Growth rate-regulated expression of the hexose transporter *HXT5* in *Saccharomyces cerevisiae*

Groeisnelheid gereguleerde expressie
van de hexose transporter *HXT5*
in *Saccharomyces cerevisiae*

(met een samenvatting in het Nederlands)

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Introduction

Yeasts are unicellular fungi that are able to produce ethanol and carbon dioxide from sugars, an ability which yeast has developed in its natural habitat, the grape juice. Already at the time of the ancient Egyptians at 3000 BC, this feature was used for preparation of food products like beer, bread and wine (1). It took until the 19th century when Louis Pasteur discovered that the yeast *Saccharomyces cerevisiae* was responsible for production of alcohol and CO_2, a process that was called fermentation (2,3). Since then, yeast has been thoroughly studied concerning its physiology and application in biochemistry and biotechnology. Molecular genetic tools provided a means to use yeast in several biotechnological applications, such as manufacturing baker's yeast, production of heterologous proteins, or production of metabolites that are used for various purposes (4). One of the metabolites with special interest is trehalose, which is not constitutively accumulated but only formed within cells during certain growth conditions to which cells may be exposed in nature. Trehalose is a non-reducing disaccharide that accumulates in bacteria, eukaryotic microorganisms like yeast, plants, insects and invertebrates, but so far not in mammals (5). Trehalose accumulation has been thoroughly studied in *Saccharomyces cerevisiae*, where it was initially thought to serve as a reserve carbohydrate (6). Nowadays, trehalose is known as a metabolite that stabilizes proteins and biological membranes under a variety of conditions, including increased temperature, hydrostatic pressure, desiccation, nutrient starvation, osmotic or oxidative stress, and exposure to toxic chemicals (5,7,8,9,10,11,12,13,14,15,16). Because trehalose has several applicable protective properties, it has become an important target for biotechnology. Trehalose may have several applications in food preparation such as dried and frozen food products, it may be used as a sweetener, in vaccine protection in hot climates, in cosmetic products as a liposome stabilizer, and may even stabilize organs that are used for transplantation (17). High trehalose contents protect cells from autolysis and increase the leavening capacity in frozen dough, making it an interesting target for baker's yeast (18,19).

Saccharomyces cerevisiae is able to consume various carbon sources, of which the fermentable monosaccharide glucose is most preferred. Yeast cells are able to grow on a wide variety of different glucose concentrations and show a great ability to adapt to changes in the environmental sugar concentration (20). Glucose, but also other carbon

sources, may serve as precursors for trehalose. When glucose is used for trehalose production, it has to be transported into the cell and converted into glucose intermediates that serve as precursors for trehalose.

Sugar metabolism

Sugar specific carriers transport sugars across the plasma membrane, before they are metabolized. On overview transport of different sugars and their subsequent metabolism is depicted in Figure 1.

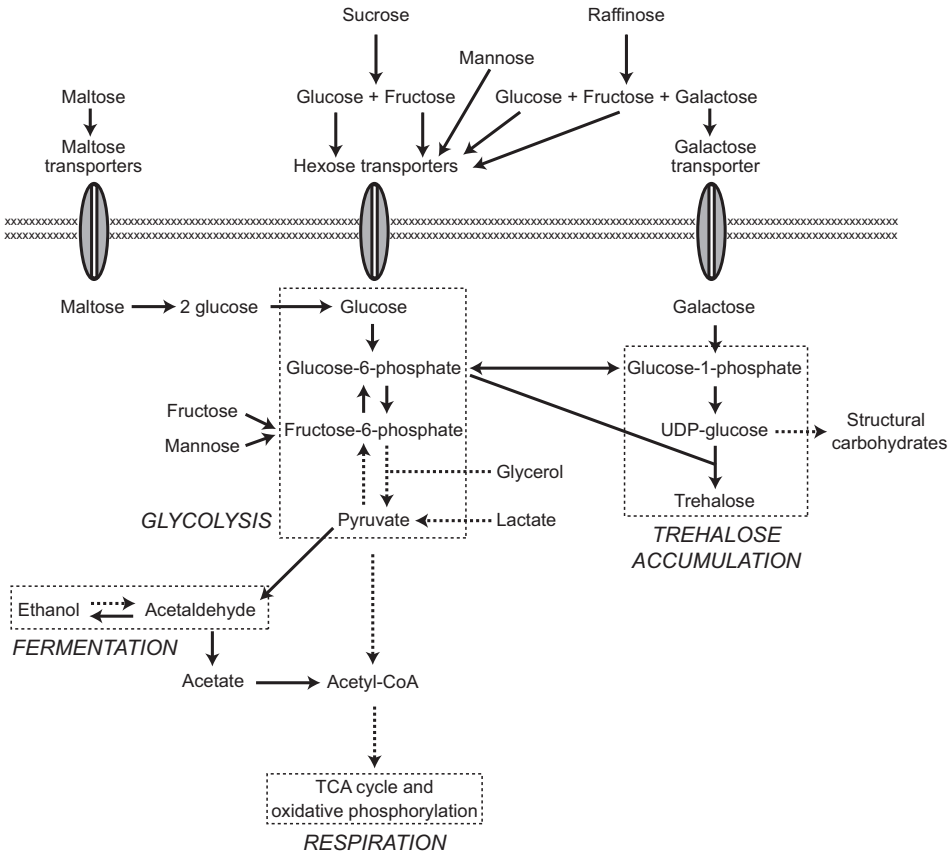

Figure 1: Overview of carbon metabolism in *Saccharomyces cerevisiae*. See text for details.

The monosaccharides glucose, fructose and mannose, are transported into cells by hexose transporter (Hxt) proteins (21,22,23,24). Upon entry, glucose is phosphorylated into glucose-6-phosphate, fructose and mannose enter glycolysis at the level of fructose-6-phosphate, and are further metabolized in glycolysis. Galactose enters glycolysis after transport by Gal2p (25,26) and conversion into glucose-1-phophate and subsequently into glucose-6-phosphate. Maltose is transported into cells by the family of maltose transporter proteins, which include Mph2p, Mph3p, Agt1p, Mal31p and Mal61p (27,28,29,30).

Glucose-6-phosphate is the precursor for UDP-glucose, and glucose-6-phosphate and UDP-glucose are used to produce trehalose. UDP-glucose is not exclusively used as precursor for trehalose, but also as precursor for structural carbohydrates, which are used as components of the cell wall. Di- and trisaccharides like sucrose, raffinose and maltose are first cleaved by glycosidases in the periplasmic space or in the cytoplasm before they are further metabolized in glycolysis. The end-point of glycolysis is pyruvate, and it depends on the growth condition whether pyruvate is used for respiration or fermentation. When cells grow in an anaerobic environment, sugars are exclusively fermented into CO_2 and ethanol. However, also when cells grow aerobically but only in the presence of a high glucose concentration, cells are able to use glucose for fermentation. This phenomenon is called the Crabtree effect (31,32). In an aerobic environment, only at low growth rates in the presence of low carbon source concentrations, sugars are completely used by respiration. During respiratory growth, pyruvate is converted into acetyl-CoA, which subsequently enters the tricarboxylic acid (TCA) cycle for conversion into CO_2, which yields NADH and FADH. In the mitochondrial respiratory chain, NADH and FADH are reoxidized to yield ATP via oxidative phosphorylation. Yeast cells are also able to grow on non-fermentable carbon sources like glycerol, ethanol, acetate and lactate, and these carbon sources are exclusively metabolized by respiration to generate ATP.

Because glucose is the most preferred carbon source, the most widely studied carbon source and because it is involved in a variety of regulatory processes within *Saccharomyces cerevisiae*, next the first step of glucose metabolism, being glucose transport, is described in more detail.

Glucose transport by hexose transporters

In *Saccharomyces cerevisiae*, a multigene family consisting of 20 genes, which include *HXT1-HXT17*, *GAL2*, *SNF3* and *RGT2*, encodes Hxt proteins (21,22,23,24). All *HXT* family members have been identified by characterization of mutant yeast strains and, after completion of the yeast-sequencing project, by sequence similarities to the known hexose transporters in yeast and various organisms (33,34). Hxt proteins have 12 putative transmembrane segments, whose amino- and carboxy-terminal regions are localized in the cytoplasm (Table 1, 23). Hxt proteins are highly homologous concerning their amino acid sequence, although the amino- and carboxy-terminal regions considerably differ in amino acid composition. However, Hxt5p is considerably larger compared to the major Hxt proteins and Hxt8-17p, because it contains a larger amino-terminal region (Table 1).

Table 1: Total number of amino acids and the number of amino acids in the potential cytoplasmic N- and C-terminal domains of the hexose transporter protein family members. Data was obtained from the Swiss-Prot database (http://www.expasy.ch/sprot).

Protein	Total no. amino acids	No. amino acids in cytoplasmic N-terminus	No. amino acids in cytoplasmic C-terminus
Hxt1p	570	60	58
Hxt2p	541	49	36
Hxt3p	567	57	58
Hxt4p	576	66	58
Hxt5p	**592**	**82**	58
Hxt6p	570	60	58
Hxt7p	570	60	58
Hxt8p	569	61	55
Hxt9p	567	56	57
Hxt10p	546	44	50
Hxt11p	567	56	57
Hxt12p	457	2	57
Hxt13p	564	52	59
Hxt14p	540	56	19
Hxt15p	567	55	59
Hxt16p	567	55	59
Hxt17p	564	52	59

Individual expression of *HXT1-4* and *HXT6-7* in the MC996 background strain, which was deleted for *HXT1-7* (*hxt* null mutant), resulted in regaining the ability to grow on glucose. Therefore, *HXT1-4* and *HXT6-7* were initially thought to encode the major Hxt proteins (35). Overexpression of *HXT5* or *HXT8-17* (except *HXT12*, which

probably encodes a pseudogene) individually in the CEN.PK background strain deleted for *hxt1-17* and *gal2* restored growth on glucose, indicating that Hxt5p, Hxt8-11p and Hxt13-17p are also able to transport glucose (36). Hxt8-11p and Hxt13-17p do not seem to play an important role in glucose uptake, because these genes are expressed to very low levels under physiological growth condition (24,37). On the contrary, it is described later in this thesis that *HXT5* is expressed during specific growth conditions. Hxt5p may indeed contribute to glucose (and fructose) transport, because transport capacity for these hexoses was measured (38,39). *SNF3* and *RGT2* encode plasma membrane proteins that are involved in sensing the amount of glucose to induce expression of specific *HXT* genes (40,41), and the product of *GAL2* is involved in transport of galactose (25,26).

The affinity for glucose of the major Hxt protein was determined by individual expression of these transporters in the *hxt* null strain (42). It appeared that *HXT1* and *HXT3* encode transporters with a low affinity for glucose (K_m= 50-100 mM) and are probably required during growth at high glucose concentrations. *HXT2* encodes transporter with bi-phasic uptake kinetics (K_m= 1.5mM and 60 mM). *HXT4* encodes a Hxt protein with moderate affinity for glucose (K_m= 9 mM) and *HXT6* and *HXT7* encode proteins with high affinity for glucose (K_m= 1-2 mM). Thus, Hxt2p, Hxt4p, Hxt6p and Hxt7p are probably required for glucose transport when low glucose levels are present. The major Hxt proteins also have affinity for fructose and mannose (42). Hxt5p has moderate affinity for glucose (K_m= 10 mM), moderate to low affinity for fructose (K_m= 40 mM) and low affinity for mannose (K_m >100 mM) (38). It should however be noted that most of the data on the kinetics of glucose transport was obtained from individual expression of *HXT* genes in the *hxt* null mutant, so that the results may not reflect the *in vivo* functions of the Hxt proteins. A single Hxt protein might behave differently in terms of affinity, which may be modulated by means of interaction between different Hxt proteins. Furthermore, the missing *HXT* genes may be important for regulation of expression of other *HXT* genes (24,43).

Transcriptional regulation of the major hexose transporter genes

Expression of the major *HXT* genes is mainly regulated by the extracellular glucose concentration (24). In general, expression of *HXT1* is induced only by high concentrations of glucose (44,45). *HXT2* and *HXT4* expression is only induced by low glucose concentrations (44,46). Expression of *HXT3* is thought to be induced

independent of the sugar concentration (44,47), although another study indicated that *HXT3* was not expressed at low glucose concentrations (37). Expression of *HXT6* and *HXT7*, encoding highly homologous proteins that only differ in two amino acids, is repressed by high levels of glucose, but expression of *HXT6* is only modestly induced by low glucose (48). *HXT7* expression is undetectable at high concentrations of glucose, *HXT7* becomes expressed when the glucose concentration decreases, and expression is absent upon glucose depletion (49).

In yeast, glucose regulates expression of genes by two different pathways, which are the glucose repression and the glucose induction pathway. The major component of the glucose repression pathway is the Mig1p transcriptional repressor complex, which recruits the general co-repressors Ssn6p and Tup1p to the promoters of specific genes when the extracellular glucose concentration is high (50). The serine/threonine kinase Snf1p plays an important role in the localization of Mig1p. At low glucose concentrations, Snf1p phosphorylates Mig1p, which is subsequently transported out of the nucleus, resulting in expression of glucose-repressed genes (51,52,53).

The signal for glucose induction of *HXT* expression is generated by the glucose sensors Snf3p and Rgt2p that both contain an exceptionally long, cytoplasmically localized carboxy-terminal domain. Snf3p is required for induction of certain *HXT* genes at low glucose concentrations, whereas Rgt2p exhibits this function at high glucose concentrations for other *HXT* genes (40,41). Expression of *HXT* genes is determined by a signal that is generated by Snf3p and Rgt2p that finally act on the transcription factor Rgt1p, which acts as a repressor of *HXT* transcription (24,54). Transcriptional repression activity of Rgt1p is dependent on the co-repressors Ssn6p and Tup7p (44). Another component that is required for expression of *HXT* genes is the ubiquitin ligase Grr1p, which was suggested to inactivate the repressing function of Rgt1p by ubiquitination in the presence of glucose. How ubiquitination of Rgt1p, or possibly a regulator of Rgt1p, alters the function of Rgt1p remains unclear (44,55). The proteins Std1p and Mth1p physically interact with the C-terminal domain of Snf3p and Rgt2p and are presumably involved in transmission of the glucose signal (56,57,58). In the absence of glucose, Rgt1p functions as a transcriptional repressor of *HXT1*, *HXT2*, *HXT3* and *HXT4* (24,44). Besides ubiquitination, phosphorylation of Rgt1p also provides a manner of regulation of the function of Rgt1p. At high glucose concentrations, Rgt1p becomes hyper-phosphorylated, dissociates from *HXT* promoters

and is converted into a transcriptional activator of *HXT* expression (54,59). At low levels of glucose, Rgt1p has a neutral role, neither repressing nor activating transcription. Expression of *HXT2, HXT4* and *HXT6* is subjected to glucose repression at high glucose concentrations and expression is therefore fully activated at low glucose concentrations when Rgt1p is inactive (44). Expression of *HXT7* is probably regulated similarly like expression of *HXT2*, *HXT4* and *HXT6* (49). The affinity for Rgt1p for *HXT* promoters depends on the amount of glucose available, with the highest affinity in cells grown in the absence of glucose, modest affinity in cells grown at low levels of glucose, and very low affinity in cells grown at high levels of glucose (59). Recently, it was shown that protein kinase Pkc1p, which is known to be involved in control of cell wall integrity, also plays a role in regulation of *HXT1*, *HXT2* and *HXT4* expression, whose expression was strongly decreased in cells deleted for *pkc1* (60). The authors suggested that Rtg1p might be a target of Pkc1p. Expression of *HXT5* is regulated in a completely different manner compared to expression of the major hexose transporters, as described later in this thesis.

Thus, *Saccharomyces cerevisiae* possesses a sophisticated means of regulation of *HXT* gene expression, which enables cells to grow on a wide variety of glucose concentrations. In the laboratory these conditions can be mimicked in a variety of experimental set-ups by growing yeast cells on solid media like agar or liquid media like growth in batch cultures.

Batch growth on glucose

When yeast cells are inoculated into a complete fermentable medium containing glucose as carbon source, and subsequently incubated under optimal physiological growth conditions, the cells will consume glucose and other nutrients present in the medium. During aerobic batch growth on glucose, distinct growth phases are observed. When glucose-deprived cells are inoculated on a medium containing a high amount of glucose, cells enter the lag phase, which represents a short period during which cells do not divide, although the length of the lag phase depends on the composition of the growth medium and the “history” of the culture that was used for inoculation (4). During this phase, the cells adapt their metabolism to take maximum advantage of the new enriched environment. Expression of genes encoding ribosomal proteins, proteins involved in proteins synthesis and glycolytic proteins are induced to establish subsequent growth at a higher growth rate (61,62). Expression of *HXT1* and

HXT3 is also induced during this phase, due to the high concentration of glucose in the medium (62). Expression of genes encoding proteins involved in respiratory metabolism or proteins required for the utilization of alternative carbon sources like galactose, sucrose or maltose, are repressed by general glucose repression (63,64).

During the next phase, when the cells have adapted to their new environment, cells grow logarithmically and this phase is therefore called the exponential phase of cell growth. Glucose is consumed completely by fermentation due to the Crabtree effect, and cells produce large amounts of ethanol, which accumulates in the growth medium (31,32). Expression of genes encoding proteins involved in glycolysis is highly induced, and the enzymatic activity of these proteins is increased (65,66).

When glucose starts to become exhausted, the cells transiently arrest growth and switch to a respiratory mode of energy production. This transition point is called the diauxic shift. Major changes are observed with respect to metabolite patterns and enzymatic activities (67), protein expression patterns as determined by two-dimensional gel electrophoresis (68,69,70,71), and gene expression using DNA microarrays (65,72). Due to exhaustion of glucose, many glucose-repressed genes become de-repressed in this phase of batch growth. However, other signal transduction pathways besides the general glucose-repression pathway are regulating expression of certain genes at this stage, which will become clear later in this thesis. Examples of genes whose expression is repressed, include those encoding proteins involved in glycolysis, transcription and translation. Genes encoding proteins involved in the TCA or glyoxylate cycle, and genes encoding proteins required for production of reserve carbohydrates, were induced upon entry in the diauxic shift phase (65). Notably, expression of some of these genes, including those involved in metabolism of the reserve carbohydrates trehalose and glycogen, was induced when (some) glucose and other essential nutrients were still available in the growth medium.

In the subsequent post-diauxic growth phase, when glucose is completely exhausted, cells use the accumulated ethanol as their new carbon source. Cells grow with a substantial decrease in the growth rate as compared to the exponential phase during growth on glucose. Gluconeogenesis provides metabolites for biosynthesis, energy is obtained by respiration, and protein synthesis is strongly decreased (66,71,73).

When all ethanol is finally exhausted, growth is completely impaired and the stationary phase is reached, which may take several days after inoculation on fresh medium (70,71,74). Some genes are specifically expressed in the stationary phase, for

example *PMA1* and *PMA2* (75), and members of the *SNZ* family (76,77), although the true function of these gene products in the stationary phase remains to be elucidated. After prolonged periods in the stationary phase, cells may autolyse and die.

In conclusion, during batch growth the fate of glucose during growth depends on the specific phase of growth. In general, when the growth rate is high during the exponential phase of cell growth, glucose is fully fermented. On the other hand, just prior to depletion of the available glucose in the medium, upon a decrease in the growth rate of cells, glucose can be converted into reserve carbohydrates, which is discussed later.

Other methods of cultivation

Most studies in yeast are performed by growing cells in batch cultures in shake flasks, merely because it is easy to set-up. However, batch cultures have some disadvantages: Many parameters, like the glucose and ethanol concentration, pH and oxygen availability are constantly changing and have a major impact on for example the growth rate of yeast cells. These problems are overcome by using continuous cultures, where cells grow for a prolonged period at exponential growth rates, without a lag or stationary phase. Parameters like pH, temperature, oxygen availability, CO_2 and ethanol production are carefully controlled and monitored. The chemostat consists of a culture into which fresh medium is added at a constant rate, and the culture volume is kept constant by continuous removal of culture. The change in biomass (x) is described by the difference between the rate of growth (μ) and the rate of removal of cells; $dx / dt = \mu x - Dx$. The dilution rate D (flow rate per unit volume per hour) is describes as F / V, where F is the medium flow rate and V is the culture volume. In steady state, when $dx / dt = 0$, the growth rate is equal to the dilution rate ($\mu = D$). The dilution rate controls the rate of cell growth via the concentration of the growth-limiting nutrient in the medium, which may be for example a carbon or nitrogen source. Furthermore, cells can be grown at specific dilution or growth rates, by increasing or decreasing the amount of culture medium that is pumped into the continuous culture system (78,79,80). Another method to cultivate yeast cells is by growing cells in fed-batch cultures, which also specifically allows controlling the growth rate of yeast cells by limitation for one specific nutrient (79). Furthermore, fed-batch cultures may be used to study events associated with an increased G_1 phase duration, for example gene expression (81) or accumulation of

reserve carbohydrates (81,82), when a low amount of galactose is continuously added to synchronized cells.

Reserve carbohydrates in *Saccharomyces cerevisiae*

Saccharomyces cerevisiae accumulates two different kinds of glucose stores, being trehalose and glycogen. An early study on accumulation of trehalose and glycogen showed that yeast cells accumulate these reserve carbohydrates when nutrients become limited (6). Several carbon sources, like glucose, galactose or ethanol may serve as precursors for trehalose and glycogen (see Figure 2). Both trehalose and glycogen have functions as reserve carbohydrates in yeast, and may be used as nutrient source during scarcity of nutrients. A method to demonstrate this is by performing viability assays; Upon a sudden removal of the carbon source, cells with high levels of accumulated trehalose and glycogen survived much longer compared to cells unable to produce these carbohydrates (83). Trehalose also has a specific function as a stress protectant (5,84). Various conditions that are harmful for cells result in accumulation of trehalose, including increased temperature, hydrostatic pressure, desiccation, nutrient starvation, osmotic or oxidative stress, and exposure to toxic chemicals (5,7,8,9,10,11,12,13,14,15,16). Trehalose can protect native proteins from denaturation and prevents aggregation of denatured proteins (14,85,86). Trehalose is also capable of protecting DNA and lipids (16). Furthermore, trehalose may protect membranes from desiccation to maintain membrane integrity by substituting water molecules and binding to the polar headgroups of phospholipids (87,88).

Although the nutrient availability is an important determinant for reserve carbohydrate accumulation, recent observations indicate that the accumulation of trehalose and glycogen is related to and may even be regulated by the duration of the G_1 phase of the cell cycle, or in other words the growth rate of yeast cells (81,82). A tight correlation between G_1 phase elongation and accumulation of trehalose and glycogen was observed during growth in fed-batch cultures; An increase in the G_1 phase duration was accompanied with higher trehalose and glycogen accumulation when the galactose consumption rate was lower than 20 fmol $cell^{-1}.hr^{-1}$ in haploid cells (82), and below 31 fmol $cell^{-1}.hr^{-1}$ in diploid cells (81). Lowering the galactose consumption rate resulted in a greater increase in G_1 phase duration and even higher levels of trehalose and glycogen accumulation. In haploid and diploid cells, trehalose was not accumulated above galactose consumption rates of 20 and 31 fmol $cell^{-1}.hr^{-1}$ respectively, although still

some glycogen accumulated (81,82). In conclusion, cells grown in fed-batch cultures accumulate trehalose and glycogen concomitant with an increase in the duration of the G_1 phase. Furthermore, the specific relation between growth rate and reserve carbohydrate accumulation was also observed by using nitrogen-limited continuous cultures using glucose as carbon source. Trehalose accumulation was only observed at $D \leq 0.10\ h^{-1}$, whereas glycogen accumulation gradually increased at decreasing growth rates and was also accumulated when D was $\geq 0.10\ h^{-1}$ (82).

Growth rate-regulated accumulation of reserve carbohydrates is also observed when yeast cells are grown by other cultivation methods like batch cultures in shake flasks. During exponential growth on fermentable carbon sources, like glucose, galactose or fructose, cells exhibit high growth rates and have low trehalose and glycogen levels. Several studies indicate that the trehalose accumulation pattern differs from that of glycogen (6,89,90). When glucose is still abundant in the medium, glycogen is accumulated, whereas trehalose accumulated only upon entry in the diauxic shift, when glucose was depleted from the medium (89,90), although another study showed that trehalose is also accumulated prior to glucose depletion (6). Thus, upon depletion of glucose, the growth rate of the cells decreases, resulting in elongation of the G_1 phase duration and accumulation of trehalose and glycogen. Other conditions, like growth at increased temperature, hydrostatic pressure, desiccation, osmotic or oxidative stress, and exposure to toxic chemicals are also associated with decreased growth rates of cells, which may be a general mechanism to regulate reserve carbohydrate accumulation. The proteins involved in accumulation of reserve carbohydrates are discussed below.

Proteins involved in trehalose and glycogen metabolism

Several carbon sources may be used as precursors for trehalose and glycogen (Figure 2). Upon transport, glucose is phosphorylated into glucose-6-phosphate by hexokinase (91) and converted into glucose-1-phosphate by phosphoglucomutase, encoded by *PGM1* or *PGM2* (92). Subsequently, glucose-1-phosphate is converted into UDP-glucose by UDP-pyrophosphorylase (Ugp1p, 93). Galactose may serve as precursor for trehalose and glycogen as depicted in Figure 2 (94). Non-fermentable carbon sources like ethanol or glycerol, which can be ultimately converted into glucose-6-phosphate by gluconeogenesis, are also used as precursors for trehalose. During conditions that result in accumulation of reserve carbohydrates, the flux towards the

precursors of trehalose and glycogen may be increased by induction of *PGM2* and *UGP1* expression, as well as regulation of the enzymatic activities of the enzymes (92,93,95).

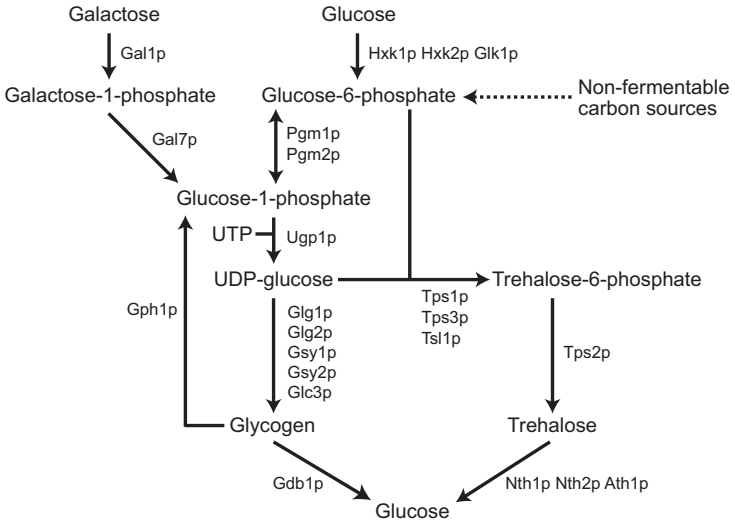

Figure 2: Overview of trehalose and glycogen metabolism in *Saccharomyces cerevisiae*. Many carbon sources can serve as precursors for trehalose and glycogen. The proteins and metabolites that are involved in trehalose and glycogen metabolism are indicated. Tps1p, Tps2p, Tps3p and Tsl1p are present in the trehalose synthase complex. For details see text.

As indicated in Figure 2, trehalose is synthesized by the conversion of glucose-6-phosphate and UDP-glucose into trehalose-6-phosphate, which is catalyzed by trehalose-6-phosphate synthase, encoded by *TPS1* (96,97). Subsequently, trehalose-6-phosphate phosphatase (Tps2p) mediates the conversion of trehalose-6-phospate into trehalose and free phosphate (98,99). Tps1p and Tps2p are part of a large protein complex called the trehalose synthase complex, which also contains the products of the *TSL1* and *TPS3* genes. Tsl1p and Tps3p are regulatory components that probably function to stabilize the complex (12,96,100). Trehalose can be degraded by the action of two types of trehalases. The first type, encoded by the *NTH1* gene (101,102), is a cytoplasmic protein (103), has its maximal activity at pH 6.8 – 7.0, and is therefore called neutral trehalase (104). *NTH2* encodes a protein with 77% similarity to Nth1p, although the cellular role of Nth2p remains unclear (105). The second type of trehalase

is found in vacuoles (103) and is encoded by *ATH1* (106). The enzyme is optimally active at pH 4.5 – 5.0 and therefore designated as acidic trehalase (104). Synthesis of glycogen involves several modification steps including initiation by auto-glucosylation of the glycogenin proteins Glg1p and Glg2p (107), elongation by Gsy1p and Gsy2p (108) and ramification by the transglucosidase Glc3p (109). Glycogen degradation is mediated by the proteins encoded by *GPH1* and *GDB1* (110,111).

The role of Tps1p in yeast

Yeast cells deleted for *tps1* display a variety of phenotypes, of which the inability to grow on rapidly fermentable carbon sources like glucose is the most profound (112). Upon addition of glucose, *tps1* deletion mutants hyperaccumulate glycolytic intermediates, and have reduced intracellular levels of inorganic phosphate and ATP (113,114). Reduction of ATP levels suggests an imbalance between the upper part of glycolysis, in which ATP is consumed, and the lower part of glycolysis, where ATP is generated. Mutations that decrease sugar transport reduce the glucose-growth defect, indicating an excessive sugar influx into glycolysis in *tps1* deletion mutants (115,116). Deletion of *hxk2* in the *tps1* deletion mutant, thereby lowering the glucose phosphorylation rate, results in regaining the ability to grow on glucose (115,117). These results imply that the system responsible for trehalose production is also important for the control of glucose influx in glycolysis (112,118). It was proposed that Tps1p acts as a direct regulator of glucose transport and phosphorylation, in conjunction with a sugar carrier and a sugar kinase to form the “general glucose-sensing complex” (96,100,112,119). The observation that Tps1p may exists both as a free form and as a component of the trehalose synthase complex supports the suggestion that Tps1p might exhibit additional regulatory functions (100). Furthermore, these observations also suggest that a link exists between glucose transport and accumulation of trehalose. Glucose, which can be transported into cells by hexose transporters, is used as a precursor for trehalose, which is accumulated during conditions when glucose becomes limited, or during conditions when trehalose is required to protect cells against harmful conditions. This glucose may be gained by lowering the amount of glucose destined for glycolysis, or by increasing the uptake of glucose, for example by increasing the amount of active glucose transporters. A system that couples glucose transport directly to accumulation of trehalose during conditions of slow growth is beneficial for the yeast cell.

Outline of this thesis

In general, trehalose accumulates in *Saccharomyces cerevisiae* upon conditions that result in a decrease in growth rates of the cells. Glucose, which is the most preferred monosaccharides for yeast, may serve as a precursor for trehalose. During growth on glucose, which is transported into cells by specific hexose transporters, trehalose is accumulated when the growth rate of cells decreases as a consequence of glucose exhaustion or conditions that are disadvantageous for growth. Earlier studies suggested that Tps1p, one of components of the protein complex involved in trehalose production, might control glucose transport. Thus, trehalose accumulation and glucose transport seem to be connected. Therefore, the aim of this study was to identify a hexose transporter that is involved in trehalose accumulation or regulation of trehalose accumulation during conditions of slow growth.

In Chapter 2, Hxt5p was identified as the hexose transporter whose expression is determined by the growth rate of cells. *HXT5* expression was induced upon conditions resulting in a decrease in the growth rate of cells, as determined by a variety of experimental set-ups and growth on different carbon sources. In contrast to the major *HXT* genes, expression of *HXT5* is not regulated by the extracellular glucose concentration. In Chapter 3, the putative regulatory elements in the promoter of *HXT5* that contribute to growth rate-regulated *HXT5* expression were identified. These results indicate that *HXT5* expression is indeed regulated in a completely different manner as compared to the major *HXT* genes. In Chapter 4, the role of Hxt5p in trehalose accumulation was investigated. *HXT5* expression correlates with the pattern of trehalose accumulation during a variety of conditions, including growth in batch, fed-batch and continuous cultures. Furthermore, expression of *HXT5* and *TPS1* is regulated by a common signal transduction pathway, being the cAMP/PKA pathway. However, upon deletion of *hxt5*, trehalose accumulation was decreased to only 80% of wildtype levels, indicating that Hxt5p may contribute to, but is not exclusively involved in trehalose accumulation. In Chapter 5, DNA microarray experiments were performed to determine genome-wide expression of cells that grow with an increased G_1 phase duration. Using this method, genes that were expressed similar to *HXT5* were identified. With this information, we elucidated whether Hxt5p is involved in other processes besides glucose transport that specifically occur when the growth rate of cells is decreased. In Chapter 6, the results of this thesis are discussed in a broader perspective.

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Chapter 2

HXT5 expression is determined by growth rates in *Saccharomyces cerevisiae*

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Abstract

In the yeast *Saccharomyces cerevisiae* hexose transporter (Hxt) proteins transport glucose across the plasma membrane. The Hxt proteins are encoded by a multigene family with 20 members, of which Hxt1-4p and Hxt6-7p are the major hexose transporters. The remaining Hxt proteins have other or unknown functions. In this study, expression of *HXT5* under different experimental set-ups is determined. In glucose-grown batch cultures, *HXT5* is expressed prior to glucose depletion. Independent of the carbon source used in batch cultures, *HXT5* is expressed after 24 hours of growth and during growth on ethanol or glycerol, which indicates that growth on glucose is not necessary for expression of *HXT5*. Increasing the temperature or osmolarity of the growth medium also induces expression of *HXT5*. In fed-batch cultures, expression of *HXT5* is only observed at low glucose consumption rates, independent of the extracellular glucose concentration. The only common parameter in these experiments is that an increase of *HXT5* expression is accompanied by a decrease of the growth rate of cells. To determine whether *HXT5* expression is determined by the growth rate, cells were grown in a nitrogen-limited continuous culture, which enables modulation of only the growth rate of cells. Indeed, *HXT5* is expressed only at low dilution rates. Therefore, our results indicate that expression of *HXT5* is regulated by growth rates of cells, rather than by extracellular glucose concentrations, as is the case for the major *HXTs*. A possible function for Hxt5p and factors responsible for increased expression of *HXT5* upon low growth rates will be discussed.

Introduction

The initial step in glucose metabolism is uptake of glucose, which is carried out by specific hexose transporter (Hxt) proteins that are localized in the plasma membrane (1,2,3,4). Uptake of glucose is mediated by facilitated diffusion. A multigene family of 20 genes, which encode different putative *HXTs*, namely *HXT1-HXT17, GAL2, SNF3* and *RGT2*, has been identified. *HXT1-4* and *HXT6-7* encode the major hexose transporters, because expression of each of these genes in the MC996 background strain deleted for *HXT1-7* allows growth on glucose (5). Overexpression of *HXT8-17* (except *HXT12)* individually in an *hxt1-17 gal2* deletion strain in the CEN.PK

background restored growth on glucose, indicating that Hxt8-11p and Hxt13-17p are also able to transport glucose (6), but expression is low under normal growth conditions (4). Recently, it was shown that Hxt5p also has glucose transport capacity (7). Expression of the major *HXTs* is mainly regulated by extracellular glucose concentrations (4). *SNF3* and *RGT2* encode plasma membrane proteins that are involved in sensing the amount of glucose to induce expression of specific *HXTs* (8,9).

HXTs encode highly homologous proteins with 12 putative transmembrane segments, with amino- and carboxy-terminal domains localized in the cytoplasm (2). The intracellular amino-terminal domains of Hxt proteins show little homology amongst each other in contrast to the remaining domains. The *HXT5* gene encodes a protein of 592 amino acids, which is approximately 20 amino acids larger compared to the major hexose transporters. The intracellular amino-terminal domains of Hxt1p, Hxt6p and Hxt7p contain 59 amino acids, those of Hxt2p, Hxt3p and Hxt4p 50, 56 and 65 amino acids respectively, but the amino-terminal intracellular domain of Hxt5p is 82 amino acids. Because Hxt5p has a longer amino-terminal domain, it is tempting to speculate that Hxt5p may have a specific function in addition to glucose transport in yeast. Furthermore, *HXT5* has a different expression pattern compared to the major hexose transporters, as determined by DNA micro array experiments. In glucose-grown batch culture experiments, expression of *HXT5* was highly induced upon glucose depletion (10), which was confirmed at the protein level by monitoring expression of Hxt5-GFP (7). Other studies indicated that expression of *HXT5* is induced by increasing the osmolarity of the growth medium after addition of NaCl or sorbitol (11,12,13,14), or by increasing the temperature (11). Increased expression of *HXT5* upon glucose depletion, temperature and osmotic up-shift suggests a specific role for Hxt5p in adaptation of cells to these new conditions. To test whether Hxt5p was essential for growth, *HXT5* was deleted, which did not result in a clear phenotype. Inoculation of stationary phase cells, which normally would have expressed *HXT5*, into fresh medium containing high levels of glucose, resulted in slightly slower growth of the *HXT5* deletion strain compared to wild type cells (7).

In this study we determined the expression of the hexose transporter *HXT5* to obtain insight in the regulation of expression and to obtain clues about a possible function for Hxt5p in addition to glucose transport. *HXT5* expression was determined under different conditions, including batch growth, fed-batch growth and well-defined growth conditions in continuous cultures. It was shown that expression of *HXT5* mRNA

and Hxt5p is not regulated by extracellular glucose concentrations, as is the case for major Hxt proteins, but merely by the growth rates of cells. Based on the unique expression profile of *HXT5* and the presence of an extended amino-terminal domain, a possible function for Hxt5p will be discussed.

Materials and Methods

Strains, media and growth conditions

The *Saccharomyces cerevisiae* strains used in this study include CEN.PK 113-7D (MATa, *SUC2, MAL2-8^c*) and was kindly provided by P. Kötter (Institut für Mikrobiologie, Johann Wolfgang Goethe-Universität, Frankfurt am Main, Germany), KY98 (MATa, *SUC2, MAL2-8^c, HXT5::GFP*) by A. Kruckeberg (E. C. Slater Institute, University of Amsterdam, Amsterdam, The Netherlands) and strain JBY20 (MATa, *SUC2, MAL2-8^c, ura3, HXT5::HA*) by J. Becker (Institut für Mikrobiologie, Heinrich-Heine-Universität, Düsseldorf, Germany). During batch culture experiments yeast cells were grown on 0.67% (w/v) yeast nitrogen base without amino acids (Difco) and 2% (w/v) of the carbon source as indicated in the text. Cells were grown at 30ºC at 180 rpm in a shaking incubator (New Brunswick Scientific). For temperature up-shift experiments cells were grown to an OD_{600} of 1.2 (± 0.1). The culture was subdivided into 15 ml falcon tubes and incubated in a water bath of 42ºC, while shaking the tubes every 10 minutes. For the osmotic up-shift experiments the cells were also grown to an OD_{600} of 1.2 (± 0.1) and concentrated 5 M NaCl was added to a final concentration of 0.7 M. Samples were directly frozen in liquid nitrogen and stored at –80ºC.

Fed-batch growth conditions

Experiments with synchronous cultures in fed-batch experiments were performed as described earlier, with some modifications (15). Cells were grown in YNB medium without amino acids with glucose as carbon source at a cell density of $1.2x10^7$ cells.ml^{-1}, and an initial extracellular glucose concentration of 0.05 mM. Glucose dissolved in YNB medium was continuously added at rates of 10 fmol and 50 fmol cell^{-1}.h^{-1} respectively. The number of cells and the external glucose concentration was monitored throughout the experiment.

Cell synchronization

Centrifugal elutriation was performed as described previously, with some modifications (16). Cells were grown until the exponential phase in YNB medium containing 1% galactose at 30ºC. $2x10^{10}$ cells were loaded in a 40 ml chamber of a Beckman J-6MI centrifuge with a JE-5.0 rotor at 30ºC and 2000 rpm. Cells were grown in the elutriation chamber on YNB 1% galactose medium. Newborn daughter cells were collected on ice, centrifuged and kept overnight on ice in YNB 1% galactose medium. The cell size was monitored with a Coulter Multizer II, and the flow rate of the elutriation was adapted to maintain a constant cell size.

Continuous culturing

Cells were grown in a 2 L BiofloIII chemostat (New Brunswick Scientific) connected to a computer controller unit with Advanced Fermentation Software (New Brunswick Scientific). Cells were inoculated in the EGLI culture medium as described previously (17) and continuous feed was connected after overnight batch growth. During the different dilution rates, the NH_4^+ concentration was adapted to 1.5 $g.l^{-1}$ to maintain nitrogen limitation, the glucose concentration in the feed was 200 mM. Steady state samples were taken as described (17).

Glucose concentration

Extracellular glucose concentrations were determined as described (18). Samples were mixed with hexokinase/glucose-6-phosphate dehydrogenase (Boehringer Mannheim), $NADP^+$ (Roche) in a 100 mM imidazole, 10 mM $MgCl_2$ buffer pH 7.0 and the conversion of $NADP^+$ into NADPH was determined using a spectrophotometer (Pharmacia Biotech).

Northern blot analysis

To isolate total RNA, yeast cells were broken with 0.45 mm glass beads in a Bead Beater (Biospec Products Inc.) in phenol and RNA extraction buffer (1 mM EDTA, 100 mM LiCl, 100 mM Tris-HCl pH 7.5, 0.5% (w/v) lithium dodecylsulfide). A phenol/chloroform extraction was performed, and total RNA was precipitated by adding 3 M NaAc pH 5.6. Samples were washed with ethanol, air-dried and suspended in DMPC-treated water. Equal amounts of total RNA (10 µg) were loaded on a 1% denaturing formamide/formaldehyde gel and RNA was separated by electrophoresis.

RNA was transferred to Hybond-N membrane (Amersham Pharmacia Biotech) and cross-linked using UV light in a UV stratalinker (Stratagene). 15 pmol of *HXT5*-specific oligonucleotides (5'-TCCCAAGGGGCCTTGATGAGCGTT-3') was labelled with T4 polynucleotide kinase (USB) and 50 μCi γ-^{32}P-ATP (Amersham Pharmacia Biotech), and purified using the QIAquick nucleotide removal kit (Qiagen). The blots were washed once in 2x SSC at room temperature, incubated for prehybridisation in hybridisation mixture (1 mM EDTA, 7% SDS, 0.5 M $NaPO_4$ pH 7.5) for at least one hour at 45ºC in a micro-4 hybridization oven (Biozym). Labelled oligonucleotides were added and hybridised overnight at 45ºC. After hybridisation the blots were washed for two minutes in 2x SSC at room temperature, twice for 20 minutes in 2x SSC, 0.1% SDS, 0.1% NaPPi at 45ºC, and once for 20 minutes with 0.5x SSC, 0.1% SDS, 0.1% NaPPi at 45ºC. After a final wash for 10 minutes in 2x SSC at room temperature, the membrane was wrapped in Saran (Dow Chemicals), and autoradiograms were developed using hyperfilm MP (Amersham). To control whether equal amounts of RNA was loaded, the gels were checked for ethidium bromide staining by UV light and the membranes were probed with an oligonucleotide against *ACT1* (5'-TGTCTTGGTCTACCGACGATAGATGGGAAG-3').

Western blot analysis

JBY20 cells were collected in Falcon tubes, frozen immediately in liquid nitrogen and stored at –80ºC. The cells were thawed on ice, collected by centrifugation, washed in ice-cold water, and resuspended in PBS containing Complete protease inhibitors (Boehringer). Equal amounts of cells were lysed by shaking vigorously with 0.45 mm glass beads in a Bead Beater. Equal amounts of protein were loaded on a 10% SDS-PAGE gel, and transferred to PVDF membrane (Roche) after electrophoresis. The membranes were blocked in 5% Protifar (Nutricia) in TBST buffer (50 mM Tris-HCl pH 7.4, 10 mM NaCl and 0.1% Tween20) for 1 hour at room temperature. The membranes were incubated with 12CA5 antibody (Roche) with TBST/0.5% Protifar for one hour at room temperature. The primary antibody was detected using peroxide-conjugated rabbit anti-mouse (Jackson Immunoresearch). Proteins were visualized by Enhanced Chemoluminescence (Renaissance).

Results

Expression of *HXT5* mRNA and Hxt5 protein in glucose-grown batch cultures

To determine whether *HXT5* expression was related to the extracellular glucose concentration, overnight-grown JBY20 cells were inoculated into fresh medium containing 2% glucose and grown in batch cultures. In JBY20 cells, *HXT5* mRNA expression was induced 9 hours after inoculation (Figure 1a). Hxt5-HA protein was expressed after 9 hours after inoculation (Figure 1b). Yeast cells had different growth rates during growth on glucose in batch cultures (Figure 1c).

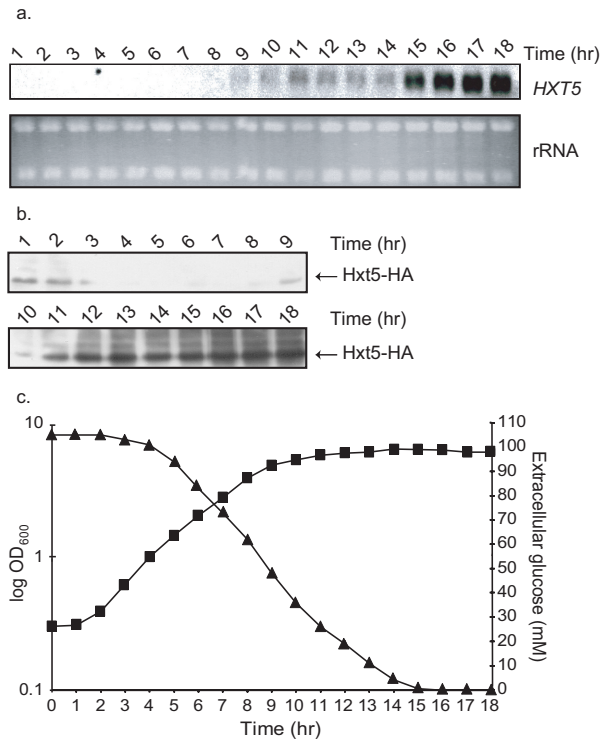

Figure 1: Expression of *HXT5* during growth on glucose in batch cultures. Strain JBY20 was grown on YNB 2% glucose in batch cultures. At the indicated time points after inoculation cells were harvested, RNA and proteins were extracted and the extracellular glucose concentration was measured. (a) Northern blot analysis of *HXT5* mRNA. (b) Western blot analysis of Hxt5-HA. (c) Optical density at 600 nm (■) and extracellular glucose concentrations (▲). Results shown are representative of at least three different experiments.

JBY20 cells exhibited fast growth during the exponential growth phase (μ=0.36 h^{-1}) and slow growth upon entry in the stationary phase approximately 9 hours after inoculation (μ=0.028 h^{-1}). No *HXT5* mRNA or Hxt5-HA was expressed during the

exponential growth phase. Expression of Hxt5-HA was observed in slowly growing cells until 2 hours after inoculation. This was probably a remainder of the Hxt5-HA that was expressed in the overnight-grown cells used for inoculation, and probably not the result of *de novo* synthesis, because *HXT5* mRNA was not expressed at these time points. Furthermore, JBY20 cells grown exponentially overnight did not show expression of Hxt5-HA at the same optical densities (data not shown).

To establish whether the increased expression was related to the extracellular glucose concentration, extracellular glucose levels in the medium were measured. Extracellular glucose levels decreased, starting from 105 mM at inoculation and decreasing to 0.55 mM after 15 hours of growth, and to 0.03 mM after 18 hours of growth (Figure 1c). *HXT5* expression was already initiated 9 hours after inoculation when still 48 mM glucose was present. However, when glucose was depleted (<1 mM) 15 hours after inoculation, *HXT5* expression was maximally induced. However, Hxt5-HA expression remained constant from approximately 12 hours after inoculation. Therefore, regulation of *HXT5* expression by glucose alone seems unlikely, but a parameter that correlated with increased expression of *HXT5* after 9 hours of growth was a decrease in growth rate of the cells.

Expression of *HXT5* on different carbon sources

To investigate whether *HXT5* expression was dependent on growth on glucose, CEN.PK 113-7D cells were grown in batch cultures on YNB media containing different carbon sources. On easily fermentable carbon sources, including glucose, galactose, fructose, mannose, sucrose and maltose, cells exhibited high growth rates during the exponential phase of cell growth. Cells growing on non-easily fermentable carbon sources, i.e. ethanol and glycerol, exhibited low growth rates during the exponential phase of cell growth (Table 1).

Carbon source	μ (h^{-1}) exponential growth
Glucose	0.35
Galactose	0.19
Fructose	0.34
Mannose	0.30
Ethanol	0.10
Sucrose	0.33
Maltose	0.26
Glycerol	0.01

Table 1: Growth rates of CEN.PK 113-7D cells growing exponentially on YNB medium with 2% of different carbon sources in batch cultures.

Independently of the carbon source that was used, *HXT5* was expressed after 24 hours of growth when cells entered the stationary phase and exhibited low growth rates (Figure 2). Cells growing exponentially 6 hours after inoculation on medium containing the carbon sources glucose, galactose, fructose, mannose, sucrose and maltose did not express *HXT5*. Expression of *HXT5* was observed during the exponential phase of cell growth, when cells were grown on ethanol or glycerol (Figure 2). These results indicate that glucose is not a determining factor in the regulation of *HXT5* expression, but a decrease in growth due to the carbon source was accompanied by induction of *HXT5* expression.

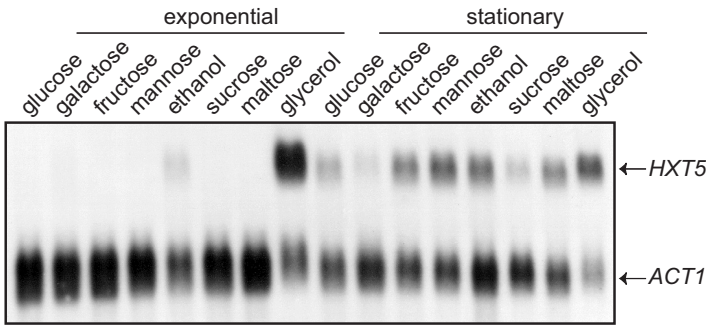

Figure 2: Expression of *HXT5* mRNA during the exponential and stationary phase of growth on different carbon sources. Strain CEN.PK 113-7D was grown on YNB medium containing 2% of each different carbon source indicated. During the exponential and stationary phase of batch growth cells were harvested and mRNA was extracted. Subsequently, expression of *HXT5* mRNA was determined by Northern blot analysis. Results shown are representative of at least three different experiments.

Expression of *HXT5* during environmental changes leading to low growth rates

Changing growth conditions by increasing the temperature or osmolarity creates conditions that influence the growth rate of cells. When the temperature of the medium of cells growing exponentially on YNB 2% glucose was changed from 30ºC to 42ºC over a 90-minute-period, growth rates decreased from μ=0.36 h^{-1} to μ=0.14 h^{-1}. Cells growing exponentially at 30ºC did not express *HXT5*. Increasing the temperature to 42ºC induced expression of *HXT5* (Figure 3a). To determine whether expression of

Hxt5 protein followed the expression pattern of *HXT5* mRNA, expression of Hxt5-HA during temperature up-shift was determined. Hxt5-HA was expressed 20 minutes after temperature up-shift and was present throughout the treatment (Figure 3b). The extracellular glucose concentration remained higher than 60 mM, indicating that increased expression of *HXT5* was not a result of glucose depletion.

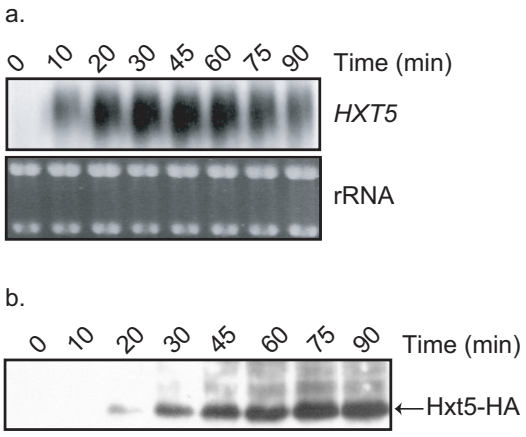

Figure 3: Expression of *HXT5* upon temperature up-shift to 42ºC. CEN.PK 113-7D cells and JBY20 cells were grown at 30ºC on YNB 2% glucose in batch cultures until the exponential phase. Then, the temperature of the medium was increased to 42ºC and at the indicated time points culture samples were harvested. mRNA was extracted from CEN.PK 113-7D cells and proteins were extracted from JBY20 cells. (a) Northern blot analysis of *HXT5* mRNA. (b) Western blot analysis of Hxt5-HA. Experiments were performed at least three time and similar results were obtained: representative blots are shown.

Stress and hence a lower growth rate was also introduced by adding NaCl to a final concentration of 0.7 M to cells growing exponentially on YNB 2% glucose (μ=0.35 h^{-1} for the non-treated and μ=0.25 h^{-1} for the treated cells). This switch in external conditions was accompanied by a transient expression of *HXT5* (Figure 4a). This transient induction is not due to glucose limitation, as the glucose concentration remained higher than 60 mM throughout the experiment. During the switch of external conditions the pattern on the Hxt5-HA protein level was different from that of *HXT5* mRNA, as the protein was present throughout the experiment, and *HXT5* mRNA was

expressed transiently (Figure 4b). Again, the results of these experiments indicate that a decrease in the growth rate of cells correlates with increased expression of *HXT5*.

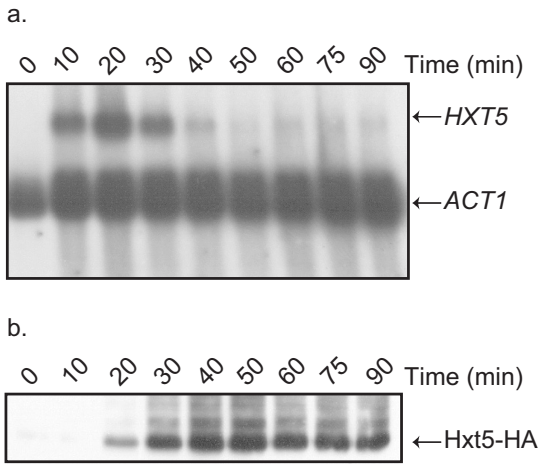

Figure 4: Expression of *HXT5* after increasing the osmolarity to 0.7 M NaCl. CEN.PK 113-7D cells and JBY20 cells were grown on YNB 2% glucose in batch cultures until the exponential phase. The osmolarity of the medium was increased to 0.7 M NaCl by adding a concentrated solution of NaCl and at the indicated time points culture samples were harvested. mRNA was extracted from CEN.PK 113-7D cells and proteins were extracted from JBY20 cells. (a) Northern blot analysis of *HXT5* mRNA. (b) Western blot analysis of Hxt5-HA. Experiments were performed at least three time and similar results were obtained: representative blots are shown.

Expression of *HXT5* during low growth rates in fed-batch cultures

To study the effect of G_1 phase elongation, and hence low growth rates, on expression of *HXT5*, Hxt5-GFP tagged cells that were synchronized early in the G_1 phase, were grown in fed-batch cultures. Low growth rates were induced by growing the cells on 10 fmol glucose $cell^{-1}.h^{-1}$, while 50 fmol glucose $cell^{-1}.h^{-1}$ was used to induce high growth rates (15). At a consumption rate of 10 fmol glucose $cell^{-1}.h^{-1}$, *HXT5* mRNA was detected. No *HXT5* mRNA was detected at a consumption rate of 50 fmol glucose $cell^{-1}.h^{-1}$ (Figure 5a). Fluorescence studies confirmed that at the consumption rate of 10 fmol glucose $cell^{-1}.h^{-1}$, Hxt5-GFP was incorporated in the plasma membrane within two hours, whereas at the consumption rate of 50 fmol glucose $cell^{-1}.h^{-1}$ even after eight hours of growth no fluorescence was observed (data not shown).

Even under these conditions the external glucose concentration did not have a regulatory function in *HXT5* expression. During the experiment extracellular glucose levels of the culture growing on 10 fmol glucose cell^{-1}.h^{-1} remained at a concentration of approximately 0.1 mM (Figure 5b). The fast growing cells with a consumption rate of 50 fmol glucose cell^{-1}.h^{-1} initially have higher extracellular glucose levels, varying between 0.2 and 0.4 mM. After 5 hours of growth, the extracellular glucose concentration diminishes to between 0.1 and 0.15 mM. These concentrations were also found in the culture growing at a consumption rate of 10 fmol glucose cell^{-1}.h^{-1} (Figure 5b). The cells growing at higher growth rates did not express *HXT5* and Hxt5-GFP after 5 hours, even when the extracellular glucose concentration is comparable to concentrations of the slowly growing culture. During slow growth in fed-batch cultures the parameter that correlated with increased expression of *HXT5* was again a decrease of the growth rate, and the growth rate *per se* might therefore regulate the expression of *HXT5*.

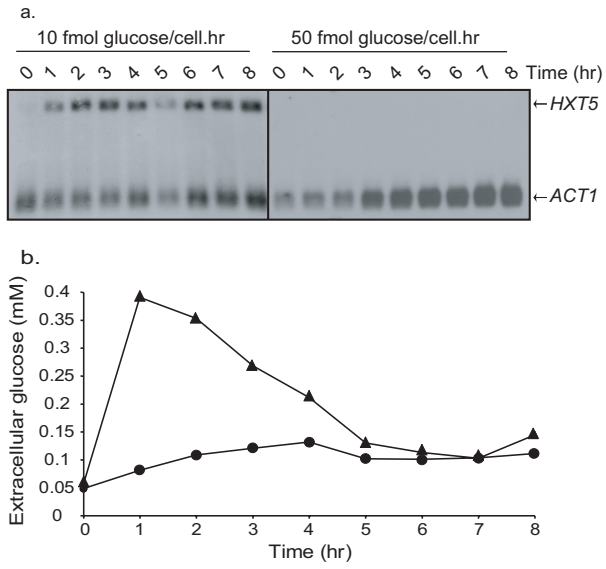

Figure 5. Expression of *HXT5* in synchronized cells in fed-batch cultures. KY98 cells were synchronized by elutriation and grown at either 10 or 50 fmol glucose cell^{-1}.h^{-1} in fed-batch cultures. At the indicated time points samples were taken and examined for *HXT5* expression and residual glucose concentration. (a) Northern blot analysis of *HXT5* mRNA. (b) Extracellular glucose concentrations of cells growing on either 10 (●) or 50 fmol (▲) glucose cell^{-1}.h^{-1} in fed-batch cultures. Results shown are representative of at least three different experiments.

Expression of *HXT5* in a nitrogen-limited continuous culture

To determine whether the growth rate was the only parameter that determines expression of *HXT5*, CEN.PK 113-7D cells were grown in a nitrogen-limited continuous culture. This experimental set-up allowed modulation of only the growth rate of yeast, by changing the dilution rate of the culture (17). Other parameters like temperature, agitation, pH and oxygen availability were constant. Also the concentration of intracellular metabolites including glucose-6-phosphate, fructose-6-phosphate, glucose-1-phosphate and ATP were constant at different dilution rates (data not shown). The concentration of the limiting compound, in this case nitrogen, was also constant, namely virtually 0 mM at all dilution rates (19,20). *HXT5* was only expressed at dilution rates lower than 0.10 h^{-1}, whereas no *HXT5* expression was observed at dilution rates higher than 0.13 h^{-1} (Figure 6). At the dilution rate of 0.10 h^{-1} *HXT5* was expressed to a lower extent compared expression at dilution rates of 0.068 h^{-1} and 0.071 h^{-1}. The dilution rate is the only parameter that is changed in the continuous culture experiments. Low dilution rates, and hence low growth rates, result in increased expression of *HXT5*. Therefore, these results clearly indicate that the growth rate determines expression of *HXT5* in *Saccharomyces cerevisiae*.

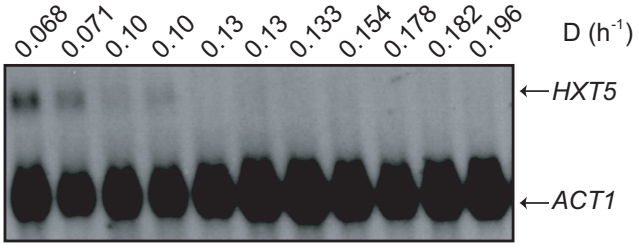

Figure 6: Expression of *HXT5* in a nitrogen-limited continuous culture. CEN.PK 113-7D cells were cultivated in a nitrogen-limited continuous culture. At the different dilution rates indicated, cells were harvested and mRNA was extracted. Expression of *HXT5* mRNA was determined by Northern blot analysis.

Discussion

To obtain clues about the function of Hxt5, and to find mechanisms that are involved in expression of *HXT5*, its expression was determined in different experimental set-ups. Batch culture experiments revealed that *HXT5* is expressed both at the mRNA and protein level when still ample glucose is available in the medium, and remained present after glucose depletion. These results are largely in agreement with earlier observations (10), however *HXT5* is also expressed prior to glucose depletion in our experiments. This suggests that Hxt5p might contribute to glucose transport, which is supported by the observation that Hxt5p is indeed able to transport glucose across the plasma membrane (7).

Independent of the carbon source in which cells are inoculated, *HXT5* is expressed after 24 hours of growth, and cells grown in ethanol or glycerol already expressed *HXT5* in the exponential phase of batch growth. These results are in agreement with earlier observations, where expression of Hxt5-GFP in cells growing on different carbon sources in batch cultures was studied (7). Increasing the temperature or osmolarity of the growth medium of exponentially growing cells resulted in increased expression of *HXT5* at both the mRNA and the protein level. These results confirm the results of various DNA micro-array experiments, which show increased expression of *HXT5* after increasing the temperature (11) or osmolarity (11,12,13,14). In fed-batch cultures *HXT5* was expressed when cells were grown at 10 fmol glucose $cell^{-1}.h^{-1}$, whereas no *HXT5* expression was observed at 50 fmol glucose $cell^{-1}.h^{-1}$. Taken together, our results indicate that during all experiments one parameter that results in induction of *HXT5* expression is in common, being a decrease in the growth rate. These results were confirmed by continuous culture experiments, which were used for modulation of only the growth rate of cells under well-defined growth conditions. *HXT5* is expressed only at dilution rates lower than 0.10 h^{-1}, and expression of *HXT5* is increased even more when the growth rate is further diminished to dilution rates of 0.068 h^{-1}. The concentration of the growth limiting substrate, in this case nitrogen, is extremely low at virtually all growth rates and is the only substrate that determines the growth rate (19,20).

Our results suggest that expression of *HXT5* is not regulated by glucose and not subjected to glucose repression. Furthermore, in a hexokinase II deletion mutant, a protein known to be involved in the regulation of glucose repression, *HXT5* expression

is not derepressed at high extracellular glucose concentrations in batch cultures (21). This result indicates that *HXT5* expression is not regulated by glucose repression. Interestingly, expression of *HXT7* is derepressed at high extracellular glucose concentrations, showing that expression of certain *HXTs* can be repressed by glucose (21). Also Snf3 and Rgt2 are not involved in regulation of *HXT5* expression, as cells deleted for *snf3* or *rgt2* still expressed *HXT5* in glucose-grown batch cultures (data not shown). The Snf3/Rgt2 pathway does regulate expression of the major *HXTs* (4). Because glucose repression and the Snf3/Rgt2 pathway are not involved in regulation of *HXT5* expression, another mechanism probably regulates expression of *HXT5*. Indeed, our results indicate that expression of *HXT5* is induced upon a decrease in growth rates of cells.

To obtain further insight how *HXT5* expression is regulated, the promoter region of the *HXT5* gene was analysed to reveal elements that might be involved in regulation of expression (22). The *HXT5* promoter contains two stress responsive elements (STREs; -472 bp and -304 bp relative to the translation initiation site respectively), two HAP2/3/4/5 binding sites (-854 bp and -785 bp respectively) and one PDS element (-544 bp) (23,24,25). Surprisingly, the promoter of *HXT5* appears to be homologous with the promoter of *GSY2*, encoding glycogen synthase 2, which is involved in glycogen synthesis (26). The promoter of *GSY2* also contains two STREs, two putative HAP2/3/4/5 complex binding sites and one PDS element. Furthermore, expression of *GSY2* exhibits a similar expression pattern as *HXT5* (27), indicating that *HXT5* and *GSY2* expression could be regulated in a similar matter. The involvement of low growth rates in *HXT5* expression does not exclude the involvement of these elements in the promoter of *HXT5* in determining expression of *HXT5* during low growth rates. Furthermore, the transcriptional elements may even be activated under conditions that cause low growth rates to induce expression of *HXT5*.

Cell cycle duration is a well-organized process of which environmental conditions are the main regulators. It was shown that cell cycle duration could be greatly elongated by growing cells in fed-batch cultures on low amounts of carbon source. Concomitantly, trehalose and glycogen were accumulated in these cells (15). Similar results were obtained from cells that were grown in continuous cultures, where also an increase in cell cycle duration is accompanied with elevated trehalose and glycogen levels (28). Surprisingly, we observed that *HXT5* was expressed whenever trehalose was accumulated during growth in batch cultures, fed-batch cultures and

continuous cultures (see Chapter 4). Furthermore, genome-wide analysis of stressful conditions including temperature up-shift, adding chemical compounds that are hazardous for cells and increased osmolarity, revealed that expression of *HXT5* is induced concomitantly with genes involved in reserve carbohydrate metabolism (11). None of the other hexose transporter has this specific expression pattern. Furthermore, Hxt5p is structurally different compared to the major Hxt proteins, because it contains a larger intracellular amino-terminal domain. Taken together, these observations suggest a specific role for Hxt5p in the accumulation or metabolism of reserve carbohydrates. The precursor for trehalose is glucose and Hxt5p may specifically regulate uptake of glucose that is designated for production of trehalose during conditions that induce low growth rates. Furthermore, it was postulated that Tps1p, a protein involved in trehalose synthesis, might function as a direct regulator of glucose transport, probably by interacting with a hexose transporter (29). In our opinion, Hxt5p seems a good candidate to interact with Tps1p, thereby regulating accumulation of trehalose.

In conclusion, our results indicate that expression of *HXT5* is determined by growth rates of cells, and not dependent on the extracellular glucose concentration. The promoter of *HXT5* contains putative regulatory elements, which may contribute to expression of *HXT5* during low growth rates. The extended amino-terminal domain of Hxt5p and the unique expression pattern of *HXT5* during various kinds of conditions leading to low growth rates that are concomitant to accumulation of trehalose, suggest a role for Hxt5p in accumulation of this reserve carbohydrate besides glucose transport.

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Chapter 3

HXT5 expression is under control of STRE and HAP elements in the *HXT5* promoter

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Abstract

Hexose transporter (Hxt) proteins transport glucose across the plasma membrane in the yeast *Saccharomyces cerevisiae*. Recently, we have shown that expression of *HXT5* is regulated by the growth rate of the cells. Because gene expression is regulated by binding of specific transcription factors to regulatory elements in the promoter of genes, the presence of putative regulatory elements in the promoter of *HXT5* was determined by computer-assisted analysis. This revealed the presence of two putative stress responsive elements (STREs), one putative post-diauxic shift (PDS) element and two putative Hap2/3/4/5p (HAP) complex binding elements. The involvement of these elements was studied by using mutations in a *HXT5*-promoter *LacZ* fusion construct. Growth during various conditions that result in low growth rates of yeast cells revealed that the STRE most proximal to the translation initiation site seemed to be involved in particular in regulation of *HXT5* expression during growth at decreased growth rates, whereas the HAP elements seemed to be required during growth on non-fermentable carbon sources. The PDS element and to a lesser extent the other STRE showed particular involvement in regulation of *HXT5* expression during growth on ethanol. A possible mechanism by which expression of *HXT5* could be regulated by the transcriptional regulatory elements in the promoter is discussed.

Introduction

Hexose transport (Hxt) proteins, encoded by the *HXT* gene family consisting of 20 members, transport glucose across the plasma membrane of the yeast *Saccharomyces cerevisiae* (1,2,3,4). Expression of the major hexose transporter (*HXT*) genes, which include *HXT1,2,3,4,6* and *HXT7*, is regulated by the extracellular glucose concentration (4). In contrast, expression of *HXT5*, which encodes a Hxt protein that is also able to transport glucose (5), is determined by the growth rate of cells and not by the extracellular glucose concentration. In batch cultures, *HXT5* is expressed upon a decrease in the growth rate of cells when glucose is still available in the medium, and expression was maximally induced upon glucose depletion (6). During batch growth on glucose, cells grow exponentially by fermentation of the available glucose, and ethanol is produced (7).

When glucose becomes exhausted, cells use the accumulated ethanol as their new carbon source and continue to growth with decrease growth rates in this so-called diauxic shift phase. Several studies revealed major changes in the pattern of gene expression, with the common feature that genes encoding components of the glycolytic pathway and cell growth machinery are repressed, and glucose-repressed genes are de-repressed (8,9,10,11). However, a subset of genes that is characteristic for this phase was already induced when glucose and all other nutrients were still available in the medium, and expression was induced far in advance of other so-called diauxic and stationary phase events (12,13,14). Examples include genes involved in the synthesis of reserve carbohydrates (for review see 15) and the general stress-response gene *CTT1* (16). Expression of the hexose transporter *HXT5* was also induced when still ample glucose was present in the medium (6). Therefore, expression of *HXT5* is as well an example of a gene whose expression is induced prior to the diauxic shift.

Expression of genes is regulated by the binding of transcription factors to specific binding sites in the promoter region. Therefore, expression of *HXT5* is possibly regulated by transcription factors that induce expression upon a decrease in the growth rate of cells. In the present study we identified five putative regulatory elements in the promoter of the *HXT5* gene by computer-assisted analysis. These include two stress responsive elements (STREs, 17,18,19,20), one PDS element (21) and two Hap2/3/4/5p (HAP) complex binding sites (22). These putative regulatory elements were mutated by site-directed mutagenesis to determine whether these elements were involved in the growth-rate regulated expression of *HXT5*. We provide evidence that expression of *HXT5* upon a decrease in growth rates was fully dependent on the presence of one STRE. In addition, the two HAP elements seemed to be involved in regulation of *HXT5* expression during growth on non-fermentable carbon sources. The PDS and the remaining STRE element are important during growth on ethanol. Possible mechanisms that regulate *HXT5* expression will be discussed in the scope of these results.

Materials and Methods

Plasmids and site-directed mutagenesis

Plasmid BM3555 contained 1342 bp upstream of the translation initiation site of the *HXT5* gene (kindly donated by M. Johnston, Washington University, St. Louis,

MO, USA). This plasmid was used as template to mutate the putative regulatory elements by site-directed mutagenesis via inverse PCR (23). Primers used to introduce mutation in the STRE1, STRE2, PDS, HAP1 and HAP2 elements are indicated in Table 1.

Table 1: List of primers (forwards and reverse) used to introduce mutations in the putative regulatory elements in the promoter of *HXT5* on plasmid BM3555. The introduced mutations are underlined.

Primer name	Primer Sequence (5'-3')
STRE1 for	GGGCATGGGTTAATTAGTTTAGATCTCACGGAGTAAACAAGAAAGG
STRE1 rev	CCTTTCTTGTTTACTCCGTGAGATCTAAACTAATTAACCCATGCCC
STRE2 for	CCCCGTGGCTGTGCACATAGATCTACAGTAAGTAATTCAC
STRE2 rev	GTGAATTACTTACTGTAGATCTATGTGCACAGCCACGGGG
PDS for	CTGTTACTTCCCGGATTAAGATCTGCATTCTAACTCTGTGCGC
PDS rev	GCGCACAGAGTTAGAATGCAGATCTTAATCCGGGAAGTAACAG
HAP1 for	CCGATACGGGAATATCTCGAGCAATACTTATCACTTCGG
HAP1 rev	CCGAAGTGATAAGTATTGCTCGAGATATTCCCGTATCGG
HAP2 for	GCAATATTTACCTTATTTTCTCGAGTACTTTTCTATACGC
HAP2 rev	GCGTATAGAAAAGTACTCGAGAAAATAAGGTAAATATGGC

Inverse PCR was performed using *Pfu* Turbo DNA polymerase (Statagene, La Jolla, CA, USA) and the following parameters: initial denaturation for 30 s at 95ºC, then 18 cycles of subsequent denaturation for 30 s at 95ºC, annealing for 1 min at 55ºC and elongation for 18 min 36 s at 68ºC. The amplified product was digested with *DpnI* and transformed into *E.coli* cells (*DH10α*). The *BamHI/EcoRI* promoter fragments were subcloned into YIp357R (24) and introduction of the desired mutation was confirmed by sequencing (BaseClear, Leiden, The Netherlands). Using the procedure described above, mutations of both STRE and both HAP elements were obtained. The plasmids were restricted with *StuI* for targeting as a single-copy integration into the *ura3* locus (25) during subsequent transformation into strain CEN.PK 115-7D by electroporation (26). Integration was confirmed by Southern blotting.

Stains, media and growth conditions

During batch culture experiments, yeast cells were grown on 0.67% (w/v) yeast nitrogen base without amino acids (Difco, Becton, Dickinson and Company, Sparks, USA) and 2% (w/v) of the carbon source as indicated in the text. The strains used during this study are indicated in Table 2.

Table 2: List of strains used during this study.

Yeast strain	Genotype	Source
CEN.PK 115-7D	*MATa, SUC2, MAL2-8^c, ura3-52*	P. Kötter
RKY01	*MATa, URA3::HXT5pr-LacZ*	This study
RKY02	*MATa, URA3::ΔSTRE1-HXT5pr-LacZ*	This study
RKY03	*MATa, URA3::ΔSTRE2-HXT5pr-LacZ*	This study
RKY04	*MATa, URA3::ΔSTRE1/2-HXT5pr-LacZ*	This study
RKY05	*MATa, URA3::ΔPDS-HXT5pr-LacZ*	This study
RKY06	*MATa, URA3::ΔHAP1-HXT5pr-LacZ*	This study
RKY07	*MATa, URA3::ΔHAP2-HXT5pr-LacZ*	This study
RKY08	*MATa, URA3::ΔHAP1/2-HXT5pr-LacZ*	This study

Cells were grown at 30ºC at 180 rpm in a shaking incubator (New Brunswick Scientific, Nijmegen, The Netherlands). For batch growth experiments, the strains were inoculated on YNB 2% glucose medium and grown overnight, until the OD_{600} of the cells was 0.50 (± 0.1). For temperature up-shift experiments cells were grown overnight to an OD_{600} of 1.0 (± 0.1) on YNB 2% glucose medium. The culture was subdivided into 15 ml falcon tubes and incubated in a water bath of 42ºC, while shaking the tubes every 10 minutes. For the osmotic up-shift experiments the cells were grown overnight to an OD_{600} of 1.0 (± 0.1) on YNB 2% glucose medium and 5 M NaCl was added to a final concentration of 0.5 M. Samples were treated as described below.

Continuous culturing

Continuous culturing was performed as described earlier (6). Cells were grown at dilution rates of 0.07 and 0.17 h^{-1}.

β-galactosidase assays

Samples were directly centrifuged, washed with PBS, resuspended in 0.1 M Tris/Triton X-100 buffer pH 7.0 and stored at -80ºC to permeabilize the cells. Upon thawing at 4ºC, Z-buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 0.25 mM DTT, pH 7.0) with o-nitrophenyl β-galactoside (ONPG, 4 mg/ml in 0.1 M potassium phosphate buffer, pH 7.0) was added to initiate the reaction, which was terminated by addition of 1.0 M Na_2CO_3. The conversion of ONPG into galactose and o-nitrophenyl, which is a yellow product, was measured on a spectofotometer at OD_{420} (Pharmacia Biotech, Cambridge, England). Miller Units are defined as OD_{420} x 1,000/OD_{600} x t (min) x volume (ml) (27).

Results

Identification of putative regulatory elements in the promoter of *HXT5*

Recently, we showed that expression of *HXT5* mRNA and protein is induced upon a decrease in the growth rate of cells, independent of the extracellular glucose concentration (6). Because expression of genes is regulated by binding of specific transcription factors to regulatory elements in their promoter, the promoter of *HXT5* was analysed by computer-assisted analysis to determine whether putative regulatory elements were present (28). Five putative regulatory elements were identified, that may be involved in transcriptional activation of *HXT5* expression in response to a decrease in the growth rate of cells (Figure 1).

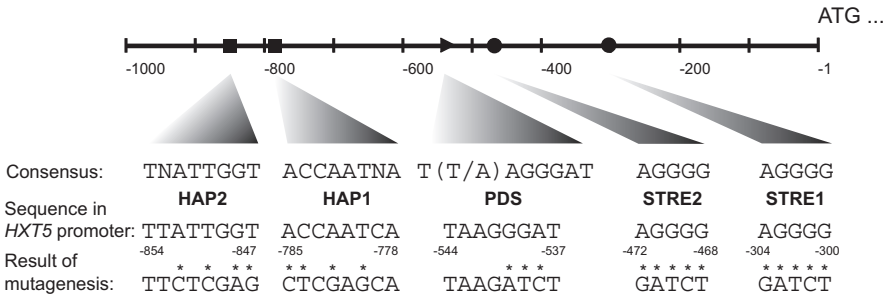

Figure 1: Putative regulatory elements in the promoter of *HXT5* and the result of site-directed mutagenesis. The coordinates indicated are relative to the translation initiation site. Mutagenesis results in (partial) replacement of the consensus sequence by unrelated sequences.

Two sequences had striking resemblance to STREs (stress responsive elements), which have the core consensus sequence AGGGG (18). The two STREs were located at the positions –304 and –472 bp relative to the translation initiation site. Furthermore, one sequence matched with the conserved sequence of the PDS (post-diauxic shift) element (core consensus T(T/A)AGGGAT) at –544 bp (21). Finally, two Hap2/3/4/5p (HAP) complex binding sites at the positions –785 and –854 bp matched the core consensus ACCAATNA (27,29), although the putative HAP site most distal to the translation initiation site at –854 bp was present in the reversed orientation (TNATTGGT). In order to determine whether these elements play a role in regulation of

HXT5 expression, we used site-directed mutagenesis to inactivate the putative STRE, HAP and PDS elements in a *HXT5*-promoter *LacZ* fusion construct, as described in materials and methods. The putative elements were mutated individually or as combinations where either both STREs or both HAP binding sites were mutated resulting in wildtype, STRE1, STRE2, STRE1/2, PDS, HAP1, HAP2 and HAP1/2 *HXT5*-promoter *LacZ* fusion constructs (Figure 1). We chose specifically for site-directed mutagenesis instead of deletion of the putative regulatory elements to avoid a possible alteration of the promoter structure. The *LacZ* expression constructs were transformed into yeast, resulting in strains carrying single-copy integrations of the wildtype and mutated *HXT5*-promoter *LacZ* construct, as indicated in Table 2.

Expression of *HXT5*-promoter *LacZ* fusion constructs during batch growth on glucose

To establish the effect of mutations of the putative regulatory elements in the promoter of *HXT5*, cells containing single-copy integrations of wildtype and mutated *HXT5*-promoter *LacZ* constructs (strains RKY01-RKY08) were grown in batch cultures containing YNB 2% glucose. During the first 6 hours after inoculation, when cells grow at a maximal growth rate (data not shown), virtually no β-galactosidase activity was measured in all strains. After approximately 6 hours of growth β-galactosidase activity increased in all transformed strains, except those containing a mutation in the STRE1 element in the *HXT5*-promoter *LacZ* construct, indicating an important role for STRE1 in regulation of *HXT5* expression (Figure 2). At this time point, the growth rate of all transformed strains started to decrease, whereas glucose was still present in the medium (data not shown). Thus, the STRE1 element may be important for regulation of *HXT5* expression when the growth rate of cells decrease. Mutations of the STRE2, PDS, HAP1 and HAP2 elements did not affect expression of the *HXT5*-promoter *LacZ* fusion construct during the remaining growth on glucose until 24 hours after inoculation (Figure 2), suggesting that these elements do not contribute to *HXT5* expression under these conditions. Mutation of both HAP elements resulted in a similar induction of *HXT5*-*LacZ* expression compared to the wildtype *HXT5*-*LacZ* construct until 9 hours after inoculation. After this time-point, however, glucose was depleted from the medium (data not shown) and β-galactosidase activity of the *HXT5*-promoter ΔHAP1/2-*LacZ* fusion construct remained constant during subsequent growth (Figure 2). The latter results suggest that the HAP elements are also involved in regulation of *HXT5*

expression during batch growth on glucose, especially when glucose is depleted from the medium.

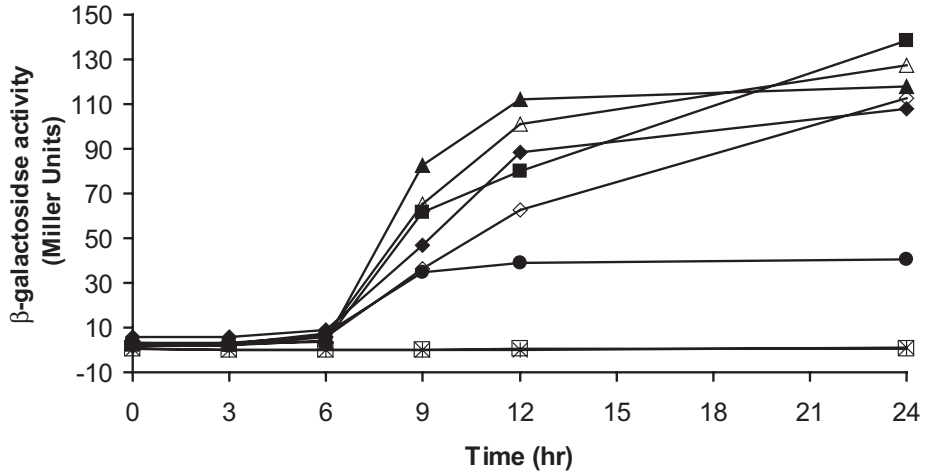

Figure 2: Effect of mutations in the putative regulatory elements in the promoter of *HXT5* on the expression of a *HXT5*-promoter *LacZ* construct during batch growth on glucose. Strain CEN.PK 115-7D was transformed with YIp357R derivative plasmids to obtain single copy integrations of the wildtype or mutated promoter region of *HXT5* fused to *LacZ*. The strains RKY01-RKY08 were inoculated on YNB containing 2% glucose and grown for 24 hours in shake flasks. Samples for β-galactosidase determination were taken every 3 hours. Symbols indicate integrated *HXT5*-promoter *LacZ* constructs containing either wildtype (■) or mutations in the putative STRE1 (□), STRE2 (◆), STRE1/2(✳), PDS (◇), HAP1 (▲), HAP2 (△) and HAP1/2 (●) elements.

Expression of *HXT5*-promoter *LacZ* fusion constructs during growth at increased temperature or osmolarity

It was shown that expression of *HXT5* was induced upon a decrease in the growth rate of cells by increasing the temperature or the osmolarity of the growth medium (6). Earlier studies indicated that STREs were absolutely required for induction of expression upon an increase in the temperature or osmolarity for many genes, for example for *GSY2* (30) and *GPH1* (31). In order to determine whether the STREs were also required for regulation of *HXT5* expression during increased temperature or

osmolarity, RKY01-RKY04 cells were grown at 30ºC to the exponential phase of cells growth. Subsequently, the cells were shifted to 37ºC or to medium containing 0.5M NaCl for 90 minutes, and samples were taken at the indicated time-points. During growth at 37ºC (Figure 3a) and 0.5M NaCl (Figure 3b), β-galactosidase activity of the wildtype and mutated STRE2 *HXT5*-promoter *LacZ* constructs increased, whereas no expression of the mutated STRE1 and STRE1/2 *LacZ* constructs was observed. These results indicated that the STRE1 element is important in regulation of *HXT5* expression when cells the growth rate is decreased at conditions of increased temperature or osmolarity.

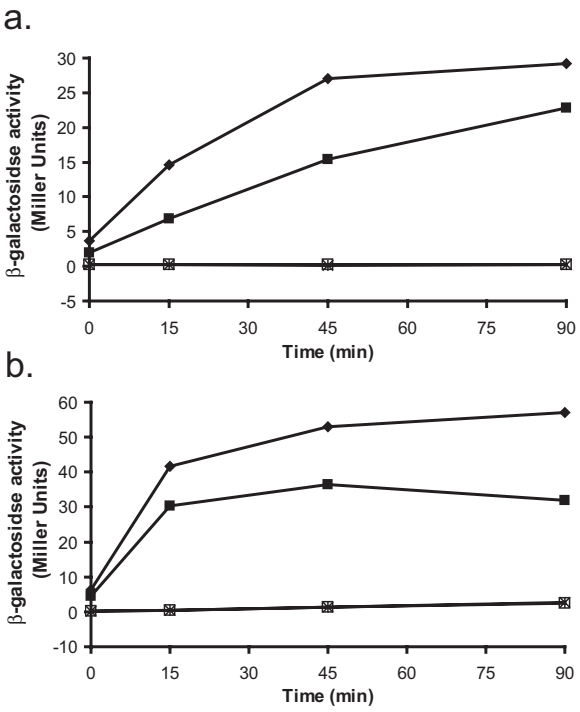

Figure 3: Expression pattern of *HXT5*-promoter *LacZ* fusion constructs containing wildtype or mutated STRE1, STRE2 or STRE1/2 upon increase of temperature or osmolarity of the growth medium. The yeast strains RKY01-RKY04 were grown at 30ºC on YNB 2% glucose in batch cultures until the exponential phase. Then, the temperature of the medium was increased to 37ºC or osmolarity was increased to 0.5M NaCl by adding concentrated NaCl. At the indicated time points culture samples were taken for β-galactosidase of the culture grown at increased temperature (a) or osmolarity (b). Symbols indicate integrated *HXT5*-promoter *LacZ* constructs containing either wildtype (■) or mutations in STRE1 (□), STRE2 (◆), STRE1/2(∗).

Expression of *HXT5*-promoter *LacZ* fusion constructs during batch growth on ethanol or glycerol

Growth on the non-fermentable carbon sources ethanol and glycerol results in a substantial decrease in the growth rate of cells, and thereby in induction of *HXT5* expression (6). To determine whether any of the putative regulatory elements identified in the promoter of *HXT5* are involved in the regulation of *HXT5* expression during growth on ethanol or glycerol, the RKY01-RKY08 strains were grown for 24 hours on YNB medium containing these non-fermentable carbon sources. All strains had reached similar optical densities and therefore had similar growth rates after 24 hours of growth (data not shown). β-galactosidase activity was always highest in strains containing the wildtype *HXT5*-promoter *LacZ* constructs in cells grown for 24 hours on YNB 2% ethanol or glycerol medium. Therefore, β-galactosidase activity of the mutated *HXT5*-promoter *LacZ* constructs is indicated as the percentage of expression of the wildtype construct (Figure 4). During growth on ethanol, strains containing the mutated HAP1 or HAP2 *HXT5*-promoter *LacZ* constructs had a 2-fold decrease in β-galactosidase activity compared to the wildtype *HXT5*-promoter *LacZ* construct, whereas β-galactosidase activity was even decreased 3-fold when STRE2 was mutated. β-galactosidase activity was again completely inhibited in strains containing mutated STRE1 and STRE1/2 *HXT5*-promoter *LacZ* constructs. Strikingly, mutation of the PDS element and both of the HAP elements together resulted 5-fold decrease in β-galactosidase activity (Figure 4a). These results suggest that the PDS and the HAP elements play an additional role next to STRE1 in the regulation of *HXT5* expression during growth on ethanol.

Growing the transformed strains for 24 hours on medium containing YNB 2% glycerol revealed that β-galactosidase activity was similar for the wildtype, the mutated STRE2 and HAP2 constructs (Figure 4b), indicating no involvement of these elements during growth on glycerol. β-galactosidase activity was diminished to about two-fold in the mutated HAP1 and PDS *HXT5*-promoter *LacZ* strains. Expression was again completely inhibited in the strains containing mutations in the STRE1 element, and a 4-fold decrease in β-galactosidase activity compared to the wildtype construct was observed when both HAP elements were mutated (Figure 4b). Furthermore, the loss of one HAP element can be complemented by the remaining HAP element, as the decrease in β-galactosidase activity was only severe when both HAP elements were mutated. Therefore, these results indicate that during batch growth on the non-fermentable carbon sources ethanol and glycerol the STRE1 element again plays a crucial role in the

regulation of *HXT5* expression, whereas the HAP elements again seem to serve an additional role. Furthermore, the experiments revealed a role for the PDS element and possibly STRE2 specifically during batch growth on ethanol.

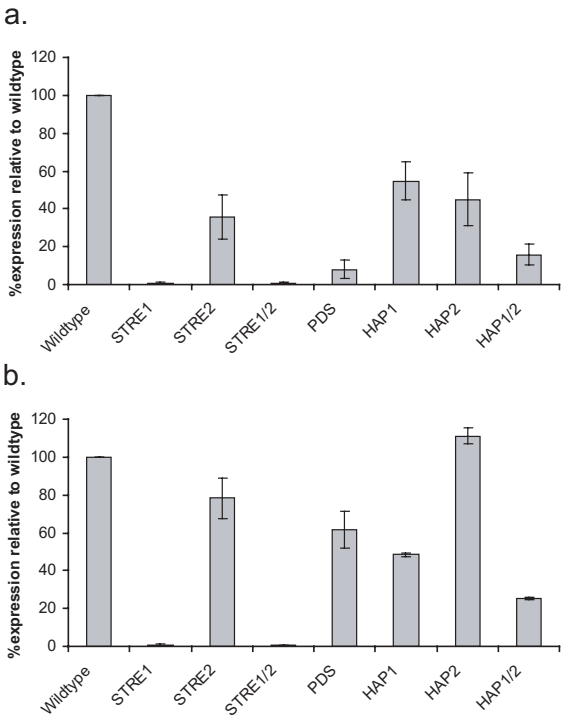

Figure 4: Effect of mutations in the putative regulatory elements on the expression *HXT5* after 24 hr of batch growth on YNB medium containing non-fermentable carbon sources. RKY01-RKY08 strains were grown overnight on YNB 2% glucose, washed and inoculated at an OD600 of 0.25 on YNB medium containing either 2% ethanol or 2% glycerol. After 24 hr of growth, samples were taken for β-galactosidase assay for growth on ethanol (a) or glycerol (b). The experiments were performed four times and the mean β-galactosidase activity, indicated as percentage expression of wildtype, of the results is indicated. Error bars represent standard deviation (n=4).

Expression of *HXT5*-promoter *LacZ* in a nitrogen-limited continuous culture

Our earlier studies indicated that expression of *HXT5* was specifically induced at dilution rates of 0.10 h^{-1} and lower during growth in nitrogen-limited continuous cultures (6). β-galactosidase activity of the wildtype and mutated *HXT5*-promoter *LacZ* constructs was determined during a fixed growth rate of 0.07 and 0.17 h^{-1} in a nitrogen-limited continuous culture. Similar to expression of *HXT5* mRNA, the wildtype *HXT5*-promoter *LacZ* construct was highly expressed at a dilution rate of 0.07 (Figure 5a), and severely decreased at a dilution rate of 0.17 h^{-1} (Figure 5b). β-galactosidase activity was about 2-fold decreased in the strains transformed with the mutated PDS and STRE2

construct at a dilution rate of 0.07 h^{-1}, indicating that the PDS and STRE2 element could play a role in regulation of *HXT5* expression during low growth rates. Mutation of the HAP1 element resulted in a slight decrease in β-galactosidase activity compared to the wildtype promoter, whereas β-galactosidase activity was more decreased in the strain containing a mutated HAP2 *HXT5*-promoter *LacZ* construct, when grown at a dilution rate of 0.07 h^{-1} (Figure 5a). Mutation of both HAP elements resulted in a 3-fold decrease in β-galactosidase activity compared to the wildtype promoter at a dilution rate of 0.07 h^{-1} (Figure 5a), which was also observed during batch growth experiments on glucose, ethanol and glycerol. β-galactosidase activity was inhibited in all transformed strains when the growth rate was increased to 0.17 h^{-1} (Figure 5b).

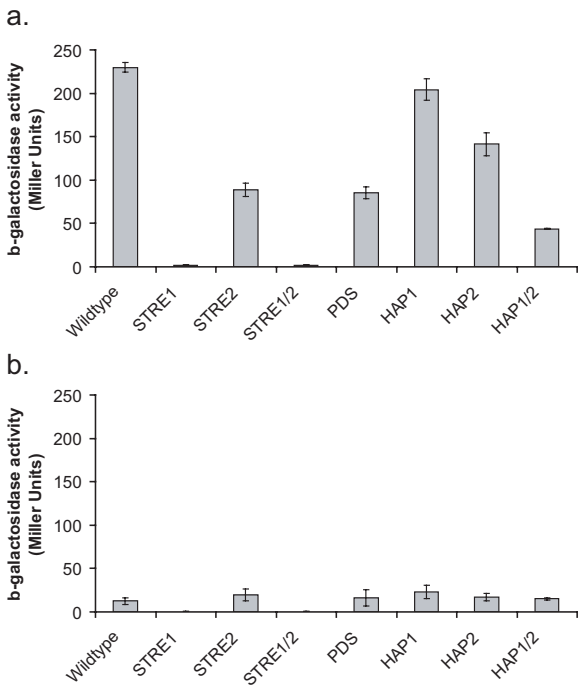

Figure 5: Contribution of the putative regulatory elements on the expression *HXT5* as measured by β–galactosidase activity of a *HXT5*-promoter *LacZ* construct during low or high growth rates in a nitrogen-limited continuous culture. Strains RKY01-RKY08 were grown specifically at low growth rates of D=0.07 h^{-1} or high growth rates of D=0.17 h^{-1}. Results shown are the mean of six β-galactosidase assays of two independent steady-states at the two different dilution rates. Error bars represent the standard deviation (n=4).

Once again, expression was virtually absent at a dilution rate of 0.07 h^{-1} in the strains transformed containing a mutation in STRE1, indicating that the STRE1 element in the *HXT5* promoter is extremely important in the regulation of *HXT5* expression during low growth rates. Finally, these results indicate that the regulation of *HXT5*

expression by the growth rate of cells is in particular determined by the STRE1 element in the promoter of *HXT5*, and to some extent by the HAP elements.

Discussion

In this study we have investigated whether putative regulatory elements in the promoter of *HXT5* are involved in the regulation of *HXT5* expression, whose expression is determined by the growth rate of cells (6). Computer-assisted analysis revealed the presence of two STREs, one PDS element and two Hap2/3/4/5p complex binding sites. These putative regulatory elements were mutated by site-directed mutagenesis, fused to *LacZ*, and transformed into yeast to determine the contribution of these elements in the growth rate-regulated expression of *HXT5*. The results from the batch culture experiments suggest that the STRE1 element is involved in induction of *HXT5* expression upon a decrease in the growth rate of cells, because β-galactosidase activity was not induced upon a decrease in the growth rate in the strain containing the *ΔSTRE1-HXT5*-promoter *LacZ* fusion construct. Furthermore, during other conditions used in this study to decrease the growth rates of cells and expression of *HXT5* (6), β-galactosidase activity was completely absent when the STRE located most proximal to the translation initiation site (STRE1) was mutated in the *HXT5*-promoter *LacZ* fusion construct. These results indicate that the STRE1 element plays an important role in growth rate-regulated *HXT5* expression. In addition, the HAP elements also play an important role in regulation of *HXT5* expression. In batch cultures, β-galactosidase activity in the HAP1/2 mutant was not further increased upon glucose depletion. In the next period of growth, cells may utilize ethanol that was accumulated as a result of fermentation of the available glucose (data not shown). It was shown previously that *HXT5* mRNA expression was fully induced when glucose was depleted from the medium (6). The results of the present study suggest that this full induction is mediated by the HAP elements present in the *HXT5* promoter. Furthermore, the HAP elements are also involved in regulation of *HXT5* expression during batch growth for 24 hours on the non-fermentable carbon sources ethanol and glycerol and during low growth rates in continuous cultures, when ethanol was produced (data not shown). Apparently, the HAP elements are involved in regulation of *HXT5* expression during growth on non-fermentable carbon sources. Mutation of the HAP1 and HAP2 elements individually

also influenced expression β-galactosidase activity to some extent, but never showed the substantial decrease in β-galactosidase activity when both HAP elements were mutated. These results suggest that, when one HAP element is mutated, the remaining one can take over the other's function. β-galactosidase activity of the mutated STRE2 and PDS element was decreased approximately 2-fold in the continuous culture experiments and more substantial after growth for 24 hours on ethanol, especially for the PDS mutation. This increase in β-galactosidase activity was not observed during batch growth on glucose and during growth on glycerol. Thus, the PDS and STRE2 element could be specifically involved in regulation of *HXT5* expression when ethanol is present in the medium. In conclusion, expression of *HXT5* is mediated by the STRE1 and both HAP elements in the promoter of *HXT5*. STRE1 seems to be involved in the induction of *HXT5* expression during conditions that decrease the growth rate of cells. The HAP elements seem required for maximal induction of *HXT5* expression when glucose is depleted from the medium, or during growth on non-fermentable carbon sources, as well as the PDS element during growth on ethanol.

Because the regulatory elements involved in *HXT5* expression are now identified, a prediction can be made about the transcription factors that bind to the regulatory elements in the *HXT5* promoter. STREs are bound and activated by the transcription factors Msn2p and Msn4p (32,33), which may bind to STRE1 in the promoter of *HXT5* and could therefore be the most important transcription factors involved in regulation of *HXT5* expression. The Hap2/3/4/5p complex binds to HAP elements (22) and could therefore bind to the HAP elements in the promoter of *HXT5* during growth on non-fermentable carbon sources. Activity of the transcription factor Gis1p that binds to PDS elements (34,35), might be specifically regulated by low growth rates during growth on ethanol.

A major question that remains, concerns the regulation of *HXT5* expression by the growth rate. The observation that the STRE1 element is extremely important in the regulation of *HXT5* expression suggests that pathways upstream of Msn2/4p could be involved. In this respect, the availability of cyclic AMP (cAMP) may play a prominent role. When extracellular glucose concentrations decrease below a certain level, resulting in decreased growth rates of cells, the level of cAMP decreases (36). This decrease results in down-regulation of the cAMP-regulated cAMP protein kinase A (PKA) signalling pathway. This pathway is involved in many cellular processes, including

nutrient sensing, carbohydrate storage and stress responses (18,37,38). PKA activity may also control the growth rate, mediated by regulation of translation of the G_1 cyclin *CLN3*, as cAMP increases Cln3 protein levels (39). The PKA pathway has a negative effect on the induction of a large number of genes at the diauxic transition of growth, for instance *CTT1*, *DDR1*, *HSP12*, *TPS2* and *GSY2* (30,40,41). Most of the genes that are induced during the diauxic or just prior to the diauxic shift posses one or more STREs in their promoter region (17,18,19,20). The activity of the PKA pathway controls the localization of Msn2/4p. Low cAMP levels and hence low activity of the PKA pathway results in nuclear localization of Msn2/4p, which could subsequently lead to activation of STRE-driven gene expression (42). Therefore, our results suggest that the PKA pathway could regulate expression of *HXT5* by acting on STRE1 via Msn2/4p. Furthermore, the PKA pathway also regulates expression of genes containing PDS elements, for example *SSA3* (34). Activity of Gis1p, the transcription factor thought to mediate regulation of PDS expression, is under regulation of Rim15p, whose activity is negatively regulated by the PKA pathway (34,43). These observations might explain the decrease in β-galactosidase activity upon mutation of the PDS element in the *HXT5*-promoter *LacZ* construct during growth on ethanol. Finally, the HAP elements are also important factors involved in regulation of *HXT5* expression. Activity of the Hap2/3/4/5p complex, which probably binds to the HAP elements in the *HXT5* promoter, is dependent on the Hap4p subunit, whose transcription is repressed by glucose (11,44,45). Thus, the HAP elements may also contribute to *HXT5* expression specifically when glucose is absent, i.e. upon depletion of glucose during batch growth, and during growth on non-fermentable carbon sources. Indeed, *HXT5* was induced to maximal extent when glucose was depleted from the medium in batch growth cultures, although expression was already induced earlier upon a decrease in growth rate of cells when glucose was still present (6). This growth rate-regulated induction might therefore be under control of a transcription factor that binds to STRE1, whereas the full activation of *HXT5* expression might be under the control of the Hap2/3/4/5p binding complex that binds to the HAP elements in the *HXT5* promoter.

In conclusion, our data show that mutation of one of the STREs present in the promoter of *HXT5* completely abolishes expression of a *HXT5*-promoter *LacZ* construct during various conditions that result in a decrease in the growth rate of cells. These conditions might be associated with decreased activity of the PKA pathway. In that way, expression of *HXT5* could be under control of the PKA pathway, which represses

expression of genes containing STREs in their promoter during optimal growth conditions. Therefore, we suggest that the PKA pathway may be involved in the regulation the growth rate, and in regulation of expression of genes, including *HXT5*, that are induced upon a decrease in the growth rate. Furthermore, the Hap2/3/4/5p complex is involved in regulation of *HXT5* expression during conditions of glucose depletion, and growth on non-fermentable carbon sources, whereas the PDS element could have a regulatory role in *HXT5* expression during growth on ethanol.

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Chapter 4

The role of Hxt5p in trehalose accumulation in *Saccharomyces cerevisiae*

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Abstract

The reserve carbohydrate trehalose accumulates under conditions when cells grow with a low growth rate, which also results in expression of the hexose transporter *HXT5*. In the present study, the role of Hxt5p in trehalose accumulation was determined. During various growth conditions, including batch growth, fed-batch growth and low growth rates in a nitrogen-limited continuous culture, *HXT5* was expressed concomitantly with trehalose accumulation. An important protein involved in trehalose accumulation is Tps1p, which is believed to interact with hexose transporters to control glucose influx. During batch growth on glucose, *HXT5* was the only hexose transporter that remained expressed upon glucose depletion when trehalose accumulation still continued and *TPS1* remained expressed. By using a *ras2* mutant, it was demonstrated that expression of *HXT5* and *TPS1* is regulated by a common regulatory pathway, being the cAMP/PKA pathway. These observations suggest that Hxt5p plays a role in trehalose accumulation. However, trehalose accumulation was affected, but not completely abolished, in a strain deleted for *hxt5*. Furthermore, no direct physical interaction between Hxt5p and Tps1p was determined. These finding suggest an indirect interaction, which was supported by the observation that Hxt5p was phosphorylated under conditions when trehalose accumulates. The possible involvement of the protein kinase Kns1p in this process is discussed.

Introduction

In *Saccharomyces cerevisiae*, hexose transporter (Hxt) proteins mediate transport of glucose across the plasma membrane (1, 2, 3, 4). The Hxt proteins are encoded by a multigene family consisting of 20 members. It was shown that *HXT1-4* and *HXT6-7* encode the major Hxt proteins (5), whose expression is regulated by extracellular glucose concentrations (4). Hxt5p, which was thought not to contribute to glucose transport, was however found to be able to transport glucose and enabled cells to grow, albeit slowly, when expressed solely in cells deleted for the major *HXT* genes (6, 7). Moreover, expression of *HXT5* was not determined by the extracellular glucose concentration, but by the growth rate of cells (8). Hxt5p is also structurally different as

compared to the major hexose transporters, as it contains a longer cytoplasmic localized N-terminal domain (Swiss-Prot, http://www.expasy.org/sprot/).

When glucose is inside the cell, it will be immediately phosphorylated by hexokinase into glucose-6-phosphate (G-6-P). Subsequently, G-6-P enters glycolysis during conditions when growth is favourable (9), or is used for formation of the reserve carbohydrates trehalose and glycogen when growth conditions are unfavourable or when glucose becomes scarce (10, 11, 12). Trehalose production is mediated by a protein complex consisting of Tps1p, Tps2p, Tps3p and Tsl1p (13, 14). The *TPS1* gene encodes trehalose-6-phosphate synthase, which converts UDP-glucose and glucose-6-phosphate into trehalose-6-phosphate (13,15). Trehalose-6-phosphate phosphatase (Tps2p) mediates the conversion of trehalose-6-phospate into trehalose and free phosphate (16,17). Tsl1p and Tps3p are regulatory components that probably function to stabilize the complex (13,14,18). Tps1p can be present in the cell either in complex with Tps2p and Tps3p/Tsl1p (14,18) or as a monomeric form (13,14). Strains deleted for *tps1* are unable to grow on the fermentable carbon sources glucose and fructose (19), and do not accumulate trehalose (20). It is considered that Tps1p itself, or its product trehalose-6-phosphate, might restrict glucose influx into glycolysis (21). For instance, it was proposed that Tps1p could have an inhibitory effect on sugar influx during growth on glucose by interacting with hexose transporters and/or with hexokinase (13,14,21).

Since the precursor of trehalose is glucose, which is transported by hexose transporters, and the suggestion that glucose transports may interact with Tps1p, we suggested that the hexose transporters are specifically involved in the accumulation of trehalose. Our results show that during various growth conditions when trehalose accumulated, concomitantly the hexose transporter *HXT5* is expressed, and that expression of *HXT5* and *TPS1* is regulated by the cAMP/PKA pathway, which suggest that Hxt5p is involved in trehalose accumulation. However, trehalose accumulation was affected, but not completely abolished, in cells deleted for *hxt5* compared to wildtype cells after 24 hours of growth. In order to reveal the mechanism behind the involvement of Hxt5p in trehalose accumulation, we sought to determine whether Hxt5p and Tps1p interacted physically, which was, despite various efforts, not established. However, the observation that Hxt5p is phosphorylated *in vivo* suggests the involvement of a kinase and provides an indication about how Hxt5p may play a role in trehalose accumulation.

Materials and methods

Strains, media and growth conditions

The *Saccharomyces cerevisiae* strains used in this study are indicated in Table 1. Cells were grown at 30ºC at 180 rpm in a shaking incubator (New Brunswick Scientific) in 0.67% (w/v) yeast nitrogen base without amino acids (Difco) and 2% (w/v) of the carbon source as indicated in the text. Strains KT1112 and JF415 were grown in 1% (w/v) yeast extract, 2% (w/v) bacto-peptone and 1% (w/v) glucose medium in batch cultures.

Table 1: List of strains used.

Yeast strain	Genotype	Source
CEN.PK 113-7D	MAT**a** *SUC2 MAL2-8^c*	P. Kötter (Frankfurt, Germany)
MSY1	MAT**a** *SUC2 MAL2-8^c leu2-3,112 Δhxt5::LEU2*	J. Diderich (Amsterdam, the Netherlands)
KY98	MAT**a** *SUC2 MAL2-8^c HXT5::GFP*	J. Diderich (Amsterdam, the Netherlands)
JBY20	MAT**a**, *SUC2, MAL2-8^c, ura3, HXT5::HA*	J. Becker (Düsseldorf, Germany)
KT1112	MAT**a** *leu2 ura3 his3*	K. Tatchell (Shreveport, USA)
JF415	MAT**a** *leu2 ura3 ras2::HIS3*	Jean François (Toulouse, France)

Elutriation and subsequent fed-batch experiments using glucose as carbon source were performed as described (8,22). Continuous culture experiments were performed as described previously (8,23).

Northern blot analysis

Total RNA was isolated, separated on agarose gels and transferred to Hybond-N membrane (Amersham Pharmacia Biotech) as described earlier (8). Oligonucleotides directed against *HXT1-7* (24), *ACT1* (5'-TGTCTTGGTCTACCGACGATAGATGGG-AAG-3') and *TPS1* (5'-AATCTCTAGCCCAGGCCATCCGAACCACTT-3') were labelled as described (8). A probe against *U2* was obtained by PCR, using plasmid pRS313U2 as template and primers 5'-GTTTTGCAAGGAAAGG-3' and 5'-CGAGAGAAACATCAAGCG-3'. The probe was labelled with [α-^{32}P]dCTP using

prime-a-gene labelling system according to manufacturer's protocol (Promega, Madison,Wisconsin, USA). Hybridization and washing was performed as described earlier (8).

Determination of trehalose levels

The amount of accumulated trehalose was determined enzymatically as described previously (22).

Yeast 2-hybrid

DNA binding (pACT2, Clontech Laboratories, USA) and transactivating vector (pGBT8, Clontech Laboratories, USA) expressing either the N-terminal, cytoplasmic domain of Hxt5p (amino acids 1-82) or full-length Tps1p were constructed as follows: The N-terminal half of *HXT5* was amplified by PCR using the primers 5'-GGGGGGGATCCTGATGTCGGAACTTGAAAACGCT-3' and 5'-CCCCGAGCTC-ATCCGACTTCGATTTCTTCTC-3', full-length *TPS1* was amplified using the primers 5'-GGGGGGATCCTGATGACTACGGATAACGCTAAG -3' and 5'-CCCCGAGCT-CTCAAGTTTTTGGTGGCAGAGGA-3'. The products were *BamHI*/*SacI* ligated into either pACT2 or pGBT8. The plasmids were transformed into strain AH109 (Clontech Laboratories, USA), plated on selective agar plates containing 2% glucose and grown at 30ºC.

Co-immunoprecipitation of Hxt5p and Tps1p

KY98 and JBY20 cells were grown overnight on YNB 2% glucose medium until glucose depletion. Cells were washed twice with water and subsequently broken with 0.45 mm glass beads in a Bead Beater (Biospec Products Inc.) in Tris buffer (0.05 M Tris.HCl, 0.15 M NaCl, 0.01 M EDTA, pH 7.5) containing Complete protease inhibitors (Boehringer), the protease inhibitor benzamidine (1 mM) and the phosphatase inhibitor NaF (1 mM). After disruption, the cell debris was transferred into a 1.5 ml reaction tube and the same volume of Tris buffer containing 1% Trition X-100, protease and phosphatase inhibitors was added. 20 µl CL-4B Protein A sepharose (Pharmacia, 0.07 µg in 500 µl Tris buffer containing 0.5% Triton X-100), precoupled to monoclonal anti-GFP or anti-HA antibody (Roche, Indianapolis, USA) or polyclonal Tps1p antibody (Kindly donated by J. Thevelein, Leuven, Belgium) was added to the cell lysate and incubated overnight at 4ºC in a rotary incubator. After incubation, the sepharose beads were washed 4 times with Tris buffer containing 0.5% Trition X-100,

protease and phosphatase inhibitors, and 20 µl 1.2x sample buffer was added. Proteins were separated using electrophoresis and Western blot was performed to determine co-immunoprecipitation of Hxt5p and Tps1p using either an antibody directed against Tps1p when Hxt5p-HA or Hxt5p-GFP was immunoprecipitated or anti-GFP or anti-HA when Tps1p was immunoprecipitated. Conditions for Western blotting were described previously (8).

Immunoprecipitation of phosphorylated Hxt5p

KY98 cells were grown overnight on YNB 2% glucose medium in shake flasks until the exponential phase (OD_{600} 0.80) and beyond glucose depletion (OD_{600} 4.80). The absence or presence of Hxt5p-GFP was confirmed by fluorescence microscopy. Cells with an equivalent of 50 OD_{600} units were centrifuged and washed twice with sterile H_2O. Cells were resuspended in a 15 ml falcon tube in YNB medium without phosphate and 500 µCi [^{32}P] carrier-free orthophosphate in dilute HCl solution (Amersham Biosciences). 2% glucose (w/v) was added to the exponentially growing cells, and no glucose was added to the glucose-depleted cells. The exponential cells were incubated for 1 hour, and the glucose-depleted cells for 4 hours in a water bath at 30ºC. After incubation, cells were disrupted in 8 cycles of vortexing with glass beads in Tris buffer (0.05 M Tris.HCl, 0.15 M NaCl, 0.01 M EDTA, pH 7.5) containing Complete protease inhibitors (Boehringer), the protease inhibitor benzamidine and the phosphatase inhibitor NaF, and further treated as described above under co-immunoprecipitation. After overnight rotation, the beads were washed for 10 times with Tris buffer containing 0.5% Trition X-100, protease and phosphatase inhibitors. After boiling the samples, a quarter of the sample was used to check for correct immunoprecipitation by Western blotting using anti-GFP antibody (Roche, Indianapolis, USA). The remaining sample was used for electrophoresis. The gel was dried and autoradiography was performed.

Results

HXT expression patterns and trehalose accumulation correlate during various growth conditions

Expression of the major hexose transporters was analyzed to establish whether any of the hexose transporters was specifically expressed during trehalose accumulation. During batch growth on glucose, *HXT1*, *HXT3* and *HXT4* were expressed until 13 hours after inoculation, although *HXT3* and *HXT4* expression was more induced 9 hours after inoculation as compared to the first 8 hours of growth (Figure 1a). *HXT2* mRNA was present only at 1 hour after inoculation. *HXT7* expression was induced 9 hours after inoculation when the growth rate of the cells decreased, but expression was repressed 15 hours after inoculation upon glucose depletion (data not shown). *HXT5* expression was also induced upon a decrease in the growth rate of cells 9 hours after inoculation, but was maximally induced 15 hours after inoculation, when *HXT7* expression was repressed (Figure 1a). Expression of the major hexose transporter *HXT6* was not observed. *TPS1* expression was induced 5 hours after inoculation and expression was maximally induced between 9 and 14 hours after inoculation. *TPS1* remained expressed until 18 hours after inoculation, when none of the major hexose transporters, except *HXT5*, were expressed (Figure 1a).

It is well known that during batch growth, yeast cells accumulate the reserve carbohydrate trehalose (10,11). Trehalose accumulation during batch growth on glucose was measured in the same samples used to determine *HXT1-7* expression. It was assumed that if one of these transporters was involved in trehalose accumulation, both accumulation and expression of the hexose transporter should occur at the same time. During batch growth on glucose, *HXT5* expression specifically correlated with the accumulation pattern of trehalose (Figure 1b). *HXT1*, *HXT3*, *HXT4* and *HXT7* expression correlated only partially with trehalose accumulation; *HXT1*, *HXT3* and *HXT4* expression was repressed 13 hours after inoculation when trehalose accumulation still continued, whereas *HXT7* expression was absent 15 hours after inoculation. After this time point, *TPS1* was still expressed, trehalose was still accumulated, and *HXT5* was the only hexose transporter that was expressed (Figure 1b).

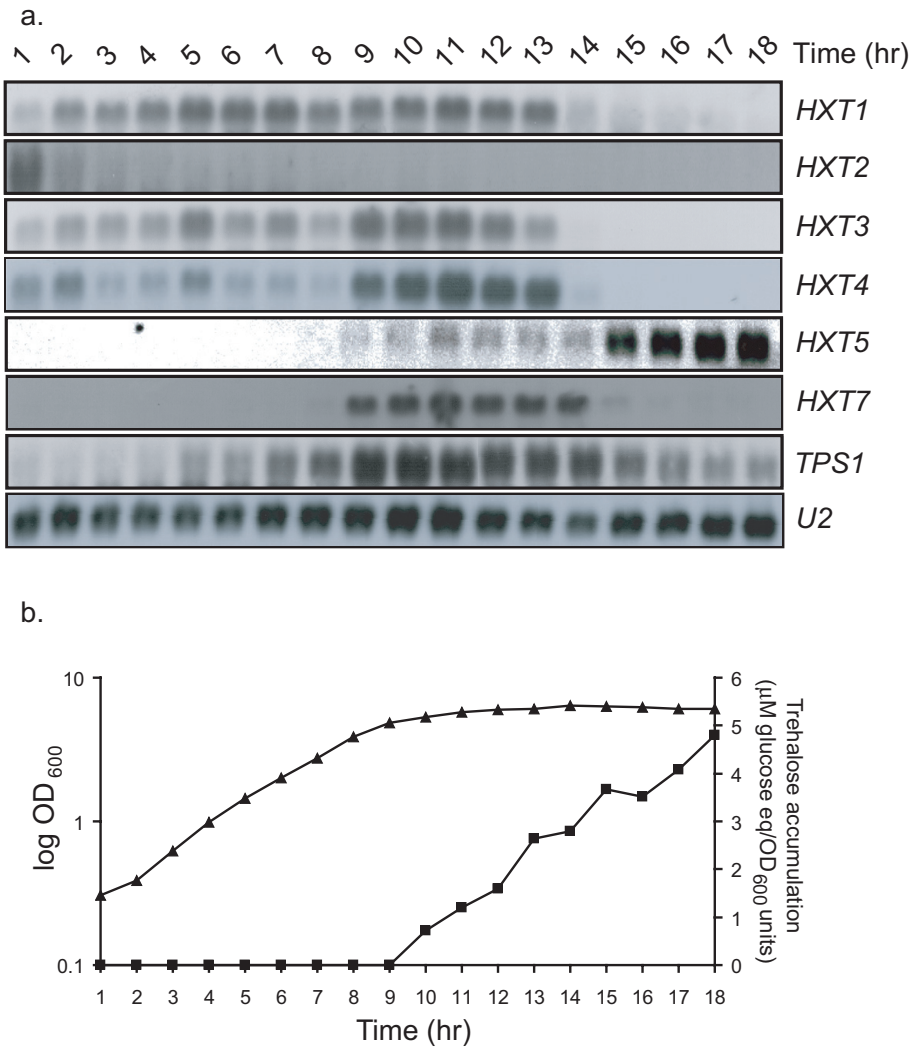

Figure 1: Expression of *HXT1,2,3,4,5,7* and *TPS1* during bath growth on glucose and accumulation of trehalose. Wildtype strain CEN.PK 113-7D was grown on YNB 2% glucose in batch cultures. At the indicated time points after inoculation cells were harvested for RNA isolation and trehalose determination. (a) Northern blot analysis of *HXT1,2,3,4,5,*7 and *TPS1* mRNA. *U*2 represents the loading control. (b) Optical density of the cells at 600 nm (▲) and the amount of accumulated trehalose (■).

In order to determine whether other growth conditions resulted in similar correlation between *HXT5* expression and trehalose accumulation, cells were grown in fed-batch cultures, using glucose as carbon source. *HXT5* expression was only observed when cells were grown at glucose consumption rates of 10 fmol $cell^{-1}.h^{-1}$, whereas no *HXT5* expression was observed during growth at 50 fmol $cell^{-1}.h^{-1}$ (Figure 2a). Trehalose was only accumulated when cells were grown at glucose consumption rates of 10 fmol $cell^{-1}.\ h^{-1}$, which again indicated a correlation between *HXT5* expression and trehalose accumulation (Figure 2b).

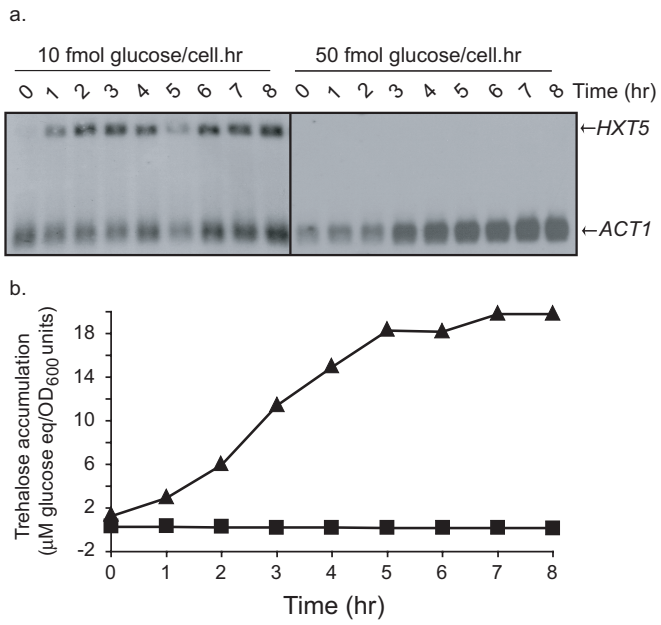

Figure 2: *HXT5* expression and trehalose accumulation during growth in fed-batch cultures. Wildtype strain CEN.PK 113-7D was grown in fed-batch cultures with glucose consumption rates of either 10 fmol $cell^{-1}.h^{-1}$ or 50 fmol $cell^{-1}.h^{-1}$. (a) Northern blot analysis of *HXT5* expression, including *ACT1* as loading control. (b) Trehalose accumulation during glucose consumption rates of 10 fmol $cell^{-1}.h^{-1}$ (▲) or 50 fmol $cell^{-1}.h^{-1}$ (■).

Finally, to show the relation between growth rates, *HXT5* expression and trehalose accumulation, cells were grown at different dilution rates in a nitrogen-limited continuous culture. *HXT5* expression and trehalose accumulation only occurred at dilution rates lower than 0.10 h^{-1}, whereas no *HXT5* expression or trehalose accumulation was observed when the dilution rate 0.13 h^{-1} or higher (Figure 3). The correlation between *HXT5* expression, *TPS1* expression and trehalose accumulation suggests that Hxt5p plays a role in accumulation of trehalose.

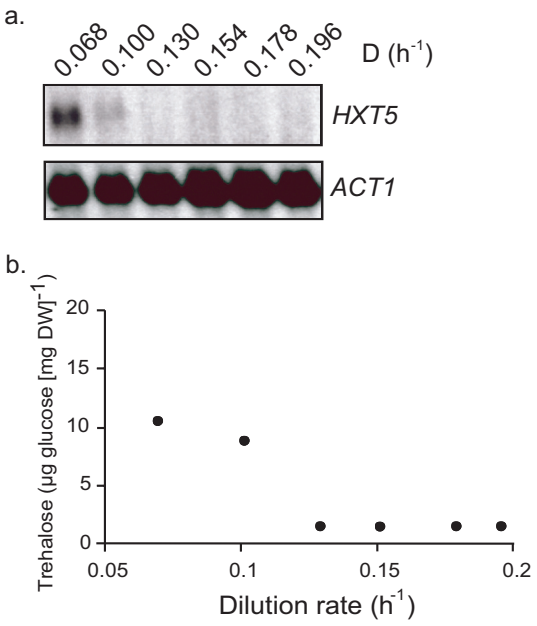

Figure 3: Expression of *HXT5* and trehalose accumulation during growth in a nitrogen-limited continuous culture. Wildtype strain CEN.PK 113-7D was grown in a nitrogen-limited continuous culture at various dilution rates. At the indicated dilution rates, cells were harvested, RNA was extracted and the amount of trehalose accumulation was determined. (a) Northern blot analysis of *HXT5* expression. *ACT1* is included as loading control. (b) Trehalose accumulation pattern (●).

Expression of *HXT5* and *TPS1* is regulated by a common regulatory pathway

If Hxt5p is involved in trehalose accumulation, it is expected that expression of *HXT5* and *TPS1* is regulated in a similar manner. It is known that the activity of the cAMP/PKA pathway is involved in regulation of trehalose accumulation. Inactivation of this pathway, for example by deletion of the *ras2* gene, results in hyperaccumulation of trehalose, even during conditions when normally no trehalose is accumulated (25,26). In order to establish whether *HXT5* and *TPS1* expression was influenced by the activity of the cAMP/PKA pathway, cells deleted for *ras2* and the corresponding wildtype strain were grown in batch cultures containing YP 1% glucose. In the wildtype strain KT1112, *HXT5* expression was induced after 7 hours of growth upon a decrease in the growth rate (Figure 4a/b), whereas increased expression of *TPS1* was observed earlier (Figure 4b). However, in the corresponding strain deleted for *ras2*, expression of both *HXT5* and *TPS1* was induced after 2 hours of growth, which was not observed in the wildtype strain (Figure 4b). After 4 hours, expression of *HXT5* and *TPS1* decreased in the *ras2* deletion mutant. These results suggest that a common regulatory pathway, being the cAMP/PKA pathway, regulates expression of *HXT5* and *TPS1*.

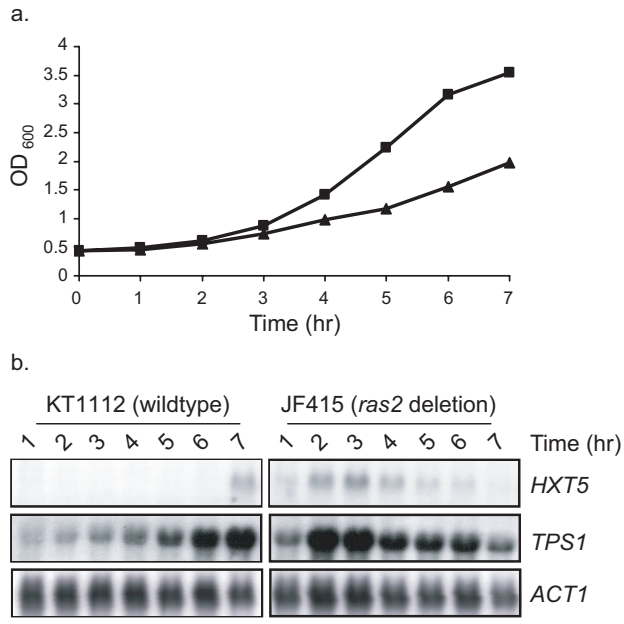

Figure 4: Expression of *HXT5* and *TPS1* in wildtype and *ras2* deletion strains during batch growth on YP 1% glucose. Wildtype strain KT1112 and strain JF415 deleted for *ras2* were grown in batch cultures containing YP 1% glucose. Samples were taken every hour to determine (a) OD600 of the KT1112 wildtype cells (■) and JF415 deleted for *ras2* (▲) and (b) expression of *HXT5* and *TPS1* as determined by Northern blot analysis. *ACT1* expression was used as loading control.

However, we have shown previously that expression of *HXT5* was determined by the growth rate of yeast cells (8). Thus the induction of *HXT5* in the *ras2* deletion mutant might not be the result of decreased activity of the cAMP/PKA pathway, but merely the result of the decrease in growth rate. Therefore, the wildtype strain and the strain deleted for *ras2* were grown in a nitrogen-limited continuous culture, which allows to determine whether decreased activity of the cAMP/PKA pathway and not a decrease in the growth rate is responsible for the induction of *HXT5* expression. *HXT5* expression in strain KT1112 was similar as observed for strain CEN.PK113-7D (Figure 3a), as *HXT5* was only expressed at low dilution rates of 0.07 h^{-1}, and not at dilution rates of 0.17 and 0.27 h^{-1}. In the *ras2* mutant, *HXT5* expression was higher at D=0.07 h^{-1} compared to wildtype levels. Furthermore, *HXT5* was still expressed at D=0.17 and 0.27 h^{-1} compared to *HXT5* expression in the wildtype strain, although expression was lower compared to D=0.07 h^{-1} in the *ras2* mutant (Figure 5). On the other hand, *TPS1* was expressed to some extent in the wildtype strain at D=0.07, 0.17 and 0.27 h^{-1}, but in the *ras2* deletion mutant *TPS1* expression was much more increased compared to the wildtype strain at these dilution rates (Figure 5). These results indicate that expression of *HXT5* and *TPS1* is regulated in a similar manner, being the cAMP/PKA pathway.

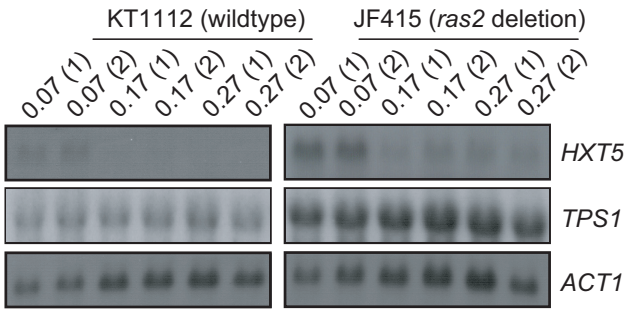

Figure 5: Expression of *HXT5* and *TPS1* in wildtype and *ras2* deletion strains during growth in a nitrogen-limited continuous culture. Wildtype strain KT1112 and strain JF415 deleted for *ras2* were grown in a nitrogen-limited continuous culture at dilution rates of 0.07, 0.17 and 0.27 h^{-1}. At each steady state, samples were taken *in duplo* to determine *HXT5* and *TPS1* expression, indicated as (1) and (2). *ACT1* expression was determined as loading control.

Trehalose accumulation is affected in a *hxt5* deletion mutant

In order to determine the involvement of Hxt5p in trehalose accumulation, a strain deleted for the *hxt5* gene was used to determine whether accumulation of trehalose was affected. Overnight-grown CEN.PK113-7D (Wildtype) and *hxt5* deletion (MSY1) strains were inoculated on YNB 2% glucose medium and grown in batch cultures. After 24 hours trehalose accumulation was measured, which revealed that the amount of trehalose accumulated in a strain deleted for *hxt5* was 80% of the amount of trehalose accumulated in the wildtype strain (Figure 6).

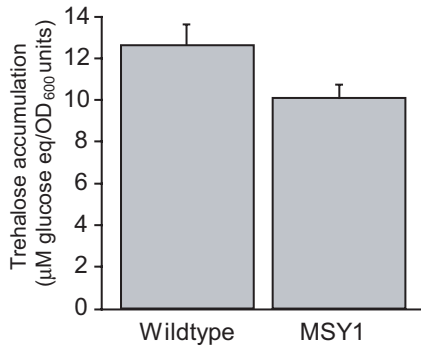

Figure 6: Accumulation of trehalose in a *hxt5* deletion strain. Strain MSY1, which is deleted for *hxt5*, and its isogenic wildtype strain CEN.PK 113-7D were inoculated at equal optical densities on YNB medium containing 2% glucose. After 24 hours, cells were harvested and trehalose accumulation was measured. (S.E.M., n=6, is indicated).

The observation that accumulation of trehalose is affected in the *hxt5* deletion strains suggests an involvement of Hxt5p in trehalose accumulation. However, trehalose is still accumulated in the *hxt5* deletion strain, which demonstrates that trehalose accumulation is not exclusively dependent on Hxt5p in the mutant strain. On the other hand, other hexose transporters may take over the specific function of Hxt5p, when *hxt5* is deleted.

No physical interaction between Hxt5p and Tps1p

It was suggested that Tps1p could be specifically involved in regulation of glucose influx into cells, probably by acting directly on the hexose transporters (21,27). Because our results indicate a correlation between *HXT5* expression and trehalose accumulation, similar regulation of *HXT5* and *TPS1* expression, and because trehalose accumulation is affected in a *hxt5* deletion strain, we hypothesized that Tps1p might specifically interact with Hxt5p, by binding to the extended cytoplasmic N-terminal domain of Hxt5p. Despite various efforts to demonstrate an interaction between Hxt5p and Tps1p using co-immunoprecipitation experiments and yeast 2-hybrid studies, no physical interaction between Hxt5p and Tps1p was found (data not shown). It is therefore likely to suggest that, if Hxt5p is involved in trehalose accumulation by interacting with Tps1p, this interaction is established not by direct but possibly by indirect interaction.

Hxt5p is phosphorylated during a condition that results in trehalose accumulation

It was reported recently that Hxt5p is part of a protein complex that associates with the serine/threonine protein phosphatase Sit4p (28,29), which suggests that Sit4p may dephosphorylate Hxt5p, and that Hxt5p may be phosphorylated during certain conditions. Therefore, cells containing GFP-tagged Hxt5p were grown into the exponential phase and overnight until glucose depletion, and labelled [^{32}P] orthophosphate was added to determine phosphorylation of immunoprecipitated Hxt5p-GFP. During exponential growth, a condition known not to express *HXT5* mRNA and protein (8), no Hxt5p-GFP was immunoprecipitated and no phosphorylated Hxt5p-GFP was detected (Figure 7). After overnight growth until depletion of glucose, a condition that results in expression of Hxt5p (8), Hxt5p-GFP was immunoprecipitated and phosphorylated (Figure 7). This result shows that Hxt5p can be phosphorylated *in vivo*.

Although the exact role of Hxt5p phosphorylation remains unknown, it might be important for a regulatory role of Hxt5p in trehalose accumulation.

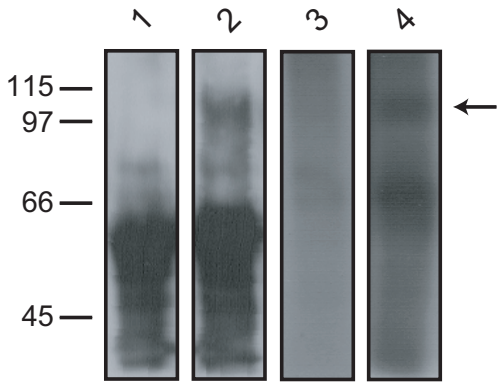

Figure 7: *In vivo* phosphorylation of Hxt5p. Strain KY98 was grown into the exponential phase or overnight until glucose depletion. [^{32}P] orthophosphate was added to the cells as described in materials and methods. After incubation, Hxt5p-GFP was immunoprecipitated and detected by Western blot (lane 1 and 2). Phosphorylated Hxt5-GFP was determined using autoradiography (lane 3 and 4). Immunoprecipitates of exponentially growing cells are indicated in lane 1 and 3, those of the glucose-depleted cells in lane 2 and 4. The protein size is indicated on the left (kDa). The arrow indicates the position of Hxt5p-GFP, which runs at the appropriate height (approximately 97 kDa).

Discussion

During batch growth on glucose, yeast cells accumulate the reserve carbohydrate trehalose when the amount of glucose in the medium becomes scarce (10,11), a condition that results in decreased growth rates of cells. On the other hand, trehalose is also accumulated during various other growth rate-decreasing conditions, for example growth at higher temperatures (30) or growth on ethanol (31). The relationship between the growth rate and trehalose accumulation was demonstrated recently (32).

Several observations in this study suggest that the hexose transporter Hxt5p plays a role in trehalose accumulation in *Saccharomyces cerevisiae*. Initially, a correlation was observed between trehalose accumulation and expression of the hexose

transporter *HXT5* during various growth conditions that all result in a decreased growth rate of cells. These conditions include growth on glucose in batch cultures, in fed-batch cultures and growth in nitrogen-limited continuous cultures. Other conditions, for example growth at elevated temperatures or growth on non-fermentable carbon sources, also resulted in expression of *HXT5* and accumulation of trehalose (data not shown). During batch growth on glucose, *HXT5* expression was induced when expression of *TPS1*, encoding a key protein involved in trehalose accumulation, was up-regulated. From this moment, trehalose was accumulated in the cells. Furthermore, when glucose was depleted from the growth medium, none of the major hexose transporters was expressed, whereas *HXT5* and *TPS1* were still expressed and trehalose accumulation still continued. This observation provides an indication that Hxt5p might play a role in trehalose accumulation.

We also provided evidence that expression of *HXT5* and *TPS1* is regulated by a common regulatory signal transduction pathway, being the cAMP/PKA pathway. It was shown that expression of *HXT5* was regulated by a Stress Responsive Element (STRE) in the *HXT5* promoter (Chapter 3). The promoter of *TPS1* contains 6 STREs, which may be important for regulation of *TPS1* expression (33). Furthermore, the activity of the cAMP/PKA pathway controls expression of STRE-controlled genes (34,35), which may control expression of both *HXT5* and *TPS1*. Indeed, increased expression of *HXT5* and *TPS1* was observed in mutant yeast strain with constitutive low activity of the cAMP/PKA pathway. During growth on glucose in batch cultures, however, increased expression of *HXT5* and *TPS1* was transient in the *ras2* deletion mutant, but were expressed in a similar pattern, suggesting co-regulated expression. During growth of the *ras2* deletion mutant in a nitrogen-limited continuous culture, *HXT5* remained expressed at D=0.17 and 0.27 h^{-1}, although expression was lower as compared to growth at D=0.07 h^{-1}. An explanation for this observation might be that full expression is dependent on activation of STREs but also HAP elements in the *HXT5* promoter (Chapter 3). Probably, at high dilution rates the HAP2/3/4/5 complex is not involved in regulation of expression and *HXT5* expression is not fully activated. Indeed, the level of *TPS1* expression was similar during growth at D=0.07, 0.17 and 0.27 h^{-1}, probably because the HAP2/3/4/5 complex is not involved in regulation of *TPS1* expression, because HAP elements are not present in the *TPS1* promoter**.** However, the observation that expression of *HXT5* and *TPS1* is regulated in a similar manner, strongly suggest

that their functions may be connected and that Hxt5p is involved in trehalose accumulation by cooperating with Tps1p.

The ultimate experiment to show that Hxt5p is indeed involved in trehalose accumulation is by determining the amount of trehalose accumulation in a strain deleted for *hxt5*. However, trehalose accumulation was only 20% decreased in a *hxt5* deletion strain compared to wildtype cells during 24 hours of batch growth on glucose. If only Hxt5p was involved in trehalose accumulation, it was expected that trehalose accumulation was completely abolished in the *hxt5* deletion strain. Thus, other factors or other hexose transporters probably also play a role in trehalose accumulation. Indeed, involvement of other hexose transporters in trehalose accumulation may be partially explained by the results of the batch growth experiments using glucose as carbon source. These experiments revealed that from that time-point after inoculation when trehalose accumulated, expression of *HXT5* was induced, but *HXT1*, *HXT3*, *HXT4* and *HXT7* were also expressed (Figure 1a). Because the available glucose, which is likely to be transported into the cells by these transporters, may also serve as precursor for trehalose, these other transporters may also contribute to trehalose accumulation. However, upon depletion of glucose, trehalose accumulation still continued and *HXT5* was the sole hexose transporter that was expressed. From this moment, cells consume accumulated ethanol that could serve as a precursor for trehalose (31) and Hxt5p might serve a regulatory role in trehalose accumulation from this moment, because Hxt5p does not seem to serve a direct role in ethanol uptake or metabolism.

It was proposed previously that Tps1p might regulate glucose influx into cells, probably by interaction with a hexose transporter (21,27). Because trehalose accumulation correlated with expression of *HXT5*, and *HXT5* expression was co-regulated with expression of *TPS1*, we suggested that Tps1p might specifically interact with Hxt5p to regulate glucose uptake and subsequently trehalose accumulation. Because Hxt5p contains a longer cytoplamic N-terminal domain compared to the major Hxt proteins, we speculated that this domain could form an interacting domain for Tps1p. However, direct interaction between the cytoplamic N-terminal domain of Hxt5p and Tps1p was not measured, as determined by yeast-2-hybrid studies. Also, no direct interaction was measured either between full-length Hxt5p and Tps1p as determined by co-immunoprecipitation. These results suggest that a possible role of Hxt5p in trehalose accumulation is probably not mediated through direct interaction between Hxt5p and Tps1p. However, large scale yeast 2-hybrid studies showed that Hxt5p interacts with the

product of *YLL020C* (36,37). *YLL020C* is part of the ORF encoded by *YLL019C* (38), which encodes the serine/threonine kinase Kns1p belonging to the LAMMER kinase family (39). Surprisingly, studies conducted to identify protein complexes by mass spectrometry revealed an interaction between Tps1p and Kns1p (29). Also, the expression profiles of *HXT5* and *KNS1* are similar as determined by DNA microarray studies during a variety of conditions that result in low growth rates of cells (40). Furthermore, it was observed in our study that Hxt5p-GFP is phosphorylated *in vivo* upon glucose depletion, a condition known to result in trehalose accumulation and *TPS1* expression. Thus, Kns1p might be the protein kinase responsible for Hxt5p-GFP phosphorylation. Kns1p might also phosphorylate Tps1p and in that way Kns1p could function as a docking protein to bring Tps1p and Hxt5p in each other's proximity. The observation that the same kinase may phosphorylate both Tps1p and Hxt5p, implies a possible importance of both proteins within the same process, being accumulation of trehalose.

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Chapter 5

Identification of genes that are expressed similar to *HXT5* upon an increase in G_1 phase duration

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Abstract

It is known that a decrease in the growth rate of cells results in expression of the hexose transporter *HXT5*. Here, we describe the analysis of a genome-wide expression array of *Saccharomyces cerevisiae* growing at a low growth rate of 19 fmol galactose $cell^{-1}.h^{-1}$ in fed-batch cultures to identify genes with similar transcription profiles as *HXT5*. This approach may provide indications about additional functions of Hxt5p besides glucose transport. Genes encoding proteins involved in trehalose accumulation were expressed similar to *HXT5*. This observation strengthens our hypothesis that Hxt5p plays a role in trehalose accumulation. Also genes encoding proteins involved in respiration, protein folding, stress responses, and genes encoding proteins involved in metabolism of carbon that were not present in the growth medium, were similarly expressed. The role of Hxt5p in any of these processes remains unclear. Results from this and other genome-wide analysis studies suggest that the genes that were expressed similar like *HXT5* are part of a group of genes whose expression is merely induced as a result of a decrease in the growth rate of cells. The promoter regions of many of these genes were enriched in so-called stress responsive elements (STREs), suggesting these elements are important in mediating induction of genes in response to decreased growth rates. Because it is known that the protein kinase A (PKA) pathway regulates expression of STRE-controlled genes, this pathway might be specifically involved in growth rate-regulated gene expression.

Introduction

During optimal growth conditions when essential nutrients are not limiting, cells grow with maximal growth rates and produce as much offspring as possible. When a nutrient source becomes limited, or when cells are exposed to stress conditions, cells change their cellular components and metabolism to adapt to these circumstances, and the growth rate of cells decreases. Conditions that result in decreased growth rates induce accumulation of the reserve carbohydrates trehalose and glycogen (1,2,3,4).

Accumulation of trehalose in particular might not be the result of a change in the environmental condition as such, but seems regulated by a general mechanism. It was observed for haploid cells that trehalose is only accumulated at low growth rates

(5). This was amongst others demonstrated by using continuous cultures, where trehalose was only accumulated when the growth rate was lower than 0.1 h^{-1}. Furthermore, in fed-batch cultures trehalose accumulation was only observed when the G_1 phase duration was longer than 160 minutes, and accumulation of trehalose increased with increasing G_1 phase duration (5). Similar regulation of trehalose accumulation was observed in diploid cells (2). A decrease in the growth rate of cells is the result of an increase in the G_1 phase duration of the cell cycle, because the duration of the S, G_2 and M phase remains nearly constant (6). Therefore, it was concluded that the G_1 phase duration, and hence the growth rate of cells, determines whether trehalose is accumulated in *Saccharomyces cerevisiae* (5)

On the other hand, regulation by the growth rate is not limited to accumulation of trehalose, but may also be a regulatory mechanism for expression of specific genes. We have shown previously that expression of the hexose transporter *HXT5* is regulated by the growth rate (7), and not by the extracellular glucose concentration, which regulates expression of other genes belonging to the hexose transporter family (8). Several observations suggest that product of the *HXT5* gene is possibly involved in accumulation of trehalose; the expression pattern of *HXT5* specifically correlates with trehalose accumulation during various growth conditions, and trehalose accumulation is affected in a *hxt5* deletion mutant (Chapter 4). It was suggested that trehalose synthase, encoded by *TPS1*, which is a protein involved in trehalose accumulation, interacts with a hexose transporter and a sugar kinase to control glucose influx, thereby forming the so-called "general glucose-sensing complex" (9,10). We reasoned that Hxt5p could be the specific hexose transporter in this complex and could interact with Tps1p. However, physical interaction between Hxt5p and Tps1p was not observed using co-immunoprecipitation and yeast two-hybrid studies. By identifying genes with expression patterns similar to *HXT5*, other members of the putative complex may be identified, because the proteins encoded by genes with similar expression patterns may be involved in similar process. A method to determine expression of virtually all genes present in the yeast genome is DNA microarray analysis (11,12).

The general procedure to perform genome-wide analysis is to compare two conditions to identify genes whose expression is specifically induced or repressed at a certain condition, for example growth at higher osmolarity versus growth at normal osmolarity (13,14,15). However, another selection providing much more insight in the cellular behavior during growth at a low growth rate is by using the *HXT5* expression

pattern as selection criterion for all expression data. This approach revealed some of the typical events that occur during growth at decreased growth rates, and provides information about a possible function of Hxt5p besides glucose transport. In the present study we show that 72 genes were similarly expressed as *HXT5*, which included genes encoding proteins involved in trehalose accumulation, including *TPS1*, *TPS2* and *TSL1*, suggesting again an involvement of Hxt5p in trehalose accumulation. Also, the hexokinase encoded by *GLK1* exhibited a similar pattern, which may be the specific sugar kinase component of the general glucose-sensing complex. On the other hand, also other genes exhibited expression pattern similar to expression of *HXT5*. These mainly include genes encoding proteins involved in the tricarboxylic acid (TCA) cycle, genes that respond to stress, genes encoding proteins involved in protein folding and genes encoding proteins that are required for growth on carbon sources, which were actually not present in the growth medium used to elongate the G_1 phase. The actual role of these genes with respect to Hxt5p or low growth rates of cells is discussed.

It is known that genes with similar expression patterns often have common regulatory elements in their promoter. It was observed that genes with expression patterns similar to *HXT5* were enriched in stress responsive elements (STREs) in their promoter region, suggesting that expression of genes that are expressed like *HXT5* is regulated in a similar manner during conditions that result in decreased growth rates of cells.

Materials and methods

Elutriation of cells

Wildtype strain CEN.PK 113-7D was used during this study. Cells were pre-grown in batch cultures on YNB medium (Difco) containing 1% galactose until the exponential phase of cell growth (OD 1.0). Synchronized cells were obtained as described earlier (7).

G_1 phase elongation

To elongate the G_1 phase duration, synchronized cells were grown in fed-batch cultures as described earlier (7), with some modifications. Cells were grown in YNB medium without amino acids with galactose as carbon source at a cell density of $1.2x10^7$ cells.ml^{-1}, and an initial extracellular galactose concentration of 0.15 mM.

Galactose dissolved in YNB medium was continuously added at rates of 19 fmol $cell^{-1}.h^{-1}$. Progression through the cell cycle was monitored by determining the percentage of budded cells. The end of the G_1 phase was indicated as the point, where 50% of the cells were budded. Samples were taken at 30, 90, 150 and 210 minutes after inoculation, immediately frozen in liquid nitrogen and stored at -80°C.

mRNA extraction and Northern blot analysis

The samples were defrosted on ice, and total RNA was isolated as using phenol/chloroform extraction as described earlier (7). Northern blot analysis was performed as described using *HXT5*- and *ACT1*-specific oligonucleotides (7).

^{33}P CTP-labelled cDNA synthesis

cDNA was produced and radioactively labeled using total mRNA as template; 4 µg RNA (measured at 260 nm) was mixed with 2 µl OligodT (1µg/µl) to a final volume of 10 µl. The following components were added: 6 µl first strand buffer (Life Technologies, Breda, The Netherlands), 1 µl 0.1 M dithiothreitol, 1.5 µl of a mixed solution containing 100 mM of dATP, dGTP and dTTP, 300 units Superscript II reverse transcriptase (Life Technologies) and 100 µCi [^{33}P]CTP (Amersham Biosciences, Roosendaal, The Netherlands). The mixture was kept at 37°C for 90 min and next 70 µl STE (0.1 M NaCl, 10 mM Tris.HCl, 1 mM EDTA, pH 8.0) was added. The newly synthesized cDNA was purified by passage through a Sephadex G-50 column (Amersham Biosciences), washed with 350 µl STE and finally eluted with 500 µl of STE. Prior to hybridization, the labeled cDNA was denatured by heating to 100°C for 3 min.

Filter hybridization

Yeast GeneFilter microarrays (ResGen, Invitrogen, Breda, The Netherlands) were washed for 5 min with boiling 0.5% SDS. The membranes were prehybridized for 4 h with 5 ml MicroHyb solution (ResGen) and 5 µl Oligo-dA (ResGen) at 42°C in a roller incubator (Thermo Hybaid, Landgraaf, The Netherlands). The labeled cDNA probes were added to the filters and incubated overnight at 42°C. The next day, the filters were rinsed twice with 2x SSC (diluted from 20x SSC which contains 3 M NaCl, and 0.3 M Na-citrate, pH 7.0) and 1% SDS for 20 minutes at 50°C. Subsequently, the filters were washed with 0.5x SSC, 1% SDS for 15 minutes at room temperature. The filters were then transferred to a humid 3-layer Whatman filter, wrapped in Saran foil

and placed against a Phosphor screen (Molecular Dynamics, Sunnyvale, CA, USA). After 144 hours of exposure the screen was scanned by a phosphorimager SI (Molecular Dynamics) coupled to a computer at 50 μm resolution using Image-Quant 5.1 (Molecular Dynamics). The samples were labeled and hybridized *in duplo* to different microarray filters.

Spot validation and data analysis

The intensities of the spots were monitored using ImaGene® 4.2 software (BioDiscovery, Marina del Rey, CA, USA). The signal mean value was subtracted by the background mean, and the resulting value was normalized against the total signal of an individual membrane on which the spot was located. Spots were flagged either automatically by the software package if the spot was negative, or of low or poor quality. Spots were flagged by hand if the normalized spot value was below 0.1.

On average of two independent microarray experiments, *HXT5* expression was induced 5.28-fold from 30 to 90 minutes after inoculation, decreased 1.84-fold from 90 to 150 minutes and was modestly induced with 1.28-fold from 150 to 210 minutes after inoculation. When these exact parameters were used to determine which genes were expressed like *HXT5*, no genes exhibited the same expression pattern. Therefore, the criteria for genes with expression patterns similar to *HXT5* were set less stringent. Genes were regarded as coinduced with *HXT5* when expression was, on average of two microarray hybridizations or in one independent microarray hybridization, 2-fold induced between 30 and 90 minutes, and 1.5-fold reduced between 90 and 150 minutes. Furthermore, expression should be at least 2-fold decreased or absent in synchronous cells grown at high growth rates on galactose (16). The data was analyzed using Microsoft Excel 2000.

Computer-assisted analysis of promoter regions

The promoter regions of induced genes were analyzed using RSA tools (17). To discover unknown patterns in the promoters of the induced genes, oligonucleotide analysis was used to reveal the presence of certain motifs. In order to search for the presence of known putative regulatory elements in the promoters of induced genes, upstream sequences were retrieved without prevention of overlap with upstream ORFs. In the promoter regions of the majority of genes demonstrated previously to be STRE controlled, the majority of STREs were present between -600 to -100 from the start

codon ATG (18). Therefore, in our queries only the sequences -600 to 100 upstream of the translation initiation site were included to determine presence of STREs, whereas the sequences -1000 to -1 upstream were used to determine the presence of HAP elements. As query patterns, CCCCT was used to determine the presence of STRE elements, and ACCAAYNA for HAP elements.

Results and discussion

The G_1 phase of the yeast cell cycle, and hence the growth rate of cells, can be regulated by growing cells in fed-batch cultures by controlled addition of low amounts of galactose (2,5). Synchronized cells were grown at 19 fmol galactose $cell^{-1}.h^{-1}$ to increase the G_1 phase duration to 210 minutes (Figure 1).

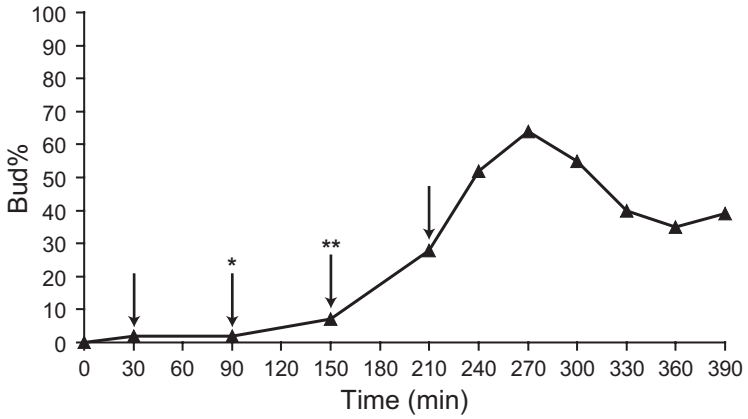

Figure 1: Elongation of G_1 phase duration. Synchronized cells were grown in fed-batch cultures upon addition of 19 fmol galactose $cell^{-1}.h^{-1}$ (▲) to elongate the G_1 phase duration to approximately 210 minutes. The G_1 phase duration was determined as the time point from inoculation where 50% of the cells were budded. Arrows indicate the time points at which samples for DNA microarray analysis were taken. The time points where *CLN1* and *CLN2* expression were maximally induced during growth at 1% galactose (*) and growth at 19 fmol galactose $cell^{-1}.h^{-1}$ (**) is indicated.

This is an increase in G_1 phase duration of approximately 90 minutes as compared to cells grown in the presence of 1% galactose, which have a G_1 phase duration of 120 minutes (5). The G_1 phase duration was determined as the time point from inoculation

where 50% of the cells were budded. Samples were taken early in the G_1 phase (30 and 90 minutes after inoculation), in mid-G_1 (150 minutes after inoculation) and at the end of the G_1 phase (210 minutes after inoculation).

It is known that *HXT5* is expressed when the growth rate of cells decreases (7). To confirm *HXT5* expression in our samples, and thereby also justifying that the growth rate of the cells was indeed decreased, expression of *HXT5* was initially determined by Northern blot analysis. This revealed that *HXT5* was indeed expressed, however not at constant levels (Figure 2a). At 30 minutes, expression was low, expression was induced at 90 minutes, and expression decreased and remained constant at 150 and 210 minutes after inoculation (Figure 2a).

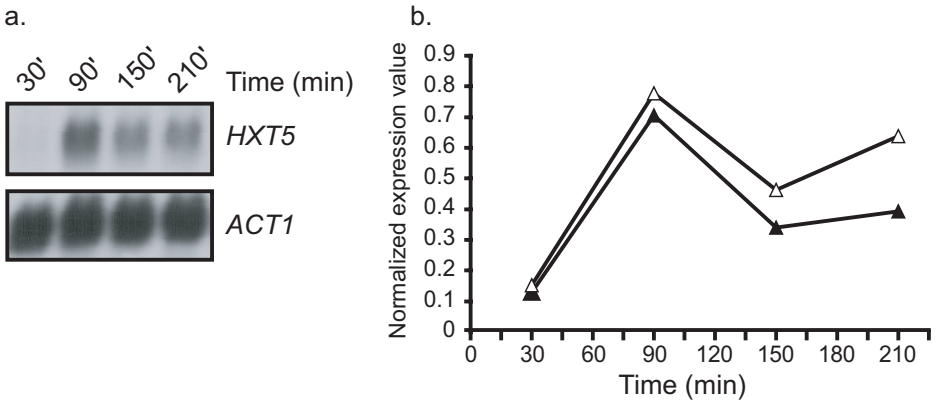

Figure 2: Expression of *HXT5* during growth at low growth rates as determined by Northern blot and DNA microarray analysis. Synchronized cells were grown in fed-batch cultures upon addition of 19 fmol galactose $cell^{-1}.h^{-1}$ to elongate the G_1 phase duration. (a) Cells were harvested at the indicated time points, mRNA was isolated and subjected to Northern blot analysis. Oligonucleotides that specifically detect *HXT5* or *ACT1* mRNA were used for hybridization. (b) Expression pattern of *HXT5* in duplicate DNA microarray experiments.

Subsequently, DNA microarray analysis was performed to identify genes that exhibited an expression profile similar to *HXT5*, which provides insight in the cellular behavior during growth at low growth rates, and may also provide information about additional functions of Hxt5p besides glucose transport. Next, the proper criteria were selected to identify genes that were expressed similar to *HXT5*. Genes whose expression

was 2-fold induced between 30 and 90 minutes after inoculation, and 1.5-fold decreased between 90 and 150 minutes after inoculation were initially identified. Furthermore, expression should also be induced 2-fold or more as compared to genome-wide expression data from synchronized cells grown at 1% galactose, which results in high growth rates of cells. This data set was available from another study and will be published separately (16). From a total of 6144 open reading frames (ORFs) analyzed, 72 genes showed an expression pattern that was similar to the expression pattern of *HXT5*. Increasing the 2-fold induction between 30 and 90 minutes after inoculation or the 1.5-fold reduction in expression between 90 and 150 minutes after inoculation always resulted in a similar set of genes (data not shown). This justified that the selection criteria applied were appropriate.

Expression of *HXT5* as determined by Northern blot and DNA microarray analysis was similar, and showed that the results of the DNA microarray experiments were indeed a reflection of expression at the mRNA level (Figure 2b). This was also concluded from the expression data of the cyclins *CLN1* and *CLN2*. It was shown previously that expression of *CLN1* and *CLN2* occurred just prior to bud emergence, and *CLN1* and *CLN2* expression was delayed in cells grown at low growth rates compared to cells grown at a higher growth rate (2). In our study, expression of *CLN1* and *CLN2* was induced at 150 minutes after inoculation of the cells (Figure 1). Synchronized cells grown at high growth rates already expressed *CLN1* and *CLN2* to maximal levels after 90 minutes (Figure 1), indicating that the growth rates of the cells used in this study were indeed decreased.

In order to categorize the identified genes, over-represented gene groups encoding proteins involved in common biological processes were found by using the SGD Gene Ontology Term Finder and applying a cut-off of $p<0.01$. This analysis revealed that most of the co-induced genes have functions in carbohydrate metabolism, protein folding, response to stress, energy pathways, coenzyme metabolism, lipid metabolism, and a large group of proteins with unknown biological functions (Table 1). The functions of these proteins will be described below.

Table 1: Gene groups with common biological processes found to be over-represented ($p<0.01$) according to the SGD Gene Ontology Term Finder (http://db.yeastgenome.org/cgi-bin/SGD/GO/goTermFinder) among the transcripts that were expressed similar to *HXT5*. Some genes may be present in several groups. Only over-represented main- and subgroups are shown.

Common biological process	Co-regulated genes
Carbohydrate metabolism Tricarboxylic acid cycle	*ATH1 CYB2 FUM1 GLK1 GRE3 GUT2 JEN1 KGD2 LSC1 LSC2 MDH1 PGM2 SDH2 TKL2 TPS1 TPS2 TSL1*
Response to stress	*AHA1 ATH1 DDR2 GRE3 GRX1 GTT1 HSP104 HSP26 HSP42 HSP78 TPS1 TPS2 TSL1 YGP1 YJL144W*
Protein folding	*AHA1 HSP104 HSP26 HSP78 MPD2 SBA1 SSE2*
Energy pathways Energy derivation by oxidation of organic compounds	*ADH3 ATH1 FUM1 ISF1 KGD2 LSC1 LSC2 MDH1 SDH2 TKL2 TSL1*
Coenzyme metabolism	*COQ4 GUT2 LSC1 LSC2 PNC1 TKL2*
Lipid metabolism Fatty acid oxidation	*FAA1 POX1 TES1*
Biological process unknown	24 ORFs (not shown)

The identified genes may be roughly divided into four categories: I) Genes encoding proteins that are involved in trehalose metabolism. II) Genes that encode proteins that are known to be required for growth at low growth rates. III) Genes that encode proteins involved in protein folding, which is part of a larger group of genes known to respond to stress. IV) Genes encoding proteins that are involved in metabolism of carbon sources, which were not present in the growth medium.

From the first group, four genes with expression patterns similar like *HXT5* encode proteins involved in trehalose metabolism, a process in which Hxt5p may be involved (Chapter 4). These include *TPS1*, encoding trehalose phosphate synthase (19,20), *TPS2* encodes trehalose-6-phosphate phosphatase (21,22), *TSL1* encodes the regulatory subunit of the trehalose phosphate synthase complex (23) and *ATH1* encodes acidic trehalase, which is involved in trehalose degradation (24). Indeed, it was observed previously that an increase in G_1 phase duration results in accumulation of trehalose during growth at 19 fmol galactose $cell^{-1}.h^{-1}$ (5) and similar amounts of trehalose were measured in the cells used for the DNA microarray experiments (data not shown). Expression of *PGM2*, encoding phosphoglucomutase was also induced. This protein converts glucose-6-phosphate to glucose-1-phosphate, which is an intermediate

required for trehalose production (25). It is described that Tps1p might regulate sugar influx in conjunction with a hexose transporter and a sugar kinase, to form the "general glucose-sensing complex" (9,10). Glucokinase, encoded by *GLK1* (26), was also expressed similar to *HXT5*. Therefore, Glk1p and Hxt5p seem logical candidates to form the components of this complex during conditions of slow growth. Our results indicate that expression of *HXT5* and *TPS1* is similar during low growth rates on galactose, a carbon source that is not transported by Hxt5p (27). Expression of *HXT5*, *TPS1* and *GLK1* was also induced after 30 minutes of ethanol stress (28), a condition that also results in accumulation of trehalose (29). Hxt5p and Glk1p do not seem have to clear functions in the metabolism of ethanol. Thus, the putative complex consisting of Hxt5p, Tps1p and Glk1p might have a regulatory role in accumulation of trehalose, independent of the carbon source present in the growth medium.

It is known that cells grow completely respiratory during sugar limitation in fed-batch cultures and low growth rates in continuous cultures (30,31,32). During G_1 phase elongation in this study cells also exhibited respiratory growth, because genes involved in respiratory metabolism were expressed similar to expression of *HXT5*, and fall within the second group of genes. It is not clear whether Hxt5p is actually involved in respiratory processes. From this group, 6 genes encode mitochondrial proteins that function in the tricarboxylic acid (TCA) cycle. The TCA cycle is involved formation of NADH that is subsequently reoxidized to NAD^+ via mitochondrial respiration to yield ATP (33,34). These genes include *FUM1* encoding fumarate hydratase (35), *KGD2* encoding 2-oxoglutarate dehydrogenase (36), *LSC1* and *LSC2* encoding the alpha and beta subunit of succinyl-CoA synthetase respectively (37) *MDH1*, encoding malate dehydrogenase (38), and *SDH2* encoding succinate dehydrogenase (39). Genes encoding proteins involved in coenzyme metabolism, which may be closely linked to respiratory growth, were also expressed similar like *HXT5*. Maintenance of intracellular redox balance by controlling the NADH/NAD^+ ratio is extremely important during growth, and the proteins encoded by the following genes may be involved in maintaining this balance. These genes include *GUT2*, encoding mitochondrial glycerol-3-phosphate dehydrogenase (40). *COQ4* encodes a protein involved in biosynthesis of coenzyme Q (ubiquinone), which is part of the mitochondrial electron transport chain (41,42). *PNC1* encodes a protein with protein involved in NAD^+ metabolism (43,44). *TKL2* encodes an enzyme with transketolase activity, which is needed for biosynthesis

of aromatic amino acids (45). It is known that amino acid synthesis results in production of NADH (46).

The third group consists of genes that are known to be induced by various so-called "stress" conditions. Surprisingly, a large number of these genes are involved in folding of proteins. These include the heat shock genes *HSP26* and *HSP42*. These genes encode two proteins with high similarity that may exhibit chaperone activity (47,48). *HSP78* encodes a mitochondrial heatshock protein (49) and *HSP104* encodes a protein that is required for disassembly of aggregates of denatured proteins (50,51,52). The protein encoded by *SSE2* is a HSP70 family member and is thought to exhibit chaperone activity (53). Three other genes with chaperone-like functions include *AHA1* encoding a co-chaperone that activates Hsp82p ATPase activity (54,55), and *SBA1* that encodes a putative co-chaperone of Hsp82 (56). *MPD2* encodes a potential protein disulfide isomerase, which also functions in protein folding (57,58). The actual function of the several heatshock genes that are induced upon an increase in the G_1 phase duration remains quite puzzling. During other conditions that result in a decrease in growth rate, for example heat shock or ethanol stress, expression of *HSP* genes was also induced and under those conditions their physiological role seems more evident (28,59). Hsp proteins have functions in preventing protein denaturation and aggregation of unfolded proteins, processes that occur during heat or ethanol stress (60). However, denaturation of proteins is not known as a response to nutrient limitation, which was used here to elongate the G_1 phase duration. Hsp78, which is present in the mitochondrion, might be specifically required for proper folding of proteins required for respiration. However, the actual physiological role of all the other chaperones during growth at a low growth rate remains unclear, as well as a possible role of Hxt5p in these processes.

Other genes that were recognized as to respond to stress were *DDR2*, encoding a protein with unknown functions, whose expression is induced upon a variety of stress conditions, including DNA damage (61). *GRX1* encodes a glutaredoxin, which displays glutathione peroxidase activity and is involved in resistance to hydroperoxides (62). *GTT1* encodes a protein involved in glutathione metabolism (63). The product of *YGP1* is a secreted glycoprotein with unknown functions, whose expression is amongst others induced upon nutrient limitation (64) and *YJL144w* encodes a protein of unknown function, which is expressed under conditions of hyperosmotic stress (65). Again, the growth conditions that were used to elongate the G_1 phase duration are not known to cause stress conditions like DNA damage, oxidative or osmotic stress, which are known

to induce expression of these genes. It is not likely that Hxt5p plays a role in any of these processes. Apparently, another mechanism than the actual stress itself might be responsible for the induction of these genes. The *GRE3* gene, which responds to osmotic stress (66), encodes an aldolase reductase with NADPH specificity (67), which catalyzes reduction of xylose to xylitol (68,69). Because wildtype yeast strains are unable to grow on xylose, the true function of Gre3p in xylose metabolism remains unclear. Recently, it was shown that Hxt5p was able to transport xylose (70) and *HXT5* expression was induced in a recombinant yeast strain with a higher ability to utilize xylose (71). Therefore, the products of *HXT5* and *GRE3* may have connecting functions specifically during decreased growth rates. Expression of genes involved in trehalose metabolism (*TPS1*, *TPS2*, *TSL1*, *ATH1*, *PGM2*) were also shown to respond to stress conditions. Indeed, trehalose accumulation has an important physiological function during growth at low growth rates, by serving as a reserve carbohydrate (2) or as a protectant of proteins and membranes during stress conditions (72,73).

The fourth category includes genes encoding proteins involved in metabolism of carbon sources that were not present in the growth medium. Three genes of this group function in lipid metabolism; *FAA1*, which encodes long-chain fatty acid CoA ligase, may be involved in the import process of exogenous fatty acids (74,75). Furthermore, Faa1p is required for transcriptional regulation of *POX1* (75), encoding acyl-CoA oxidase, which functions in fatty acid beta-oxidation in the peroxisome (76,77,78). Another peroxisomal protein belonging to this group is *TES1*, encoding acyl-CoA thioesterase, which is probably involved in fatty acid oxidation (79). *JEN1*, encoding a lactate transporter (80) and *CYB2*, which encodes a protein involved in the conversion of lactate into pyruvate (81) were expressed similar to *HXT5*, although lactate was not present in the growth medium. The function of these genes during low growth in the absence of their substrates is unknown. Maybe yeast cells induce expression of these genes during growth at low growth rates to be prepared to changing conditions, in case these carbon sources are available in a new environment. It is noteworthy to mention that Hxt5p was suggested to function as a reserve transporter, which might be involved in the initial uptake of glucose when glucose becomes available again. This was based on the observation that glucose-depleted cells deleted for *hxt5* have a slightly increased lag phase upon inoculation on a fresh glucose medium, compared to wildtype cells grown in the same way (27). In this respect, induction of the genes encoding chaperones, which was observed in our study, may

have a similar function as well, by rapidly enabling folding of newly synthesized proteins when growth conditions suddenly improve. Whether this is really the case remains to be elucidated.

Results of other studies may provide indications about why the genes found to be expressed similar to *HXT5* upon an increase in the G_1 phase duration were specifically induced. Previously, the existence of the family of environmental stress response (ESR) genes was described, which corresponds to a cluster of genes that have similar expression patterns under a variety of stress conditions, for example heat shock, osmotic shock, growth into the stationary phase and nitrogen depletion (59). One of the ESR genes is *HXT5*. Among the 300 genes up-regulated in the ESR, 13 genes were expressed like *HXT5* during an increase of G_1 phase duration in our study. These genes include *ATH1*, *GLK1*, *GRX1*, *GTT1*, *HSP104*, *HSP26*, *HSP42*, *HSP78*, *PGM2*, *SSE2*, *TPS1*, *TPS2* and *TSL1*. Expression of *HXT5* was also induced in other genome-wide expression studies, including addition of salt (13,14,15), cell damaging agents (82,83) or high amounts of ethanol (28). Surprisingly, many of the genes that were expressed like *HXT5* in our study were also induced together with *HXT5* in those studies (Table 2). It is known that all conditions that result in expression of those genes and *HXT5* have a common feature, being a reduction in the growth rate of cells. We have shown earlier that expression of *HXT5* is determined by the growth rate of cells (7). Thus, the results of these studies and our present study indicate that growth rate-regulated expression of genes is not limited to expression of *HXT5* alone. Expression of several genes was induced upon a decrease in the growth rate of cells in this study, and they do not seem to have a clear function during the conditions that were used in this study. Most strikingly, several *HSP* genes encoding proteins involved in protein folding were induced, although it is unlikely that a decrease in the growth rates of cells in our experimental set-up results in protein denaturation or protein aggregation. Thus, the genes that are expressed similar to *HXT5* may be part of a common set of genes whose expression is merely induced upon a decrease in growth rate of cells, independent of the specific condition that results in the decrease in the growth rate of cells. Apparently, not all genes that are induced, exhibit a clear physiological or metabolic function during every slow growth-inducing condition, but are merely expressed as a consequence of the decrease of the growth rate of cells.

Table 2: Expression of *HXT5* co-regulated genes in other studies. Most of the genes that were expressed similar to *HXT5* upon an increase in G_1 duration are also expressed like *HXT5* during a variety of other conditions, as determined by DNA microarray analysis. (1, ref 13), (2, ref 14), (3, ref 15), (4, ref 59), (5, ref 82), (6, ref 83), (7, ref 28).

	0.4M NaCl (1)	0.7M NaCl (2)	1M NaCl (3)	ESR (4)	Sulfometuron methyl (5)	Cell damaging conditions (6)	Ethanol stress (7)
ADH3							
AHA1							
ATH1				x			
COQ4					x		
CYB2		x			x		
DDR2		x	x		x		
FAA1		x	x			x	
FUM1							
GLK1	x	x	x	x	x		x
GRE3	x	x	x				x
GRX1		x	x	x			
GTT1				x		x	
GUT2							
HSP104	x	x	x	x	x		x
HSP26		x	x	x	x		x
HSP42	x	x	x	x	x		x
HSP78	x		x	x	x		x
HXT5	x	x	x	x	x	x	x
ISF1						x	
JEN1						x	
KGD2						x	
LSC1			x				
LSC2					x	x	
MDH1					x	x	
MPD2							
PGM2		x	x	x	x		x
PNC1		x	x				
POX1							
SBA1							
SDH2					x	x	
SSE2		x	x	x	x		
TES1							
TKL2							
TPS1	x	x	x	x			x
TPS2		x	x	x	x		x
TSL1	x	x	x	x			x
YGP1		x	x		x		x
YJL144W						x	

Genes with similar expression patterns often have common regulatory elements in their promoter region. These elements are bound by similar transcription factors, which was observed earlier for ribosomal proteins (84) and stress-induced genes (85). The genes that were expressed similar to *HXT5* might therefore contain similar regulatory elements in their promoters. Indeed, 36% of the genes that were expressed similar to *HXT5* contain two or more STREs in their promoter region, in comparison to

only 11% of all ORFs, and 74% of the genes in this study contained one or more STRE in their promoter region versus 36% of all ORFs (Table 3, ref 17). This suggests that a significant part of the transcriptional response to a decrease in the growth rate was mediated by STRE-regulated expression.

Table 3: % of genes that were expressed similar to *HXT5* that contained STREs in their promoter region, as determined by RSAT (17). 72 genes exhibited an expression pattern similar to *HXT5*. The consensus sequence for STREs is CCCCT.

No. STREs:	**1**	**2**	**3**	**4**	**5**	**6**	**Total no. genes**
No. genes (% of total no. genes)	27 (38%)	9 (12%)	10 (14%)	3 (4%)	2 (3%)	2 (3%)	53 (74%)

The involvement of STREs in growth rate-regulated expression was shown for *HXT5*, because mutation of one of the two STREs present in the *HXT5* promoter completely abolished *HXT5* expression (Chapter 3). Furthermore, expression of a subset of these genes were also found to be dependent on functional STREs, including *GLK1*, *HSP104*, *HSP42*, *HSP78* (18), *DDR2* (86), *HSP26* (87) and *TPS1*, *TPS2* and *TSL1* (88). It is known that the activity of the PKA pathway plays an important role in regulation of expression of STRE-controlled genes (89). Many of the conditions that induce *HXT5* expression, and at the same time expression of other STRE-controlled genes, all have in common that the growth rate of cells is decreased. Therefore, the growth rate may in some way regulate the activity of the PKA pathway, and by that expression of STRE-regulated genes. It seems likely that the growth rate and the activity of the PKA pathway are connected, because *HXT5* was expressed in a mutant strain with constitutive low activity of the PKA pathway during growth at high growth rates in a continuous culture (Chapter 4). However, not all genes that were expressed like *HXT5* contained STRE elements in their promoter region, indicating that expression of growth-rate regulated genes is not entirely dependent on the presence of STREs in the promoter. Those genes with expression patterns similar to *HXT5* that were involved in respiration were enriched in HAP2/3/4/5 complex binding sites. It is known that expression of many respiratory genes is transcriptionally regulated by the carbon source and that the HAP2/3/4/5 complex binding site regulates expression of these genes (90,91). Thus, other regulatory mechanism may also be involved in growth-rate

regulated expression of genes, although expression of the majority of genes seems to be regulated by STRE-mediated expression.

Conclusion

In this study, genes were identified that exhibited expression patterns similar to *HXT5* during an increase in the G_1 phase duration by using DNA microarray analysis. Of those genes, only a small number may actually give indications about additional functions of Hxt5p, which were genes encoding proteins involved in trehalose metabolism, as we previously suggested that Hxt5p is involved in this process. Other co-induced genes provided no clear indications for additional functions of Hxt5p besides glucose transport. These include genes encoding proteins involved in respiration, genes encoding chaperones, stress-induced genes, and genes involved in carbon metabolism. Genes that were expressed similar to *HXT5* in this study were also co-induced during various other slow growth-inducing conditions. Cells may be prepared to rapidly and specifically respond to harmful conditions by inducing a common set of genes encoding proteins with protective properties. The trigger for this induction might be a decrease in the growth rate of cells. Because a high percentage of these genes contain STREs in their promoter region, this element may be important for growth rate-regulated expression of genes, which is likely to be coordinated by the PKA pathway.

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Chapter 6

General discussion

General discussion

In this thesis regulation of expression, and the function of the hexose transporter Hxt5p was examined to reveal the involvement of a specific hexose transporter in accumulation of the reserve carbohydrate trehalose during conditions of slow growth in *Saccharomyces cerevisiae*. In Chapter 2 it appeared that the growth rate of cells determines expression of the hexose transporter *HXT5*. This was concluded from the observations that *HXT5* expression initiated during conditions that result in a decease in the growth rate of cells, and from the observation that *HXT5* was only expressed at low dilution rates in continuous cultures. However, the growth rate by itself is probably not involved in regulation of *HXT5* expression, because growth rate is in fact a quite arbitrary term. Basically, yeast cells can grow in two ways, by proliferation or cell growth. Proliferation is cell division, which leads to an increase in cell number, whereas growth is macromolecular synthesis, which leads to an increase in cell mass or size. In this thesis, we have always regarded the increase in cell number as the parameter for growth rate. There is also a connection between the increase in cell size and cell number, because in general cells divide when cells reach a critical cell size per cell.

What determines the growth rate of cells? In principle, the growth rate is determined by the concentration of the growth limiting nutrient, which is described by the Monod equation: $\mu = \mu_{max}.S / (K_s+S)$ (1). In this equation, μ is the specific growth rate of cells, μ_{max} is the maximum growth rate for growth on the limiting nutrient source, S is the concentration of the limiting nutrient and K_s is the Monod coefficient, which corresponds to the nutrient concentration at which μ is half of its maximum. During batch growth on glucose, the growth rate μ is virtually equal to maximal growth rate μ_{max} when the concentration of glucose is high or not limiting, whereas the growth rate μ decreases when glucose becomes limiting and all other essential nutrients remain present. In addition, the growth rate will be influenced independent of the availability of nutrients by certain environmental conditions that are harmful for cells, like growth at increased temperature or osmolarity (2). By examining the influence of nutrients on the growth rate, it appeared that the cell cycle is largely determined by the duration of the G_1 phase. The G_1 phase of the cell cycle can be greatly elongated, whereas the other phases, S, G_2, M, remain fairly constant (3). There is a connection between the growth rate, or the G_1 phase duration, and the carbon consumption rate of cells, which was

observed both for haploid (4) and diploid cells (5). It was observed for haploid cells that the G_1 phase duration was greatly increased below a galactose consumption rate of 20 fmol $cell^{-1}.hr^{-1}$, and increased even more upon a further decrease of the galactose consumption rate. At galactose consumption rates higher than 20 fmol $cell^{-1}.hr^{-1}$, the G_1 phase duration remains fairly constant in length (4). Also during these conditions, the concentration of the growth limiting condition determines the growth rate, although it is quite remarkable that large changes in the length of the G_1 phase are due to relatively small changes in the galactose consumption rate. The intriguing question now is whether a system exists that senses the nutrient availability, signals this towards the cell that responds by growing at a particular growth rate, and ultimately regulates expression of *HXT5*?

From the results in Chapter 3, it is concluded that so-called stress responsive elements (STREs) in the promoter of the *HXT5* gene play an important role in growth rate-regulated expression of *HXT5*. It was however observed that two HAP2/3/4/5 complex binding sites are also involved in regulation of *HXT5* expression during glucose depletion or during growth on non-fermentable carbon sources. It is known that two signal transduction pathways, being the cyclic AMP/protein kinase A (cAMP/PKA) and the target of rapamycin (TOR) pathway, are involved in regulation of the translocation of Msn2p and Msn4p to the nucleus, where they bind to STREs present in the promoters of genes and activate transcription (6,7). The cAMP/PKA and TOR pathways probably control binding of Msn2/4p to the 14-3-3 anchor proteins Bmh1p and Bmh2p, which retain Msn2/4p in the cytoplasm during conditions when cells exhibit high growth rates (8). It was shown in Chapter 4 that the cAMP/PKA pathway is involved in regulation of *HXT5* expression. Furthermore, we and others have shown that expression of *HXT5* is specifically induced upon addition of rapamycin, a compound that specifically inhibits activity of the TOR pathway (our unpublished data, 9,10,11). In response to nutrient availability the cAMP/PKA pathway and/or the TOR pathway may be involved in regulation of the growth rate of cells, or the growth rate might regulate the activity of the cAMP/PKA and/or TOR pathway. The cAMP/PKA pathway is involved in a variety of processes, including reserve carbohydrate accumulation, cell growth and cell division, and regulation of gene expression by controlling Msn2/4p and Gis1p (12,13,14). The TOR pathway is besides controlling the translocation of Msn2/4p to the nucleus and thereby expression of STRE-regulated genes, also involved in control of cell growth and cell division, and accumulation of reserve carbohydrates

(8,15,16,17). The cAMP/PKA pathway and the TOR pathway probably act independently, because glycogen accumulation still occurred in a hyperactive cAMP/PKA mutant upon addition of rapamycin (15). An overview of the components that are involved in the regulation of the cAMP/PKA pathway and downstream components of this pathway is depicted in Figure 1, and are shortly described below (for review see 12,18,19,20).

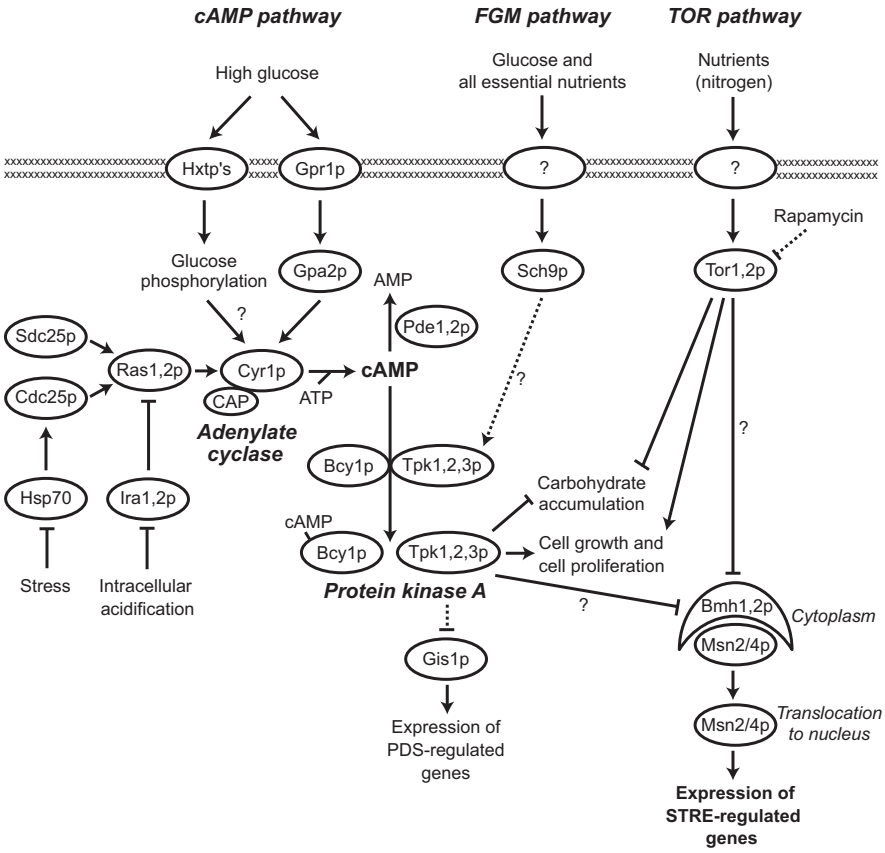

Figure 1: Overview of the PKA pathway and additional pathways that are involved in controlling the localization of Msn2/4p. For details see text.

The activity of the PKA pathway is regulated by transient activation, or by sustained activation. The first components of the cAMP/PKA pathway that were cloned were the *RAS1* and the *RAS2* genes. Ras2p is active when GTP is bound, and becomes inactive when GTP is hydrolysed to GDP. The exchange of GDP for GTP is mediated by the Cdc25p and Sdc25p, and Ira1p and Ira2p stimulate exchange of GTP for GDP. A downstream target of Ras2p is adenylate cyclase, encoded by *CYR1*. CAP/Srv2p associates with Cyr1p, and is required for activation of adenylate cyclase by Ras. Adenylate cyclase mediates the conversion of ATP into cAMP. The target of cAMP is the cAMP-dependent protein kinase A, known as PKA. It consists of a regulatory subunit, which is encoded by *BCY1*, and three catalytic subunits, encoded by *TPK1*, *TPK2* and *TPK3*. In non-growing cells, or cells that grow with a low growth rate, PKA is an inactive tetramer consisting of two regulatory subunits and two catalytic subunits. In response to extracellular signals that result in increased cAMP levels, cAMP binds to the regulatory subunits. Subsequently, a conformational change in the regulatory subunits results in a decreased affinity for the catalytic subunits, which are released and present in a free and active conformation. Hydrolysis of cAMP by Pde1p and Pde2p restores PKA to the resting, inactive state. Three mechanisms are probably involved in transient up-regulation of the activity of the cAMP/PKA pathway. Firstly, intracellular acidification seems to serve upstream of the cAMP/PKA activity during conditions of nutrient depletion (21). It is believed that intracellular acidification blocks activity of Ira1p and Ira2p, which results in a long lasting increase in cAMP levels and increased activity of the cAMP/PKA pathway. It was suggested that intracellular acidification might be involved controlling the maintenance of a proper intracellular pH and ATP levels during conditions of carbon starvation (22). Secondly, it was suggested that Cdc25p is activated by the HSP70 heat shock protein Ssa1p, which binds directly to Cdc25p, and thereby controls activity of the cAMP/PKA pathway (23). The authors proposed that after stress conditions, transient accumulation of unfolded proteins occurs, which reduces the availability of HSP70 for Cdc25p and reduces the activity of the cAMP/PKA pathway. Thirdly, a system exists that specifically activates the cAMP/PKA pathway in response to addition of glucose to glucose-deprived cells, which results in a transient increase in cAMP levels. This system involves the proteins Gpr1p and Gpa2p. For proper activation of adenylate cyclase by this pathway, glucose transport and glucose phosphorylation are also required (24). There seems to exist a system that is responsible for maintaining a sustained high activity of the cAMP/PKA

pathway during growth on glucose or other rapidly fermented sugars. This pathway, called the fermentable growth-medium-induced (FGM) pathway, has been proposed on basis of physiological data that showed that only a complete growth medium containing glucose or a related fermentable carbon source is able to maintain the glucose effect of targets of the cAMP/PKA pathway (25). For example, starvation for nitrogen in cells growing on glucose results in a slow increase in the cAMP level rather than a decrease, although phenomena associated with decreased activity of the cAMP/PKA pathway were observed (26). Down-regulation of targets of the cAMP/PKA pathway is apparently also regulated in a cAMP independent manner, suggesting the involvement of an additional mechanism that regulates cAMP/PKA activity, being the FGM pathway. It was shown that the protein kinase Sch9p, which has high homology to the catalytic subunits of PKA (27), is involved in activation of the FGM pathway (28). The FGM pathway may control the activity PKA directly, or the downstream targets of the cAMP/PKA pathway. It remains however unclear how glucose and other rapidly fermentable sugars are sensed and activate the FGM pathway. Thus, it seems that transient activation of PKA is mediated by cAMP, whereas the FGM pathway mediates sustained activation of PKA.

In yeast, a molecular machinery consisting of cyclins and cyclin-dependent kinases (CDKs) regulates progression through the cell cycle, and is therefore an important regulator of the growth rate of cells. In some way the availability of nutrients should be connected to the cell cycle machinery in order to regulate the length of the G_1 phase, which is determined by the time when cells enter the passage from the G_1 to the S phase. Cdc28p, a CDK, is activated throughout the cell cycle by binding of different cyclins, and controls the major events of the yeast cell division cycle (29). Transition from the G_1 to the S phase is mediated by a peak in expression of *CLN1* and *CLN2* (30,31,32). The timing of this event is regulated by Cln3p, whose abundance is regulated by transcription and translation (33,34,35). Translation of Cln3p is regulated by activity of the cAMP/PKA and TOR pathway, and in addition *CLN3* mRNA may be kept in an untranslated state, which is mediated by Whi3p (15,36,37,38).

Thus, the nutrient availability is involved in controlling the growth rate of cells, which may be governed by the cAMP/PKA, the TOR and the FGM pathway. At the same time, these pathways are involved in expression of STRE-regulated genes. It is therefore an interesting question whether the growth rate really regulates expression of *HXT5* and STRE-regulated genes. If this is the case, it would mean that certain

components of the cell cycle machinery, e.g. cyclins, would also function upstream of Msn2/4p. In other words, the cAMP/PKA pathway might regulate expression of *HXT5* and the growth rate separately. By experiments described in Chapter 4, the growth rate and the activity of the cAMP/PKA pathway was uncoupled by using continuous cultures. A *ras2* deletion mutant, which always exhibits low PKA activity, and has decreased growth rates in batch cultures as compared to wildtype cells, was grown at low and high dilution rates in nitrogen-limited continuous cultures. In this mutant, *HXT5* remained expressed even during growth at high dilution rates, which was not observed in wildtype cells. If a high growth rate *per se* results in repression of *HXT5* expression, this would also have occurred in the *ras2* deletion mutant, unless Ras2p or a pathway controlled by Ras2p is involved in *HXT5* expression. This suggests that the cAMP/PKA pathway, and not the growth rate which is determined by the activity of the cAMP/PKA pathway, is important for regulation of *HXT5* expression. However, if Ras2p or the cAMP/PKA pathway was directly involved in regulation of the growth rate of cells, *ras2* deleted cells would probably be unable to grow at high dilution rates, and cells should be washed out during growth in continuous cultures. Because the latter does not occur, additional mechanisms besides the cAMP/PKA pathway should exist to regulate the growth rate of cells, which subsequently determines expression of *HXT5*. Additionally, the TOR pathway may regulate the growth rate of cells, as well as the FGM pathway. However, other components may be involved in controlling the growth rate as well.

This is best discussed when we know some additional regulatory features of the cAMP/PKA pathway, concerning the dynamics of reserve carbohydrate accumulation as observed for synchronized cells. This provides a link between accumulation of reserve carbohydrates and regulation of the growth rate of cells. Similar like the relation between the G_1 phase duration and the carbon availability, the accumulation of reserve carbohydrates and in particular trehalose also correlated with the availability of the carbon source. A tight correlation was observed between the duration of the G_1 phase and the level of reserve carbohydrate accumulation; upon a longer duration of the G_1 phase, the maximal level of accumulation increased (4,5). Moreover, when the G_1 phase duration was specifically elongated, the accumulation pattern of trehalose and glycogen proved to be quite dynamic. During conditions when the G_1 phase is elongated, trehalose levels gradually increased during progression through the G_1 phase. However, at a sudden point towards the end of the G_1 phase, the

levels of accumulated reserve carbohydrates suddenly dropped (5). This decrease was suggested to be required for a proper progression to the S phase, where the liberated glucose would ultimately result in increased sugar flux and increased *CLN1* and *CLN2* expression, making progression to the S phase possible. This also prompted the hypothesis that accumulation of reserve carbohydrates by themselves caused the increase in G_1 phase duration. Indeed, cells unable to synthesize trehalose and glycogen have a short G_1 phase duration of 120 minutes when grown at galactose consumption rates of 20 fmol $cell^{-1}.hr^{-1}$ and lower, whereas wildtype cells increased their G_1 phase duration to maximally 520 minutes during the same conditions (39). Thus, accumulation of reserve carbohydrates is also involved in regulation of the growth rate of cells. In wildtype cells, trehalose and glycogen probably decrease the amount of carbon source that may be used for G_1 phase progression. In cells unable to synthesize reserve carbohydrates, signalling through the cAMP/PKA pathway may be increased because of higher carbon availability, resulting in higher translation of Cln3p and faster progression through the G_1 phase.

A recent study revealed the mechanism responsible for the characteristic behaviour of reserve carbohydrates in the G_1 phase at low growth rates (40). In this study, cells were grown in fed-batch cultures under glucose-limiting conditions, resulting in extracellular glucose concentrations that were always lower than 0.17 mM, thereby not activating the Gpr1-Gpa2 sensing system or the FGM pathway, which both exhibit maximum activation at glucose concentrations of about 20 mM (24,40). It was observed that at the end of the G_1 phase, just prior to the S phase, intracellular cAMP levels increase, and decrease again subsequently in the G_2 and M phase (40). This increase in cAMP was suggested to transiently activate the cAMP/PKA pathway. A decrease in the oxygen tension was observed, which is known to be associated with a rise in glycolytic flux and occurrence of overflow of metabolism. This increase in glycolytic flux is probably due to breakdown of trehalose and glycogen, because increased PKA activity results in increased trehalase activity and increased breakdown of glycogen by inhibiting its accumulation and stimulating its degradation (41). Thus, a transient increase in cAMP might be important for progression through cell cycle during growth at low growth rates, which suggests that cells may not progress through the cell cycle if there is no transient increase in cAMP. Indeed, a transient increase in intracellular cAMP levels was also observed at high extracellular glucose concentrations, but now in the M phase of the cell cycle (40). A transient increase in the

cAMP levels during the cell cycle might be required for proper progression through the cell cycle, and the nutrient availability, inherent to the growth rate of cells, might determine in which phase of the cell cycle this occurs. It remains however unknown how this sudden increase in cAMP was generated, but the possibility that components upstream of Cyr1p are involved were excluded (40). Maybe, this transient cAMP increase is also involved in regulation of expression of *HXT5*. It is known that sudden addition of a high amount of glucose results in a transient increase of cAMP, which is caused by the activation of the Gpr1p-Gpa2p system (24), and will probably also occur in synchronized cells early in the G_1 phase. During subsequent growth at high growth rates, intracellular cAMP levels are higher compared to growth at low growth rates (40), and the transient increase in cAMP was observed again in the M phase, as if the cells might build in a control to govern the growth rate. Thus, the increase in intracellular cAMP might be important for governing the growth rate and subsequent expression of genes. During growth at low growth rates at low nutrient availability, something else might happen; the initial peak in cAMP is not observed, thereby rendering the cAMP/PKA pathway inactive, and resulting in expression of *HXT5*. Throughout the G_1 phase, the levels of cAMP remains low, thereby still inducing *HXT5* expression. However, at the end of the G_1 phase, the increase in cAMP might transiently decrease *HXT5* expression, although this has not been demonstrated experimentally.

If accumulation of reserve carbohydrates itself has a major impact on the growth rate of cells, other factors that are important for the regulation of reserve carbohydrate accumulation may be important for regulation of the growth rate as well. In this respect, as we suggested that Hxt5p is involved in trehalose accumulation, Hxt5p itself may play a role in regulation of the growth rate by controlling to some degree trehalose accumulation. The suggestion that Kns1p might phosphorylate Hxt5p (Chapter 4) and Tps1p indicates that this kinase may be important in this process. Remarkably, expression of *KNS1* was similar to expression of *HXT5* during a variety of conditions that result in low growth rates (42). Phosphorylation of Hxt5p and Tps1p by Kns1p might render Hxt5p and Tps1p more active, or might induce a conformational change so that Hxt5p and Tps1p may interact when both proteins are phosphorylated. Subsequently, the glucose flux may be driven towards accumulation of trehalose. At the G_1/S transition, trehalose is no longer accumulated, maybe because the activity of Kns1p is cell cycle-regulated and decreases at the G_1/S transition. It will be interesting to determine whether the activity of Kns1p is under control of the PKA pathway.

In order to change the sugar flux from glycolysis towards reserve carbohydrate accumulation during low growth rates, other changes in the control of metabolism have to occur as well. The flux has to be changed towards glucose-1-phosphate and UDP-glucose, by stimulating expression of *PGM1*, *PGM2* and *UGP1*, or by rendering these enzymes more active. Recently, the PAS kinases Psk1p and Psk2p were identified to have a role in the regulation of changing the flux towards and from glycolysis and reserve carbohydrate accumulation (43). Psk1p and Psk2p were originally identified to be required for growth on galactose at 39ºC (Gal^{ts}). Interestingly, cells grown on galactose exhibit lower maximal growth rates as compared to growth on glucose, and during growth at 39ºC the growth rate is decreased compared to growth at 30ºC. Thus, the Psk proteins may be involved in regulation the growth rate of cells, or regulation of certain processes that are involved in growth rate regulation. A suppression screen revealed that overexpression of *PGM1* or *PGM2* rescued the Gal^{ts} growth defect, thus Psk1p and Psk2p are somehow involved in the control of the interconversion of glucose-6-phosphate to glucose-1-phosphate. Furthermore, it was shown that Psk2p was able to phosphorylate Ugp1p, thereby probably rendering it inactive. This was concluded because trehalose and glycogen accumulation was increased in exponentially growing cells deleted for *psk1* and *psk2*. Thus, low growth rates might result in inactivation of Psk1p and Psk2p, dephosphorylation and activation of Ugp1p and pushing the flux towards UDP-glucose and reserve carbohydrate accumulation. During high growth rates, Psk1p and Psk2p are probably active, and direct the sugar flux towards glycolysis. The signal that regulates the activity of PAS kinases is however unknown. It would be interesting to determine whether the activity of PAS kinase in cell cycle regulated during low growth rates, thereby modulating the carbon flux during the G_1 phase.

During conditions of slow growth expression of a number of genes is specifically induced, and activity of certain proteins may be modulated to drive the carbon flux away from glycolysis towards other metabolic routes, like accumulation of reserve carbohydrates. These changes are well coordinated by the cAMP/PKA, TOR and the FGM pathway, and additionally by proteins like Psk1/2p, and may also be controlled by Hxt5p.

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Summary

Glucose, which is transported into yeast cells by hexose transporter (Hxt) proteins, is the most preferred carbon source for the yeast *Saccharomyces cerevisiae*. A multigene family consisting of 20 members encodes the Hxt proteins. *HXT1-4* and *HXT6-7* encode the major Hxt proteins, the remaining Hxt proteins have other or unknown functions. Hxt proteins localize to the plasma membrane, have 12 membrane-spanning domains, and their amino- and carboxy-terminal domains are present in the cytoplasm. Expression of the major *HXT* genes is regulated by the extracellular glucose concentration. Some of the major *HXT* genes are expressed when the glucose concentration is high, whereas others are expressed when the concentration is low.

Comparing the number of amino acids of all Hxt proteins revealed that the Hxt5 protein contains a longer amino-terminal domain as compared to the major Hxt proteins, which suggests that Hxt5 has additional functions besides glucose transport. In Chapter 2 we determined the expression pattern of *HXT5* to obtain insight in regulation of *HXT5* expression. It appeared that expression of *HXT5* is not regulated by the extracellular glucose concentration, but by the growth rate of yeast cells. This was concluded by growing cells in batch cultures on glucose, ethanol and glycerol, growth at a higher temperature or osmolarity, growth in fed-batch cultures and continuous cultures. The results indicated that low growth rates of cells result in induction of *HXT5* expression.

Gene expression is regulated by binding of transcription factors to regulatory elements in the promoter of genes. Therefore, the growth rate should somehow be involved in binding of specific transcription factors to the promoter of the *HXT5* gene. In Chapter 3, the putative regulatory elements in the promoter of *HXT5* that contribute to growth rate-regulated *HXT5* expression were identified. Computer-assisted analysis revealed the presence of two putative stress responsive elements (STREs), one putative post-diauxic shift (PDS) element and two putative Hap2/3/4/5 (HAP) complex binding elements. The involvement of these elements was studied by mutation of these putative regulatory elements in a *HXT5*-promoter *LacZ* fusion construct. It appeared that one of the STREs is extremely important for growth-rate regulated *HXT5* expression, whereas in addition the HAP elements are important for regulation of *HXT5* expression during growth on ethanol and glycerol.

Glucose is not only used for metabolism, but it may also serve as a precursor for the reserve carbohydrates trehalose and glycogen. Earlier studies in our laboratory have shown that trehalose in particular accumulates when the growth rate of cells is decreased. It was suggested in literature that Tps1, one of components of the protein complex involved in trehalose production, might control glucose transport by interacting with a hexose transporter. Because Hxt5 contains a longer amino-terminal domain that may serve as an interaction domain for Tps1, and expression of *HXT5* and accumulation of trehalose are both regulated by the growth rate of cells, the role of Hxt5 in trehalose accumulation was investigated in Chapter 4. It appeared that *HXT5* was indeed expressed when trehalose accumulated during low growth rates in glucose-grown batch cultures, fed-batch cultures and continuous cultures. Furthermore, expression of both *HXT5* and *TPS1* was found to be regulated by the cAMP/PKA signal transduction pathway. In addition we observed that Hxt5 was phosphorylated upon glucose depletion, a condition that results in trehalose accumulation. Other studies indicated that the kinase that is likely to phosphorylate Hxt5 binds physically to Tps1. These observations strongly suggested that Hxt5 plays a role in trehalose accumulation. However, trehalose accumulation was affected, but not completely abolished, in a strain deleted for *hxt5*. Furthermore, no direct physical interaction between Hxt5 and Tps1 was determined as determined by yeast 2-hybrid studies and co-immunoprecipitation. Therefore, the exact role of Hxt5 in trehalose accumulation remains to be further explored.

To establish whether Hxt5 is involved in other processes, genes that were expressed similar to *HXT5* during growth at a low growth rate in fed-batch cultures were identified using DNA microarray analysis. This approach was chosen, because genes that are similarly expressed may be involved in similar processes. From a total of 6144 genes, 72 genes exhibited an expression pattern similar to *HXT5*. These included genes encoding proteins involved in trehalose accumulation, which strengthens our hypothesis that Hxt5 plays a role in trehalose accumulation. Also genes encoding proteins involved in respiration, protein folding, stress responses, and genes encoding proteins involved in metabolism of carbon that were not present in the growth medium, were similarly expressed. How Hxt5 is involved in any of these processes remains to be determined. It appeared that the genes that were identified in our study were also similarly expressed in a variety of other studies that all had one common feature, being a reduction in the growth rate of cells. We suggested that cells might be pre-

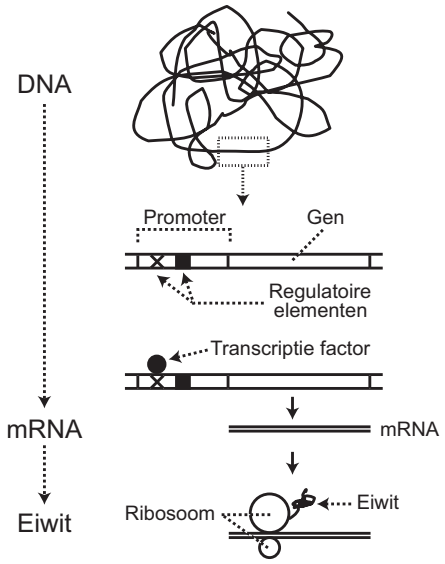
programmed to rapidly and specifically respond to harmful conditions by inducing a common set of genes encoding proteins with protective properties. Expression of these genes is triggered by a reduction in the growth rate of cells. A high percentage of these genes contain STREs in their promoter region. This regulatory element may therefore be involved in growth rate-regulated expression of genes. Because it is known that the cAMP/PKA pathway is involved in STRE-mediated gene expression, this pathway seems important for regulation of growth rate-regulated gene expression in *Saccharomyces cerevisiae*.

In Chapter 6, the results of this thesis are discussed in a broader perspective, and we explained which factors and processes might contribute to regulation of the growth rate and growth rate-regulated expression of genes in *Saccharomyces cerevisiae*.

Samenvatting

Gisten zijn ééncellige organismen. Er zijn veel verschillende soorten gisten, waarvan *Saccharomyces cerevisiae*, oftewel bakkersgist, de bekendste is. Gisten worden al sinds de tijd van de oude Egyptenaren gebruikt voor de bereiding van bier, brood en wijn, omdat ze in staat zijn ethanol (alcohol) en koolzuur te produceren. Om te kunnen groeien en overleven hebben gisten voedingsbronnen als stikstof en koolstof nodig. Voorbeelden van koolstofbronnen zijn ethanol en glycerol en de suikers glucose en galactose. Gisten hebben een voorkeur voor bepaalde koolstofbronnen, omdat deze makkelijk te gebruiken zijn voor productie van energie, een proces dat metabolisme heet. Van alle koolstofbronnen wordt glucose het makkelijkst gemetaboliseerd en gisten hebben daardoor een voorkeur om te groeien op glucose. Op het moment dat gisten in aanraking komen met glucose, zal de samenstelling van de gistcel zó veranderen, dat glucose zo snel en efficiënt mogelijk gemetaboliseerd kan worden. Om dit te bewerkstelligen zijn eiwitten nodig. Om te begrijpen hoe die eiwitten gemaakt worden, volgt nu een stukje "basiskennis" (Figuur 1).

Gistcellen bevatten, net als bijvoorbeeld menselijke cellen, DNA. Op het DNA, dat erg groot is, zijn kleine afzonderlijke stukjes aanwezig, genaamd genen die de informatie bevatten om eiwitten te maken. In *Saccharomyces cerevisiae* zijn 6144 genen aanwezig en van die genen kunnen in principe uiteindelijk evenzoveel eiwitten worden gemaakt, die het "werk" in de cel doen. Van een gen wordt eerst het zogenaamde boodschapper RNA of messenger RNA (mRNA) gemaakt. Dit mRNA dient vervolgens als boodschapper voor eiwitsynthese, wat gebeurt met behulp van speciale eiwitten die ribosomen heten. De aanmaak (expressie) van mRNA wordt gereguleerd door een klein stukje DNA, de zogenaamde promoter, dat voor een gen ligt. Aan kleine gebiedjes in de promoter, de regulatoire elementen, kunnen transcriptie factoren binden, wat eiwitten zijn die reguleren of een gen tot expressie wordt gebracht (Figuur 1).



Figuur 1: Van DNA tot eiwit. In de promoter van genen zijn regulatoire elementen aanwezig, waaraan transcriptie factoren kunnen binden. Als dit gebeurt, kan het gen worden vertaald in boodschapper RNA (mRNA). Het mRNA wordt gelezen door ribosomen die zorgen voor aanmaak van het eiwit.

Glucose, de meest geprefereerde koolstofbron, kan niet zomaar door het omhulsel (membraan) van een gistcel heenkomen. Het wordt specifiek de cel in getransporteerd door eiwitten die hexose transporters (Hxt's) heten. Deze eiwitten zijn aanwezig in het membraan van de gistcel. Zie hiervoor de kaft van mijn proefschrift: De paarse stipjes aan de rand van de grote ronde cel geven de locatie van het Hxt5 eiwit in de membraan van de cel aan. Er zijn 17 verschillende *HXT* genen die 17 verschillende Hxt eiwitten kunnen maken in *Saccharomyces cerevisiae*. Niet alle Hxt's even belangrijk. Alleen Hxt1,2,3,4,5,6 en Hxt7, de zogenaamde "hoofd" Hxt's, dragen daadwerkelijk bij tot glucose transport, *HXT8-17* komen bijna niet tot expressie. Expressie van de "hoofd" hexose transporters wordt bepaald door de hoeveelheid glucose die aanwezig is in de omgeving van de gistcel. Ze worden niet allemaal tegelijk tot expressie gebracht. Sommige van de "hoofd" *HXT* genen komen tot expressie als er veel glucose, en andere als er weinig glucose aanwezig is.

In Hoofdstuk 2 is aangetoond dat expressie van *HXT5* niet door de glucose concentratie, maar op een andere manier gereguleerd wordt, namelijk door de snelheid

waarmee gistcellen groeien, kortweg de groeisnelheid. Deze term is gedefinieerd als de tijdsduur om van één cel twee cellen te maken, wat in bakkersgist gebeurt door middel van celdeling. *HXT5* komt niet niet tot expressie wanneer de groeisnelheid hoog is, maar juist wel bij lage groeisnelheden. Dit is uitgezocht met behulp van verschillende opgroeimethoden. Zo komt *HXT5* tijdens groei op glucose in batch culturen, waarbij de hoeveelheid glucose na verloop van tijd wordt opgebruikt, niet tot expressie wanneer cellen groeien met een maximale groeisnelheid. *HXT5* komt pas tot expressie nadat de groeisnelheid na verloop van tijd afneemt. Ook komt *HXT5* tot expressie tijdens groei op ethanol of glycerol, koolstofbronnen waarop de groeisnelheid laag is. Een hoge temperatuur of aanwezigheid van een hoge concentratie zout leidt ook tot een verlaging van de groeisnelheid en expressie van *HXT5*. De concentratie glucose in het groeimedium is dan nog steeds hoog. In fed-batch culturen, waarbij de groeisnelheid door middel van zeer gedoseerde toevoeging van de hoeveelheid suiker gereguleerd kan worden, is *HXT5* expressie alleen aangetoond bij lage groeisnelheden, onafhankelijk van de concentratie glucose in het groeimedium. In continu culturen, waarmee de groeisnelheid heel specifiek gereguleerd kan worden, komt *HXT5* alleen tot expressie bij lage groeisnelheden en is expressie weer onafhankelijk van de glucose concentratie. De conclusie van dit onderzoek was dat expressie van *HXT5* gereguleerd wordt door de groeisnelheid.

Uiteindelijk moet de groeisnelheid op de één of andere manier aangrijpen op transcriptie factoren, omdat deze eiwitten expressie van genen reguleren. Daarom is in Hoofdstuk 3 uitgezocht welke regulatoire elementen in de promoter van het *HXT5* gen betrokken zijn bij *HXT5* expressie. Door middels van een computeranalyse werden 5 mogelijke regulatoire elementen in de promoter van het *HXT5* gen geïdentificeerd, te weten 2 stress responsive elements (STREs), 1 post-diauxic shift (PDS) element en 2 Hap2/3/4/5 transcriptie factor binding (HAP) elements. Het is bekend dat de transcriptie factoren Msn2 en Msn4 binden aan STREs, Gis1 bindt aan PDS en het HAP2/3/4/5 complex bindt aan HAP elementen. Deze transcriptie factoren kunnen zo expressie van genen reguleren, die deze regulatoire elementen in de promoter bevatten. Door nu de mogelijke elementen zó te veranderen (=muteren), dat de transcriptie factoren niet meer kunnen binden, kon de rol van de afzonderlijke elementen in de regulatie van *HXT5* expressie worden bestudeerd. Het bleek dat één van de STREs uitermate belangrijk is voor groeisnelheid gereguleerde *HXT5* expressie. Op het moment dat dit element gemuteerd was, kwam *HXT5* niet meer tot expressie tijdens groeicondities die

resulteerden in een langzame groeisnelheid. Verder bleek dat de HAP elementen van belang zijn bij regulatie van *HXT5* expressie tijdens groei op ethanol en glycerol. Omdat één van de STREs zo belangrijk is voor regulatie van *HXT5* expressie, en omdat het uit de literatuur bekend is dat de transcriptie factoren Msn2 en Msn4 onder controle staan van de protein kinase A (PKA) signaal transductie route, suggereerden wij dat deze route een belangrijke rol zou kunnen spelen bij groeisnelheid gereguleerde *HXT5* expressie.

Glucose kan door cellen worden gemetaboliseerd, maar daarnaast onder bepaalde omstandigheden ook worden opgeslagen in de gistcel als de reservestoffen trehalose en glycogeen. Eerder onderzoek in ons laboratorium heeft uitgewezen dat de opbouw van met name trehalose, net als expressie van *HXT5*, ook door groeisnelheid gereguleerd wordt. Verder is in de literatuur gesuggereerd dat één van de eiwitten die betrokken is bij trehalose opbouw, genaamd Tps1, de opname van glucose zou kunnen reguleren door te binden aan hexose transporters. Het Hxt5 eiwit is wat betreft zijn structuur ook nog eens verschillend van de andere Hxt eiwitten, omdat het een langere, unieke "staart" bezit die zich in de binnenkant van de cel bevindt. Ons idee was dat Tps1 hier specifiek aan zou kunnen binden en vervolgens de naar binnen getransporteerde glucose direct in de richting van trehalose opbouw zou dirigeren. In Hoofdstuk 4 is de mogelijke rol van Hxt5 in trehalose opbouw dan ook onderzocht. In glucose gegroeide batch culturen kwam het expressie patroon van *HXT5* geheel overeen met het opbouw patroon van trehalose, terwijl het expressie patroon van de andere "hoofd" *HXT* genen niet of maar gedeeltelijk overeen kwam. In fed-batch culturen kwam *HXT5* alleen tot expressie én werd trehalose opgebouwd bij een lage groeisnelheid, terwijl dit voor beide processen niet het geval was bij een hoge groeisnelheid. Ook in continu culturen was er alleen *HXT5* expressie en trehalose opbouw bij lage groeisnelheden. Verder is aangetoond dat expressie van zowel *HXT5* als *TPS1* gereguleerd wordt door de PKA signaal transductie route. Al deze bevindingen suggereerden in sterke mate dat Hxt5 betrokken is bij de opbouw van trehalose in gistcellen. De eerder gesuggereerde binding tussen Hxt5 en Tps1 kon niet worden aangetoond, dus de manier waarop Hxt5 betrokken zou kunnen zijn bij trehalose opbouw is (nog) onbekend. Verder wezen de resultaten erop dat Hxt5 niet exclusief betrokken is bij trehalose opbouw in gist; in een giststam die geen Hxt5 meer kan aanmaken werd nog steeds 80% van de hoeveelheid trehalose opgebouwd ten opzichte van een stam die Hxt5 nog wel bevat.

Om erachter te komen of Hxt5 eventueel betrokken is bij andere processen in gist, is in Hoofdstuk 5 onderzocht welke genen hetzelfde expressie patroon als *HXT5* hebben tijdens langzame groei in een fed-batch cultuur. Genen die op hetzelfde moment tot expressie komen, zouden immers iets met elkaar te maken kunnen hebben. Met behulp van de DNA microarray techniek kan het expressie patroon van alle 6144 genen in gist tegelijk worden bepaald. Hiermee werd aangetoond dat 72 genen een expressie patroon hadden dat gelijk was aan het expressie patroon van *HXT5* tijdens langzame groei. Deze genen werden vervolgens onderverdeeld in groepen met gemeenschappelijke functies. 1). Genen die betrokken zijn bij trehalose opbouw, wat dus weer een betrokkenheid van Hxt5 bij trehalose opbouw suggereert. 2). Genen die betrokken zijn bij respiratie, een proces dat zorgt voor energie productie tijdens langzame groeisnelheden. 3). Genen die betrokken zijn bij de vouwing van eiwitten. Deze genen behoren tot een grotere groep genen, waarvan bekend is dat ze tot expressie komen tijdens "stress"condities. 4). Genen die betokken zijn bij metabolisme van koolstofbronnen die verrassenderwijs niet aanwezig waren in het groeimedium. Ook was er een aantal genen waarvan de functie (tot nu toe) nog niet geïdentificeerd is. Hoe en of Hxt5 betrokken is bij deze processen, is onbekend. Het was echter opvallend dat veel van de genen die hetzelfde expressiepatroon als *HXT5* hadden in onze studie, ook tegelijk met *HXT5* tot expressie kwamen na toevoeging van verschillende hoeveelheden zout, verhoging van de temperatuur of het toevoegen van chemicaliën die schadelijk zijn voor de cel. Dit was uitgezocht in andere studies waarin op dezelfde manier het totale expressie plaatje van genen was onderzocht. Tussen al die studies en de onze is één overeenkomst, namelijk dat de condities alle leiden tot een verlaging van de groeisnelheid van cellen. Dus *HXT5* zou samen met de in onze studie geïdentificeerde genen tot een gemeenschappelijke groep van genen kunnen behoren die tot expressie wordt gebracht als de groeisnelheid van cellen wordt verlaagd, ongeacht de conditie die resulteert in een verlaging van de groeisnelheid. Het was verder opvallend dat een hoog percentage van de genen met hetzelfde expressie patroon als *HXT5* één of meerdere STREs bevatte. In Hoofdstuk 3 is aangetoond dat dit element enorm belangrijk is voor groeisnelheid gereguleerde expressie van *HXT5*. Omdat bekend is dat de PKA signaal transductie route een belangrijke rol speelt bij expressie van STRE-gereguleerde genen, en deze route van belang is voor regulatie van *HXT5* expressie, zou de PKA signaal transductie route in het algemeen een belangrijke rol kunnen spelen in groeisnelheid gereguleerde genexpressie in *Saccharomyces cerevisiae*.

In Hoofdstuk 6 zijn de bevindingen uit het proefschrift in een breder perspectief bediscussieerd. Tevens is geprobeerd om te verklaren hoe groeisnelheid exact gereguleerd kan worden en hoe groeisnelheid kan aangrijpen op expressie van genen in *Saccharomyces cerevisiae*.

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List of publications

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Curriculum vitae

De schrijver van dit proefschrift werd geboren op 18 oktober 1975 in Gouda. In 1994 behaalde hij zijn VWO diploma aan het Coornhert Gymnasium in Gouda. In datzelfde jaar startte hij met zijn studie Biologie aan de Universiteit Utrecht, met als afstudeerrichting Fundamentele Biomedische Wetenschappen. Tijdens zijn studie werd een eerste stage gelopen bij de vakgroep Moleculaire Celbiologie aan de Universiteit Utrecht (Dr. C.M.J. Sagt en Prof. Dr. Ir. C.T. Verrips). Een tweede stage werd gelopen bij de sectie Cellulaire Biochemie van het Nederlands Kanker Instituut in Amsterdam (Dr. B.N.G. Giepmans en Prof. Dr. W.H. Moolenaar). In juni 1999 werd zijn studie voltooid, waarna bij de vakgroep Moleculaire Celbiologie aan de Universiteit Utrecht begonnen werd aan het promotie onderzoek dat in dit proefschrift beschreven staat (Prof. Dr. J. Boonstra, Prof. Dr. Ir. C.T. Verrips en Prof. Dr. A.J. Verkleij). Vanaf september 2003 is de schrijver werkzaam als post-doctoraal onderzoeker bij de sectie Fungal Genomics van het laboratorium voor Microbiologie aan de Wageningen Universiteit (Prof. Dr. Ir. A.J.J. van Ooyen).