensitive to NCR. Included among this sample of genes was **CAN1** which has long been accepted to be NCR-insensitive. Expression of most of these genes also increased to a greater or lesser extent in a **dal80** mutant. On the other hand, expression of most, but not all, of the catabolic genes required a functional **GLN3** gene product. Most notably, expression of the **UGA1** gene was highly sensitive to NCR, increased markedly in a **dal80** deletion mutant, and occurred at nearly wild-type levels in a **gln3** deletion mutant. These observations support the suggestion that UAS_{NTR} is the cis-acting site through which NCR is exerted. They also suggest that if NCR is exerted through the **GLN3** gene product, there must be an alternate route for this physiological response as well.

Finally, expression of the **DAL4**, **DAL7**, **DUR1,2**, and **DUR3**, genes in **S. cerevisiae** is induced by the presence of allophanate, the last intermediate of the allantoin degradative pathway. Analysis of the **DAL7** 5'-flanking region has identified an element, designated the **DAL** upstream induction sequence (**DAL UIS**), required for response to inducer (MCB 9:3231-3243). The operation of this cis-acting element requires functional **DAL81** and **DAL82** gene products (J. Bact. 173:255-261 & MCB 11:1161-1166). Since this element participates in a transcriptional response to the allantoin-pathway specific signal molecule, allophanate, the protein binding to it would be expected to be pathway specific. The observation that **dal81** mutants exhibit a pleiotropic loss of **UGA1** induction in addition to that of the **DAL** system genes argued against **DAL81** protein binding directly to the **DAL7 UIS** element. **DAL82** product, which appears to be allantoin pathway-specific, on the other hand, was a reasonable candidate for such a binding function. These hypotheses were tested using electrophoretic mobility shift assays (EMSAs) for protein binding. To establish specificity of protein binding to the **UIS** element we determined its structure using saturation mutagenesis. A dodecanucleotide sequence is the minimum required for response of reporter gene transcription to inducer. There are two copies of the sequence in the 5' flanking region of the **DAL7** gene, both of which function. There are also functional copies of the **DAL UIS** element upstream of all inducible allantoin pathway genes. Using EMSA and footprinting assays with **DAL82** protein produced in **E. coli**, we demonstrated that **DAL82** encodes the **DAL UIS** binding protein. This work is supported by NIH grant GM-35642 and FRD grant 11-2-2-237.

PARTICIPATION OF SPECIFIC AND PLEIOTROPIC FACTORS IN THE REGULATION OF ARGININE METABOLISM IN *S.CEREVISIAE*.

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ARGRI, ARGRII and ARGRIII proteins control the expression of arginine anabolic and catabolic genes. They repress the synthesis of five anabolic enzymes (encoded by *ARG5,6, ARG8, ARG3* and *ARG1* genes) and induce that of two catabolic ones (encoded by *CAR1* and *CAR2* genes). We have shown that they are also required in vitro, to observe a protein/DNA complex with the arginine anabolic and catabolic promoters, in the presence of arginine. By creation of mutations, DNase 1 protection footprinting and by gel shift assays, we have defined in anabolic and catabolic promoters two inverted repeated sequences ("arginine boxes") required for regulation by arginine. These regions share homology with the P(PAL) sequence, which is the target of MCM1 protein. In vitro binding experiments to these promoters reveal that transcription factor MCM1, involved in transcription of cell-type-specific genes, is part of the ARGR regulatory complex. The participation of MCM1 in the regulation of arginine metabolism is confirmed by the behaviour of *mcm1-gcn4* mutant, which is affected in the repression of arginine anabolic genes and in the expression of the catabolic ones. MCM1 action requires the integrity of the ARGR proteins and acts through the "arginine boxes" present in all the promoters regulated by the ARGR system.
Whereas ARGRI and ARGRII are specific regulatory factors of the arginine metabolism, ARGRIII plays a pleiotropic function in the cell. Disruption of the gene leads not only to loss of arginine regulation but also to a lethal phenotype at 37°C. At 29°C, the growth of the disrupted strain is reduced. This strain is sterile and the homozygous *argRIII::URA3* diploid does not sporulate. Expression of

cell-type specific genes (*MFα, STE2*) is reduced, but MCM1 expression remains unaffected. By gel retardation assays with crude protein extracts, we show that ARGRIII seems to be required for the binding of MCM1 to its DNA target sequences. However, it is well established that pure MCM1 is able to interact with DNA , in the absence of additional factors. Therefore, we propose that ARGRIII could titrate an inhibitor preventing MCM1 to interact with DNA. We have already proposed a similar hypothesis for the role of ARGRIII in arginine regulation.

The arginine catabolism is also regulated negatively by three other proteins called CAR80, CAR81 and CAR82. Mutations in these genes do not lead to a negative phenotype, which renders difficult the cloning of these genes by growth complementation. Using a technique of transposon mutagenesis, we have created a *car80* mutation by Ty insertion. This Ty element contains the plasmid pian7 and the *SupF* gene, allowing its specific detection and its recovery from yeast. The complete *CAR80* gene was then cloned by colony hybridization. This gene encodes a 836 amino acid protein, with a Zinc-finger motif in its C-terminal end. Disruption of the gene leads to constituve expression of arginine catabolic genes, to slow growth and to a sporulation defect. The *CAR80* gene is allelic to the *UME6* gene, as shown Cooper's group. Our sequence is 100% identical to that of *UME6*, as determined by the group of R. Easton-Esposito (personal communication).

All these pleiotropic factors involved in arginine metabolism (MCM1, ARGRIII, CAR80) are part of a set of accessory proteins which play a role in transcription by assisting gene-specific regulatory proteins, such as ARGRI and ARGRII.

Transcriptional control of anabolic and catabolic threonine deaminases in yeast. Steen Holmberg, Institute of Genetics, University of Copenhagen, DK-1353 Copenhagen, Denmark.

Regulation of the yeast genes encoding enzymes involved in amino acid biosynthesis is very different from the regulation of the corresponding genes in bacteria. When amino acids are present in the growth medium, bacteria turn off the transcription of the genes for their amino acid biosynthetic enzymes. Under similar growth conditions, yeast cells maintain a significant level of transcription from amino acid biosynthetic genes, often referred to as the basal level.

The *ILV1* gene of *Saccharomyces cerevisiae* encodes the enzyme for the first committed step in isoleucine biosynthesis and is regulated by the general control system for amino acid biosynthesis. Deletion analysis of the *ILV1* promoter revealed a GC-rich element important for the basal level expression. This *cis*-acting element, called $ILV1_{BAS}$, is functional independently of whether GCN4 protein is present. Furthermore, unlike the situation at *HIS4*, the magnitude of GCN4-mediated derepression is independent of $ILV1_{BAS}$. The element has homology to the consensus REB1-binding sequence CGGGTARNNR. Gel retardation assays showed that REB1 binds specifically to this element. REB1-binding sites normally situated in the *SIN3* promoter and in the 35S rRNA promoter can substitute for the *ILV1* REB1 site. Furthermore, a *SIN3* REB1 site containing a point mutation that abolishes REB1 binding does not support *ILV1* basal level expression, suggesting that binding of REB1 is important for the control of *ILV1* basal level expression. Interestingly, an ABF1-binding site can also functionally replace the *ILV1* REB1-binding site. A mutated ABF1 site that displays a very low affinity for ABF1 does not functionally replace the *ILV1* REB1 site. This suggests that ABF1 and REB1 may have related functions within the cell.

Although the REB1-binding site is required for the *ILV1* basal level expression, the site on its own stimulates transcription only slightly when combined with the *CYC1* downstream promoter elements, indicating that another *ILV1* promoter element functions in combination with the REB1 site to control high basal level expression. A poly(dA) element situated between the *ILV1* REB1 site and the uppermost transcription initiation site also displayed a poor UAS activity of its own. However, additional insertion of the REB1 site upstream of the poly(dA) element enhances synergistically the effect on transcription of this weak activator. This effect of REB1 is strongly distance dependent. In available data bases, many yeast promoters containing a REB1 site in front of a poly(dA) element were found. Such a combination may provide constitutive or high basal level expression to these genes.

Strains devoid of a functional ILV1 protein have a requirement for isoleucine. Phenotypic suppression of *ilv1* can occur by activation of a catabolic threonine/serine deaminase, encoded be the *CHA1* gene, i.e. the catabolic deaminase is able to substitute for the anabolic deaminase. Expression of the *CHA1* gene is transcriptionally induced by serine (80-100 fold), and to a lesser extent by threonine, and allows the yeast to grow on media with serine or threonine as sole nitrogen source. Deletion analysis has identified a *cis*-acting element in the *CHA1* promoter, UAS_{CHA}, needed for induction. This

sequence confers serine induced expression to a heterologous promoter. We have isolated a recessive mutation, designated *cha4-1*, that prevents cells from utilizing serine/threonine as sole nitrogen source. The *cha4-1* mutation reduces basal level expression of *CHA1* (aspargine as nitrogen source) 8-10 fold and eliminates inducible expression. Furthermore, serine induced expression of a heterologous promoter through UAS_{CHA} depends on the presence of *CHA4*. The *CHA4* gene has been cloned and sequenced. The CHA4 protein has a deduced length of 648 amino acids, including an N-proximal (residue 43 to residue 70) motif known as the $Zn(II)_2Cys_6$ binuclear cluster, suggesting that *CHA4* encodes a DNA binding protein. Furthermore, four acidic regions of 25 - 40 amino acids can be recognized. Gel retardation experiments employing an *E. coli* produced truncated version of CHA4 (residue 1 to residue 174) and wild-type and mutated forms of UAS_{CHA} demonstrate that CHA4 specifically binds to UAS_{CHA} *in vitro*.

As mentioned above, induction of *CHA1* leads to suppression of *ilv1*. We have identified a recessive mutation, *sil2*, and a dominant mutation, *SIL3*, that both suppress *ilv1* by inducer independent, constitutive transcription of *CHA1*. Interestingly, gap-repair analysis has demonstrated that *sil2* and *SIL3* both are alleles of *cha4*. We have cloned six alleles of *SIL3* and two alleles of *sil2* and are in the process of localizing the mutated site in the CHA4 protein in order to identify functional domains.

TRANSLATIONAL AND TRANCRIPTIONAL REGULATION OF GENE EXPRESSION: THE GCN4 MODEL IN YEAST

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One important feature in biological systems is their ability to reprogram gene expression in response to environmental, hormonal or intercellular signals. Yeast cells adapt to amino acid limitation by increasing the levels of transcription of genes encoding amino acid biosynthetic enzymes. This increase is mediated by the binding of the GCN4 transcriptional activator at a specific DNA site located within the promoter region of these genes. Underlying this transcriptional regulatory mechanism is a translational control system necessary to mediate the response. When amino acids are limited, translation of the GCN4 mRNA is increased while the rates of the overall protein synthesis are reduced. The regulated step in this response is the formation of preinitiation complexes between the 40S ribosomal subunit and the initiator met tRNA (43S complexes).

All evidence indicate that the amino acid starvation signal is transduced to the protein synthetic machinery through the mediation of the GCN2 protein kinase. This protein kinase when activated (quantitatively and qualitatively) phosphorylates the translation initiation factor eIF2. This phosphorylation then results in the reduction of the formation of 43S preinitiation complexes. The translational activation of the GCN4 mRNA under these conditions is due to the unusual structure of this message. Its 5' untranslated region contains four small open reading frames which engage the 40S ribosomal subunits to repeated rounds of translational reinitiation events. This accounts for the low levels of translational initiation at the GCN4 coding AUG when the growth conditions are normal. When the recharging of the 40S subunits with met tRNA is limited (i.e.under amino acid limitation conditions) these reinitiation events are reduced and thus more 43S complexes can establish translation initiation events at the GCN4 coding AUG.

Translational activation of the GCN4 mRNA is necessary but not sufficient for the transcriptional enhancement of the amino acid biosynthetic genes. We have recently revealled that the GCN4 mediated transcriptional activation requires the function of the GCN5 protein. This protein is a transcriptional coactivator which shares a region of similarity with other proteins such as SNF2/SWI2, SPT7, STH1 in yeast, Brm1,Fsh1 in Drosophila and FSH1,CCG1 in mammals. The requirement for the GCN5 protein correlates with the ability of the GCN4 protein to gain access to its target and preliminary evidence suggests that the GCN5 protein affects the chromatin structure. Finally, the GCN5 protein is a general coactivator since its function is required for maximal levels of transcription of genes regulated by the HAP2/HAP3/HAP4 complex. The possible interactions of the GCN5 protein with these activators, elements of the chromatin and the core transcriptional machinery is under investigation.

Sixth session

THE *SSL1* and *SSL2* SUPPRESSOR GENES OVERCOME THE INHIBITORY EFFECT OF A STEM-LOOP MUTATION IN THE *HIS4* LEADER POST-TRANSCRIPTIONALLY AND RENDER YEAST CELLS HYPERSENSITIVE TO UV-LIGHT.

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We initiated a genetic reversion analysis to identify components of the translation initiation complex that mediate ribosomal binding/scanning of mRNA. A 36 bp sequence with perfect dyad symmetry was inserted into the *HIS4* leader region which resulted in a His- phenotype. A genetic reversion analysis of this strain has identified eight unlinked suppressors genes (*SSL*) of the stem-loop mutation which restore *HIS4* expression.

The *SSL2* suppressor gene encodes an essential 95-kDa protein that contains motifs characteristic of it being a member of the superfamily of RNA helicases. *SSL2* is 54% identical at the amino acid level to the human gene *ERCC-3 (XPBC)*. *ERCC-3* is a gene which is correlated with the disease xeroderma pigmentosum and Cockayne's syndrome, ailments resulting from the inefficient repair of damaged DNA. A synthetically derived allele of *SSL2* designed to resemble the human disease allele conferred UV light sensitivity to yeast cells. In contrast, the *SSL2* suppressor allele is as resistant to UV-irraditation as wildtype cells. Also, *SSL2* does not suppress *HIS4* by any obvious mechanism related to DNA repair but rather via a post-transcriptional mechanism.

The *SSL1* gene encodes an essential protein with a calculated M_r-value of 52-kDa. The *SSL1* gene contains eight Cys-X_2-Cys-like sequences located in the carboxyl end of the protein, suggesting that this protein could play a role in nucleic acid binding. The mechanism of *SSL1* suppression is not related to either increasing the level of *HIS4* transcription nor altering the start position of the *HIS4* transcript. Instead, the mechanism of *SSL1* suppression is post-transcriptional and results in a 3-5 fold increase in *HIS4* translational expression despite the stem-loop mutation in the *HIS4* leader region. *SSL1* suppressor strains which are conditional for growth on enriched medium show an aberrant polysome profile at the restrictive temperature, indicating that *SSL1* protein is involved in translation initiation. In agreement with this, cell-free extracts made from these Ts- strains also display an inherent thermolabile defect in their ability to translate exogenously added mRNA. These studies indicate that the *SSL1* gene product is essential for translation initiation in yeast. Interestingly, the *SSL1* strains that exhibit an in vivo and in vitro defect in translation are also hypersensitive to UV irradiation. To explain these diverse phenotypes, we propose a model whereby *SSL1* and *SSL2* gene products function in translation initiation and control the expression of a gene product that is necessary for the DNA repair process.

POSTERS

A complex combination of specific and pleiotropic regulatory proteins determine the expression of the GABA utilization pathway genes in *Saccharomyces cerevisiae*

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In *S. cerevisiae*, transcription of the *UGA* genes involved in utilization of 4-aminobutyric acid (GABA) as a nitrogen source is induced in the presence of GABA. In addition, it is subject to nitrogen catabolic repression (NCR) when a preferred nitrogen source is available.

UGA3 and UGA35 are two trans-acting factors required for GABA induction of the *UGA* genes. UGA3 is an inducer-specific factor: it specifically regulates expression of the GABA-inducible genes. In contrast, the positive regulation mediated by UGA35 extends to other inducible nitrogen pathway genes. Specifically, we have shown that *UGA35* is identical to the *DURL* and *DAL81* genes previously identified as positive regulators of allophanate-inducible genes, including the *DUR* genes of the urea degradation pathway and some of the *DAL* genes for allantoin utilization. In the allophanate induction process, the UGA35/DURL/DAL81 pleiotropic factor acts in conjunction with DURM/DAL82 which, like UGA3, appears to be an inducer-specific factor. The *UGA3* and *UGA35/DURL/DAL81* gene products both contain a GAL4-type zinc-binding domain and other features found in some DNA-binding transcriptional activators. The two proteins mediate GABA induction through an 18-nucleotide, partially palindromic, upstream activating sequence: this UAS_{GABA}, present in one or two copies in the 5'-flanking regions of the *UGA* genes, directs GABA-dependent reporter gene transcription in a UGA3- and UGA35/DURL/DAL81-dependent fashion.

The UAS_{GABA} present upstream from the *UGA1* and *UGA4* genes act cooperatively with other proximal UASs. These UASs contain the sequence GAT(A/T)A at their central core and are shared by multiple, constitutive or inducible nitrogen pathway genes. It was shown by others that the product of the *GLN3* gene is required for expression supported by $UASs^{GATA}$ isolated from the *DAL5* and *DAL7* genes. Expression conferred by a cluster of $UASs^{GATA}$ located upstream from the *UGA4* gene is partially dependent on GLN3, as is induction of *UGA4* in response to GABA. Experiments will be presented indicating that another pleiotropic factor, in addition to GLN3, is required for transcription supported by the clustered $UASs^{GATA}$ of *UGA4*. The deduced GLN3 sequence contains a particular type of zinc finger, called GATA zinc-finger. This motif, present in highly conserved duplicate in the vertebrate GATA family of transcription factors, directs binding to GAT(A/T)-containing sequences. The *UGA43* gene (recently shown to be identical to *DAL80*) is yet another *S. cerevisiae* gene encoding a GATA zinc-finger protein. It encodes a negative regulator of differentially induced nitrogen pathway genes, including *UGA4*: in *uga43/dal80* mutant cells, *UGA4* is constitutively expressed at high levels. The UGA43/DAL80 factor was shown to exert strong negative control over the clustered $UASs^{GATA}$ from *UGA4*.

In the presence of a preferred nitrogen source like ammonium ions, the expression of the *UGA* genes is subject to NCR. This repression requires the integrity of the *URE2/GDHCR* gene and glutamine as an effector. Positive regulation exerted by the UAS_{GABA} seems to be unaffected under conditions of NCR. In contrast, like the $UASs^{GATA}$ from the *DAL5* and *DAL7* genes, the $UASs^{GATA}$ of the *UGA* genes are extremely sensitive to NCR. This repressive effect requires both URE2/GDHCR and glutamine.

Expression of the *UGA43/DAL80* negative regulatory gene is also extremely sensitive to NCR, so that repression by UGA43/DAL80 does not occur under conditions of NCR. This profile of expression suggests the following role for UGA43/DAL80: upon release from NCR, the newly synthesized UGA43/DAL80 proteins take over from the NCR exerted upon the $UASs^{GATA}$, thereby preventing these UASs from conferring expression in the absence of inducer.

Is the RAS-cAMP signaling pathway involved in the control of catabolite repression in Saccharomyces cerevisiae?.

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Study of the RAS-cAMP pathway in S. cerevisiae has attracted considerable interest in the last years, because this yeast contains two RAS genes highly homologous of the human ras genes, which are involved in cellular oncogenic transformation. Whereas in humans the effector of RAS proteins remains unknown, in S. cerevisiae, RAS proteins are specific modulators of adenylate cyclase. The RAS-cAMP pathway appears to work as nutrient transmission signal specifically triggered by glucose and related sugars. S. cerevisiae is a fermentative yeast, and the signal behaves as a sensor that detects the presence of external fermentable carbon sources and subsequently promotes a change in cellular metabolism: from a respiratory-gluconeogenic fashion to a fermentative-anaerobic fashion. In turn, glucose exerts a drastic repression on a set of genes that code for enzymes required for hydrolysis of carbohydrates, gluconeogenic enzymes and enzymes involved in mitochondrial respiration (glucose or catabolite repression).

The possible relation between glucose repression and cAMP in yeasts is still disputed. The most extended claim stands that low levels of cAMP do not correlate with the repressive effect, as it occurs in prokaryotes. However, conclusive evidence has recently proven an essential role of cAMP on the transcriptional control of several genes such as ADH2, CTT1 or UB14.

We have measured the existence of fluctuations in the cAMP signal under different physiological conditions of repression as well as in mutants altered in the mechanism of glucose repression. Our results can be summarized as follows: (i) In glucose-grown stationary cells and glycerol-grown exponential cells (derepressed cultures), addition of glucose triggers a rapid and transient cAMP signal within 15 seconds. This signal is followed by a cAMP-dependent phosphorylation cascade that modifies the activity of certain key enzymes. However no cAMP increase is detected after glucose addition to glucose-grown exponential cells (repressed cultures). (ii) In mutants unable to undergo derepression (cat1, cat3), the glucose-induced cAMP signal is absent provided that there is no limitation of ATP synthesis. (iii) In mutants lacking glucose repression such as hxk2 or hex2, the cAMP increase is always recorded in cultures growing actively on glucose. Interestingly, the mutant cat80 belonging to this group, is deficient in all the cAMP elevations so far known, but still exhibits a certain cAMP basal level. We have found in the genetic background of this strain another mutation termed lcr1, which is responsible of this phenomenon. The mutation lcr1 appears to be allelic to CDC35-CYR1 (adenylate cyclase). (iv) Mutants with drastic reduction of cAMP-dependent protein kinase activity (tpk^w strains) display a huge of cAMP hyperaccumulation and are deficient in glucose repression of mitochondrial respiration.

All results here presented are consistent with the existence of a glucose-repressible protein in the RAS-cAMP pathway, protein that has not yet been identified. However, our data do not mean that the RAS-cAMP signal is responsible for the induction of catabolite repression, rather they simply suggest that both systems share a common initiation point and that cAMP might play a role in some step of the mechanism of repression.

CLONING AND CHARACTERIZATION OF TWO GENES (GUS1 AND GUS2) CODING FOR GLUTAMATE SYNTHASE ACTIVITIES IN SACCHAROMYCES CEREVISIAE.

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In several microorganisms,glutamate biosynthesis can be achieved through the action of the NDAP-dependent glutamate dehydrogenase (NADP-GDH) or, through the concerted action of glutamate synthase (GOGAT) and glutamine syntetase (GS). It has been previously shown that S. cerevisiae has a GOGAT activity composed of a large and a small subunit (169,000 and 61,000 daltons respectively).
However previous data suggested that this yeast had two GOGAT activities (GOGAT A and B),one should be the already described dimeric enzyme ,and the other one would be a novel GOGAT enzyme,not described so far.(1)
Using Romex o (gdh,gus1,gus2,ura39), a mutant strain altered in both GOGAT A (GUS1) and B (GUS2) (1),we were able to obtain by complementation:
a) a serie of Ycp50 plasmids all containing a 4.5 Kb yeast genomic fragment, coding for a 1.5Kb mRNA.This gene corresponds in our opinion to the small subunit of GOGAT B (GUS2)(2).We are currently studying ,by Northern analysis,the transcriptional control of GUS2 gene expression.
b) recentely we isolated a new plasmid (pGA31) which also complements the lack of GOGAT activity of the mutant.Biochemical chacterization of the yeast transformants obtained indicated that we had complemented gus1 mutation. pGA31 recognizes on Northern a 5.5 Kb mRNA.Our previous studies on proteins associated with GOGAT activities permitted the identification of a polypeptide of about 200KDs copurifying with the dimeric enzyme already described.We hypotize that the insert present in pGA31 codes for a second glutamate synthase, never described in yeast, constituted as in Neurospora or in plants by a single polypeptide of high molecular weight.
We are currentely sequencing both genes and doing gene disruptions.

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ISOLATION IN *SCHIZOSACCHAROMYCES POMBE* OF A HOMOLOG OF THE *SACCHAROMYCES CEREVISIAE CIF1* GENE

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Saccharomyces cerevisiae cif1 mutants do not grow on glucose or fructose, although growth on galactose or non-fermentable carbon sources is not impaired (Navon et al. (1979) *Biochemistry* 18, 4487-4499). These mutants show several regulatory defects, such as the absence of catabolite inactivation of fructose-1,6-bisphophatase or the lack of cAMP response to glucose. The *CIF1* gene has been isolated (González et al. (1992) *Yeast* 8, 183-192) and it encodes a protein of 495 aminoacids. It has been recently shown that CIF1 is the small subunit of the trehalose-6P synthase complex (Vuorio et al. (1992) *Yeast* 8, S626). The sequence of *CIF1* shows no significant similarities with any other sequences present in the databanks except for a few stretches in the sequence of the product coded by an open reading frame of *Methanobacterium thermoautotrophicum* whose function is unknown (Ostergaard et al. (1987) *Syst. Appl. Microbiol.*9, 199-209).

Based on this slight similitary, degenerated oligonucleotides coding for two common regions were synthesized and used as primers in a PCR amplification experiment with genomic DNA from *Schizosaccharomyces pombe*. A 250 bp fragment was amplified and sequenced. The DNA sequence of the isolated fragment from *Sch. pombe* was 68% identical to the equivalent region in the *S. cerevisiae CIF1* gene.

Using this 250 bp fragment as probe, we screened a *Sch. pombe genomic* banck constructed on YEp13 (Broach et al. (1979) *Gene* 8, 121) by colony filter hybridization and found two overlapping clones. *S. cerevisiae cif1* mutants transformed with these plasmids can grow on glucose and fructose and other defects caused by this mutation are also eliminated. The effect of a disruption of this gene in *Sch. pombe* is being examined.

Yeast ADP/ATP carrier proteins - enzymatic properties and transcriptional control.

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In *Saccharomyces cerevisiae* the mitochondrial ADP/ATP carrier protein is encoded by three distinct nuclear genes: AAC1, AAC2 and AAC3 (1). The reason for the existence of multiple genes encoding proteins functioning in the adenine nucleotide translocation through the mitochondrial inner membrane is not readily apparent. The AAC genes are differentially regulated by environmental factors (oxygen), and it has been shown that only one of the genes (AAC2) is enough to supply sufficient amount of the ADP/ATP translocating activity to support both fermentative and nonfermentative growth of the yeast cells (2).
In order to elucidate the function of the separate proteins (75%-90% homologous) we created the whole set of the aac null mutants including the triple mutant, completely lacking the AAC proteins (3). All three AAC genes have been cloned into the yeast shuttle vector YPN2 under the yeast GAL promotor and expressed in the triple disruptant. Using this system we were able to isolate three types of mitochondria harboring separately the three AAC proteins and assay the ADP/ATP translocation activity of each of them. Herewith we showed that the three AAC proteins exhibit similar enzymatic characteristics (4).
Northern analysis of the AAC transcripts in the wildtype showed that the AAC genes are differentially regulated in response to the presence of oxygen. Whereas AAC1 and AAC2 are induced by oxygen, AAC3 is tightly repressed. To examine the regulation of the AAC3 gene, we isolated the gene with a more extensive upstream region (1000 bp) and determined the sequence. An AAC3-lacZ reporter was constructed and 5'deletions were introduced into the AAC3 promoter. The expression of the reporter gene was then studied in the presence and absence of oxygen and in different mutant strains. Deletion of the -230 to -120 upstream region resulted in a 100 fold increase of the AAC3-lacZ expression under aerobic conditions. A more comprehensive analysis of the AAC3 regulating elements provided evidence that a heme induced repression factor ROX1 (5) acts through a hypoxic consensus sequence similar to that found in the regulatory region of another hypoxic gene, ANB1 (6).

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ISOLATION AND PARTIAL CHARACTERIZATION OF MUTANTS RESISTANT TO CATABOLITE REPRESSION BY GLUCOSE IN *Candida utilis*.

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In *Saccharomyces cerevisiae*, a set of mutants deregulated for maltose and invertase expression has been isolated. Many of them, lacked or had altered the gene *hex 2*, which codes for a hexokinase (HK PII). This gene seems to have an important role in the scheme of control of catabolism of mono and disaccharides in glucose presence (recently also hexokinase PI has been related with catabolite repression). In other yeast genera the hexose-phosphorylating enzymes seem to be pieces in the control mechanisms of catabolite repression.

We have studied the relation between hexose-phosphorylating enzymes levels and catabolite repression in *Candida utilis*. Two hexokinases, one of them with very low specific activity, and one glucokinase were partially purified and characterized. However we couldn't relate the hexokinase levels with repression conditions.

To study further the problem we have tried to obtain derepressed mutants for disaccharides consumption. We have isolated spontaneous mutants using the industrial *Candida utilis* IGC 3092 as parental strain with the classical selection methods developed for *Saccharomyces cerevisiae*. Three of these mutants strains were able to growth in a bad invertase substrate medium (raffinose) in the presence of 2-deoxyglucose, due to high levels of invertase. The mutants were able to express high levels of maltase in presence of glucose, not been necessary maltose as inductor (total deregulation of maltose permease and maltase expression control). They didn't show diauxic behaviour when grown in a maltose-glucose medium. All of this mutants had active hexokinase and were able to growth in a fructose medium. This could mean that the failure in the catabolite repression control isn't in the HK level. Work on the enzymatic characterization of these mutants are in progress.

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Control of gene expression and salt tolerance in S.cerevisiae
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Salt stress induces the expression of many genes in plants and fungi. Some of these genes may be crucial components of salt adaptation and their genetic manipulation could improve salt tolerance in crop plants. We are utilizing the yeast *S. cerevisiae* as a model system for salt tolerance and the control of gene expression by salt in plants. We have identified two salt induced genes, HAL1 and HAL3 , which upon overexpression improve the growth of yeast under salt stress. Both genes are conserved between yeast and plants, and encode soluble proteins without significant homologies in data banks. The HAL3 protein,however, contains a carboxyl-terminal acidic domain shared by some transcription factors, suggesting that it could mediate signal transduction during salt adaptation. In adition we have identified two genes not yet isolated which modify the expression of HAL1 and HAL3. Overexpression of HAL4 results in constitutive expression of HAL1, and mutation of HAL5 causes constitutive expression of HAL3. The expectations that novel transcription factors and signal transduction pathways participate in the response of yeast and plant cells to salt stress heve been fulfilled.

Coordinate regulation of glycogen metabolism and its control by signaling pathways depending on RAS-cAMP and SNF1 in the yeast Saccharomyces cerevisiae.

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Glycogen is a major carbohydrate reserve in *S. cerevisiae* whose regulation and function are still poorly understood. During growth on glucose, glycogen begins to accumulate rapidly in late exponential phase, well before complete exhaustion of glucose from the medium. This rapid synthesis coincides with an increase in the activity of both glycogen synthase and glycogen phosphorylase, the key enzymes in the biosynthesis and the biodegradation of the polysaccharide, respectively, and with an activation of glycogen synthase and inactivation of glycogen phosphorylase by a mechanism of dephosphorylation. As in mammalian system, the dephosphorylation of yeast glycogen synthase involves the combination (probably as a form of complex) of protein phosphatase I (PP-1) encoded by *GLC7/DIS2S1* and the product of *GAC1* which codes for a regulatory subunit of PP-1 similar to the regulatory subunit of PP-1 from rabbit skeletal muscle. Remarkably, *GAC1* is induced at the time that glycogen accumulates, together with the increase in enzyme activities for glycogen synthase, glycogen phosphorylase and branching enzyme (*GLC3*), suggesting a coordinate regulation of glycogen metabolism at the level of gene expression.

The RAS-cAMP signaling pathway strongly influences glycogen metabolism. Mutants that have increased levels of cAMP or cAMP-dependent protein kinase (cAPK) have reduced levels of glycogen whereas mutant deficient in cAMP hyperaccumulate glycogen. While the activities of glycogen synthase and glycogen phosphorylase are controlled by reversible phosphorylation, it is not yet clear whether cAMP-dependent protein kinase directly phosphorylates these two enzymes. We found that at least part of the regulation by the RAS-cAMP pathway occurs at the level of protein synthesis since *GAC1* and *GPH1* transcripts do not accumulate in a mutant that has a constitutively activated cAPK.

The *SNF1* gene encodes a protein kinase which is required for derepression of many glucose repressible enzymes. The phenotype of a *snf1* null mutant is that commonly associated with an overactivation of the RAS-cAMP pathway. In particular, this mutant fails to accumulate glycogen. However, this phenotype is not restored by the disruption of the three genes encoding the catalytic subunit of cAPK, suggesting that *SNF1* acts through a cAMP-independent pathway. We raise the hypothesis of a role of *SNF1* protein kinase in the control of the dephosphorylation of glycogen synthase and glycogen phosphorylase since preliminary results indicate that glycogen synthase is less active and glycogen phosphorylase is more active in a *snf1* null mutant than in the control strain.

The regulatory factors HAP1, and HAP2 from *Saccharomyces cerevisiae* operate on oxygen induction and catabolite repression of the cytochrome c gene from *Kluyveromyces lactis*

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The cloning and sequencing of the KlCYC1 gene, allowed us to study the regulation of this gene homologous to the CYC1 gene from *S. cerevisiae*. There is a sequence $CCG(N_6)CCG$ at position -258 in the KlCYC1 upstream region which fits with the consensus sequence found in UAS1A and UAS1B from CYC1 (where binds the transcription factor HAP1), and also with the UAS1B from CYC7, through oxygen induction is exerced. A second sequence TTGGTTTGTTG is a common motive respect to the consensus sequence descrived for the UAS 2 from the CYC1, HEM1 and COX6 genes. The activation of the CYC1 gene in a non-fermentable carbon source is produced by the binding of a heterotrimeric complex, formed by HAP2,HAP3 and HAP4, to the UAS2 sequence.

Northern blot analysis of the KlCYC1 showed the existence of two transcripts, 0.89 and 1.28 Kb. in size which presented a double regulation:

- Catabolite repression was found in KlCYC1 since levels of expression were increased when cells were grown in a non fermentable carbon source as glycerol respect to those growing on glucose .
- Oxygen regulation (heme mediated). In aerobic cultures, heme increased the transcription of the gene; this was thrue for both glucose or glycerol growing cells. The gene was not expressed when cells were shifted to anaerobic conditions; nevertheless, when cultures were suplemented with heme we obtained similar levels to the aerobic ones without heme.

Heterologous expression studies were carried out in *Saccharomyces cerevisiae* strains ZW 13, wild type, and ZW10, carrying the CYP1-16 mutation The CYP1-16 allele is a mutant of HAP1(CYP1) which causes an increased expression of CYC7, and a decrease in CYC1 gene expression under anaerobic conditions (Zitomer et al. 1987). Both strains were transformed with a centromeric plasmid containing the KlCYC1 gene. The KlCYC1 expression in the ZW13 strain growing in aerobic or anaerobic conditions was higher than in the HAP1 mutant strain ZW10; in both strains aerobic expression was higher than that corresponding to anaerobic conditions. Heterologous expression was also studied in BWG1-7a (wild type) and LPY22 (Δhap1) strains (from L. Guarente). Cells from these strains were transformed with KlCYC1 and growth on lactate and glycerol compared; as expected LPY22 cells had a lower capacity to grow.

HAP2 from *S. cerevisiae* is essential for KlCYC1 expression since cells from J01-1a (Δ hap2),also isogenic to BGW1-7a, transformed with KlCYC1 were unable to grow on lactate or glycerol.

Taking together data from the upstream KlCYC1 region and heterologous expression, it seems clear that HAP1 and HAP2 from *S. cerevisiae* control the expression of the KlCYC1 gene. Regulation by oxygen and carbon source in KlCYC1 suggests that homologous factors to HAP1 and HAP2 operate to regulate its expression.

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Catabolite inactivation of heterologous fructose-1,6-bisphosphatases and fructose-1,6-bisphosphatase-ß-galactosidase fusion proteins in *Saccharomyces cerevisiae*

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Addition of glucose to *Saccharomyces cerevisiae* growing on non-sugar carbon sources inactivates fructose-1,6-bisphosphatase (FbPase). This phenomenon of catabolite inactivation is not observed in other yeasts such as *Schizosaccharomyces pombe*.

We have found that the enzyme of *Sch. pombe* or *E. coli* expressed in *S. cerevisiae* is not sensitive to the inactivation by glucose.

In order to determine sequences responsible for the susceptibility of *S. cerevisiae* FbPase to degradation we have constructed fusion proteins between different fragments of the *FBP1* gene from *S. cerevisiae* and the *Lac Z* gene from *E. coli*. When the fusion protein contains only the 19 N-terminal aminoacids from *FBP1*, the ß-galactosidase activity is not affected by the addition of glucose. A fusion containing the N-terminal half of FbPase (176 aminoacids) is inactivated after an initial lag of 30 min, at a rate similar to that of the *S. cerevisiae* FbPase.

An examination of the sequence of these 176 aminoacids showed the motif RAELVNLVG...KK...K similar to the "destruction box" described for the ubiquitin-dependent proteolysis of cyclins and the sequence QKKLD that could direct the import of the protein into the vacuole. The change of the last sequence to QKNSD or the removal of the putative "destruction box" did not affect the inactivation of the corresponding fusions proteins. We have tested also the inactivation of the *S. cerevisiae* FbPase in *pra1* mutants (lacking the vacuolar protease A) and *ubr1* (lacking a protein required for ubiquitin-dependent proteolysis by the N-end rule) without finding any difference with a wild type strain.

These results suggest that there are regions in *S. cerevisiae* FbPase located in its N-terminal half able to destabilize the protein and that neither the degradation by the N-end rule nor by vacuolar proteases are the unique mechanisms responsible for FbPase inactivation in *S. cerevisiae*.

GCN5 PROTEIN IS REQUIRED FOR THE FUNCTION OF TWO DISTINCT YEAST TRANSCRIPTIONAL ACTIVATORS.

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gcn5 mutant strains exhibit poor growth under conditions of amino acid starvation. It is known that such conditions require the action of the GCN4 transcriptional regulator. We have shown that the GCN5 gene product is required to promote normal transcriptional regulation of some of the biosynthetic genes which are under the GCN4 control.

gcn5 mutant strains also exhibit reduced growth rates when grown in nonfermentable carbon sources. Such conditions require the derepression of respiratory genes under the control of the HAP2/HAP3/HAP4 heterotrimer transcriptional activator. GCN5 protein function is required for achieving maximal levels of transcription of such genes.

Our results suggests two possible functions for the GCN5 protein : (i) it is required for the transcription activating function of the aformentioned transcriptional activators, or (ii) it facilitates their access to or interaction with their DNA binding sites. The latter maybe achieved by an alteration of the chromatin structure as a result of the GCN5 function.

Interestingly GCN5 protein shares a similar region with other yeast, Drosophila and human proteins (FSH1H; fsh1; CCG1; SNF2/SWI2; STH1; brm and SPT7). It has been suggested that many of these proteins are involved in transcriptional regulation through the alleviation of the barrier imposed by chromatin structure.

Experiments that can provide us evidence to dinstinguish among the possible functions of GCN5 are now in progress.

GLUTAMINE SYNTHESIS: A REGULATORY SIGNAL CONTROLLING GLUCOSE CATABOLISM IN *Saccharomyces cerevisiae*

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In *S. cerevisiae*, glutamine can be catabolized through glutaminase, w-amidase pathway and glutamate synthase. When this yeast is grown aerobically, the main pathway for glutamine degradation is glutaminase B. We have obtained mutants altered in this enzyme. These strains are unable to degrade glutamine, and thus accumulate this amino acid and are unable to grow on glutamine as sole nitrogen source. The physiological characterization of these mutants showed that glutamine accumulation results in a lowered glutamine synthetase activity and in an increased ATP: ADP ratio which reduces glucose metabolism. When a wild type strain was studied it was found that when glutamine synthetase activity is decreased, the ATP:ADP also rises, diminishing glucose metabolism. We propose that,through the action of glutaminase B and glutamate aminotransferase, a wild type strain degrades glutamine to 2- oxoglutarate, thus furnishing substrates that feed the TCA cycle., The regulatory role of glutamine synthesis and degradation in controlling carbon metabolism will be discussed.

TRANSCRIPTIONAL CONTROL OF S-PHASE ENTRY IN THE FISSION YEAST *SCHIZOSACCHAROMYCES POMBE*

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We have been using the fission yeast *S. pombe* as a model system to study the molecular processes that control passage through the G_1 and S-phases of the cell cycle. In particular we are studying two cell cycle genes, *cdc22+* and *cdc10+*.

cdc22+ encodes the large subunit of ribonucleotide reductase, and is periodically expressed during the cell cycle at the onset of S-phase. We have identified elements upstream of *cdc22+* resembling MCBs (*Mlu*I cell cycle boxes) previously identified in *Saccharomyces cerevisiae*. We have shown that MCBs can drive periodic expression of a reporter gene in *S. pombe*, strongly implicating MCBs in the control of periodic expression of genes.

We have identified a protein complex that specifically binds to MCBs, which we have called DSP1 - for DNA synthesis control in *S. pombe*. DSP1 is related to DSC1, an MCB binding activity previously identifed in *S. cerevisiae*. Furthermore, we have shown that the *cdc10+* gene product, $p87^{cdc10}$, is part of this complex.

In summary, $p87^{cdc10}$ is part of a transcription complex that controls expression of *cdc22+*, a gene required for S-phase.

Mechanisms underlying the activation of *Saccharomyces cerevisiae* plasma membrane H^+-ATPase

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Ethanol concentrations (up to 8% (v/v)) which affect *S. cerevisiae* growth induce the *in vivo* activation of plasma membrane H^+-ATPase (1). The activity of this membrane enzyme purified from cells grown with and without ethanol measured as the rate of ATP hydrolysis was inhibited by ethanol present in the assay mixture. Cells grown without ethanol and then incubated (for 5 to 15 minutes) with 6% (v/v) of ethanol showed an increase of H^+-ATPase activity which was greater when glucose (30 g/l) was present in the incubation medium.

In order to elucidated the role of an increased transcription level of *PMA1* or/and *PMA2* genes (2 isogenes that codes for H^+-ATPase plasma membrane), two gene fusions between the *PMA1* or *PMA2* promoters and the *lacZ* gene (from *E. coli*) were used (constructed by Supply, P., Capieaux, E., van Dick, L. and Goffeau, A.). The efficiency of *PMA1* promoter decreased in cells grown in the presence of ethanol. On the contrary, *PMA2* promoter efficiency was 2-3 fold higher in cells grown with 6% (v/v) of ethanol as compared with cells grown in its absence. The inhibition of the *PMA1* transcription was confirmed based on the lower mRNA concentrations determined by Northern blotting. It was not possible to detect any hybridization signal for mRNA of *PMA2*, due to the associated low transcription level that is 500-600 times lower than *PMA1*. Therefore, H^+-ATPase activation can not be associated with an increase of gene expression. Some correspondence could exist between the increase of the activity of H^+-ATPase and the *PMA2* expression in cells grown with ethanol. Under these conditions *PMA1* expression is inhibited. Activation of expression of *PMA2* appears to be a response to stress in *S. cerevisiae*. However, physiological conditions capable to turn on significantly *PMA2* gene remain to be elucidated.

Plasma membrane H^+-ATPase activation was thought to be possibly dependent on post-translation modifications such as H^+-ATPase phosphorylation by a casein kinase present in the plasma membrane that is co-purified with the H^+-ATPase (2). Nevertheless, a decrease in the activity (50%) of casein kinase in cells grown in the presence of ethanol was observed and the activation of H^+-ATPase can not be explained by the increase of the phosphorylation activity of this kinase protein. However, a higher phosphorylation level of H^+-ATPase in response to ethanol can not be overruled.

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YEAST AS A MODEL FOR STUDYING TRANSLATION

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Protein synthesis is an important pathway in the process of gene expression and there are numerous examples of control of gene expression that occur at the level of translation. The rate of protein synthesis can be regulated by the efficiency of translation of the mRNA, a process that involves different initiation factors. The overall pathway of initiation of protein synthesis is known, but many molecular details of how the initiation factors function are lacking. The process of translation is conserved between yeast and larger eukaryotes, with many of the proteins of the translational apparatus sharing 30 to 60% sequence identity and functional interchangeability. Moreover, the relative genetic tractability of the yeast *S. cerevisiae* makes it an ideal eukaryote for the study of translational regulatory mechanisms.

We demonstrated that yeast eIF-5A, encoded by two essential genes named TIF51A and TIF51B, is active in the mammalian methionyl-puromycin assay. Furthermore, human eIF5A cDNA supports yeast growth in the double null background, confirming that the yeast gene products are functionally identical to mammalian eIF5A. The mammalian cDNA encoding eIF2α complements a SUI2 knock out, thereby demonstrating a functional conservation. Experiments for the *in vivo* complementation of yeast SUI3 with eIF-2β are in progress.

A detailed study of the function of yeast eIF5A shows that either gene product supports growth in both aerobic and anaerobic conditions. The protein is partially phosphorylated, but no functional difference is seen *in vitro*. Depletion of eIF5A in yeast causes growth to cease, but the translation rate continues at 70% of wild type cells and polysomes are affected very little. This surprising result suggests that eIF5A is not essential for translation of most mRNAs. However, it may be required for a small class of essential mRNAs or for some other function in the cell.

We have begun experiments to biochemically purify and characterize yeast initiation factors and to reconstitute a protein synthesis system *in vitro*. Spheroplast lysates are capable of mRNA-dependent protein synthesis and when diluted are stimulated by addition of the ribosomal high salt wash fraction (HSW). This HSW has been further fractionated on FPLC Mono Q and Mono S columns, giving fractions that stimulate translation of total yeast mRNA or specific capped mRNA up to 7 fold. This assay may allow us to purify the active components in the HSW. Using the same procedure with a temperature sensitive yeast mutant, *prt1-1*, a highly active system dependent upon the PRT-1 initiation factor was obtained. Another approach is to use the methionyl-puromycin assay , a reaction that includes eIF-3, eIF-5, eIF-5A, eIF-1A, eIF-2 and mammalian ribosomes. The yeast HSW can replace the mammalian factors.

GALACTOSE INHIBITION OF THE CONSTITUTIVE TRANSPORT OF HEXOSES IN Saccharomyces cerevisiae.

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The relationship between the pathways of glucose and galactose utilization in Saccharomyces cerevisiae has been studied. Galactose (wich is transported and phosphorylated by inducible systems) is a strong inhibitor of the utilization of glucose, fructose and mannose (wich have the same constitutive transport and phosphorylation systems). Conversely, all these three hexoses inhibit the utilization of galactose, though with poor efficiency. These cross-inhibitions only occur in yeast adapted to galactose or in galactose-constitutive mutants.

The efficiency of galactose as inhibitor is even greater than the efficiencies of each of the other three hexoses to inhibit the utilization of each other. Phosphorylation is not involved in the inhibition and the transport of sugars is the afected step.

The cross-inhibitions between galactose and either glucose, fructose or mannose do not implicate utilization of one hexose at the expense of the other, as it occurs in the mutual interactions between the latter three sugars. It seems that, by growing the yeast in galactose, a protein component is synthesized, or alternatevely modified, that once bound to either galactose or any one of the other three hexoses (glucose, fructose or mannose), cross-interacts respectively with the constitutive or the inducible transport systems, impairing their function

Interactions between Glucose Uptake, Glucose Repression and Glycolysis in *TPS1* and *tps2/grr1/cat80* Mutants of *Saccharomyces cerevisiae*

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Δ*tpi1* mutants of *S. cerevisiae*, deficient in triosephosphate isomerase (*TPI*) are strongly inhibited in growth by glucose. Selection for phenotypic suppression yielded mutants in five complementation groups, designated *TPS1* through *tps5* (tpi suppressors). One complementation group, *TPS1*, was defined by dominant mutations while *tps2* and *tps5* were recessive and allelic to *grr1/cat80* (1) and to *hex2/reg1/srn1* mutants previously described.

TPS1 and *tps2/grr1* mutants displayed a number of glucose-specific defects (2,3): Reduction in glucose uptake, glucose repression and utilization. In both mutant types, the glucose uptake defect was apparently caused by a drastic reduction of high-affinity uptake. We could show that in this genetic background high-affinity transport is dependent on *SNF3* function (4). However, Northern analysis of *SNF3* transcription with derepressed wild-type and mutants cells revealed no significant difference in *SNF3* expression. We followed expression of a functional and membrane-located SNF3::lacZ fusion protein on multicopy and single copy plasmids. The level of ß-galactosidase activity in *TPS1* and *tps2/grr1* cells was similar and slightly decreased compared to wild-type cells. Also, we did not find a significant difference in the subcellular distribution of SNF3::lacZ activity between wildtype and these mutants. These data support the idea that the effects of both *TPS1* and *tps2* mutations on *SNF3* function may rather be caused by discrete post-transcriptional alterations of transporter proteins or of some yet unidentified components of the sugar uptake system. In both mutants the glycolytic flux is reduced by about 30-40% on high (2%) and about 90% on low glucose (0.1%) which is consistent with the observed reduction in low- and high-affinity transport and with the growth phenotype on glucose and raffinose, resp.

TPS1 and *tps2/grr1* mutants are released from glucose repression for a subset of normally glucose-repressible functions (e.g. invertase, α-glucosidase, cytochrom oxidase and hexokinase PI). Surprisingly, expression of invertase seems to be glucose-inducible in these mutants. The growth defects of *TPS1* and *tps2/grr1* cells can be restored by two kinds of multicopy suppressors: Overexpression of glucose transporter genes *SNF3* and *HXT1* lead to an elevated glucose transport, but the defect in glucose repression was not affected. Another set of plasmids also affected hexose transport, however suppression was apparently mediated by titration effects of plasmid sequences. From these data we conclude that

- **Glucose uptake is limiting for metabolism**
- **Glucose repression is not affected significantly by the glycolytic flux**
- ***TPS1* and *tps2/grr1* mutations interfere with the function of glucose transporters through post-transcriptional effects. This interference seems to be responsible for the defect in glucose repression**

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EXPRESSION OF ENA1 AND ENA2 GENES OF Saccharomyces cerevisiae

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The ENA locus of Saccharomyces cerevisiae is a tandem array of four genes, determining the Na^+, Li^+ and high pH tolerance in yeast (Haro, 1991). The coding regions of these genes are very similar, and they encode P-type ATPases, which probably pump K^+, Na^+ and Li^+. Disruption of the four genes gives rise to a strain very sensitive to Na^+, Li^+ and high pH, lacking Na^+ and Li^+ effluxes. Testing the activity of the two first genes of the tandem array (ENA1 and ENA2) in the disrupted strain revealed that ENA1 was an inducible very active gene, while ENA2 showed a very low activity.

To characterize the expression of ENA1 and ENA2 genes their 5' non-coding regions were fused to the reporter gene lacZ of Escherichia coli. Plasmids carrying these constructs were used to transform the wild type strain DBY746. In the strain bearing the ENA1-lacZ fusion the ß-galactosidase activity increased more than ten fold when cells had grown in media with Na^+, Li^+ or at high pH. The regulatory region of ENA1 was studied by nested deletions of its 5' non-coding region from base -1385 (numbered from the ATG). Deletions up to a region around -750 decreased the expression induced by Na^+, Li^+ or high pH, without affecting the low expression in the absence of the inducer. When deletions extended up to a region around -300 the expression of the gene was constitutive and high, decreasing it dramatically when deletion approached -100. We conclude that there is a region around -750 related to transcriptional activation by Na^+, Li^+ and high pH and an operator element around -300. ENA2-lacZ fusion showed that the expression of this gene was very low and constitutive.

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Reversible Inactivation of Nitrate Reductase by Heat-Shock in the Yeast Hansenula anomala: Association of the Inactive Enzyme with Mitochondria.

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Treatment at 40ºC, resulted in a rapid and reversible inactivation of the yeast H.anomala NADPH-NR activity. However, the inactive enzyme retains non-physiological activities such as MV_r-NR and $FMNH_2$-NR. Inactive NR pelleted at 12000 x g for 30 min with difference to the active enzyme, which remains in the supernatant. Analysis of the pellet, containing inactive NR, by ultracentrifugation in a continuous metrizamide gradient showed the association of inactive NR with mitochondria. Similary, in non heat-shocked cells around 10 % of the total NR is inactive and associated with mitochondria, in contrast to the solubility of the active enzyme. In vitro inactive NR was partially dissociated from mithocondrias at pH 11.5. as well as by CHAPS 15 mM.

Abbreviations: NR,nitrate reductase; MV_r, methyviologen reduced; PAGE, polyacrilamide gel electrophoresis; PMSF, phenylmethanesulphonyl fluoride; CHAPS, 3-{(3-cholamidopropyl)dimethylammonio}-1-propanosulfonate

Molecular analysis of the glucose sensing system of *Saccharomyces cerevisiae* cells.

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Glucose is the preferred carbon and energy source for yeast. Addition of glucose to a culture growing on a non-fermentable carbon source results in a rapid switch to glucose metabolism together with a wide variety of regulatory phenomena. These include rapid post-translational effects and long-term effects at the transcriptional level. In the past, mutants were isolated by various groups (1,2) with no apparent defect in the enzymes involved in glycolysis, but unable to grow on glucose or fructose. These mutants also lacked the glucose induced regulatory responses, like the cAMP signal, inactivation of FBPase, activation of plasma membrane ATPase a.o. A gene required for these phenomena has been isolated by complementation of both the *fdp1* mutant (1) and the allelic *byp1-3* mutant (2) and is called *GGS1* for General Glucose Sensor. The *fdp1* mutation is a missense mutation in the N-terminus of the protein while the *byp1-3* mutation is a nonsense mutation in the second half of the protein. Deletion of the *GGS1* gene results in absence of growth on fermentable carbon sources and in deficiency of glucose-induced regulatory responses. Addition of glucose causes, as in the *fdp1* and *byp1-3* mutants, hyperaccumulation of sugar phosphate, intracellular acidification and depletion of ATP, phosphate and downstream intermediates of glycolysis, indicating an important role in sugar uptake or phosphorylation.
Diminishing the hexokinase activity in *ggs1* strains by deleting the *HXK2* gene resulted in strains that grew again on glucose. This is in agreement with the idea that the inability of the *ggs1* mutants to regulate initiation of glycolysis causes the growth deficiency. Most of the regulatory phenomena deficient in *ggs1* mutants, were also restored by the deletion of *HXK2*. These includes glucose induction of pyruvate decarboxylase, glucose induced cAMP-signalling, glucose induced inactivation of fructose-1,6-bisphosphatase and glucose induced activation of the potassium transport system (3). However, both the *ggs1Δ* as well as the *ggs1Δ hxk2Δ* mutant lack detectable trehalose and trehalose-6-phosphate synthase activity. These data fit with the recent cloning of *GGS1* as a subunit of the trehalose-6-phosphate synthase/phosphatase complex. A model explaining the requirement of trehalose synthesis for a metabolic balance of sugar phosphates and free inorganic phosphate during the transition from respirative to fermentative metabolism will be presented.

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