The effect of altered expression of transcriptional regulators of catabolism on the transcription profile and physiology of Saccharomyces cerevisiae

Schuurmans, J.M.

Chapter 1

Introduction

General introduction

Mankind has been using yeast for several millennia. Historians have traced the roots of the first alcoholic fermentations back to African, Egyptian and Sumerian tribes, some 6000 years ago (Jackson, 1977). In the old world the art of making alcohol-containing beverages and bread spread from Egypt to Greece and Rome, but in the Far East it had been developed even earlier. However, it took several thousand years before the agent causing alcohol production was discovered and described. The first yeast cells to be observed were identified by Antonie van Leeuwenhoek in 1680 using his self-built microscope but at that time yeast was not seen as a living organism and not thought to be responsible for the fermentation process (Schierbeek, 1963). It took another 100 years, before the Frenchman Lavoisier first described the fermentation process (1789) in the form of a chemical equation: "le mout de raisin = acide carbonique + alkool" (Barnett, 1998).
After this, the concept of yeast being a living organism developed, and between 1855 and 1875, the French chemist Louis Pasteur unequivocally showed yeast to be the organism solely responsible for the fermentation of malt sugars into alcohol and carbon dioxide. At last, fermentation was no longer a "magical" phenomenon and yeast became a popular organism to study (Barnett, 2000). Around 1881 Emile Hansen isolated the first yeast for use in "single cell" cultures from a production fermentation of the Carlsberg brewery in Denmark. It was called *Saccharomyces carslbergensis*, which is a yeast very closely related to *Saccharomyces cerevisiae* (Barnett, 2000). Eduard Buchner, a German physiologist and chemist demonstrated in 1897 for the first time a cell-free fermentation by use of extracts from yeast cells. These experiments, together with studies in muscle, stood at the basis of the discovery of glycolysis (also known as the Emben-Meyerhof-Parnas pathway, named after its discoverers (Barnett, 2000)), a series of chemical reactions responsible for the conversion of glucose to pyruvate. The end product of glycolysis serves as the substrate that is finally converted to ethanol and carbon dioxide in the fermentation proper.

Hexose Transport

Before anything else, the very first obligate step in sugar catabolism is transport of the sugar molecules, and here we will limit ourselves to glucose, over the plasma membrane. *Saccharomyces cerevisiae* transports glucose, but also other hexoses such as fructose and mannose, over the membrane by means of facilitated diffusion. This transport step exerts a high degree of control on the glycolytic flux and has been considered for long the sole rate-determining step for sugar metabolism (Oehlen, et al., 1994). Not only is this transport step crucial for energy- and carbon metabolism; the availability and transport process of glucose are also important factors for a large set of extensive and complex regulatory mechanisms, which trigger large alterations in the transcript profile of the cell in response to the presence of glucose. This regulatory program is known as glucose repression (or induction) and is described in the next paragraph. As in nature *S. cerevisiae* typically grows on decomposing fruits, it needs to be able to adapt to very large changes in sugar concentration, from amounts as high as 1.5 M glucose, down to micromolar

concentrations (Barnett, 1976). Whereas facilitated diffusion is most suitable as the mechanism for uptake of extracellular glucose into the yeast cell when the extracellular glucose concentration is high and fairly constant, it is far from ideal for low ambient glucose concentration. One may wonder why this particular yeast species does not have energy driven (e.g. proton symport) systems available for the transport and intracellular accumulation of this sugar as this would be beneficial to the cell at low sugar conditions (van Urk, et al., 1989b). Interestingly, baker's yeast does possess sugar symporters for disaccharide sugars such as maltose (Lagunas, 1993). To accommodate optimal transport against steep concentration gradients, *S. cerevisiae* has developed a large diversity of hexose transport proteins (Hxtp), each with their own specific kinetic properties and environment-dependent (transcriptional) regulation (Kruckeberg, 1996). The expression of these hexose transporters is tightly regulated by the availability of the corresponding substrate, to allow for optimal transport rates at each sugar concentration.

In *S. cerevisiae* a large family of hexose transporters has been identified on the basis of sequence analyses and similarities to sugar transporters of other organisms. This family (the HXT family) consists of 20 hexose transporter (-like) proteins (Hxt1p - Hxt17p, Gal2p, Snf3p and Rgt2p). A dendrogram of sequence similarity of this family of transporters is shown in figure 1 (taken from A. Kruckeberg (Kruckeberg, 1996)). This figure suggests that the large diversity in hexose transporters of *S. cerevisiae* may have arisen from several duplication events throughout evolution. The *HXT* family in turn belongs to a large transporter super-family, called the major facilitator super-family (MFS). The MFS consists of a large variety of transporters for a large range of metabolites in eukaryotes and prokaryotes (Marger and Saier, 1993).

Sequence alignment of the transporters from the MFS family showed particular conservation throughout that part of the sequence that encodes 12 putative trans-membrane helices (Kruckeberg, 1996). The N- and C- terminal regions of the different proteins differ considerably in length and sequence; nevertheless they all are predicted to be located on the cytosolic side of the cytoplasmic membrane.

Glucose transport kinetics of whole cells is complicated by the presence of so many transporters. In wild-type cells under de-repressing conditions a glucose-repressible high-affinity component with a K_m of about 0.7 mM and of approximately 2 mM under glucose-repressed conditions, as well as a low-affinity component with a K_m of approximately 25 mM (under de-repressed conditions) and of approximately 45 mM (under repressed conditions) has been observed (Bisson and Fraenkel, 1984; Coons, et al., 1995). In view of the complexity of glucose transport, it is noteworthy that the uptake rate of glucose is fairly constant during batch cultivation with a starting concentration of glucose of 100mM. Yet, the affinity for glucose increases as glucose concentrations decrease (Walsh, et al., 1994). Modulation of the expression level of various transporters allows for these changes and is brought about in various ways, *e.g.* by changing the expression levels of *HXT* transcripts, encoding transporters with different affinities (Reifenberger, et al., 1997), by removal of the transporter proteins from the cytoplasmic membrane and subsequent inactivation of these hexose transporters (Bisson and Fraenkel, 1984; Horak and Wolf, 1997), by modulating the affinity of a specific transporter (Reifenberger, et al., 1997). Mutant analysis showed that deletion (or mutation) of any single Hxt protein does not lead to a significant phenotype, with the exception of *GAL2* and *SNF3* (Boles and Hollenberg, 1997).

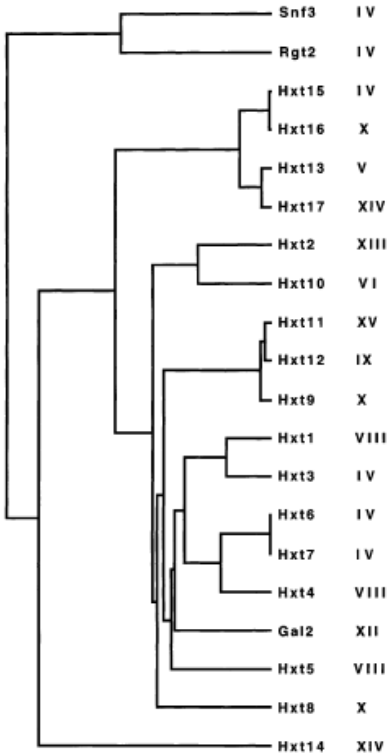

Figure 1: Dendrogram of the sequence similarity among the yeast hexose transporter proteins. The dendrogram shows the clustering relationship of the sequences based on their mutual similarity; the length of the horizontal branches is inversely proportional to the sequence similarity of the sequences at each branching point. The sequences were clustered by the GCG PILEUP program, and the dendrogram was plotted by the GCG FIGURE program (Genetics Computer Group 1994). The chromosomal location of each gene is shown next to the name of the corresponding transporter protein. (taken from (Kruckeberg, 1996))

These latter two proteins do not allow the cells to grow on galactose nor on low concentrations of glucose. Growth on glucose is fully abolished, even when this sugar is present at high concentrations, only when *HXT1 to HXT7* are all deleted (Reifenberger, et al., 1995). In such a "*hxt null*" strain no significant rate of glucose transport can be detected. Reintroduction of either *HXT1, -2, -3, -4, -6 or -7* restores growth on glucose, although not at all glucose concentrations, for every transporter (Reifenberger, et al., 1995). These results show that these seven Hxt proteins are the major proteins used for transport of glucose, and it further illustrates that non-facilitated diffusion through the membrane does not contribute significantly to glucose uptake (Gamo, et al., 1995). These 'single-*HXT* strains' allowed the biochemical characterization of the respective transporters (i.e. with respect to K_m and V_{max}) (Reifenberger, et al., 1997; Reifenberger, et al., 1995). Further details about the members of the Hxt family are described in detail below, with the exception of Snf3p and Rgt2p, which are discussed in the next section

under glucose signaling. Table 1 summarizes some key features of the various hexose transporters. Further details about each transporter follow below.

Reintroduction of *HXT1* into the *hxt null* strain restores growth on high concentrations of glucose (or fructose, mannose), but not on low concentrations of glucose, which is consistent with its low-affinity. *HXT1* expression is highly induced at high levels (>200 mM) of glucose, but not by galactose. Expression is also induced during chemostat growth at very high dilution rates ($\mu > 0.36$), as revealed by Northern blot analysis (Diderich, et al., 1999). Additionally, *HXT1* expression is induced by hyperosmotic stress (addition of 0.1 M NaCl or sorbitol) (Hirayama, et al., 1995). This induction is controlled by the Hog1p pathway. Activation of this pathway leads to the formation of glycerol inside the cell, in order to protect the cells against the osmotic stress. Taken together, these properties make Hxt1p an excellent glucose transporter when yeasts are growing on freshly decomposing fruits, where glucose (or fructose) concentrations can be as high as 1.5 M.

As seen in figure 1, the closest relative of Hxt2p is Hxt10p. The genes encoding these two proteins are 72% identical, but only in the coding regions. Hxt2p has been identified as a medium- to high- affinity transporter, with shifting affinities for glucose, depending on the extracellular glucose concentration. At a glucose concentration of 100 mM, an apparent K_m value of approximately 10 mM was observed. In contrast, a strain that only contained Hxt2p grown at low glucose concentrations expressed biphasic uptake kinetics, with a high affinity component (K_m = 1.5 mM) and a low affinity component (K_m = 60 mM) (Reifenberger, et al., 1997). Interestingly, reintroduction of *HXT2* in the *hxt null* strain restored growth on very low glucose concentrations, whereas that is not the case for *HXT4*, a transporter with a similar affinity for glucose. During chemostat growth *HXT2* is induced at low to medium growth rates, peaking between a μ of 0.15 and 0.20 h^{-1} (Diderich, et al., 1999). In this study no induction on batch growth was observed. Strangely this has to do with growth also, as there is no detectable *HXT2* when cells are grown from 100 mM glucose to glucose exhaustion, but there is a large amount of *HXT2* expressed when cells are shifted to a new medium with low glucose concentrations. The complexity of the regulation of *HXT2* expression goes even further as its expression is both activated and repressed by glucose (Ozcan and Johnston, 1995; Wendell and Bisson, 1994). At high glucose levels, *HXT2* is repressed by the Mig1p repressor protein (Nehlin and Ronne, 1990). More generally speaking, Mig1p is involved in the glucose-dependent expression of a number of genes and is discussed below. At low levels of glucose, Mig1p is released from the promoter region. This process requires Snf1p (Celenza and Carlson, 1986) via the Snf1p kinase that regulates the activity of Mig1p (see below). Adding to the complexity once more, this release itself is not sufficient, as *HXT2* expression in the absence of glucose is also repressed by the Rgt1p repressor. Only on low concentrations of glucose, neither of the two pathways is active and this therefore results in *HXT2* expression. Interestingly, the Snf1p-Mig1p pathway seems to control most of its expression, as in a *snf1Δ* mutant, there is continuous repression by Mig1p regardless of the glucose concentrations (Ozcan and Johnston, 1995) , whereas in a *rgt1Δ* mutant expression is constitutive in the absence of glucose , but still effectively repressed at high levels of glucose (Boles and Hollenberg, 1997). Unlike Hxt2p, Hxt10p despite its high similarity doesn't seem to be able to transport glucose at all (or very little)(Reifenberger, et al., 1995) and will not be discussed here.

Hxt3p is most closely related to Hxt1p with 86.4% identity. Reintroduction of Hxt3p in the *hxt null* mutant restores growth at high glucose concentrations exclusively, consistent with its low-affinity. During glucose-limited chemostat growth, *HXT3* is only expressed at or near the μ_{max} of *S. cerevisiae*, and in batch cultures only in the very first phase of growth (Diderich, et al., 1999). Expression of *HXT3* is regulated by the Snf3p-Rgt2p pathway and, surprisingly, is independent of the Snf1p pathway.
Hxt4p is most closely related to Hxt6p and Hxt7p with 83.4% identity to both. Reintroduction of *HXT4* in the *hxt null* mutant restored growth on intermediate and high glucose concentrations, but not on 5mM (low) glucose. Under glucose-limited chemostat conditions, however *HXT4* is expressed at high growth rates (0.36 h^{-1}) and in batch culture only in the first phase of growth on glucose, which doesn't seem to fit with the affinities reported (Diderich, et al., 1999). Expression of *HXT4* is sufficient to suppress the growth defects of a *snf3Δ* mutant. Regulation of *HXT4* expression appears to be similar to that of *HXT2*, as it is induced under low glucose conditions and repressed at high glucose concentrations. Although the promoter sequence of *HXT4* contains a Mig1p and a Rgt1p binding site, it seems that *HXT4* is repressed solely by Mig1p, although this could be strain dependent (Ozcan and Johnston, 1996). Unlike *HXT2,* expression of *HXT4* in the *hxt null* mutant restores a near wild-type growth rate on ~250 mM glucose, whereas upon expression of *HXT2* in the *hxt null* mutant, growth on high glucose concentrations was strongly inhibited.
A rather enigmatic putative glucose transporter is Hxt5p. Interestingly, it is repressed by high-, intermediate-, as well as very low glucose concentrations. Only in the absence of glucose (i.e. growth on an ethanol/glycerol mixture), *HXT5* will become expressed, be it not at a very high level when *S. cerevisiae* is grown in batch cultures. The only condition reported to result in high expression of *HXT5* is sporulation, as determined by a GFP-tagged Hxt5p. Reintroduction of *HXT5* in the *hxt null* mutant does not restore growth on glucose, and these cells can only be grown on a non-fermentable carbon source or sugars requiring different transporters (like galactose or maltose). Upon growth on maltose and galactose, however, *HXT5* is also repressed. Affinities for glucose were determined by growing the corresponding strain on an ethanol/glycerol mixture, until sufficient *HXT5* was expressed, after which glucose uptake was measured. An apparent K_m value for glucose of 11 mM was determined (Diderich, et al., 2001b). In glucose-limited chemostat cultures *HXT5* is expressed at a dilution rate of 0.1 h^{-1}, but not at 0.05 h^{-1}. The expression at 0.1 h^{-1} is rather inconsistent with the findings that *HXT5* is repressed by glucose (Diderich, et al., 1999). Deletion of *HXT5* does not result in a clear phenotype , although a slightly longer lag phase was observed after transfer from glucose exhausted medium to fresh medium, and the *hxt5Δ* strain resumed with pseudo-hyphal growth instead of the normal budding that the wild-type displays (Diderich, et al., 2001b).
Hxt6p and Hxt7p are the most closely related hexose transporters in yeast, with only two amino acid differences. These two residues are positioned outside the 12 membrane spanning domains and do not show up in any of the other hexose transporters, making these two proteins virtually identical in their membrane intrinsic domains. Interestingly, strains with a *HXT6/7* chimaeric gene have been reported. This chimaera can also arise spontaneously by intrachromosomal rearrangement between highly related genes (Boles and Hollenberg, 1997). Reintroduction of one or both of these genes restores growth on low concentrations of glucose (5mM) (Reifenberger, et al., 1997). During normal growth

HXT7, however, is far more abundant then *HXT6* and in glucose-limited chemostat cultures *HXT7* is expressed at all growth rates, whereas *HXT6* was only found at intermediate growth rates (0.15-0.28 h^{-1}) (Diderich, et al., 1999). Both genes are strongly repressed by glucose concentrations exceeding 25 mM, whereas Hxt6p in addition seems to be degraded post-transcriptionally by high glucose (Boles and Hollenberg, 1997). *HXT6* and *HXT7* are strongly expressed when there is no glucose available in the environment (for example during growth on substrates like maltose, ethanol and galactose, etc.), most likely to immediately be able to resume glucose consumption when this preferred substrate becomes available. Both genes are regulated by Snf3p; however, only *HXT6* requires Snf3p for maximal expression during growth on low glucose concentrations.

Hxt8p does not contribute significantly to glucose transport. Only overproduction of Hxt8p can partially restore growth on glucose in the *hxt null* strain, which qualifies it as a functional hexose transporter that is not used to a significant extent under the growth conditions reported in literature.

With respect to Hxt9p, Hxt11p and Hxt12p the functionality seems somewhat like that of Hxt8p. These are three hexose transporters that again do not contribute to the transport of glucose. Interestingly, these transporters are not only very similar in their coding region; the similarities extend into the entire promoter sequence as well. It has been found that these three transporters are not regulated by glucose at all but seem to be involved in pleiotropic drug resistances (Nourani, et al., 1997). This is a phenotype closely related to the mammalian multidrug resistance phenotype (Balzi and Goffeau, 1995). This phenotype causes the rapid expression of plasma membrane-bound non-proton ATP-ases, which confer resistance to various drugs by acting as efflux pumps. Expression of genes, involved in this response in yeast, is under control of Pdr1p and Pdr3p, and indeed *HXT9* and *HXT11* are under control of these very same proteins. Hxt9p, Hxt11p and Hxt12p, however, cause sensitivity to various drugs. A Pdr3p mutation, which dramatically increases *HXT9* and *HXT11* expression, can still not restore growth on glucose in the *hxt null* mutant, which is a further indication that these genes are most likely not functional hexose transporters, but rather multi-drug efflux pumps (Nourani, et al., 1997).

Very little if anything is known about Hxt13p, Hxt14p, Hxt15, Hxt16p and Hxt17p. *HXT13, HXT15, HXT16* and *HXT17* are closely related, whereas *HXT14* is the most distantly related member of the hxt family (see figure 1). Deletion of either one or more of these proteins does not show a noticeable phenotype, and no significant expression of any of these was detected. The only exception to this is *HXT13*, which was found to be regulated by the Hap2p regulator, although no function has been assigned (Boles and Hollenberg, 1997).

Gal2p, although a member of the hxt family, predominantly transports galactose. Of the 17 Hxts, it is most closely related to Hxt6p and Hxt7p. Expression of *GAL2* is strongly induced by galactose and repressed by glucose. Induction is controlled by the Gal11p-Gal80p-Gal4p pathway, which is initiated by binding of intracellular galactose to the Gal1p galactokinase. This, however, requires galactose to be present inside the cell, for *GAL2* expression to start. It has been shown that the glucose transporters can transport small amounts of galactose, thus transferring the initial signal intracellularly. Similarly, the Gal2p can transport glucose. *GAL2* is repressed by the Snf1p pathway and Gal2p is

furthermore inactivated by endocytosis into the vacuole and subsequent proteolysis when glucose is present (Horak and Wolf, 1997).

Table 1: Properties of the major Hexose Transporters of *S. cerevisiae*

Transporter	ORF Name	Protein Length	Affinity
HXT1	YHR094C	570 a.a.	Low (K_m ~ 100mM)
HXT2	YMR011W	541 a.a.	Moderate to High (K_{m1} ~ 1.5 mM, K_{m2} ~ 60 mM)
HXT3	YDR345C	567 a.a.	Low (K_m ~ 60 mM)
HXT4	YHR092C	576 a.a.	Moderate (K_m ~ 9 mM)
HXT5	YHR096C	592 a.a.	Moderate (K_m ~ 11 mM)
HXT6	YDR343C	570 a.a.	High (K_m ~ 1-2 mM)
HXT7	YDR342C	570 a.a.	High (K_m ~ 1-2 mM)
GAL2	YLR081W	574 a.a.	High (K_m ~ 2 mM)

Glucose Signaling (repression and induction)

Saccharomyces cerevisiae can use a large variety of compounds as carbon and energy source, ranging from polysaccharides, monosaccharides such as glucose, fructose, mannose and galactose, linear alcohols such as ethanol, to organic acids like acetate and lactate. However, glucose is by far the preferred carbon and energy source for *Saccharomyces cerevisiae*, as reflected by its intricate and complex mechanisms to detect, adapt and finally metabolize this sugar. In this paragraph the main sensors and regulators that play a role in recognizing and responding to different amounts of glucose will be discussed. These are the Ras-cAMP-, the Snf1-, the Snf3- and Rgt2-pathway. Although these are discussed here as separate pathways, they most likely mutually communicate, which leads to additional, emerging properties of the entire regulatory network and creates a dynamic system of regulation and responses to glucose, and as such one might even consider them to all be part of one large system.

The Ras-cAMP Pathway

One of the major mechanisms of glucose signaling is the Ras–cAMP Pathway (see also figure 2). Ras proteins are monomeric GTPases that can act as molecular switches, depending on whether GDP (inactive) or GTP (active) is bound. These proteins are always accompanied by GAPs (GTPase Activating Proteins), which stimulate the hydrolysis of GTP to GDP, thus rendering the GTPase in an inactive state and GEFs (Guanine Exchange Factors), which stimulate the dissociation of GDP and the binding of GTP, rendering the GTPase active. The two Ras proteins in *S. cerevisiae* are encoded by

RAS1 and *RAS2*. Deletion of either of these has no effect on growth of glucose, however, deletion of both causes a growth arrest in the G1 phase of the cell cycle (Tatchell, et al., 1984; Toda, et al., 1985). Several important effectors mediate the activity of the Ras proteins in *S. cerevisiae. CDC25,* an essential gene in yeast (which also causes G1 cell cycle arrest, upon deletion (Hartwell, et al., 1973)), is a GEF for the Ras proteins and activates them (Cameron, et al., 1988). When Ras is activated, it initiates the production of cAMP in the cell by the adenylate cyclase (*CYR1*) (Matsumoto, et al., 1982). On the other hand *IRA1* and *IRA2* inactivate Ras proteins by stimulating GTP hydrolysis, thereby lowering cAMP levels (Tanaka, et al., 1991; Tanaka, et al., 1989). Additionally, cAMP levels are modulated by the cAMP phosphodiesterases (*PDE1* and *PDE2*) (Nikawa, et al., 1987).

When glucose is added to cells growing on a non-fermentable carbon source (or when they are in the stationary phase), a signal is generated by one or more sensors. These signals converge on membrane-bound Ras. This subsequently triggers a rapid, but transient increase in cAMP levels within 1-2 minutes and returns to normal within 20 minutes (Thevelein, et al., 1989).

Protein Kinase A (PKA) is a heterotetrameric protein consisting of 2 regulatory subunits and 2 catalytic subunits (Toda, et al., 1987). The cAMP spike that has been triggered by the Ras proteins, leads to the dissociation of the 2 regulatory subunits (encoded by *BCY1*) from the 2 catalytic subunits (encoded by the genes *TPK1*, *TPK2* and *TPK3*). This results in an active PKA, allowing for the start of a phosphorylation cascade. Active PKA is thought to phosphorylate many regulatory proteins in the nucleus and thereby initiate a wide-spread change in the transcriptional network needed for adaptation to the availability of glucose (Griffioen and Thevelein, 2002). Interestingly, the Ras-cAMP pathway is associated with many more functions and as such appears to be a hub for regulation of transcription in response to many factors, such as thermotolerance (Zhu, et al., 2000), stress resistance , glycogen accumulation (Smith, et al., 1998) and aging (Lin, et al., 2002). Another G-protein has been found to contribute to the adenylate cyclase activity and subsequently the activation of PKA. This protein, consisting of an α, β and presumably a γ subunit (if analogous to other G-proteins), can also, like the monomeric Ras, bind to guanine nucleotides. The α subunit Gpa2p and its GAP Rgs2p have been found to act in the glucose signaling cascade (Versele, et al., 1999). β subunits (Gpb1p and Gpb2p) have been identified by interaction with Gpa2p, however so far no γ subunit has been found (Harashima and Heitman, 2002). The Gpbp1 and Gpb2p proteins are not β-subunits in the classical sense, instead they are proteins with a different structure, also identified as the Kelch-repeat proteins Krh1p and Krh2p (Batlle, et al., 2003). These proteins facilitate the binding of Bcy1p to the Tpk subunits, and are capable of bypassing the cAMP dependent regulation of PKA (Peeters, et al., 2006). Also the Krh1p and Krh2p can bind to the Ira1p and Ira2p proteins, inhibiting Ras inactivation and thus stimulating cAMP production. Deletion of *RAS2* and *GPA2* leads to a severe synthetic growth defect which can be restored by removal of *PDE1* and *PDE2*. It has been proposed therefore that the essential missing part is the cAMP synthesis needed to trigger PKA activity (Xue, et al., 1998). Indeed expressing activated Ras and/or activated Gpa2 shows the full response even in absence of glucose (Wang, et al., 2004). Gpa2p presumably is activated by Gpr1p, a membrane-bound g-protein coupled receptor (Xue, et al., 1998). Deletion of *GPR1* causes the same effects as deletion of *GPA2* and in combination with *RAS2*

deletion causes a severe synthetic growth defect. This indicates that Gpr1p is the only receptor coupled to Gpa2p to activate the cAMP response. As expression of either Ras or Gpa2 in their activated form causes the same effect as addition of glucose, this raises the question as to how they are activated by glucose. Gpr1p is a membrane bound protein and therefore could directly interact with glucose and as such activate Gpa2 to activate the cAMP signaling cascade.

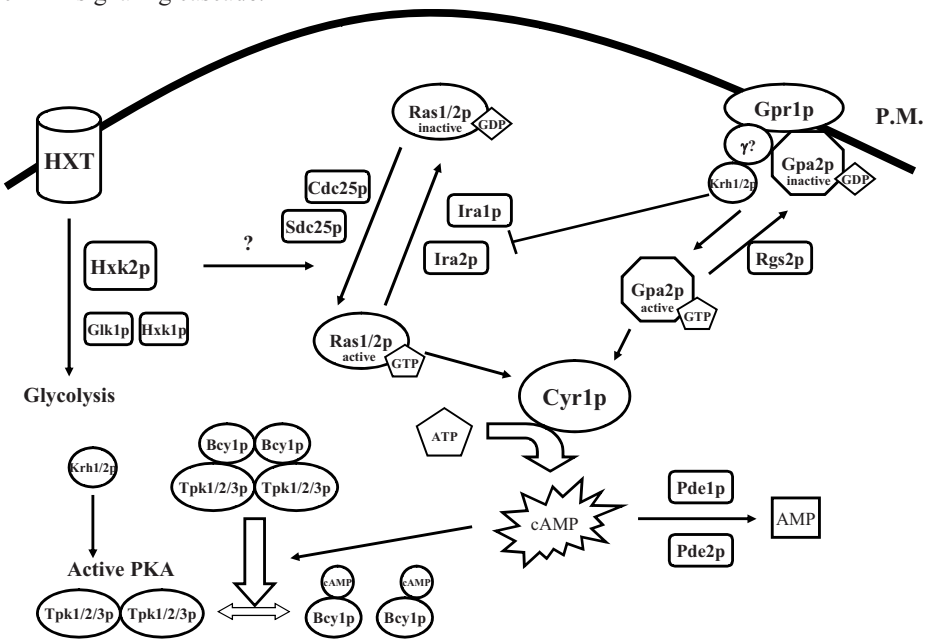

Figure 2 : Cytoplasmic events during PKA Signaling. Glucose regulation is mediated through the intracellular signaling molecule cAMP. Cyr1p (adenylate cyclase) responds to both Gpa2p and Ras1/2p. In GTP-bound form Ras1/2p and Gpa2p bind independently to Cyr1p and stimulate production of cAMP. RasGEFs (Cdc25p and Sdc25p) and RasGAPS (Ira1p and Ira2p) activate and deactivate Ras, respectively. The signal generated by glucose transport and/or phosphorylation stimulates Ras1/2p activation in a still unknown way. The receptor protein Gpr1p acts upstream of Gpa2p in glucose signaling and is a member of the *G p*rotein-*c*oupled *r*eceptor (GPCR) superfamily. Gpa2p has a high similarity to the mammalian G_α subunits of heterotrimeric G proteins, and based on this similarity the putative G_β subunits Gpb1p and Gpb2p were detected via their interaction with Gpa2p. However, a corresponding γ subunit has yet to be identified (shown as a question mark). Rgs2p is a GAP for Gpa2p; a GEF for Gpa2p has not yet been identified. Phosphodiesterases (Pde1p and Pde2p) antagonize glucose signaling via conversion of cAMP to AMP. cAMP activates the PKA tetramer by binding to the regulatory subunit Bcy1p, triggering a conversion and releasing the catalytic subunits of PKA, namely Tpk1p, Tpk2p and Tpk3p. The catalytic domain is then ready to phosphorylate corresponding target proteins (adapted from (Rolland, et al., 2002)).

On the other hand, Gpr1p does not activate Ras signaling. Ras signaling appears to be activated by phosphorylated intracellular glucose as a glucose-phosphorylation deletion mutant (*hxk1Δhxk2Δglk1Δ* mutant) does not cause an increase of Ras2-GTP protein, the activated form of Ras2p. It has been suggested that phosphorylated glucose inhibits the RasGAP proteins Ira1p and Ira2p (Colombo, et al., 2004).

Clearly, many more experimental data is needed before we can unravel the precise mode of action by glucose on the Ras and Gpa2 proteins or their regulatory GEFs and GAPs. A schematic overview of mode of action is shown in figure 2.

The glucose sensors Snf3p and Rgt2p

Another way of sensing glucose by *S. cerevisiae* is by means of the 2 glucose receptors Snf3p and Rgt2p. As outlined above, *SNF3* and *RGT2* are considered as members of the hexose transporter family despite the fact that they respond to the availability of glucose rather than transporting it and thereby mainly regulate the expression of the main 7 hexose transporters in yeast (*HXT1-7*) (Ozcan, et al., 1996a). One piece of evidence that they are sensors and not transporter comes from the observation that expression of either of these proteins in the *hxt null* mutant (*hxt1-7Δ*) does not restore growth on glucose. Additionally two dominant mutations in these proteins (*RGT2-1* and *SNF3-1*) cause constitutive expression of *HXT1* to *HXT4*, even in the absence of glucose, which normally is not associated with their expression (Ozcan, et al., 1996a). Both sensors are required for the glucose induction or glucose repression of the various hexose transporters (see figure 3). Snf3p acts as a high-affinity sensor, sensing low levels of glucose. It is required for the induction of *HXT2* and *HXT4*, but not for the induction of *HXT1,* a low affinity glucose transporter that is expressed under high glucose conditions. This is also supported by the observation that *SNF3* is repressed at high glucose concentrations and cannot exert its function under those conditions. Rgt2p acts as the low affinity sensor, sensing high levels of glucose. It is required for the maximal induction of *HXT1*, but has no function in inducing *HXT2* and *HXT4* at low glucose concentrations (Ozcan and Johnston, 1999).
These 2 sensors consist of two functional domains, a predicted 12 trans-membrane spanning domains and a long C-terminal intracellular tail, which is not present in other hexose transporters(Marshall-Carlson, et al., 1990). The trans-membrane domains are similar to the functional hexose transporters and almost certainly enable the sensors to bind glucose. However, the divergence from the functional hexose transporters might cause the inability to transport glucose (Ozcan and Johnston, 1999).
Upon glucose binding on the outside, the C-terminal tail generates a signal (of which the precise nature is unknown) that presumably consists of a conformational change in the C-terminal tail, in order to interact with the next signaling proteins in the signaling pathway. Interestingly swapping the long C-terminal tail onto the Hxt1p or Hxt2p protein restores the induction of hexose transporters in a *snf3Δrgt2Δ* mutant (Ozcan, et al., 1998). After glucose binding the signal is transduced to Grr1p, possibly via Mth1p/Std1p. Grr1p is an inhibitor of a repressor Rgt1p (mentioned below). Mutants in Grr1p were originally identified as strains resistant to 2-deoxyglucose (a non metabolize-able form of glucose) (Bailey and Woodword, 1984). These mutants were defective in various aspects of glucose repression such as the repression of maltase, invertase and mitochondrial cytochrome c oxidase. Grr1p appears to have more functions then repressing the genes involved in glucose metabolism as this mutation causes pleiotropic effects, such as elongated cell morphology and increased sensitivity to stress factors, i.e. osmotic stress and nitrogen starvation (Bailey and Woodword, 1984; Flick and Johnston, 1991).

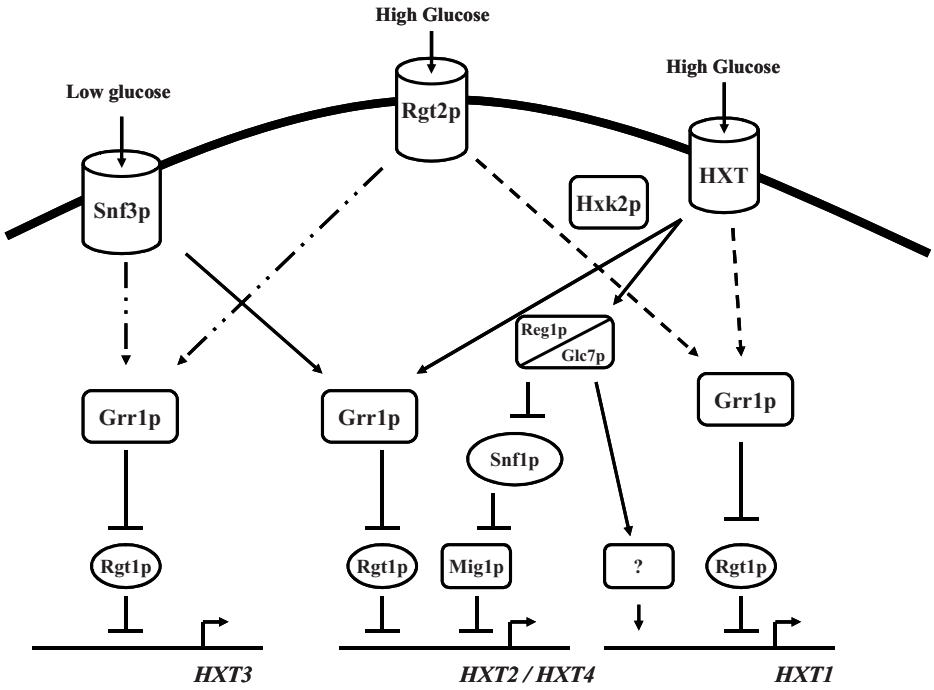

Figure 3: Three different modes of modulation of the expression of individual *HXT* genes by different levels of glucose. Mode 1: At high glucose concentrations, signals are generated by glucose transport itself and by the low-affinity glucose sensor Rgt2p (- - -). The signal from the transporters is mediated by the Reg1p/Glc7p phosphatase complex and an unknown activator, which induces expression of *HXT1*. Additionally, the signal generated by Rgt2p activates Grr1p, which inactivates the Rgt1p repressor allowing expression of Hxt1. Mode 2: At both low and high levels of glucose signals are generated by both Snf3p and Rgt2p (-..-) which activate Grr1p, which in turn inactivates Rgt1p, allowing expression of *HXT3*. No other ways of regulation of *HXT3* expression by Snf3 and Rgt2p has been found so far. Mode 3: At intermediate to low levels of glucose, a signal (---) is generated by Snf3p, which activates Grr1p, which in turn inactivates Rgt1p, allowing expression of *HXT2* and *HXT4.* An other signal (---) generated via the Snf1p pathway, generated by Hxk2p is also required to allow expression of *HXT2* and *HXT4* (adapted from (Ozcan and Johnston, 1999)).

A *grr1Δ* strain is unable to induce *HXT* genes in response to glucose and is impaired in glucose uptake (Ozcan, et al., 1994; Vallier, et al., 1994). This is due to the inability to inactivate the transcriptional regulator Rgt1p. Overexpression of *RGT1* in a *grr1Δ* mutant restores the expression of *HXT* genes, restoring their lowered transport capability (Marshall-Carlson, et al., 1991; Ozcan and Johnston, 1995). However, it does not restore the altered morphology of these mutants, which shows that Grr1p acts upon other proteins as well.

Rgt1p is a transcriptional regulator of *HXT* expression. It is a bi-functional protein that can act as a repressor of transcription in the absence of glucose and as an activator under high level glucose conditions (Ozcan, et al., 1996b). At low concentrations of glucose it acts neither as a repressor nor as an activator. During repression Rgt1p is associated with Ssn6p/Tup1p, a repressor complex that associates with many other repressors, including

Mig1p. At low concentrations of glucose it dissociates from this complex losing its functionality. . High concentrations of glucose convert Rgt1p into an activator, in which function it is independent of Ssn6p/Tup1p (Ozcan, et al., 1996b). Figure 3 summarizes the regulation by the two glucose sensors upon the various hexose transporters.
HXT3 is activated by both low and high levels of glucose, conditions that result in Grr1p activation and hence Rgt1p inactivation. It has been reported that the presence of glucose may leading to a 10-fold increase in expression (Ozcan and Johnston, 1995). *HXT2* and *HXT4* are two moderate affinity transporters that are induced only under low levels of glucose. Snf3p activates Grr1p, relieving repression by Rgt1p. An additional signal, possibly intracellular glucose or glucose-6-phosphate, deactivates Reg1p, a regulatory protein involved in Snf1p kinase activity. In its activated form, Snf1p kinase phosphorylates Mig1p rendering it inactive. These two converging signals then allow for full induction of *HXT2* and *HXT4* (Ozcan and Johnston, 1999). Also at high glucose concentrations relief of repression by Rgt1p via Rgt2p signalling to Grr1p occurs. Rgt1p is then converted into an activator of transcription instead, furthermore the high amounts of glucose coming into the cell lead to the activation of a regulator, which is yet to be identified. What is known that this activation requires Hxk2p and Reg1p.

General glucose repression pathway (Snf1p pathway)

As mentioned before, intracellular glucose or phosphorlyated glucose appears to be an important factor in the initiation of glucose signaling. Either of these compounds is implied in Ras2 activation and also seems to play a role in hexose transporter regulation. In de-repressed cells intracellular glucose can reach detectable levels, which can reduce transport activity by 50%. This elevation of intracellular glucose and/or the reduction of the transport activity may trigger the glucose response (Teusink, et al., 1998).
Yeast has 3 hexokinases, of which only *HXK2* is abundantly expressed in cells growing on glucose. This particular hexokinase also appears to be one of the bases of the glucose repression/induction machinery (see figure 4) (Moreno and Herrero, 2002). In glucose grown cells approximately 14% of Hxk2p is nuclear as has been shown by various techniques (Herrero, et al., 1998). It implies that Hxk2p might have a direct regulatory function on at the level of DNA. Indeed, it has been shown that Mig1p (a repressor of transcription) can bind to Hxk2p, and Mig1p even appears to mediate the nuclear localization of Hxk2p to the nucleus (Ahuatzi, et al., 2004).
Additionally, interactions with Med8p have been described. Med8p is a mediator protein and can bind directly to DNA to either positively or negatively act on glucose regulatory elements in the promoter regions (Chaves, et al., 1999).
Whether the action is positive or negative is most likely determined by the proteins it is associated with. The mode of action of Hxk2p, in this way shows many similarities with Gal1p, the galactokinase, which phosphorylates galactoses and activates the transcription factor Gal4p by binding to Gal80p a repressor protein of Gal4p (Zenke, et al., 1996).
Here too, the exact signaling role of Hxk2p still hasn't been elucidated and contradictory reports have been made. The deletion of a small N-terminal fragment (residue 7-16) was reported to abolish nuclear localization of Hxk2p and restored repression of invertase.

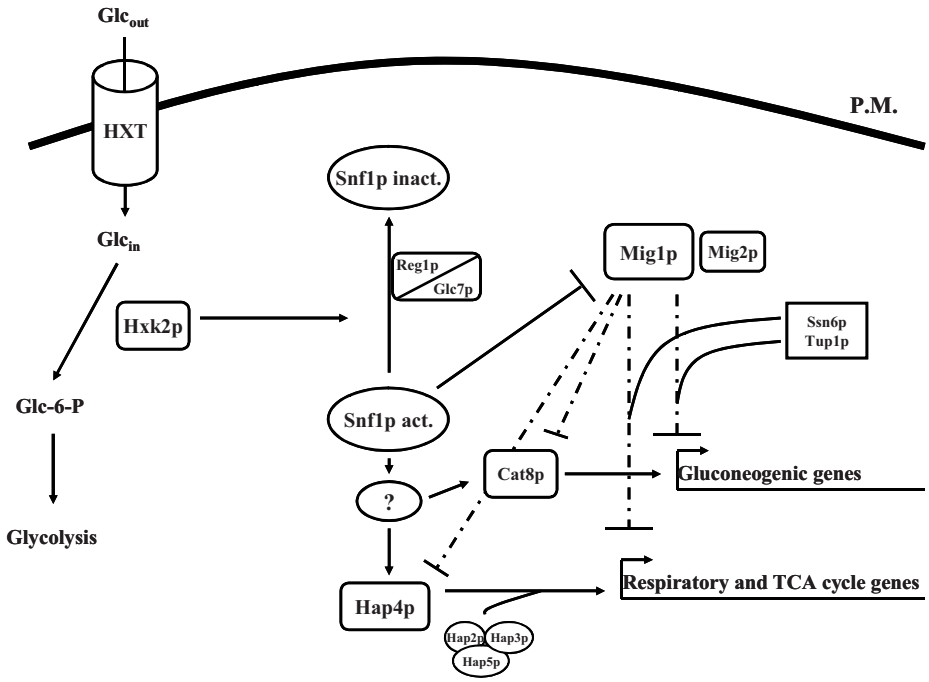

Figure 4: Simplified view of the glucose signaling pathway in *S. cerevisiae.* Extracellular glucose is transported into the cell by the various hexose transporters. Intracellular glucose is phosphorylated by hexokinase. Upon phosphorylation by Hxk2p, a signal is created which presumably indirectly affects Snf1p kinase, as direct interactions between Hxk2p and Snf1p or Reg1p/Glc7p have never been shown. An active Snf1p, at low glucose concentrations, inactivates the Mig1p and Mig2p repressor proteins, thereby relieving repression of many genes needed for respiration and TCA cycle activity and when glucose is depleted also genes needed for gluconeogenesis. Hap4p is an activator of respiratory- and TCA cycle genes and is also controlled by activity of the Mig1p and Mig2p repressors. A direct interaction between Snf1p and Hap4p and/or Cat8p hasn't been shown, but a still unknown factor might facilitate this activation.

The catalytic activity of this truncated Hxk2p was reported not to be affected (Rodriguez, et al., 2001). In contrast, deletion of residues 1-16, resulted in the opposite result, abolished glucose repression and correct localization (Mayordomo and Sanz, 2001).
Hxk2p is considered in transferring a signal to the Glc7p phosphatase and the Snf1 kinase. No direct interactions have been shown between Hxk2p and either of these proteins however, which once more illustrates the need to find out exactly how Hxk2p signaling works. Not only would the top end of the Snf1p pathway then be unraveled, it might give insight as to how Hxk2p signaling is linked to the PKA and Snf3-Rgt2p pathways.
SNF1 was identified in two screens, one for regulatory factors in the glucose response, designated *CAT1* at that time, and in a screen for mutants that could ferment glucose, but not sucrose, and designated *SNF1* (Sucrose Non- Fermenting)(Carlson, et al., 1981; Ciriacy, 1977; Entian and Zimmermann, 1982). Deletion (or inactivation) of *SNF1* leads to various defects in the utilization of galactose, maltose, sucrose and non-fermentable carbon sources, and as such it was recognized as a very important regulator of the glucose

repression response. *SNF1* was shown to encode a serine/threonine protein kinase (Celenza and Carlson, 1986). Additionally, in the same screen *SNF3* (discussed above) and *SNF4* were found (discussed above). The phenotypes of *snf1Δ* and *snf4Δ* are nearly identical. A hyperactive allele of *SNF1,* can however partially restore the defect of the *snf4Δ* strain (Celenza, et al., 1989). Further studies showed that Snf4p co-immuno precipitates with Snf1p and is required for maximal Snf1p activity. This led to the conclusion that Snf4p is an activator subunit of the Snf1p complex (Estruch, et al., 1992). More proteins were identified that act together with Snf1p. Three proteins, namely Sip1p, Sip2p and Gal83p strengthen Snf4p and Snf1p association and appear to form a bridge between the two proteins, as each of the three proteins have a Snf1p binding internal region and a Snf4p binding C-terminal region (Jiang and Carlson, 1997). In another search for mutants showing consecutive expression of *SUC2*, many proteins were found, of which most have been mentioned already: Snf1p, Ira1p, Ras2p and Hxk2p (Neigeborn and Carlson, 1987). In addition, another gene was identified, later named *GLC7*. This gene encodes the yeast homolog of the mammalian protein phosphatase I (PPI) (Feng, et al., 1991). Glc7p appears not only to have a function in glucose repression but also to play a crucial role in other cellular processes as well, including cell cycle progression through G2/M phase (Bloecher and Tatchell, 2000), actin organization (Chang, et al., 2002), sporulation (Ramaswamy, et al., 1998) and glucose induced degradation of fructose-1-6-bisphosphatase (Cui, et al., 2004). For a specific function PPI needs a subunit, as PPI itself shows hardly any substrate specificity. Most of these subunits have been identified. Reg1p is needed for glucose repression (Tu, et al., 1996), Gac1p and Pig1p are needed for storage carbohydrate metabolism (Cheng, et al., 1997; Stuart, et al., 1994), Reg2p and Sds22p are used in the cell cycle progression (Frederick and Tatchell, 1996; Peggie, et al., 2002), Scd5p for actin reorganization (Chang, et al., 2002)and Gip1p for sporulation (Tu, et al., 1996).

Reg1p (first identified as the gene *HEX2*) regulates glucose repression by Glc7p as is shown in various ways. Both originally identified alleles of *glc7* and *reg1*, are involved in transcription of the gene *SUC2*, encoding invertase (Neigeborn and Carlson, 1987). Combinations of these alleles showed no synergy in release of glucose repression but overexpression of *REG1* suppresses the repression defect (constitutive expression) caused by the *GLC7-T152K* allele (Tu and Carlson, 1995). This creates a balance between 2 complexes, one that phosphorylates the target proteins (discussed below) involved in repression and one that de-phosphorylates them. Under high glucose conditions Snf1p is in an auto-inhibited form as the regulatory domain is bound to the catalytic domain (Jiang and Carlson, 1996; Sanz, et al., 2000). When glucose concentrations become lower, Snf4p will compete for the regulatory domain and its binding will release the catalytic domain allowing it to phosphorylate its target proteins (Jiang and Carlson, 1996). When glucose is present again, PPI then presumably de-phosphorylates Snf1p, resulting in the release of Snf4p and conversion back into the auto-inhibited state. Interestingly, this de-phosphorylation was reported to occur not in the nucleus but in the cytoplasm, and it is postulated that Snf1p shuttles in and out of the nucleus to exert its functions (Dombek, et al., 1999). This also may explain the role of the two 14-3-3 like proteins, Bmh1p and Bmh2p in glucose regulated gene expression (Ichimura, et al., 2004) as they are known to respond to PKA signaling and might act as mediators for Reg1p-Glc7p function on Snf1p (Dombek, et al., 2004).

Several downstream repressors have been identified that are phosporylated by Snf1p. The first identified was Mig1p (Multicopy Inhibitor of *GAL* expression) (Nehlin and Ronne, 1990). Deletion of Mig1p abolishes repression of numerous genes known to be glucose repressed (Klein, et al., 1998). Deletion of Mig1p in a *snf1Δ* mutant suppresses many *snf1Δ* mutant specific defects and therefore Mig1p is presumed to function directly downstream of Snf1p, as Snf1p also interacts with Mig1p (Vallier and Carlson, 1994). Upon phosphorylation of Mig1p by Snf1p, the repressor can no longer bind to DNA and loses its repressor function allowing gene expression of glucose repressed genes (Treitel, et al., 1998).

Mig2p, Mig3p, Nrg1 and Nrg2p are 4 additional Snf1p regulated repressors. Mig2p appears to have exactly the same function as Mig1p although in a less pronounced way as there are much less Mig2p molecules present than Mig1p (Klein, et al., 1999). The function of mig3p is unclear so far. The protein is very similar to Mig1p and can bind to Mig1p binding sites, but physiologically relevant target genes of Mig3p have not been identified. Mig3p was found in a screen for multicopy suppressors of Rad53-GFP toxicity and as such could be involved in the DNA damage response, but no clear evidence has been found so far (Dubacq, et al., 2004).

Nrg1p and Nrg2p are, like Mig1p, zinc-finger proteins. Nrg1p was identified in a screen for factors mediating repression of glucose-regulated genes (Park, et al., 1999) whereas Nrg2p was identified as a binding partner of Snf1p in a 2-hybrid screen (Vyas, et al., 2001). Both these proteins show glucose-dependent repression of a heterologous reporter gene and they are both able to interact with Snf1p although neither of them appears to be phosphorylated by Snf1p (unlike the Mig proteins) (Berkey, et al., 2004; Vyas, et al., 2001). They are implied to have functions in various processes such as glucose repression, filamentous invasive growth and biofilm formation but this vague notion is all that can be said about these proteins since their regulatory relationship with Snf1p nor their functioning still needs to be elucidated (Vyas, et al., 2001).

The Hap2/3/4/5p complex is a global regulator complex activating genes involved in the TCA cycle and oxidative phosphorylation (de Winde and Grivell, 1993; Forsburg and Guarente, 1989). The Hap4p regulator of this complex is under control of the Snf1p pathway and plays an important role in induction of genes needed for respiration. The Hap2p, Hap3p and Hap5p members of this complex are continuously expressed and as such do not appear to control the amount of expression of genes needed for respiration. In contrast, the Hap4p regulator is responsive to glucose via the Sn1p pathway, by means of Mig1p and Mig2p (Gancedo, 1998). When glucose is abundant, the *HAP4* gene is repressed by Mig1p and Mig2p, as well as the genes involved in the TCA-cycle and oxidative phosphorylation (shown in figure 4). When glucose concentrations become lower, Sn1p phosphorylates Mig1p, allowing expression of *HAP4* and respiratory genes. Hap4p subsequently binds to the Hap2/3/5p complex to form a transcriptional activator, which further activates genes containing a CCAATCA like promoter element (Dang, et al., 1994). This promoter element is present in many of the genes (*e.g.* the *QCR* genes) encoding for enzymes that catalyze respiration. Overexpression of *HAP4* generally results in an increase in respiration by activating a whole set of genes (Blom, et al., 2000; van Maris, et al., 2001).

Not only does Snf1p interact with repressors of transcription, it also regulates (at least) three transcriptional activators (Cat8p, Sip4p and Adr1p) needed for proper utilization of

non-fermentable carbon sources. Cat8p and Sip4p both bind specifically to a set of genes containing the carbon source responsive elements (CSRE) (Vincent and Carlson, 1998). Although both proteins are closely related structurally and can both be phosphorylated by Snf1p, Cat8p seems to be the most important one as a *cat8Δ* mutant causes defective growth on non-fermentable carbon sources, whereas deletion of *SIP4* shows no detectable phenotype (Hedges, et al., 1995; Lesage, et al., 1996). Cat8p activates the expression of a wide array of genes needed for utilization of non-fermentable carbon sources, such as genes required for ethanol utilization (*ACS1* and *ACR1*) (Bojunga, et al., 1998), genes needed for gluconeogenesis (Hedges, et al., 1995), the glyoxylate cycle (Haurie, et al., 2001), lactate utilization and isocitrate metabolism (Bojunga and Entian, 1999). Interestingly it also regulates the activity of *SIP4*, which could explain why *cat8Δ* shows a phenotype and *sip4Δ* does not (Vincent and Carlson, 1998). Under glucose repressing conditions, the *CAT8* promoter is bound by Mig1p and as such it needs two subsequent phosphorylations by Snf1p to be activated, the first being the phosphorylation of Mig1p, to relieve its binding of the promoter sequence of *CAT8* and the second being phosphorylation by Snf1p on Cat8p itself to become active (Randez-Gil, et al., 1997). Upon addition of glucose, Cat8p is de-phosphorylated by PPI and becomes inactive again. As a consequence Mig1p will bind to the promoter and expression will also stop. A third factor is Adr1p which works together with Cat8p for maximal expression of a small group of genes (*e.g. ADH2, ACS1* and *ALD6*). Although proper activation of Adr1p requires Snf1p, its regulatory mechanism is virtually unknown (Young, et al., 2003).

Yeast performance in industrial applications

As *S. cerevisiae* is used worldwide for many applications, such as the making of bread, the brewing of beer and the production of fine chemicals and specific proteins, decades of research have been devoted to understand and optimize the performance of yeast, both during these processes and in the production phase of the yeast in large scale fermentors. One of the most important quality parameters is the "raising power" or fermentative capacity. Fermentative capacity is defined as the ability to make ethanol and CO_2 under anaerobic conditions, which occurs during the raising process of bread dough. Research by P. van Hoek showed that the fermentative capacity of an industrial yeast is far from optimal at low growth rates (see figure 5 (Van Hoek, et al., 1998)).
Due to the nature of the production process in fed-batch cultivations, the growth rates of yeast is very low at the end of the production process, expressing only one-third of the capacity found at high growth rates.. Many attempts have been made to increase the fermentative capacity. One of these attempts focused on increasing the expression of genes involved in glycolysis but generally speaking to no avail or with only limited success. With further insight in metabolic control analysis and hierarchical regulation, it could be understood that aiming for increased cellular capacity by enhancing one catalytic step in a multi-step reaction sequence may not be a proper approach: shifts in control of other steps will result in only moderate improvement of the overall fluxes (Smits, et al., 2000).

In this thesis we took another approach, instead of attempting to increase the fermentative capacity we have looked at strains with increased respiration capacity, allowing for higher yields on the used carbon source (glucose) during the production process (which is aerobic), while maintaining the fermentative capacity of the wild-type at various growth rates. Rather than focusing on individual enzymes in glycolysis, we have sought to achieve increased cell production by studying the effects of general glycolytic regulators. Several strains have been investigated, a *hxk2Δ* strain, a *HAP4* ↑ strain, a *mig1Δmig2Δ* strain and the *hxk2ΔHAP4* ↑ strain. Although one might intuitively assume that strains with an increased respiration might be impaired in fermentation, it is shown that this is not always the case.

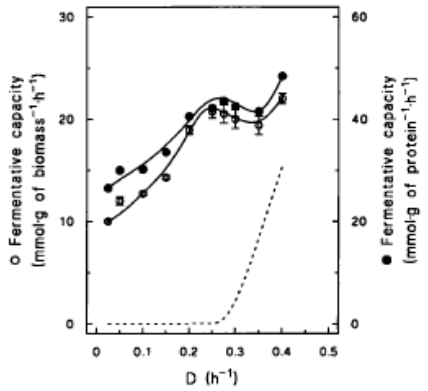

Figure 5: Effect of specific growth rate on the fermentative capacity of *S. cerevisiae.*
Effect of specific growth rate on the fermentative capacity of *S. cerevisiae* DS28911, expressed as millimoles of ethanol produced per gram of dry yeast biomass (left) and expressed per gram of cell protein (right). Fermentative capacity was assayed anaerobically under a CO_2 atmosphere in complete mineral medium supplemented with 2 % (wt/vol) glucose. The dashed line indicates the specific rate of ethanol production ($q_{ethanol}$; millimoles of ethanol per gram of dry yeast biomass per hour) in chemostat cultures. Data are presented as the means and standard deviations of results from duplicate assays at different time points in the same steady-state chemostat cultures. Taken from (Van Hoek, et al., 1998).

Outline of this Thesis

This thesis describes the effects of various alterations in the expression of global catabolic regulators on the catabolism of *S. cerevisiae.* As *S. cerevisiae* is an organism that is often used in food and beverage applications, this organism has been studied extensively. However, the balance between respiration (the preferable mode of metabolism during yeast production processes) and fermentation (which is preferable during beer and bread production) cannot yet be tuned sufficiently to make these industrial processes proceed optimally. Attempts along these lines to increase the performance of yeast by over-expression of single enzymes were only moderately successful (Smits, et al., 2000). By changing the expression level of global regulators, a whole set of genes, often encoding all the enzymes required for an entire metabolic

pathway, can be affected. This could change the performance of that particular module as a whole, and thereby the overall metabolism of the organism as such.

Chapter 2 describes the effects of alteration of the expression of two key regulators in central carbon metabolism, *HXK2* and *HAP4*. Both mutants, when either *HXK2* is deleted or *HAP4* is overproduced show an increase in respiration at high glucose concentrations, as compared to the wild-type. A transcriptome analysis of these mutant strains indeed revealed many increases in the transcript level of enzymes primarily involved in the TCA-cycle and in oxidative phosphorylation. These findings are consistent with the observed alterations in metabolic fluxes. Additionally, significant effects on transcription of genes involved in metal ion homeostasis were observed. It is not clear however, whether these are primary or secondary effects.

In Chapter 3 the results are presented of growth experiments of the strains described in Chapter 2 in a chemostat. As these strains show increased respiration, their ability to ferment under anaerobic conditions was tested in an off-line assay. Increased respiration might imply decreased fermentation; however, at low growth rates no effects were observed in the rate of fermentation of these strains. At the critical dilution rate, the growth rate at which the wild-type strain switches to respiro-fermentative metabolism, the performance of the *hxk2Δ* strain was largely reduced, whereas the *HAP4* ↑ strain showed the same capacity as the wild-type. In contrast, the *hxk2Δ* strain performed well on maltose (which is the most abundant sugar in bread dough) under all conditions. To investigate the molecular mechanisms responsible for these changes, transcript profiles, enzyme activities and the sugar-transport capacity of these strains were measured. When the cells were grown on glucose, only the activity of hexokinase was found to be significantly affected, even though this activity should still allow a wild-type flux. With maltose as the catabolic substrate, however, there was a very clear difference between transport capacity of the *hxk2Δ* and wild-type strains.

In chapter 4, a detailed analysis is presented of transcript profiling experiments performed with the Hap4 over-expression strain, under a large range of physiological conditions. This analysis shows a set of specific transcription factors of which their targets genes have been changed in expression in response to the different conditions and some which are specific for the *HAP4* ↑ strain. As expected we found the target genes of *HAP4* to be significantly upregulated in the *HAP4* ↑ strain under conditions, where glucose repression is present (high glucose). Additionally we found the target genes for Mig1p to be changed when comparing conditions with glucose repression to conditions in which the cells are de-repressed. For genes involved in iron and zinc homeostasis, changes were found as well, specific for the *HAP4* ↑ strain as well as condition dependent. Especially genes under control of Zap1p (zinc activator protein) were changed significantly. *HAP4* ↑ cells under batch conditions, downregulate genes need for zinc homeostasis, although the cause of this event is largely unknown. Target genes of Cat8p were found to be upregulated in de-repressed conditions. The target genes of transcription factors Msn2-4p were significantly present under nutrient-limited conditions, combined with low-growth rates.

Chapter 5 describes a theoretical study on the relationship between the Monod constant K_s and the affinity constant K_m of the transporter. It is shown that it is very well possible that the former has a lower value than the latter i.e. the cell as a whole may have a better affinity for a limiting substrate than the transporter of that substrate. The two constants turn out to be related by the control of the transporter on the specific growth rate. It is shown further that the unexpected result is confirmed by the experimental data for *Saccharomyces cerevisiae* grown in glucose-limited chemostats.

Chapter 6 summarizes the most important findings reported in this thesis and discusses the implications of these findings and possible future research.