REGULATION OF THE *TPS1* GENE EXPRESSION IN *SACCHAROMYCES CEREVISIAE*

Georgia Gladys Bihl

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Supervisor: Professor H.J.J. van Vuuren

DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and has not previously in its entirety or in part been submitted at any university for a degree.

G.G. Bihl ..... 8 March 1999

SUMMARY

For many years the disaccharide trehalose was thought to be simply a carbon and energy reserve in the yeast *Saccharomyces cerevisiae*. However, a positive correlation between the trehalose content and thermotolerance, freeze tolerance, desiccation resistance, osmotolerance and exposure to toxic chemicals was observed in yeast as well as in other organisms. Therefore, trehalose is now considered to be a stress protectant rather than an energy reserve. This property of trehalose as a possible stress protectant has been used by the baking and brewing industries to increase the freeze tolerance of yeast. However, there is still a need in both industries to further optimise the freeze tolerance of yeast and to use the protective function of trehalose to the fullest extent.

Genes encoding enzymes involved in the trehalose metabolic pathway have been manipulated in order to increase trehalose levels in yeast cells. However, it has been shown that higher trehalose levels do not necessarily increase the stress resistance of yeast. It therefore seems that factors other than high trehalose levels are responsible for stress resistance in yeast and these factors could function either in combination with or independently from trehalose.

The focus of this study was to unravel the regulation of the *TPS1* gene that encodes the first enzyme of the trehalose biosynthesis pathway, namely trehalose-6-phosphate synthase. A deletion of the *TPS1* gene leads to various phenotypes, including defects in trehalose synthesis and defective growth on glucose. This implies additional functions for the *TPS1* gene and prompted us to investigate the transcriptional regulation of the gene.

Based on previous results, two control mechanisms were investigated: an autoregulatory mechanism and regulation by stress responsive elements. Autoregulation implies that the gene product regulates expression of its own gene by interacting with the DNA sequences either within the promoter or within the open reading frame (ORF). The latter hypothesis was investigated by scanning the ORF for possible DNA-protein interactions and by expression studies with different truncation constructs of the *TPS1* gene under glucose shift conditions. Our results, however, showed no evidence for an autoregulatory mechanism.

The correlation between trehalose content and stress conditions led us to investigate the possibility of stress-regulated expression of the *TPS1* gene. Stress responsive elements (STRE) have been identified in several stress-related genes where they confer stress-induced expression. Six putative STRE elements were identified in the promoter of the *TPS1* gene. Gel retardation experiments and competition studies were used to show that nuclear proteins bind specifically to the STRE elements in the *TPS1* gene promoter. Mutation analysis of these elements further showed that the STRE element closest to the ATG (STRE1) played a prominent role in regulating expression of the gene. However, this does not explain the various phenotypes observed for the *TPS1* mutant. Further research is required to elucidate the various aspects concerning the control mechanisms involved in expression of the *TPS1* gene.

OPSOMMING

Vir baie jare is die disakkaried trehalose as 'n energie- en koolstofbron in die gis *Saccharomyces cerevisiae* beskou. 'n Positiewe verband is egter tussen die trehalose-inhoud en weerstandbiedenheid teen hitte, vries, wateraktiwiteit (A_w), osmotiese druk en blootstelling aan toksiese chemikalieë in giste sowel as in ander organismes, waargeneem. Dit het daartoe aanleiding gegee dat trehalose as 'n stresbeskermer eerder as 'n energiebron beskou is. Hierdie eienskap van trehalose as 'n moontlike stresbeskermer is deur die bakkers en brouers industrieë benut om die vriestoleransie van giste te verhoog. Daar is egter steeds in beide hierdie industrieë 'n behoefte om die vriestoleransie van die gis verder te optimiseer om sodoende die beskermende funksie van trehalose ten volle te benut.

Gene wat kodeer vir ensieme betrokke in die trehalose metaboliese weg, is reeds gemanipuleer om die trehalose konsentrasie in die gissel te verhoog. Dit is egter gevind dat hoër vlakke van trehalose nie noodwendig die stres-weerstandbiedendheid van giste verhoog nie. Dit blyk dus dat ander faktore vir stres-weerstandbiedendheid in giste veranwoordelik is; hierdie faktore kan in kombinasie of onafhanklik van trehalose funksioneer.

Die fokus van hierdie studie was om die regulering van die *TPS1* geen wat die eerste ensiem in die trehalose-biosintetiese weg, (trehalose-6-fosfaat) enkodeer, te ontrafel. 'n Delesie van die *TPS1* geen lei tot verskeie fenotipes, insluitende defekte in trehalose sintese en defektiewe groei op glukose. Dit impliseer addisionele funksies vir die *TPS1* geen en het daartoe aanleiding gegee dat ons die regulatoriese meganismes van die geen bestudeer het.

Twee moontlike beheermeganismes is ondersoek: outoregulering en regulering deur middel van stres-beheerelemente bekend as STREs. Outoregulering impliseer dat die geenproduk uitdrukking van sy eie geen reguleer deur interaksie met DNA sekwensies in die promoter of in die oopleesraam (OLR). Laasgenoemde moontlikheid is ondersoek deur skandering van die OLR vir moontlike DNA-proteien interaksies en uitdrukking- studies met delesie-konstrukte

van die OLR onder glukose kondisies. Geen bewyse vir outoregulering kon egter gevind word nie.

Die verband tussen trehalose-inhoud en streskondisies het daartoe gelei dat ons die moontlikheid van stres-gereguleerde uitdrukking van die *TPS1* geen ondersoek het. Stresbeheer-elemente (STRE) is reeds in ander stres-verwante gene geïdentifiseer waar dit stres-geïnduseerde uitdrukking bemiddel. Ses moontlike STRE-elemente is in die promotor van die *TPS1* geen geïdentifiseer. Elektroforetiese mobiliteits-eksperimente en kompetisie-studies is aangewend om te toon dat nukluêre proteïene spesifiek aan die STRE-elemente in die *TPS1* geenpromotor bind. Mutasie-analise van hierdie elemente het verder getoon dat die STRE-element naaste aan die ATG (STRE1), 'n prominente rol in die regulering van die geen speel. Hierdie resultate verduidelik egter nie die verskeie fenotipes wat vir die *TPS1* mutant waargeneem is nie. Verdere navorsing word benodig om die verskillende aspekte van die regulatoriese meganismes betrokke in die uitdrukking van die *TPS1* geen te verklaar.

BIOGRAPHICAL SKETCH

Georgia Gladys Bihl was born in Johannesburg, South Africa, on February 19, 1971. She matriculated in 1989 from Silver Oaks Senior Secondary High School. In 1990 she enrolled at the University of Stellenbosch where she obtained a B.Sc. degree in Microbiology and Biochemistry and an Hons. B.Sc. degree in Microbiology in 1995.

In 1995, she enrolled for a M.Sc. degree in Microbiology at the same university. During the course of her Masters degree she spent ten months in Prof. Johan M. Thevelein's laboratory in Belgium where she studied "The regulatory mechanisms controlling the *TPS1* gene" as part of her Masters degree.

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CONTENTS

GENERAL INTRODUCTION

All the genes encoding enzymes in the trehalose biosynthetic pathway in the yeast *Saccharomyces cerevisiae* have been cloned and characterised (Bell *et al.*, 1992; De Virgilio *et al.*, 1993; Londesborough and Vuorio, 1993; Panek *et al.*, 1987; Vandercammen *et al.*, 1989; Vuorio *et al.*, 1993). Synthesis of trehalose occurs in two steps and is catalysed by the trehalose-6-phosphate (Tre6P) synthase/phosphatase complex. This complex is composed of four subunits encoded by the *TPS1*, *TPS2*, *TPS3* and *TSL1* genes. Tre6P synthase encoded by the *TPS1* gene catalyses the first reaction while Tre6P phosphatase encoded by *TPS2* catalyses the dephosphorylation of Tre-6-P. The precise function of the *TSL1* and *TPS3* gene products in trehalose synthesis has not yet been elucidated (Reinders *et al.*, 1997).

Several point mutations in the *TPS1* gene have shown similar, but not identical, pleiotropic phenotypes. Besides the expected absence of trehalose synthesis, mutants no longer grow on fermentable carbon sources such as glucose, fructose and maltose. In addition, the mutant cells loose their sporulation capacity and glucose - induced signalling is also absent (Gonzalez *et al.*, 1992; Hohmann *et al.*, 1992). These phenotypes imply additional functions for either the *TPS1* gene or the trehalose biosynthesis pathway as a whole, which prompted us to investigate the regulatory mechanisms controlling expression of this gene. One of the functions assigned to trehalose is that of a stress protectant and Hazell and co-workers (1995) have shown evidence that the *TPS1* gene could modulate stress response positively and independently of trehalose synthesis.

When transcriptional regulation of the *TPS1* gene was first investigated by Winderickx *et al.* (1996), initial results showed a discrepancy between data obtained by northern blot analysis and β-galactosidase activity assays of the reporter gene fused to the *TPS1* promoter. When northern blot analyses under glucose shift conditions were carried out, expression of the *TPS1* gene was transiently repressed while the β-galactosidase assays showed complete repression under the same conditions. Possible explanations could be that

cis-acting elements other than in the upstream promoter, perhaps sequences within the open reading frame, regulate expression of this gene. In addition, the transient repression could indicate the existence of some form of autoregulation.

Six putative stress response regulatory elements (STREs) in the *TPS1* promoter have been identified. The STRE element consists of a consensus sequence CCCCT and is present in the promoters of several stress-induced genes where it confers stress-induced expression (Kobayashi and McEntee, 1993; Marchler *et al.*, 1993; Schüller *et al.*, 1994). Interestingly, the *TPS2, TPS3* and *TSL1* genes also contain STRE elements, but only the *TPS2* gene has been shown to be controlled in a STRE-dependent manner (Gounalaki and Thireos, 1994). Since trehalose is synthesized in response to stress and the levels of trehalose are partly controlled by the *TPS1* gene product under these conditions, it seemed possible that the *TPS1* gene could also be controlled in an STRE-dependent manner.

The aim of this study was to investigate the control mechanisms that play a role in the regulation of the *TPS1* gene. The following aspects were investigated:

1) Regulation of the gene by elements within the open reading frame and the possibility of an autoregulatory mechanism that regulates the *TPS1* gene.
2) STRE-dependent regulation of the *TPS1* gene.

In the following sections, different aspects concerning the function, synthesis and hydrolysis of trehalose as well as the regulation of genes in the trehalose metabolic pathway in the yeast *S. cerevisiae* will be discussed. In some instances, it has been shown that trehalose synthesis forms part of the stress response mechanism. Some aspects of general stress responses will therefore be discussed to provide an overview of how yeast senses and responds to adverse conditions.

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CHAPTER 1- LITERATURE REVIEW

Genes Involved in Trehalose Metabolism and General Stress Response in *Saccharomyces cerevisiae*

INTRODUCTION

Trehalose is a non-reducing disaccharide with a widespread occurence in the biosphere. It is present in a diversity of organisms ranging from bacteria to plants and mammals (Elbein, 1974). In the last five years extensive studies on trehalose have been done and well-documented data has shown that trehalose does not serve mainly as a storage compound as had been believed. Indeed, it has been shown that this disaccharide is involved in a number of common biological phenomena such as the acquisition of stress resistance and anabiosis and may also function as an inducer of acquired stress resistance in eukaryotes and prokaryotes (Feofilova, 1993). The work presented here focus on trehalose in yeast, but the presence and effects of trehalose in higher eukaryotes have recently received remarkable attention and I will briefly reflect on the main ideas derived from these studies.

In plants it appears that trehalose is restricted to primitive phyla of vascular plants and as a rare exception to certain resurrection plants among the angiosperms (Müller *et al.*, 1995). However, under *in vitro* conditions, trehalose seems also to be effective as a stress protectant in plants. It can reduce injuries due to freezing of plant cells in cryopreservation (Bhandal *et al.*, 1985) and the leakage of thylakoids at low temperatures (Bakaltcheva *et al.*, 1994). Whether the biosynthetic pathway for trehalose in plants is the same as in micro-organisms, still needs to be investigated. None of the corresponding enzymes in micro-organisms have been identified in plants.

An intriguing finding is that the trehalase enzyme responsible for the hydrolysis of trehalose is present in most higher plants of all major taxonomic groups (Table 1), even though plants do not commonly seem to produce or contain any trehalose. This observation was also made in mammals, which do not produce any trehalose. A trehalase from rabbit intestine has been cloned, purified and sequenced (Ruf *et al.*, 1990). Trehalase is also present in mammalian kidney (Sacktor, 1968) and in human serum (Yoshida *et al.*, 1993).

Table1. Trehalases in non-symbiotic organs of vascular plants (Müller *et al.*, 1995).

Plant Taxon	Organ or Tissue
Gymnosperms	
Picea glauca	Callus
Pinus banksiana	Callus
Angiosperms	
Dicotyledons	
Leguminosae	
Glycine max	Callus, cell culture; roots, leaves, flowers
Lathyrus odoratus	Pollen
Medicago sativa	Cell culture, roots
Phaseolus radiatus	Shoot explant
Vigna unguiculata	Callus, roots, leaves
div. other genera	Roots
Theaceae	
Camellia japonica	Pollen
Crucifera	
Arabidopsis thaliana	Callus
Brassica napus	Cell culture
Raphanus sativus	Shoot explant
Umbelliferaceae	
Daucus carota	Callus
Solanaceae	
Datura innoxia	Callus
Lycopersicon pimpinellifolium	Pollen; cell culture
Nicotiana tabacum	Callus
Solanum tuberosum	Cell culture
Convolvulaceae	
Pharbitis sp.	Cell culture
Quamoclit	Shoot explant
Phoenicea	
Asteraceae	
Helianthus annus	Cell culture
Monocotyledons	
Liliaceae	
Galtonia candicans	Pollen
Hemerocallis minor	Pollen
Lilium longiflorum	Pollen
Poaceae	
Saccharum of ficinarum	Roots, leaves, stalk tissue
Triticum aestivum	Callus
Zea mays	Cell culture
Orchidaceae	
Cymbidium sp.	Pollinia

Trehalose is a disaccharide composed of two glycosidic α-α-linked glucose residues. There are four major features by which different disaccharides can be distinguished (Mathews and van Holde, 1990): 1) The two specific sugar monomers involved. These can be of the same kind (the two glucose residues in trehalose) or they may differ (the glucose and fructose residues in sucrose). 2) The carbons involved in the linkage. Many possibilities exist, but the most common are 1→1 (as in trehalose), 1→2 (as in sucrose), 1→4 (as in lactose) and 1→6 (as in gentiobiose). 3) The order of the two monomer units if they are of different kinds. 4) The anomeric configuration of the hydroxyl group on carbon-1 of each residue. The configuration may either be α or β and has a major effect on the shape of the molecule that is recognised readily by enzymes. A schematic representation of the structure of trehalose is given in Fig. 1.

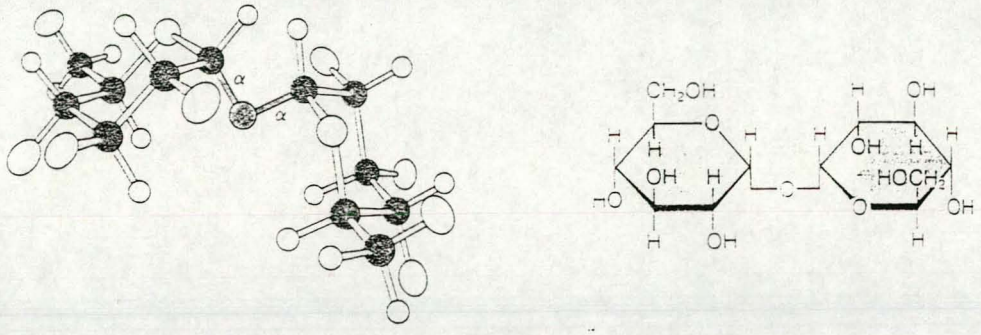

Fig. 1. Schematic representation of the trehalose molecule, showing the α-anomeric configuration of the hydroxyl group on carbon-1 (Mathwes and Van Holde, 1990).

The non-reducing (no free aldehyde group) properties of the sugar explain its stability in alkaline conditions. Trehalose is readily soluble in water and aqueous ethanol, but crystallizes in 80% ethanol as the dehydrate (Elbein, 1974). Finally, trehalose is a sweet sugar that does not cause tooth decay and can be used as a material for food and toiletries.

TREHALOSE METABOLISM

Synthesis of Trehalose

Trehalose-6-phosphate synthase phosphatase complex

In yeast trehalose is synthesized in two steps (Cabib and Lelior, 1958). In the first step, uridine diphosphate-glucose and glucose-6-phosphate combine to form trehalose-6-phosphate (Tre6P), a reaction catalysed by trehalose-6-phosphate synthase encoded by the *TPS1/GGS1* gene (Bell *et al.*, 1992; Vuorio *et al.*, 1993). The second step is catalysed by trehalose-6-phosphatase encoded by the *TPS2* gene (De Virgilio *et al.*, 1993), whereby Tre6P is dephosphorylated to form trehalose (Fig. 2).

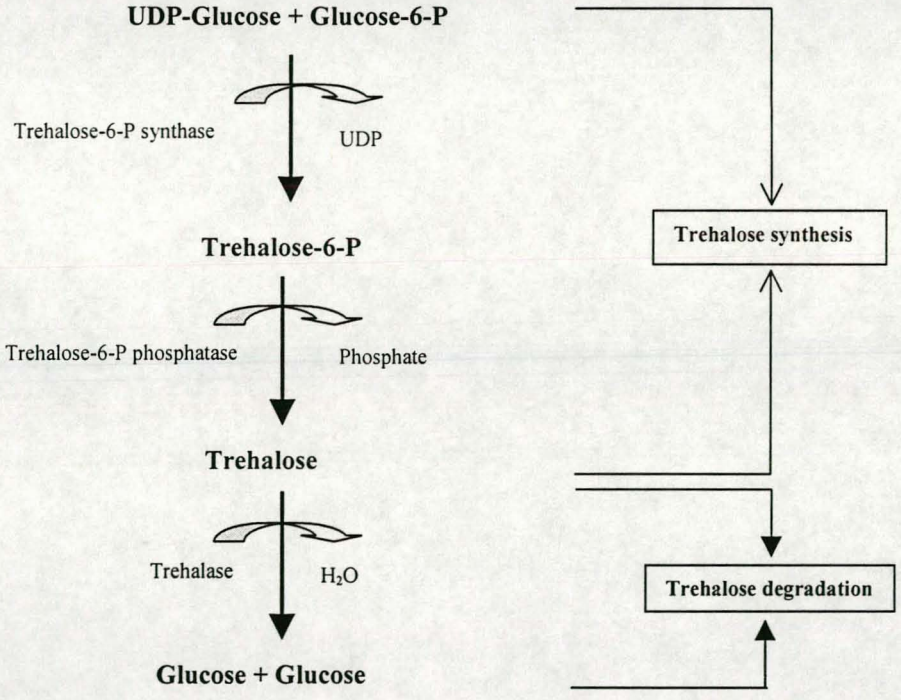

Fig. 2. Trehalose Metabolism. UDP, uridine-diphosphate; P, phosphate (Müller *et al.*, 1995).

The two enzymes, trehalose-6-phospate synthase and trehalose-6-phosphatase, have been purified by several groups and shown to form a complex with another protein encoded by the *TSL1* gene (for Tre6P synthase long chain) (Bell *et al.*, 1992; Londesborough and Vuorio, 1991;

Vandercammen *et al.*, 1989). Due to a 55% homology between a gene called *TPS3,* to the *TSL1* gene, it has been proposed that the *TPS3* gene product may constitute a fourth subunit of the complex. The intact purified protein complex has a molecular weight of about 630-800 kDa. All the genes involved in trehalose metabolism have been cloned and sequenced. The *TPS1* gene encodes a 56 kDa subunit (Bell *et al.*, 1992), the *TPS2* gene encodes a 102 kDa subunit (De Virgilio *et al.*, 1993) and *TSL1* encodes the largest subunit of 123 kDa which probably acts as regulatory subunit (Londesborough and Vuorio, 1993).

Trehalose-6-phosphate synthase and the *TPS1* gene. Firstly, it has been shown that *tps1*Δ strains lack any detectable Tre6P synthase activity (Bell *et al.*, 1992). Secondly, expression of the *TPS1* gene in *Escherichia coli* and in yeast resulted in greatly increased Tre6P synthase activity (Vuorio *et al.*, 1993). Thirdly, the *TPS1* gene complemented an *E. coli otsA* mutant that was unable to synthesize trehalose (McDougall *et al.*, 1993). Finally, it has also been shown that the expression of the *TPS1* gene in transgenic tobacco plants led to the synthesis of trehalose (Holmström *et al.*, 1996).

The *TPS1* gene was cloned independently by complementation of *fdp1*, *byp1, glc6, sst1* and *cif1* mutants encoding fructose-1, 6-bisphosphatase, phosphofructokinase, glycogen synthase, trehalose-6-phosphate synthase and catabolite-inactivation of fructose-1,6-bisphosphatase, respectively, which show a specific growth defect on rapidly fermentable sugars like glucose, fructose, and mannose (Cannon *et al.*, 1994; González *et al.*, 1992; Hohmann *et al.*, 1992; Thevelein, 1992; Van Aelst *et al.*, 1993). Since the *CIF1* gene, was reported as being essential for growth on glucose in the yeast *S. cerevisiae* (González *et al.*, 1992) and is identical to the *TPS1* gene, a second function for the latter as a general glucose sensor (GGS) has been proposed by Thevelein and Hohmann (1995). The *TPS1* gene is therefore also referred to as the *GGS1* gene. Three different models for this unexpected role of Tps1p (Ggs1p in this context) in the control of glycolytic influx have been proposed and will be discussed in detail later in the review.

Trehalose-6-phoshate phosphatase and the *TPS2* gene. The Tps2p carries the Tre6P phosphatase activity (De Virgilio *et al.*, 1993). By disrupting the *TPS2* gene, it has been shown that Tre6P phosphatase activity is lost and accumulation of high amounts of Tre6P occurs as long as a functional *TPS1* gene is present. This confirmed that *TPS2* encodes Tre6P phosphatase.

***TSL1* and *TPS3*.** Whereas it is clear that *TPS1* and *TPS2* encode Tre6P synthase and Tre6P phosphatase, respectively, the role of the *TSL1* gene product with respect to trehalose synthesis is not yet understood. Vuorio and co-workers (1993) have reported that partial degradation of Tsl1p reduced the phosphate-mediated inhibition as well as the fructose-6-phosphate-mediated activation of Tre6P synthase. It therefore seems that Tsl1p may be involved in modification of the kinetic properties of the Tre6P synthase activity. Recent studies on both *TPS3* and *TSL1* expression levels suggested that Tsl1p and Tps3p may act to some extent as interchangeable regulators of the Tre6P synthase/phosphatase complex. Nevertheless, they may also exert different regulation under specific growth conditions (Winderickx *et al.*, 1996), which will be discussed in a different section of the review. Reinders *et al.* (1997) recently showed that both Tsl1p and Tps3p can interact with Tps1p and Tps2p *in vivo,* while the latter two proteins also interact with each other. In order to clarify the specific function of these two proteins, more research needs to be done.

Biochemical characteristics of Tps1p and Tps2p

Trehalose-6-phosphate synthase has a K_m of 0.5 mM for UDP-glucose and of 3.5 mM for glucose-6-phosphate. The trehalose-6-phosphatase requires Mg^{2+} for activity and has a K_m of approximately 0.2 mM for trehalose-6-phosphate at its optimal pH of 6.0 (Vandercammen *et al.*, 1989). The intermediate of trehalose synthesis, trehalose-6-phosphate, is hardly detectable in wild-type cells and might be channelled inside the complex rather than being released into the cytosol.

Trehalose Catabolism

In fungi trehalose accumulates under adverse conditions like heat stress, desiccation, sporulation (Hottiger *et al.*, 1987a; Van Laere, 1989; Wiemken, 1990) and during periods of reduced growth in nutrient starvation conditions (Thevelein, 1984). Trehalose is rapidly mobilised by the trehalase enzyme upon resumption of growth after addition of the lacking nutrients to the starved yeast culture during the first steps in germination (Thevelein, 1984).

It has been shown that trehalases are the only enzymes responsible for trehalose breakdown in fungi (Thevelein, 1984). The biological function of the trehalases is to assist in the control of the trehalose concentration via degradation of trehalose. To date, three different trehalase genes have been identified in the yeast *S. cerevisiae*. The gene products of only two are capable of hydrolysing trehalose; no trehalase activity has been reported for the third gene product. The *S. cerevisiae* trehalases have distinct subcellular locations in the vacuoles and cytoplasm.

Trehalases have distinct names according to the optimum pH where they are functional. The trehalase which functions at an optimum pH of 6.7 to 7 is called neutral trehalase, while the acid trehalase functions at an optimum pH of 4.5 to 5.0 in the vacuoles (Londesborough and Varimo, 1984). It is interesting to note that the presence of both types of trehalases has also been reported for *Candida utilis*, *Schizosaccharomyces pombe* and *Kluyveromyces lactis* (Amaral *et al.*, 1997; Argüelles and Gacto, 1986; Carrillo *et al.*, 1992; De Virgilio *et al.*, 1991; Mittenbühler and Holzer, 1988). San-Miguel and Argüelles (1994) reported that the acid and neutral trehalases showed an opposite pattern of activity during the growth phase of the yeast *S. cerevisiae*. The activity of the neutral trehalase is high when cells are growing exponentially on fermentable sugars and decreases rapidly as cells enter respiratory and stationary phases (Lewis *et al.*, 1993; San-Miguel and Argüelles, 1994). The activity of the acid trehalase is only detected when cells enter respiratory and stationary phases or when grown on non-fermentable carbon sources such as ethanol and glycerol (San-Miguel and Argüelles,

1994). Therefore, high activity of acid trehalase corresponds to stages of the yeast life cycle when its substrate (trehalose) accumulates to appreciable levels, while the neutral trehalase is only active during the exponential growth phase when trehalose levels are low.

Neutral trehalases

***NTH1* gene.** The neutral trehalase in *S. cerevisiae* is encoded by the *NTH1* gene located on chromosome IV near the centromere (Kopp *et al.*, 1993, 1994). The native subunit of the neutral trehalase in the cytoplasm has a molecular weight of 86 kDa and the active enzyme is usually found as a dimer or a tetramer. It has a low heat stability and is the key enzyme involved in trehalose breakdown in the cytoplasm. Furthermore, neutral trehalase is specific for trehalose with an apparent K_m between 4.8 and 40 mM. It mainly requires the presence of Ca^{2+} or Mn^{2+} and is strongly inhibited by Zn^{2+} (Londesborough and Varimo, 1984). Kopp and co-workers (1994) reported two possible cAMP-dependent phosphorylation sites in Nth1p and it has been shown that its activity is regulated by a cAMP-dependent phosphorylation/dephosphorylation mechanism. It is therefore referred to as the regulatory trehalase.

***NTH2* gene.** A second neutral trehalase encoded by a gene called *YBR0106* (also referred to as *NTH2*) was identified by Wolfe and Lohan (1994). The *NTH2* gene is located on chromosome II of *S. cerevisiae*. The Nth2p shares 77% identity with the predicted amino acid sequence of the Nth1p. Even though the *NTH2* gene is expressed, it is responsible for neither neutral nor acid trehalase activity and it does not show any detectable influence on the trehalose concentration in intact cells (Nwaka *et al.*, 1995a, b). More research needs to be done to unravel the specific function of the Nth2p.

Common functions of *NTH1* and *NTH2*. Nwaka and co-workers (1995b) showed that the *NTH1* and *YBR0106* (*NTH2*) genes are necessary for the recovery of cells after heat shock. They showed a 10-fold and 3-fold increase in the expression of the *NTH1* and *NTH2* genes respectively, under heat shock conditions. This 'heat shock protein function' is consistent with the

inducibility of the neutral trehalase genes by heat stress (Nwaka *et al.*, 1995a, b). In addition the *NTH1* contains three STRE elements in its promoter while the *NTH2* gene contains four as well as a single heat shock element. STRE element refers to stress-response-element it consists of a consensus sequence of CCCCT (Gounalaki and Thireos, 1994; Kobayashi and McEntee, 1993; Machler *et al.*, 1993). The same element regulates the stress-induced expression of the *CTT1*, *DDR2* and the *TPS2* genes, respectively (discussed in following sections). The presence of these motifs in the *NTH1* and *NTH2* promoters may explain the finding of heat stress-induced expression of these genes. However, the acid trehalase gene does not contain STREs in its promoter (Nwaka *et al.*, 1995b). In 1992, Hottiger and co-workers proposed a possible interaction between neutral trehalase and the heat shock protein Hsp70p under heat shock conditions as a mechanism that protects neutral trehalase from thermal denaturation and misfolding. It therefore seems likely that the interaction of the trehalases with heat shock proteins is part of the stress response mechanism (Nwaka *et al.*, 1995a).

Acid trehalase

***ATH1* gene.** The *ATH1* gene encoding the acid trehalase from *S. cerevisiae,* has been cloned and sequenced (Destruelle *et al.*, 1995). This gene does not show any homology to any of the known trehalases and does not contain a consensus signal sequence cleavage site for vacuolar targeting. This made it unclear whether the *ATH1* gene was indeed the structural gene for acid trehalase and whether it encoded a regulatory factor. Nevertheless, Alizadeh and Klionsky (1996) confirmed that the *ATH1* gene is indeed the structural gene for the acid trehalase.

The acid trehalase from bakers' yeast has been purified and characterised (Londesborough and Varimo, 1984). The enzyme has an apparent K_m between 1.4 and 4.7 mM and seems to function independently of divalent cations. The active acid trehalase is a glycoprotein that is synthesized as an inactive precursor of 41 kDa that has to be processed proteolytically. The mature enzyme has a Mr of 220 kDa with a carbohydrate content of 86%. It

has a high heat stability, but little is known about the regulation of the acid trehalase. Since the acid trehalase is located in the vacuole and trehalose (its substrate) in the cytosol, Thevelein (1984) proposed decompartmentation of the enzyme and its substrate as the main (possible) regulatory mechanism. In addition, catabolite repression was proposed as a possible regulatory mechanism (Mittenbühler and Holzer, 1988), and will be discussed in more detail in the next section.

Role of acid trehalase. The specific function of the acid trehalase was not known until recently. The discovery that the yeast *S. cerevisiae* is able to utilise trehalose as a carbon source similar to *E. coli*, raised the question on which of the three known trehalases is responsible for growth on trehalose. Nwaka *et al.* (1996) showed that an *ath1*Δ deletion mutant does not grow on exogenous trehalose, unlike the neutral trehalase deletion mutants *nth1*Δ and *nth2*Δ. This strongly indicates a role for acid trehalase in a pathway for trehalose utilisation independent of the neutral trehalases. It therefore seems that the neutral trehalase is responsible for the hydrolysis of the endogenous cytosolic trehalose pool while the acid trehalase is responsible for hydrolysis of the external pool of trehalose in the vacuole.

In addition, it has been shown that a deletion mutation in acid trehalase confers a higher stress resistance especially with respect to dehydration, freezing and ethanol shock (Kim *et al.*, 1996). The improved stress resistance and growth exhibited by the *ath1Δ* deletion strain strongly correlates with its ability to accumulate higher levels of trehalose in both rich and minimal media. Although this phenotype has great industrial applications in the brewing, baking and wine industries, as well as for the production of fuel, more work still needs to be done to clarify and optimise the various parameters.

Acid trehalase and the trehalose transporter. A trehalose-specific transporter or carrier has been described for *S. cerevisiae* (Eleutherio *et al.*, 1993a). Similar to the acid trehalase mutant the trehalose carrier mutant is unable to grow on trehalose as a carbon source. When the trehalose carrier gene is mutated it has a low level of expression in exponentially growing cells

on glucose due to glucose repression and a high level of expression in stationary phase when the trehalose level in the cell is high (Crowe *et al.*, 1991). Since the trehalose carrier exhibits the same characteristics as the acid trehalase with regards to the metabolic regulation, a similar function for both has been (Nwaka *et al.*, 1996).

REGULATION OF TREHALOSE MOBILISATION

The regulation of trehalose mobilisation centres mainly on the regulation of the trehalases. As previously mentioned, there are two types of trehalases: a regulatory (neutral) and a non-regulatory (acid) trehalase.

Regulation via Acid Trehalase

Although the regulatory pathway controlling acid trehalase activity is largely unknown, it has been suggested that trehalose mobilisation is triggered by decompartmentation (Thevelein, 1984), because the acid trehalase and its substrate trehalose have different localisations in the vacuole and the cytosol, respectively. Thevelein (1984) suggested that the initiation of trehalose mobilisation by decompartmentation could occur by breakdown of a permeability barrier between the acid trehalase and trehalose. Gupta and co-workers (1987) also suggested decompartmentation as a possible mechanism for rapid trehalose mobilisation by acid trehalase during early spore germination in *Dictyostelium discoideum.* In addition, it has been proposed that acid trehalase is also catabolically repressed by glucose like other vacuolar hydrolases (San-Miguel and Argüelles, 1994). Results obtained by this group showed that no acid trehalase activity could be detected on media containing glucose or any other rapidly fermentable carbon source.

Regulation via Neutral Trehalase

For the neutral trehalase, phosphorylation/dephosphorylation by protein kinase A was proposed as the main triggering mechanism for trehalose

mobilisation *in vivo*. *In vitro* activation of neutral trehalase by cAMP-dependent protein kinase is well established in *S. cerevisiae* (App and Holzer, 1989; Uno *et al.*, 1983). *In vivo* changes of trehalase activity under different conditions such as nutrient starvation (Coutinho *et al.*, 1992; Durnez *et al.*, 1994), heat-induced activation of trehalase (De Virgilio *et al.*, 1991) and the drop in activity during the diauxic shift (Coutinho *et al.*, 1992) seems to be mediated by phosphorylation/dephosphorylation mechanisms. Although *in vivo* phosphorylation of neutral trehalase has never been demonstrated, the availability of the neutral trehalase gene *NTH1* allowed new approaches to obtain information on the molecular mechanism of trehalase activation, such as the identification of the two possible cAMP-dependent phosphorylation sites in the sequence of the Nth1p (Kopp *et al.*, 1994). Site-directed mutagenesis of these sites should reveal whether only one or both consensus sites are used for cAMP-dependent protein phosphorylation.

Pathways Regulating Neutral Trehalase

The regulation of trehalase through a phosphorylation/dephosphorylation mechanism could occur via two pathways: the Ras-cAMP pathway and the fermentable-growth-medium-induced pathway.

Ras-cAMP pathway. The Ras-cAMP pathway is glucose-inducible and is activated upon the addition of glucose to glucose-starved stationary phase cells, exponential phase cells growing on a non-fermentable carbon source or ascospores. The cells have to adapt their metabolism to the new nutrient supply and the Ras-cAMP pathway controls the adaptation from gluconeogenic/respirative to fermentative growth. The glucose signal is transduced through a signal transduction cascade via the RAS proteins (Boutelet *et al.*, 1985; Kataoka *et al.*, 1985; Matsumoto *et al.*, 1985), whereby protein kinase A is activated to phosphorylate its targets (Taylor *et al.*, 1990; Toda *et al.*, 1987). Trehalase, one of the potential targets of protein kinase A, would be phosphorylated and this would result in the hydrolysis of accumulated trehalose. It is important to note that only sugar phosphorylation and no further metabolism is necessary in this pathway, suggesting that the

pathway contains a glucose-repressible protein which has not yet been identified. Finally, the glucose-induced activation of trehalase is transient and absent in glucose-repressed cells.

Fermentable-growth-medium-induced (FGM) pathway. Detailed studies showed that trehalase is also activated when a missing nutrient other than a fermentable carbon source is added to starved cells e.g. nitrogen in the presence of glucose (Durnez *et al.*, 1994; Hirimburegama *et al.*, 1992; Thevelein and Beullens, 1985). Since the addition of nitrogen to nitrogen-starved cells does not induce a cAMP-signal, it was concluded that the activation of trehalase in glucose-repressed cells by other nutrients, such as nitrogen, phosphate or sulphate, is not mediated by cAMP (Durnez *et al.*, 1994; Hirimburegama *et al.*, 1992; Thevelein and Beullens, 1985). In addition, it has been observed that the addition of glucose to stationary-phase cells leads to both a transient activation of trehalase and partial mobilisation of trehalose. However, when a complete medium including a fermentable carbon source is given to the cells, trehalase is activated for a much longer time and trehalose is completely degraded (Thevelein *et al.*, 1982; Van der Plaat, 1974). The fermentable-growth-medium-induced (FGM) pathway therefore implies that the activation of trehalase by other nutrients always requires the presence of a fermentable carbon source.

Since the discovery of this pathway, no components thereof have been identified. However, a protein kinase, Sch9p, which is a homologue of the catalytic subunit of cAMP-dependent protein kinase (cAPK) was recently reported (Crauwels *et al.*, 1997). This protein is required for nitrogen activation of the FGM pathway independently of the Ras-cAMP pathway. The same group suggested that the Sch9p might be the protein kinase activated by the FGM pathway and responsible for phosphorylation of the different targets.

REGULATION OF THE GENES INVOLVED IN TREHALOSE METABOLISM

The aim of this chapter is to address various aspects of the regulation of the genes involved with trehalose metabolism.

The synthesis of trehalose is dependent on both the growth phase of the cells and on the amount of stress the cells are exposed to. In the previous section different conditions causing stress in yeast were mentioned. Before considering the regulation of the genes involved in trehalose metabolism, it will be interesting to see which are the different stress conditions that lead specifically to trehalose synthesis. Both the trehalose levels and the stress resistance are low during exponential growth on rapidly fermentable carbon sources such as glucose and fructose. A rapid increase in both is observed upon the exhaustion of glucose and both remain high during subsequent growth on ethanol. Both also increase strongly upon starvation of an essential nutrient such as nitrogen, phosphate or sulphate in a glucose-containing medium (Attfield *et al.*, 1992; Lillie and Pringle, 1980). The same pattern is followed during sub-lethal heat treatment (Hottiger *et al.*, 1987a, b) and osmotic shock (Singh and Norton, 1991).

The trehalose level in yeast is determined by the activity of the trehalose synthase complex and the degradative activity of trehalase. As already mentioned, the trehalose synthase complex consists of four subunits, namely Tre6P synthase, Tre6P phosphatase and the gene products of *TPS3* and *TSL1*. The Tre6P synthase activity is strongly enhanced by fructose-6-phosphate and inhibited by phosphate, while the Tre6P phosphatase requires phosphate for its activity (Londesborough and Vuorio, 1993). Phosphate is also an inhibitor of glycogen synthase and stimulates phosphofructokinase 1 and 2, thereby enhancing glycolysis. This suggests a role for phosphate in the regulation of carbohydrate metabolism in yeast, with high concentrations favouring glycolysis and low concentrations favouring synthesis of trehalose and glycogen (Vandercammen *et al.*, 1989).

In addition, trehalose biosynthesis is controlled via transcriptional and posttranslational regulation of the subunits of the synthase complex. It has been proposed that co-regulation of transcription of the four subunit-encoding genes by growth phase and stress is mediated by the STRE elements in their promoters. Similar to other genes containing STREs in their promoters, such as *CTT1* and *SSA3,* expression of the trehalose synthase genes is induced by heat shock, osmotic stress and nutrient starvation. They are also negatively regulated by the Ras-cAMP pathway.

Interestingly, Winderickx and co-workers (1996) showed that the presence of STRE elements in the promoters of the genes encoding subunits of the synthase complex, does not necessarily imply complete co-regulation of expression. During fermentative growth only *TSL1* follows the expression pattern of STRE-controlled genes. They are expressed when cells are nutritionally starved for one of the essential nutrients, but upon glucose addition, these genes are repressed. Expression of the three other trehalose synthase genes is only transiently down-regulated on glucose media. This confirms the observation that Ggs1p/Tps1p is required for growth on fermentable carbon sources (Neves *et al.*, 1995; Thevelein and Hohmann, 1995).

Several authors reported the strong correlation between thermotolerance and trehalose accumulation in yeast cells. When non-fermentative stationary phase cells are exposed to heat shock, huge amounts of trehalose accumulate in the cytosol. Whether the rapid accumulation of trehalose is due to the increase in intracellular pools of precursors for trehalose synthesis (UDP-glucose and glucose-6-phosphate) (Winkler *et al.*, 1991) or activation of pre-existing trehalose synthase and inactivation of trehalase at higher temperatures (Londesborough and Vuorio, 1993; Neves and François, 1992) remains to be determined. Nevertheless, all these events may contribute to the induction of trehalose synthesis by fructose-6-phosphate acting as a potent activator of trehalose synthase (Londesborough and Vuorio, 1993). Although the exact mechanism for rapid trehalose heat-induction is not yet well understood, it seems reasonable to conclude that it is readily reversible,

since trehalose accumulated upon heat shock is rapidly mobilised upon a subsequent temperature downshift (De Virgilio *et al.*, 1994; Piper, 1993; Neves and François, 1992; Nwaka *et al.*, 1994; Van Dijck *et al.*, 1995). All the genes encoding subunits in trehalose metabolism are stress-inducible and are therefore active during the heat shock response in non-fermentative stationary phase cells. However, Winderickx and co-workers (1996) reported that these genes are only transiently induced in heat shocked glucose-growing cells.

There are also some indications that the induction of trehalose and heat shock proteins might be linked. Reports have shown that the *tps1Δ* mutant does not show normal levels of heat shock proteins (Hsp) synthesis upon heat shock (Hazell *et al*, 1995). However, the pleiotropic phenotype of this mutant does not allow conclusive remarks at this stage. To further substantiate the link between heat-induction of trehalose and Hsps it has been shown that mobilisation of trehalose during a temperature shift-down is defective in strains with low Hsp70p levels. This could indicate that the induction of Hsp70p in heat shocked cells down-regulates the simultaneous trehalose induction in these cells (Hottiger *et al.*, 1992).

FUNCTIONS OF TREHALOSE

To date several functions have been assigned to trehalose, but controversies still remain. Based on the data presently available, it seems that trehalose has a dual function as a reserve carbohydrate and as a stress protectant in yeast cells.

Trehalose as Reserve Carbohydrate

For many years it has been believed that trehalose serves mainly as a reserve carbohydrate because of its presence in high amounts in spores and resting cells, its accumulation during sporulation and its mobilisation during spore

germination and resumption of growth (Elbein, 1974; Thevelein, 1984). The importance of trehalose for spore germination is clearly seen for spores that are able to germinate in distilled water, such as *Neurospora* ascospores (Sussman, 1954) and *Dictyostelium* spores (Cotter, 1975). However, the importance for spores that are able to germinate only in sugar-containing media is less obvious since the amount of glucose produced in trehalose breakdown is relatively small compared with the glucose taken up from the medium in rich germination media (Thevelein *et al.*, 1982).

Together with trehalose, glycogen is also considered to be a reserve carbohydrate and intracellular concentrations vary with the growth phase of the cell. The pattern of glycogen accumulation and utilisation correlates with the expected pattern of a source of energy. Glycogen accumulates in the late exponential phase in glucose medium when the sugar is still abundant and it is consumed during the post-diauxic phase. However, trehalose does not behave in this manner since it only accumulates after the exhaustion of glucose during the post-diauxic phase when an exogenous substrate is lacking and glycogen is slowly broken down (Lillie and Pringle, 1980). Furthermore, during sporulation glycogen is accumulated first and trehalose only later, partially at the expense of glycogen (Kane and Roth, 1974). Van Laere (1989) therefore strongly advocated the function of trehalose as a stress protection metabolite rather than an energy reserve. Wiemken (1990) even went a step further and argued for the dismissal of the idea that trehalose functions primarily as a reserve carbohydrate.

Role of Trehalose Synthesis in Controlling the Glycolytic Flux

A possible function for trehalose synthesis in the regulation of the glycolytic flux has been proposed recently (Thevelein and Hohmann, 1995) Although glycolysis was the first biochemical pathway to be discovered, the basic mechanisms for its regulation and the control of glucose influx are still not well understood. It was thought that glycolysis in yeast is regulated at the level of two irreversible steps catalysed by phosphofructokinase (PFK) and pyruvate kinase (PYK), respectively (Gancedo and Serrano,1989). Fructose 2,6-

bisphosphate (F2,6-BP) is the key allosteric effector that controls PFK under physiological conditions and is present in micromolar concentrations. Fructose 1,6-bisphosphate (F1,6-BP) is the product of the PFK reaction and an allosteric activator of PYK. F1,6-BP is a glycolytic intermediate present in millimolar concentrations. The changes in the concentrations of F2,6-BP and F1,6-BP appear to be sufficient to regulate glycolysis in response to glucose availability, and enhanced expression of PFK and PYK did not have a significant effect on glycolytic flux (Maitra and Lobo, 1971). In addition, metabolic control analysis has considered sugar uptake as a major flux-controlling step. Therefore, in an attempt to understand how glycolytic flux is controlled, deletion studies of five genes encoding putative glucose/fructose carriers, namely *SNF1*, *HXT1*, *HXT2*, *HXT3* and *HXT4* were performed. Strains with deletions of all these genes still grow on glucose, suggesting the presence of at least one, if not more, additional genes allowing glucose transport.

A subunit of the trehalose synthase complex in *S. cerevisiae*, Ggs1p/Tps1p, was implicated in the control of glucose influx into glycolysis. Deletion of the *TPS1* gene causes a pleiotropic phenotype of which one characteristic is that the cells no longer grow on glucose or any other fermentable carbon source. The addition of glucose to the *TPS1* mutants leads to an uncontrolled influx of sugar into glycolysis that results in the hyper-accumulation of sugar phoshates, depletion of ATP levels and a drastic drop in free inorganic phosphates (Pi). Due to this metabolic disorder, glycolysis arrests as a result of the absence of sugar influx and implies a role for Ggs1p/Tps1p or trehalose synthesis in controlling the influx of sugars into glycolysis.

Models for the role of Tps1p in glycolytic influx

Three models have been proposed to explain the role of the *TPS1* gene product in sugar influx into glycolysis.

The first model suggests that, apart from Ggs1/Tps1p being a subunit in trehalose biosynthesis, it forms part of a "general glucose-sensing complex." The complex consists of a sugar transporter, a sugar kinase and the

Ggs1/Tps1 protein. In this model, Ggs1p/Tps1p is localised within this complex and directly inhibits the hexokinase activity with greater efficacy than the free molecule. Furthermore, this general glucose sensing complex has two functions: 1) the transporter and the kinase interact and restrict glucose influx into glycolysis and thereby restrain the flux through glycolysis; 2) the entire complex acts as the initial trigger for several glucose-induced signalling pathways (Fig. 3) (Thevelein, 1992). It has been shown by Hohmann and co-workers (1993) that disruption of hexokinase PII encoded by the *HXK2* gene, restores growth on glucose of the *TPS1* mutant as well as several glucose-induced signalling phenomena. A function for the *TPS1* gene product in glucose signalling therefore seems unlikely, but cannot be completely excluded since a *hxk2Δ* mutation abolishes glucose repression. It is therefore not possible to test glucose repression in the *hxk2Δ* genetic background.

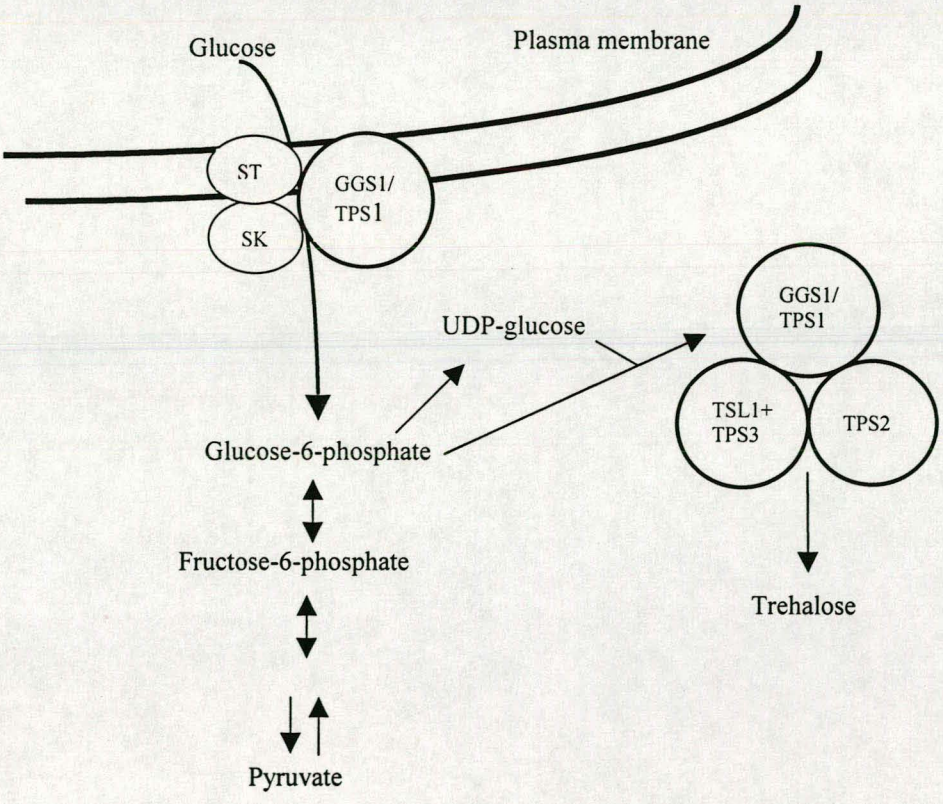

Fig. 3. In model 1 it is proposed that the *TPS1* gene has two functions: (1) as a subunit of the trehalose synthase complex, and (2) it forms part of a glucose sensing complex where it plays a role in controlling glucose transport and phosphorylation. SK, sugar kinase; ST, sugar transporter (Thevelein and Hohmann, 1995).

In the second and third model a direct link between trehalose synthesis and the control of glucose influx was proposed. In the second model trehalose synthesis serves to recover free phosphate upon the addition of glucose to derepressed wild type cells (Fig. 4).

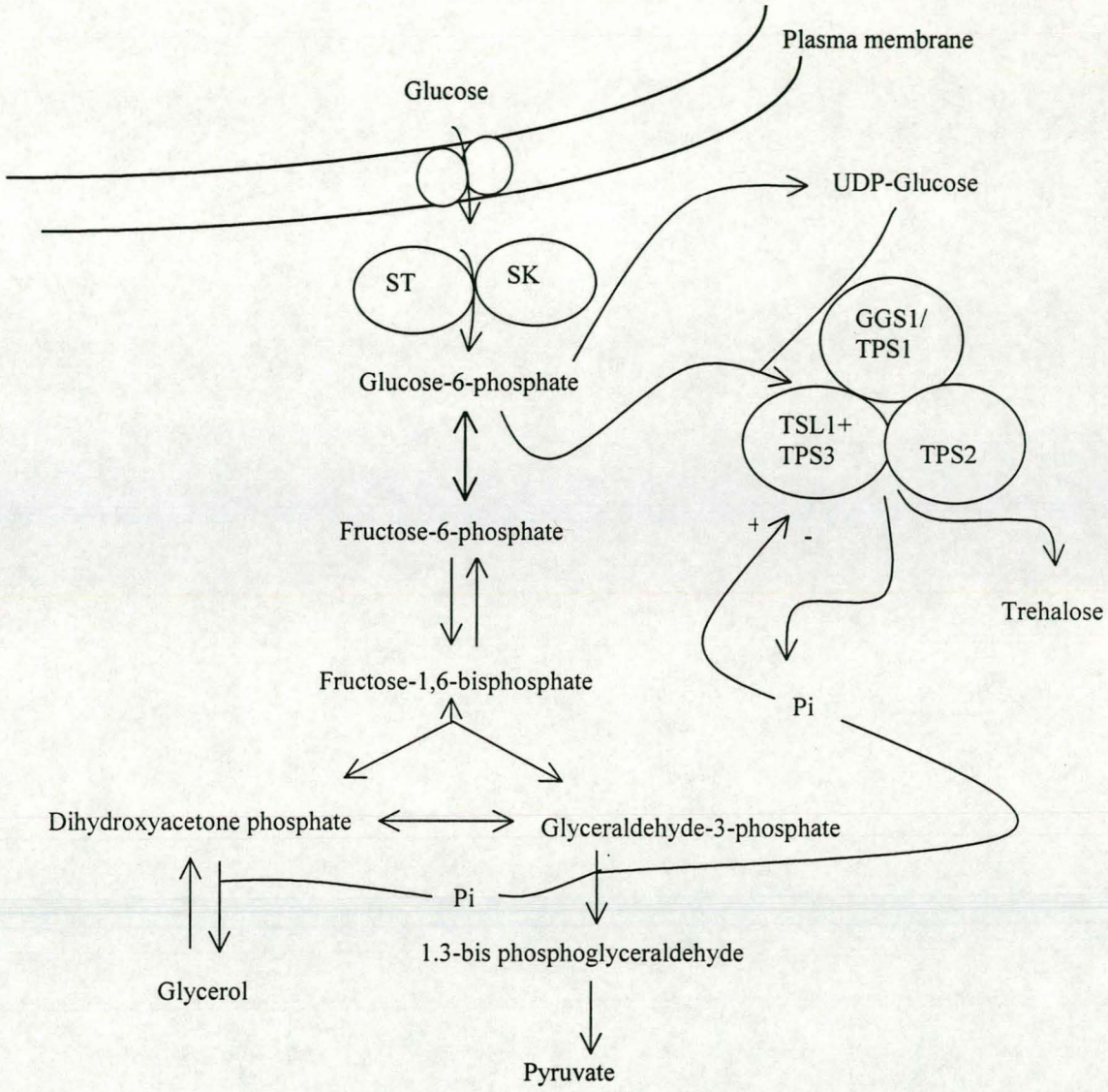

Fig. 4. Model II for *TPS1* function. It controls glucose influx into glycolysis through its function in trehalose synthesis, therefore trehalose synthesis serves as a metabolic buffer system when glycolysis overflows and inorganic phosphate drops. UDP-glucose, uridine diphosphate glucose (Thevelein and Hohmann, 1995).

Glucose uptake and phosphorylation apparently occur at a very fast rate, leading to a fast transient accumulation of sugar phosphates. This model proposes that the excess glucose-6-phosphate is channelled rapidly into trehalose synthesis and free phosphate is released which is required as a substrate for glyceraldehyde-3-phosphate dehydrogenase downstream in the glycolytic pathway. Trehalose synthesis therefore serves as a metabolic buffer controlling the intracellular inorganic phosphate level that is essential for normal flux through glycolysis (Hohmann, 1993). In the *TPS1* deletion mutant that lacks trehalose-6-phosphate synthesis, sugar phosphates accumulate continuously and the free phosphate level drops and disturbs the normal flux through glycolysis.

The third model (Fig. 5) is based on *in vitro* results showing that *TPS1* is required for the control of glucose influx into glycolysis through the synthesis of trehalose-6-phoshate that directly inhibits hexokinase activity (Blázquez *et al.*, 1993). Results obtained with the *tps2Δ* and *byp1-3 tps2Δ* mutants affected in trehalose-6-phosphate metabolism showed that trehalose-6-phosphate only plays a role in the initial adaptation to fermentative growth and that other mechanisms regulate the influx of glucose during logarithmic growth on glucose (Hohmann *et al.*, 1996). However, no unequivocal *in vivo* evidence for a role of trehalose-6-phosphate in the control of glycolysis has yet been found.

Another aspect regarding this model is the competitive nature of inhibition of hexokinase by Tre-6-P. A consequence of competitive inhibition is that Tre-6-P should be out-competed by increasing sugar concentrations that are expected to occur during partial inhibition of the hexokinases. This effect should partially be relevant during heat shock when internal glucose concentrations rise, but not those of Tre-6-P. However, no deregulation of glycolysis during heat shock occurs (Neves and François, 1992). According to these observations an important question concerning the significance of inhibition of hexokinase by Tre-6-P is how much sugar or Tre-6-P is actually exposed to the hexokinase (Hohmann *et al.*, 1996).

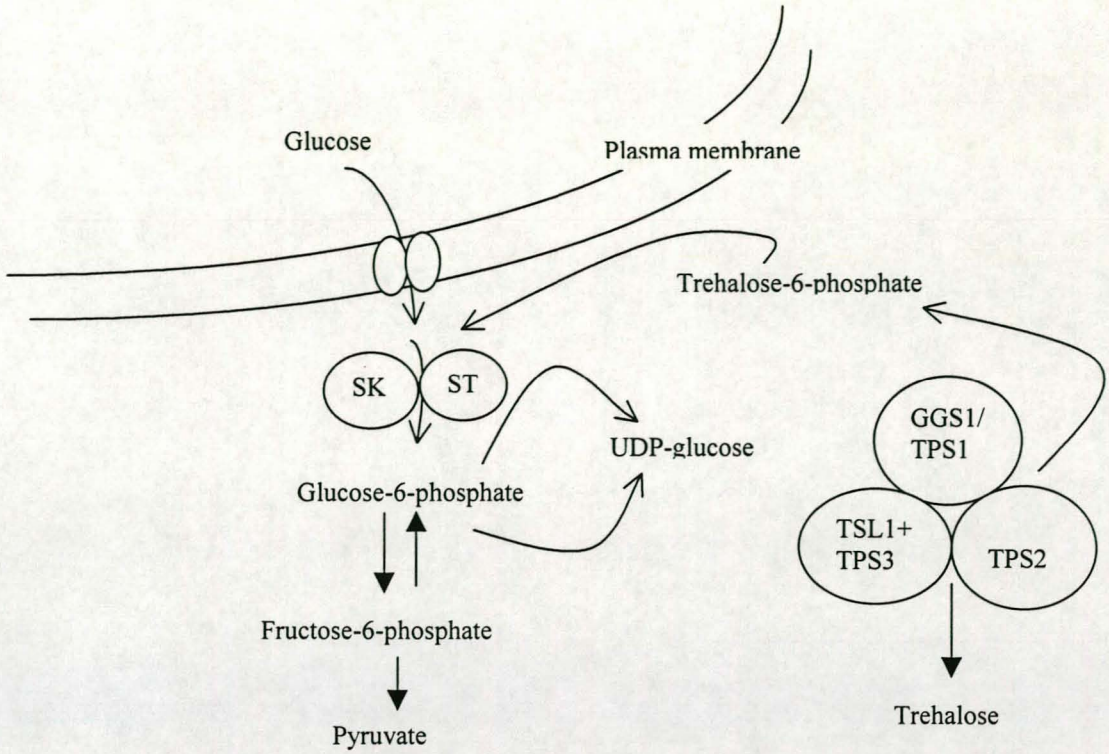

Fig. 5. Model III for the function of *TPS1* gene. Glucose influx into glycolysis is controlled by *TPS1* encoding trehalose-6-phosphate synthase that directly inhibits hexokinase activity. UDP, uridine diphosphate glucose (Thevelein and Hohmann, 1995).

Trehalose as Stress Protectant

As already mentioned, the role of trehalose as a stress protectant has been strongly advocated by some authors because of the high levels found in many survival forms of fungi, nematodes, insects and plants. These survival forms include stationary phase cells, spores and sclerotia, cysts, and dried desert resurrection plants (Thevelein, 1984). In addition, many *in vitro* and *in vivo* studies have indeed shown a close correlation between trehalose levels and stress resistance (De Virgilio *et al.*, 1994; Eleutherio, 1993b). A correlation between the intracellular trehalose content and thermotolerance has been found, not only in *S. cerevisiae* (De Virgilio *et al.*, 1991; Hottiger *et al.*, 1987a, 1992), but also in other yeasts such as *Schizosaccharomyces pombe* (De Virgilio *et al.,* 1990). It has been shown that other organisms such as *Dictyostelium discoideum* (Killick and Wright, 1972) and *Neurospora crassa* (Neves *et al.*, 1991) show similar patterns. Therefore, it has been proposed that the primary function of trehalose in yeast and other fungi, nematodes and

insects is not that of an energy source, but rather that of a stress protectant. It has further been shown that trehalose protects the cells of *S. cerevisiae* under stresses such as freezing, dessication and exposure to toxic chemicals (Van Laere, 1989).

Although evidence points towards trehalose serving as a stress protectant, it is not yet well understood how this occurs on the molecular level. Some data have indicated that trehalose accumulation may increase thermotolerance of yeast proteins by enhancing protein stability in intact cells (Hottiger *et al.*, 1987a). It could also act as a membrane-protecting agent during environmental stress conditions such as dehydration (Crowe *et al.*, 1983; Crowe *et al.*, 1984; Eleutherio *et al.*, 1993a; Gadd *et al.*, 1987; Leslie *et al.*, 1995) and influence the strength of hydrogen bonding interactions in the membrane. It has been shown that for trehalose to confer stress resistance to yeast cells, it has to be present on both sides of the plasma membrane. This is accomplished by the trehalose specific transporter in the plasma membrane that translocates trehalose from the cytosol to the extracellular environment (Crowe *et al.*, 1991; De Araujo *et al.*, 1991; Eleutherio *et al.*, 1993b). The trehalose transporter is usually synthesized in response to the exhaustion of fermentable sugars in the growth medium which coincides with an increase in cellular trehalose levels. (Crowe *et al.*, 1991; De Araujo *et al.*, 1991). The trehalose transporter seems to be important in stress resistance response since mutants in transport activity showed significantly lower stress resistance (Eleutherio *et al.*, 1993a).

Two hypotheses were proposed to illustrate the function of trehalose as a stress protectant: the water replacement hypothesis and the glass transition hypothesis. The water replacement hypothesis suggests that trehalose replaces water molecules which are hydrogen-bonded to the surface of biological macromolecules and are essential for maintaining the structure of the molecule (Crowe *et al.*, 1984). The protective effect of trehalose upon dehydration could also be ascribed to the tendency of trehalose solutions to undergo glass rather than crystal formation, which would prevent distortion of the protein structure (Burke, 1985). However, these hypotheses are not a

straightforward explanation for the stress protectant function of trehalose compared to other molecules with similar structures such as glucose, maltose and sucrose (Crowe *et al.,* 1990). Trehalose does possess a few physical properties that might give it a unique functionality different from other sugars. These properties include a high hydrophilicity, a non-hygroscopic glass formation, a very high chemical stability, a non-reducing character and the absence of internal hydrogen bond formation that results in unusual flexibility of the disaccharide bond.

Although much evidence exists to substantiate the role of trehalose as a stress protectant, one should note that this is not the only protective agent against heating or other severe conditions. Since other factors are also required. An example is the well-studied heat shock proteins that are strongly induced under heat stresses. Results have shown that mutants deficient in *HSP104* do not exhibit thermotolerance even though the accumulation of trehalose was as high as in the wild-type cells (Winkler *et al.*, 1991). Hsp12p and Hsp70p also seem to have a direct role in protecting the cells under heat stress conditions. In addition, different lines of evidence suggest a protective co-operation between some Hsps and trehalose: (i) trehalose accumulation occurs in parallel with Hsp synthesis in mutants altered in the Ras-cAMP pathway (Hottiger *et al.*, 1989), (ii) the expression level of several Hsps negatively modulates the actual content of trehalose during the heat stress and/or heat shock recovery (Hottiger *et al.*, 1992; Piper, 1993) and (iii) the *TPS1* and *TPS2* genes belong to the group of Hsps (Bell *et al.*, 1992; De Virgilio *et al.*, 1994; Gonzalez *et al.*, 1992).

Elliot and co-workers (1996) reported synergy between trehalose and Hsp104p for thermotolerance in the yeast *S. cerevisiae*. *In vitro* evidence suggests that trehalose acts as a thermoprotectant by stabilising proteins and preventing heat inactivation (Colaço *et al.*, 1992; Hottiger *et al.*, 1992), while the Hsp104p repairs heat-damaged proteins by resolubilizing the insoluble aggregates (Parsell *et al.*, 1994). The exposure of cells to certain chemicals such as exposure to N^6-(2-isopentenyl) adenosine (Coote *et al.*, 1992) and salt induces thermotolerance, but has little or no effect on trehalose.

As mentioned above, trehalose as stress protectant is also dependent on other mechanisms. Trehalose therefore forms only one part of the entire mechanism in yeast that confers stress resistance. The next section will give a broad overview on conditions that cause stress in yeast and will describe two signalling pathways that sense and react in response to the applied stress. The transcriptional control elements activated by stress conditions will also be discussed.

GENERAL STRESS SIGNALLING IN YEAST

Like all living cells in nature, yeast cells are constantly exposed to several adverse conditions including many different environmental or metabolic stresses. Factors and conditions known to cause stress in yeast include: metabolic stresses, nutritional starvation, oxygen and oxygen metabolites, high and low temperature, heavy metals, high and low osmolarity, high or low pH, DNA-damaging agents, high ethanol concentrations and dessication (Müller, *et al.*, 1995).

For survival in nature, living organisms have an intrinsic property to sense and properly respond to life threatening changes. Depending on the type of stress, different components are damaged and different mechanisms affected. All cellular components can be targets for damage. Mechanisms affected include gene expression, metabolism, cell growth, cell cycle and cell polarity (Hohmann and Mager, 1996). Yeasts have an intrinsic property of acquired stress resistance that allows the cells to withstand a severe stress condition more easily if they were previously exposed to a mild form of the same stress. In addition to acquired stress, another phenomenon observed in yeast cells is cross protection where yeast cells develop not only tolerance against a specific stress, but also against other agents. Therefore, it seems that certain aspects of the stress responses in yeast cells are shared, resulting in a certain level of cross protection. This strongly suggests the existence of an integrating mechanism that is sensing and responding to different forms of stress.

Another important aspect of stress response in yeast cells is the specific responses that include both early and late responses. A schematic representation of the early and late responses involved in mediating stresses is shown in Fig. 6.

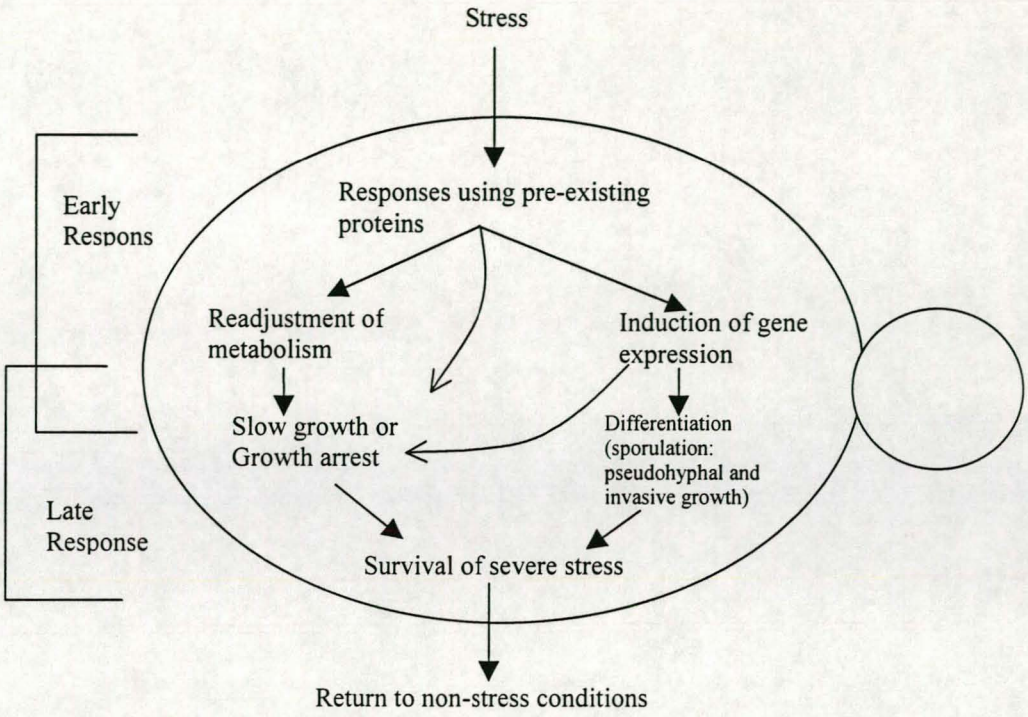

Fig. 6. Hierarchy of yeast stress responses showing early and late responses and the overlap of these responses. The overlapping region of the late and early responses means that these specific responses may occur either early or late, or both (Hohmann and Mager, 1996).

The early responses do not require the synthesis of new proteins, but involve the modulation of pre-existing proteins. Some of these changes include metabolic flux and membrane transport. In turn, these changes may require the activation of signal transduction pathways by stress signals. The early responses serve as a basis for the late responses and may lead to the induced expression of genes required for mediating the different stress responses (Schüller *et al.*, 1994). One example of the early response could be trehalose synthesis. Trehalose could provide minimal protection against sudden stress and could initiate late responses, for example the synthesis of heat shock proteins that will protect cells more permanently and effectively by allowing an adaptation to persistent stress (Ruis and Schüller, 1995).

The combination of the early and late responses therefore enables cells to adapt and resume growth under mild stressful conditions and to survive under more severe conditions. This phenomenon is known as induced stress resistance. How these early and late responses that mediate protein-regulated changes of gene transcription under stress work together, remains to be answered. In the next chapter the signalling mechanisms in yeast will be discussed.

Signal Transduction Pathways

Although the transcriptional regulation of genes involved in stress response has been studied extensively in yeast and other organisms (Mager and Moradas Ferreira, 1993) the mechanisms that sense and mediate different forms of stress are largely unknown. Two types of signalling pathways have nevertheless been identified: the Ras-cAMP pathway that is known to control responses to nutrient starvation and stationary phase entry of yeast cells, and the MAP (mitogen activated protein)-kinase pathways. The Ras-cAMP pathway negatively regulates the stress responsive elements (STREs) in promoters of stress-induced genes (Machler *et al.*, 1993; Wieser *et al.*, 1991). The MAP kinase pathway is stimulated by osmotic stress. Five different MAP kinase pathways have been identified, including the mating pheromone response pathway, the pseudohyphal development pathway, the cell integrity or PKC pathway, the sporulation pathway and the High Osmolarity Glycerol (HOG) pathway (Herskowitz, 1995; Kyriakis and Avruch, 1996; Levin and Errede, 1995; Schultz *et al.*, 1995; Treisman, 1996;). All these pathways share a cascade of three protein kinases that are referred to as the MAP-kinase (MAPK), the MAP-kinase kinase (MAPKK) and the MAP-kinase kinase kinase (MAPKKK). Since the signal transduction pathway stimulated by osmotic stress is among the best understood stress-induced signalling pathway in yeast, the review on MAP kinase pathways will only focus on this pathway.

Ras-cAMP Pathway

The Ras-cAMP pathway in yeast uses cAMP as a secondary messenger to transmit information concerning nutrient availability. High intracellular cAMP levels correspond to nutrient-rich growth conditions whereas low cAMP levels are observed during nutrient limitation and poor growth conditions. cAMP is produced as a secondary messenger upon the addition of glucose or another easily fermentable carbon source to cells grown on non-fermentable carbon source when the cells adapt to fermentative growth conditions. Rapid increases in cAMP levels are also triggered upon the addition of protonophores at low extracellular pH which causes intracellular acidification of the cells (Thevelein, 1991).

The intracellular concentration of cAMP is controlled by a complicated mechanism involving many different components. In *S. cerevisiae,* two RAS proteins encoded by the *RAS1* and *RAS2* genes, respectively, act as the functional equivalents of the mammalian G-proteins on yeast adenylate cyclase (Powers *et al.*, 1989). Adenylate cyclase is encoded by the *CYR1/CDC35* gene (Boutelet *et al.*, 1985) and is one of the targets of the RAS proteins in yeast. A third component in the pathway is the GDP-GTP exchange factor encoded by *CDC25* (Broek *et al.*,1987) that acts as an activator of the Ras-cAMP pathway. Like other G-class proteins, Ras proteins are active in the GTP-bound form and inactive in the GDP-bound form. The intrinsic GTPase-activity of the Ras proteins is stimulated by the *IRA1* and *IRA2* gene products. They form part of the Ras adenylate cyclase pathway and act as inhibitors of the pathway. Several yeast *RAS* equivalents of mammalian *ras* oncogene alleles (same mutation at the corresponding amino acid position) have been obtained (Matsumoto *et al.*, 1985). The best-studied example is the $RAS2^{val19}$ mutant. This dominant allele shows greatly reduced GTPase activity and is insensitive to the action of the Irap. Strains containing such an allele or with deletions in the *IRA* genes, show the typical phenotype of strains with an overactive Ras-cAMP pathway. The last component in the pathway is the cAMP-dependent protein kinase A (PKA). Like the other eukaryotic cAMP-dependent protein kinases A, the yeast enzyme consists of three catalytic subunits encoded by the *TPK1, TPK2* and *TPK3* genes and an

inhibitory regulatory subunit, encoded by the *BCY1* gene. The pathway with all its components is represented in Fig. 7.

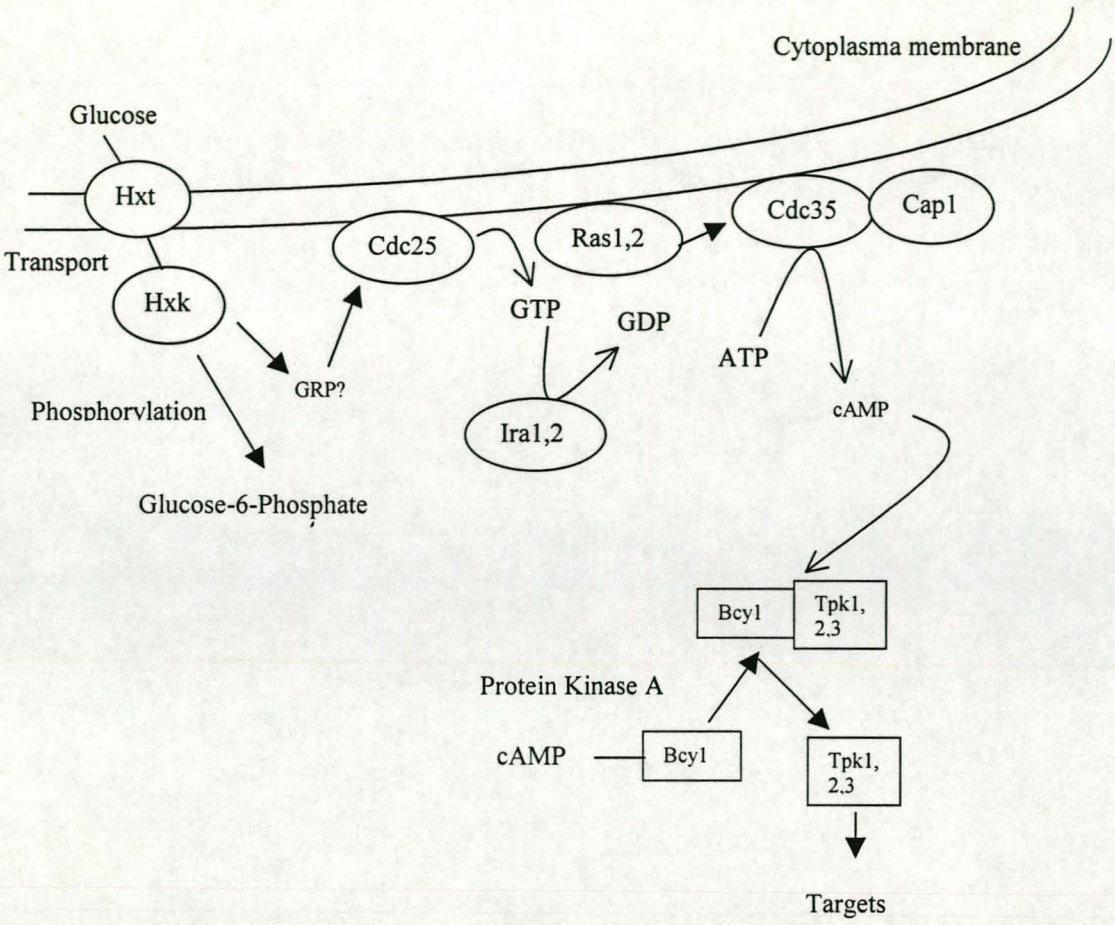

Fig. 7. The *S. cerevisiae* Ras-cAMP pathway. Upon addition of an easily fermentable carbon source, the Ras-cAMP cascade is initiated, glucose is transported into the cells and cAMP is synthesized through a number of steps. It causes the dissociation of the regulatory subunits of PKA from the catalytic subunit. This leads to the activation of PKA which mediates various regulatory processes leading to fermentative metabolism (Hohmann and Mager, 1996).

As shown in Fig. 7, the addition of glucose to cells on a non-fermentable carbon source or to stationary phase growing cells, leads to the activation of the Ras1p via the GDP-GTP exchange factor. Subsequently, the Ras1p activates adenylate cyclase which stimulates the conversion of ATP to cAMP. cAMP activates the cAMP-dependent protein kinase A by binding to its regulatory subunit, the three catalytic subunits are released and become active. This response leads to the phosphorylation of a diversity of target proteins that will mediate the metabolic switch from non-fermentative to fermentative growth.

As mentioned above, cAMP only serves as a second messenger in the nutrient response when glucose is added to glucose-starved cells causing the cAMP level to rise (Thevelein, 1991; Van der Plaat, 1974). However, in conditions where cells are starved for any of the other essential nutrients such as nitrogen, sulphate or phosphate and entry into stationary phase, the cells show specific characteristics and accumulate huge amounts of carbohydrate reserves like glycogen. Trehalose also accumulates and could function as a reserve under these conditions (Lillie and Pringle, 1980).

Meanwhile, the STRE-regulated genes like *CCT1* and *SSA3*, are activated and transcription of ribosomal protein genes is repressed. General stress resistance is enhanced, and in particular the thermotolerance (Plessit *et al.*, 1987) and resistance to cell wall lytic enzymes are high (Thevelein, 1984). The subsequent addition of glucose to these cells will again result in the increase of the cAMP levels. This will lead to the activation of the trehalase enzyme responsible for trehalose mobilisation, whereas STRE-regulated genes are repressed and the cells will start growing again.

HOG pathway

The HOG kinase pathway is an example of a MAP kinase signal transduction pathway. It mediates part of the osmostress response via the stress response elements (Schüller *et al.*, 1994) by specifically activating STRE-dependent genes upon osmotic stress. Cells of the yeast *S. cerevisiae* respond to osmotic stress with the accumulation of glycerol as compatible solute to counterbalance the osmotic pressure (Blomberg and Adler, 1992; Mager and Varela, 1993). This intracellular accumulation of glycerol is due to enhanced glycerol production and reduced glycerol permeability of the plasma membrane. The HOG-pathway functions both to regulate the increased production of glycerol upon a hyper-osmotic shock (Albertyn *et al.*, 1994) and to regulate cell morphogenesis in response to an increase in external osmolarity (Brewster and Gustin, 1994). The *GPD1* gene was identified as a target of the HOG pathway (Albertyn *et al.*, 1994) and encodes a cytosolic glycerol-3-phosphate dehydrogenase, the major enzyme responsible for

glycerol production in *S. cerevisiae*. A second identified target of the HOG pathway is the *CTT1* gene that encodes catalase T (Schüller *et al.*, 1994). Several other targets might also exist since osmotic shock is known to affect a considerable number of proteins (Varela *et al.*, 1992).

Several components of the HOG-signalling pathway have been identified, including the *HOG1* and *PBS2/HOG4* genes coding for Hog1p, a member of the MAP kinase family (Brewster *et al.*, 1993) and Pbs2p/Hog4p which is a homologue of MAP kinase kinases (Boguslawski and Polazzi, 1987; Boguslawski, 1992). Another component is the transmembrane osmo-sensor protein, Sln1p. Active Sln1p blocks activation of the signalling cascade when the medium osmolarity is low and it therefore seems to control the pathway negatively. In addition, *SLN1* disruption causes lethality due to an overactive pathway (Maeda *et al.*, 1994, 1995). A second protein, Ypd1p encoded by the *YPD1* gene in the cascade, transmits the signal from Sln1p to a third protein, Ssk1p, encoded by the *SSK1* gene. These three proteins form an osmosensing complex.

Both Sln1p and Ssk1p seem to contain a receiver domain as well as a putative output domain. Under normal growth conditions Sln1p protein kinase is active and autophosphorylates a histidine residue after which the phosphate group is transferred to an aspartate residue both internally in the protein's receiver domain and externally to a similar domain, namely the receiver domain of the Ssk1p. The phosphate group is transferred from Sln1p to Ssk1p via the Ypd1p. Phosphorylation of Ssk1p causes its inactivation and thus absence of HOG pathway stimulation. In addition, two MAPKKK homologues, Ssk2p and Ssk22p that function in the upstream part of the HOG pathway were also identified. The Ssk22p is 69% identical to Ssk2p (Maeda *et al.*, 1995) and the latter protein is controlled by the osmosensing complex Sln1p-Ypd1p-Ssk1p. Both Ssk2p and Ssk22p activate the Pbs2p upon osmotic shock. The HOG pathway and all its components are schematically presented in Fig. 8.

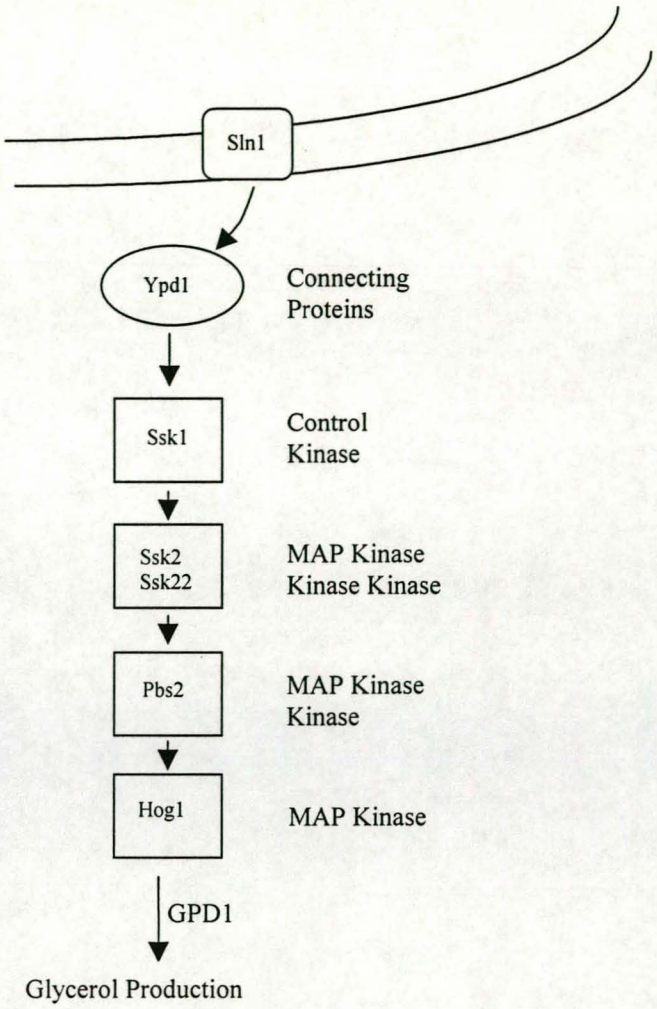

Fig. 8. Schematic representation of the HOG pathway (Hohmann and Mager, 1996).

As seen in Fig. 8, Sln1p is a transmembrane osmosensor that becomes inactive under high osmolarity. Since Sln1p is an inhibitor of Ssk1p, high osmolarity causes the activation of Ssk1p. Ssk1p contains a putative output domain that provides the link between the sensor system and the downstream components of the HOG pathway. Ssk1p directly interacts with the MAPKKK (Ssk2p and Ssk22p) as shown by two-hybrid system analysis. The MAPKKK is active and responsible for the osmostress-induced phosphorylation of Pbs2p that subsequently phosphorylates the Hog1p (Brewster and Gustin, 1994). As a result of activation of the HOG-pathway, glycerol is accumulated in order to increase the internal osmolarity and counteract cell dehydration (Blomberg and Adler, 1992; Mager and Varela, 1993).

Finally, it has been shown that HOG pathway mutants are only partially defective in osmostress-induced glycerol production. Therefore, it seems that a second HOG-independent pathway exists that also affects glycerol production. This control is probably situated at the post-translational level since a *hog1* mutant is entirely deficient in osmostress-induced expression of *GPD1* (Albertyn *et al.*, 1994).

Cis-Acting Elements and *Trans*-Acting Factors

In *S. cerevisiae* it has been shown that at least three positive transcriptional elements are activated by stress, namely the heat shock elements (HSEs), stress response elements (STREs) and AP-1 responsive elements (AREs). These elements could mediate transcriptional control of specific genes with regard to specific stressful conditions. This chapter will review what is known about these elements and will give examples of stress related genes that are specifically controlled by these elements.

Heat shock elements and the heat shock transcription factor

A particularly well-studied example of stress response is the events triggered by heat shock. When cells are exposed to a high temperature, they respond with the production of a set of proteins called heat shock proteins (HSPs). This strong induction of heat shock proteins is the main characteristic of the heat shock response. In addition, apart from several physiological changes in the cell, a large cytoplasmic pool of trehalose accumulates. The heat shock proteins are regulated on the transcriptional, translational and posttranslational level (Lindquist and Craig, 1988; Parsell and Linquist, 1994; Wu *et al.*, 1994). In *S. cerevisiae*, certain preconditions are required for cells to display the heat shock response. The response is essentially one of actively growing cells; stationary yeast cells do not show a rapid response since they are already intrinsically thermotolerant (Kirk and Piper, 1991). The response is only induced over a comparatively narrow temperature range, namely a temperature upshift to the maximum temperature at which the cells can grow (37 to 39.5°C). The lower the initial temperature of the yeast culture

and thus the larger the temperature upshift, the more the more intense the responseof cells seem to be (Kirk and Piper, 1991). The heat shock response is not only triggered by heat shock, but also by exposing cells to a variety of chemical agents such as alcohols and arsenite (Parsell and Lindquist, 1993).

The heat shock proteins are encoded by the *HSP* genes that contain one or more repeats of the heat shock response element (HSE) in their promoters. This element is a binding site for the heat shock transcription factor (HSF) and it comprises of at least three repeats of the 5 bp sequence nGAAn, nGAAnnTTCnnGAAn or nTTCnnGAAnnTTCn, each repeat comprising one half-turn of the DNA double helix (Sorger, 1991). A single HSE is sufficient to confer heat-inducible expression to a reporter gene (Kirk and Piper, 1991), although multiple HSEs act co-operatively to mediate transcription activation of heat shock genes. An unusual example of a HSE is in the promoter of the *CUP1* gene encoding metallothionein that is essential to prevent copper toxicity (Hamer *et al.*, 1985). The *CUP1* gene contains two repeats of the nGAAn-element, which is sufficient to give slight thermal induction (Yang *et al.*, 1991).

The HSF of *S. cerevisiae* is constitutively expressed and is bound to heat shock promoters as trimers (Jakobsen and Pelhem, 1988; Sorger, 1991; Wisniewski *et al.*, 1996). It has a basal activity as a transcriptional *trans*-activator even in unstressed cells, unlike the HSF in higher eukaryotes that is an inactive monomer in unstressed cells. Only when stress is induced, does the HSF trimerizes and acquires high DNA-binding affinity to become an active transcription factor (Mager and De Kruijff, 1995; Sorger, 1991).

The constant requirement for the HSF in yeast may implicate a multifunctional role for the HSF protein (Smith and Yaffe, 1991). This is not surprising, since heat shock proteins acting as chaperones (e.g., Hsp70p) are needed under all types of growth conditions and the HSF is required for basal expression of some heat shock genes (Park and Craig, 1988). In addition, the presence of the HSE in the *CUP1* promoter to which the HSF binds, suggests that the HSF is involved in transcriptional regulation of genes other than *HSP* genes (Mager and Moradas Ferreira, 1993). In yeast, heat shock merely increases

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the trans-activation activity of these promoter-bound HSF trimers and causes them to become hyperphosphorylated (Sorger and Pelham, 1988). However this hyperphosphorylation is apparently not essential for increased activity of the factor, but may serve as a means to deactivate the factor and contribute to the transient nature of the heat shock response (Flick *et al.*, 1994).

HSFs in different eukaryotes all share two regions of homology, namely a trimerization domain and a DNA binding region. For budding yeast, it has been shown that the *trans*-activating ability of HSF is localised in two separate domains located near the N-terminus and the C-terminus, respectively (Nieto-Sotelo, *et al.*, 1990; Sorger, 1990). The C-terminal *trans*-activation domain seems to overlap with a leucine zipper-like motive (Chen *et al.*, 1993). The activity of this domain was reported to be influenced by a conserved heptapeptide (CE2) (Hoj and Jakobsen, 1994), the trimerization domain, the DNA-binding domain as well as other parts of the protein. In yeast, these regions suppress the heat-induced *trans*-activating activity in non-stress conditions. It seems that free Hsp70p levels might be directly or indirectly involved in maintaining the promoter-bound yeast HSF in a state of basal transcriptional activity.

Apparently, some of the genes subject to HSF control are needed for growth at moderate temperatures, but not for resistance to more severe heat stress conditions (48 to 52°C). It has been shown in *S. cerevisiae* that there is a good inverse correlation between the thermotolerance at 50°C and the activity of protein kinase A, although protein kinase A has very little influence on HSE induction levels (Machler *et al.,* 1993). Thermotolerance at very high temperatures (48 to 50°C) is also induced by treatments that cause no heat shock element induction, like osmotic dehydration or exposure to weak organic acids (Mager and Moradas Ferreira, 1993). Therefore, it seems that, although HSE and HSF are essential for growth under conditions of moderate heat stress (27 to 39°C), they are not required for the induction of tolerance against severe stress.

Stress responsive elements (STREs) and possible DNA-binding factors

A novel regulatory element responsive to a variety of stress conditions has been identified and, is refered to as a stress response element (STRE) based on the observation that this element is activated by multiple stress factors. It consists of a consensus CCCCT sequence and is functional in both orientations. The stress responsive element is able to mediate transcription induced by various forms of stress and it therefore seems that STRE-directed gene expression may program the cell for survival under diverse conditions of stress, including extreme temperature and nutrient starvation (Ruis and Schüller, 1995). This DNA element was also identified as being essential for HSF-independent heat shock induction of a number of stress related genes such as *DDR2* (Kobayashi and McEntee, 1993), *UBI4* (Finley *et al.*, 1987), *CTT1* (Belazzi *et al.*, 1991) and *DDR2* (Kobayashi and McEntee, 1993).

Interestingly, STRE motifs are present in the promoter region of all the genes of the biosynthetic pathway of trehalose (Mager and de Kruijff, 1995; Varela *et al.*, 1995). The STRE motifs in the *TPS2* gene were shown to be functional and those in the *TPS1* gene are currently being investigated. Several authors suggested that the STRE elements are negatively controlled by cAMP-dependent protein kinase (Boorstein and Craig, 1990; Gounalaki and Thireos, 1994; Machler *et al.*, 1993; Schüller *et al.*, 1994; Varela *et al.*, 1995) but it is not yet clear whether it would exert its effect by modulating the activity of the STRE elements rather than directly transmitting any stress signal. This possible modulation would explain why the expression of STRE-controlled stress protein genes is minimal in rapidly growing cells while it is enhanced under suboptimal growth conditions or in stationary phase. A schematic representation of the external stress factors that control STREs and the possible effects triggered by STRE activation is shown in Fig. 9.

The *SSA3* gene that encodes one of the Hsp70p subunits contains a PDS element, a *cis*-acting element with a consensus sequence of AGGGA, that was proposed to be a variant of the STRE element (Ruis and Schüller, 1995). The Hsp70p serves as a chaperone under heat shock conditions.

homologous transcriptional activators that contain two CysHis zinc fingers at the C-terminus and share similarity over their entire protein (Martinez-Pastor *et al.*, 1996). It has been suggested by DNA sequence recognition analysis that Msn2p and Msn4p specifically bind the STRE sequence NAGGGG (Choo and Klug, 1994). This hypothesis was further strengthened by computer assisted molecular modelling and by using *in vitro* translated Msn2p and purified Msn2p and Msn4p expressed in *E. coli* as GST fusions. It has been shown that both proteins were indeed able to bind specifically to oligonucleotides that include STREs sequences, whereas no binding was observed with a mutated oligonucleotide (Estruch and Carlson, 1993). In addition, Martinez and co-workers (1996) have shown that Msn2p and Msn4p mediate STRE-dependent transcription by direct binding to the STRE sequences.

A double mutation in the *MSN2* and *MSN4* genes led to higher stress sensitivity compared to wild-type cells (Martinez-Pastor *et al.*, 1996). The increase in sensitivity is restricted to severe acute stress and is not observed in stationary phase cells. On the other hand, overexpression of Msn2p and Msn4p led to an improved general stress resistance and constitutive expression of the STRE regulated genes under non-stress conditions. Nevertheless, osmotic induction through the HOG pathway via the STREs is independent of the Msnps (Martinez-Pastor *et al.*, 1996). It has also been shown that mechanisms independent of Msn2p and Msn4p do exist that contribute to the high level of stress resistance in stationary phase cells. Furthermore, the absence of a growth defect exhibited by the *msn2 msn4* double mutant under mild stress conditions, suggests that mechanisms independent of STREs or Msnps exist that allow growth under unfavourable conditions after adaptation (Mirralles and Serrano, 1995). Another interesting observation in this regard is that osmotic induction via the STREs is independent of the Msnps (Martinez-Pastor *et al.*, 1996). This implies that there must be several pathways that activate transcription via STREs and that additional factors may bind to the same element to confer stress resistance.

AP-1 responsive elements (ARE)

Similar to HSEs and STREs, AP-1 responsive elements (ARE) are positive transcriptional control elements activated by stress conditions. A comparison of the AREs found in *S. cerevisiae* promoters, suggests that there may be considerable variation in the consensus ARE sequence (Wiemmie *et al.,* 1994). ARE-controlled genes may function mainly during oxidative stress and in response to toxic conditions caused by heavy metal ions (Hertle *et al.*, 1991).

AREs bind the transcription factor Yap1p encoded by the *YAP1* gene first cloned by Harshman *et al.* (1988). The *YAP1* gene was repeatedly identified in screens for genes that cause resistance to various toxic compounds in high copy number (Hertle *et al.*, 1991; Hussian and Lenard, 1991). Disruption of the *YAP1* gene causes hypersensitivity to hydrogen peroxide, suggesting a function for the gene product in the protection against oxidative stress (Schnell *et al.*, 1992; Kuge and Jones, 1994). A second gene, *YAP*2, has been cloned independently (Bossier *et al.,* 1993; Wu *et al.,*1993). The product of this gene has a similar function as Yap1p, but there are no reports of Yap2p binding to AREs and no targets for Yap2p are known.

The *YAP1* gene product of *S. cerevisiae* contains a basic-leucine zipper (B-ZIP) domain and is homologous to the mammalian transcription factors of the AP-1 family. The leucine zipper In the Yap1p dimerization domain is adjacent to a basic region (B-ZIP) that has been shown to interact directly with DNA. Gounalaki and Thireos (1994) suggested a dual function for Yap1p in the acquisition of stress tolerance: firstly, it is an indirect and general requirement for transcriptional activation of STRE-regulated tolerance genes, and secondly, it has direct involvement in the synthesis of detoxifying gene products. Three different targets of the Yap1p have been identified: the *YCF1* gene encoding Yeast Cadmium Factor 1, the *GSH1* gene encoding g-glutamylcysteine synthetase gene, and the *TRX2* gene encoding one of the two yeast thioredoxins. The latter two gene products play essential roles in oxidative stress protection by providing non-enzymatic cellular defence against reactive oxygen species whereas the Yfc1 protein seems to confer

highly elevated cadmium resistance to wild-type yeast strains (Szczypka *et al.*, 1994). Surprisingly, it was found that the Yap1p plays a role in regulating STRE activity (Kobayashi and McEntee, 1993). Since STRE-driven expression of a reporter gene was diminished in a *yap1* mutant. However, Yap1p does not bind to the STREs and therefore this effect appears to be indirect.

A schematic representation of the three known *cis*-acting elements and interaction with their corresponding *trans*-acting factors upon several stress conditions, is given in Fig. 10.

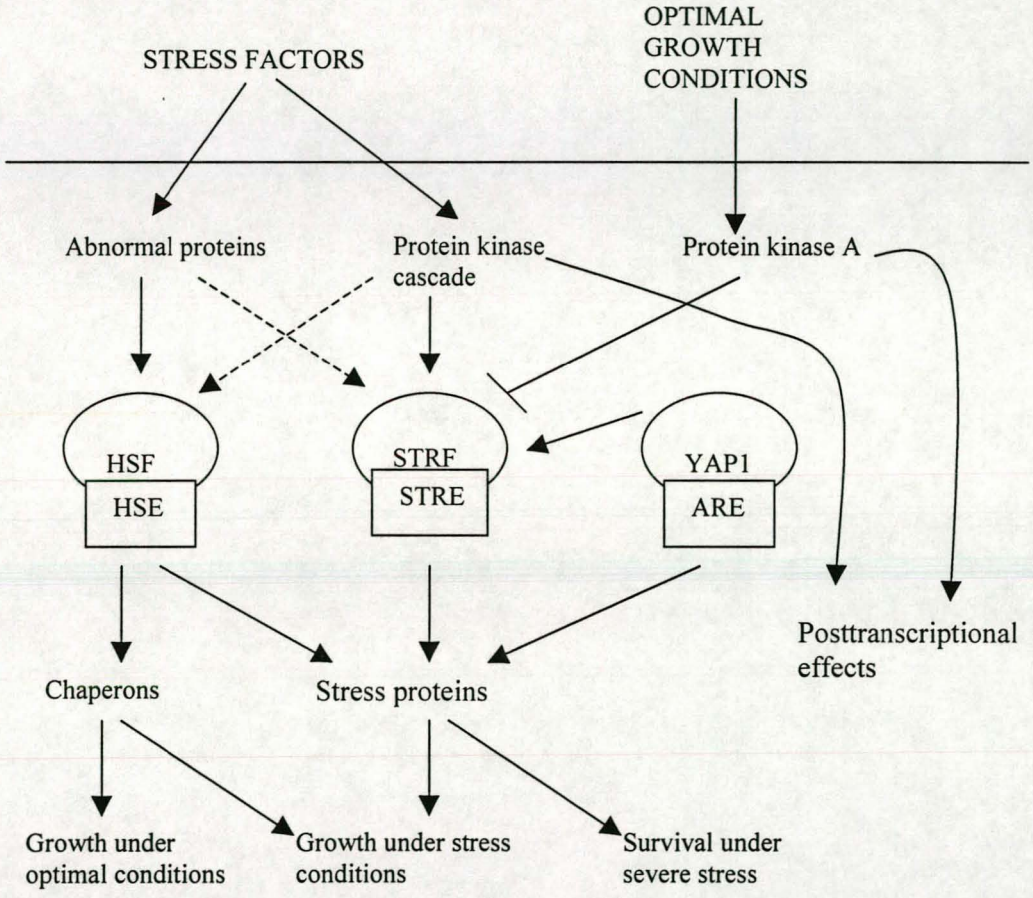

Fig. 10. Illustration of the three different transcriptional control systems activated by external stress signals (Ruis and Schüller, 1995).

INDUSTRIAL IMPORTANCE OF TREHALOSE

Yeasts have been used extensively for centuries by both the brewing and baking industries and have numerous applications in the beer, wine and fermented food industries. As mentioned previously, several functions have been assigned to trehalose in the yeast cell, and the protective function of trehalose can have great implications for several industries that produce or make use of yeast. The implications of trehalose for the brewing and baking industries will be discussed in this section.

Brewing Industries

Most brewing yeasts are either polyploid or aneuploid, unlike laboratory strains of *S. cerevisiae*, that are either diploid or haploid. Industrial yeasts may benefit from being polyploid since extra copies of a gene could improve their fermentation performance. Furthermore, polyploid yeasts could be more stable than haploid yeasts since multiple mutational events would be required in order to bring about any changes. However, polyploid yeasts can harbour non-functional recessive mutations (Delgado and Conde, 1983). This means that genetic stability is more likely to be a function of the frequency of segregational events leading to the expression of mutant genes, rather than of the frequency of mutations itself.

Current brewing yeast strains have a number of limitations that need to be addressed by the brewer. Brewing strains sporulate poorly, rarely form spores in tetrads and many of the spores are non-viable even when great efforts are made to produce ideal conditions for sporulation (Hammond, 1995). Brewing yeasts cannot be used at high temperatures and cannot ferment worts of high carbohydrate content due to their limited thermostability and tolerance to ethanol and osmotic pressure. They are inefficient at fermenting wort since the conversion of carbohydrates to ethanol is limited by excessive yeast growth and by the inability to ferment all wort sugars. Another area of concern is contamination of brewery fermentations with other micro-organisms, which could be reduced by the use of yeast possessing anti-contaminant properties.

In addition, brewers have limited control over the flavours produced by yeasts, and variation of yeast flocculation properties is also a problematic area (Hammond, 1995). All these limitations make the optimisation of brewing yeast strains a priority for brewing companies.

Recombinant DNA technology could address some of these problems, in particular the sensitivity of yeast to osmotic stress in high gravity (HG) and very high gravity (VHG) worts (Majara *et al.*, 1996a). During fermentation, the yeasts are subjected to osmotic stress, which reduces growth and increases the loss of cell viability (Casey *et al.*, 1984; Odumeru *et al.*, 1993; Thomas and Ingledew, 1992). High ethanol concentrations is a further detrimental factor in HG and VHG brewing. Recently, Majara *et al.* (1996b) showed that brewing yeast uses trehalose as an osmoprotectant in HG and VHG fermentations. They found that as the wort gravity increases, the cellular concentration of trehalose also increases and that this response was not adjunct-specific. Apart from trehalose, other low molecular weight compounds such as proline, glycine, betaine, glutamate and glutamine can also act as osmoprotectants (Thomas *et al.*, 1994).

Another problem created by high gravity worts, is the high concentration of dissolved CO_2, known as carbonation. At high concentrations, CO_2 acts as a growth-limiting factor that affects the cell permeability and therefore the uptake and utilisation of assimilable nutrients (Kruger *et al.*, 1992). Whilst the toxic effects of dissolved CO_2 in fermentation are well documented (Kruger *et al.*, 1992), it is commonly accepted that it is non-toxic to non-growing yeast. Therefore, the alleviation of high levels of dissolved CO_2 by decarbonation (removal of dissolved CO_2) during yeast handling, is often not considered important. However, carbonation induces a stress response and trehalose is synthesized in non-growing yeast, indicating that the alleviation of dissolved CO_2 in yeast handling could play a role in maintaining cell vitality.

It has been shown that trehalose accumulation occurs after the yeasts have been exposed to most types of stress such as freezing, ethanol shock, oxygenation and carbonation. It seems that in these instances, trehalose acts as a membrane stabiliser and protectant against the applied stress (Lillie and

Pringle, 1980; Odumeru *et al.*, 1993). Stress response causes significant changes in the cellular composition that may either have a direct or indirect impact on fermentative performance. Since trehalose was found to be a very sensitive and responsive compound to stress conditions, the cellular trehalose content could be used as a stress indicator in certain brewing yeast strains for fermentation optimisation (Majara *et al.*, 1996a). In addition, results have shown that it is possible to genetically manipulate the trehalose regulatory pathway in the yeast cell to increase the trehalose concentration for protection against stress exerted during the fermentation process (Thevelein, 1984; Thomas *et al.*, 1994) however, higher trehalose levels do not necessarily imply higher stress resistance since additional factors are also required to improve stress resistance. Nevertheless, brewers are trying to preserve a trehalose level that is necessary to maintain stress resistance. Since high glucose concentrations in the worts affect the stress resistance in yeast by repressing trehalose synthesis, brewers can lower the glucose concentration in the worts (Majara *et al.*, 1996a). This will ensure that trehalose synthesis occurs relatively early in the fermentation. During yeast handling and storage, the yeast trehalose content can be preserved at the levels measured at the time of yeast harvesting by keeping the yeast cold for minimal periods in the absence of entrained oxygen.

Baking Industries

Bakers' yeast is particularly suited for the requirements of the baking industries and are the most highly selected strains of the species *S. cerevisiae*. A clear correlation between the trehalose content and stress resistance in bakers' yeast has been known for a long time and the baking industry has applied it to some extent (Pollock and Holmström, 1951). This is particularly true for 'Instant Active Dry Yeast', that remains active in dried form and does not need rehydration prior to mixing with flour (Trivedi and Jakobsen, 1986). The culture conditions for commercial bakers' yeast have been optimised to obtain a high trehalose content of approximately 10% of dry weight (Gelinas *et al.*, 1989), and it may even go as high as 15 to 20% (Trivedi and Jakobsen, 1986). This is a significant difference when compared

to laboratory strains where the trehalose content contributes only 3 to 4% of the dry weight.

Improving the tolerance of microbial cells to freezing and thawing is of major concern in areas of applied microbiology such as culture collections, frozen starters and frozen doughs (Gelinas *et al.*, 1989; Yoshikawa *et al.*, 1994). Yeast in frozen dough products will normally loose approximately 50% of its activity during twelve weeks of frozen storage (Bruinsma and Geisenschlag, 1984). Much work has therefore been done to improve the quality of frozen yeast-leavened doughs. The stability of the frozen dough largely depends on the freeze-thaw resistance of the bakers' yeast (Godwin and Cathcart, 1949). For the preservation of yeast viability in frozen dough, a high trehalose content is considered to be crucial, but trehalose is rapidly mobilised during fermentation and the yeast cells loose their stress resistance. However, Meric and co-workers (1995) have recently shown that cryoresistance was not directly correlated with the initial amount of trehalose in the yeast. They have clearly shown that 4 to 5% trehalose stock at freezing is a threshold level sufficient for trehalose to ensure protection for the membrane or cytoplasm throughout the freeze-thaw sequence of events. This level is also sufficient to play a role in the recovery of metabolic activity and possible repair of damage after the freezing stress. A higher quantity of cellular trehalose does not necessarily contribute to further cryoresistance (Meric *et al.*, 1995).

As mentioned above, initiation of fermentation during dough preparation is associated with a rapid mobilisation of trehalose and a rapid loss in stress resistance. It is important to note that bread produced without fermentation prior to freezing lacks adequate taste and flavour. Other methods have therefore been considered to increase the cryoresistance of bakers' yeast. The current methods have been improved by mainly focusing on the effects of the strain, the growth phase and the growth medium composition in batch fermentation. Harvesting the cells during the stationary phase rather than in the logarithmic phase has improved the cryoresistance of several yeast strains (Kirsop and Henry, 1984). Furthermore, rapid handling of the dough is recommended to minimize yeast activity before freezing and low dough-

processing temperature to prevent freezing injury (Hino *et al.*, 1990).

Hino and co-workers (1987) reported three new strains of freeze-tolerant yeast that retain their fermentative ability and bread-leavening activity even after prolonged frozen storage of fully fermented doughs before freezing. They exhibited differences in fermentation ability as well as in gassing rate (CO_2 production) before and after freezing in several types of dough. It has successfully been shown that frozen dough made with these yeasts gave bread of equal quality as that made from unfrozen dough with ordinary bakers' yeast. However, there are some limitations in the use of these freeze-tolerant yeasts as the dough should be prepared under optimum conditions. The characteristics of these freeze-tolerant yeasts may change as soon as the culture conditions such as aeration, feed rate of sugar and other available nutrients, change during the production of yeast (Hino *et al.*, 1987). Therefore, more detailed studies should be done on the culture conditions for the effective use of these new freeze-tolerant strains.

CONCLUSIONS

This review focused on trehalose metabolism and stress signalling in the yeast *S. cerevisiae*. In yeast, stress can generally be defined as factors or conditions that negatively affect cell growth. Yeast cells are commonly exposed to such conditions and therefore have intrinsic properties to sense and respond appropriately. It has been shown that one of the ways in which yeast cells respond to certain stresses is by accumulating huge amounts of trehalose. In the first section of the review the role of trehalose metabolism, and its regulation were discussed in detail. Several functions have been assigned to trehalose including a role as a reserve carbohydrate, a role in the regulation of the glycolytic flux and a role as a stress protectant. It has been shown that trehalose protects the cells during dehydration, desiccation and freezing by maintaining the structural integrity of the membrane.

Expression of all the genes involved in trehalose biosynthesis shows

significant similarity to the expression of stress-induced genes such as *CTT1*, *DDR2*, *UBI4*, *HSP12* and others. Like the stress-induced genes, they are negatively regulated by cAMP-dependent protein kinase A, they all contain stress response elements in their promoters, and they are induced by nutrient starvation, stationary phase entrance, heat shock and other stress conditions. Interestingly, even the neutral and acid trehalase enzymes responsible for trehalose hydrolysis seem to be in one way or the other related to stress resistance. The *NTH1* gene encoding neutral trehalase contains STRE elements in its promoter and, together with a second possible neutral trehalase gene *NTH2,* seems to be necessary for the recovery of cells after heat shock. It is therefore tempting to suggest that genes encoding enzymes in the synthetic pathway as well as trehalose hydrolysing enzymes form part of the response to stress in yeast.

The final section focused on certain aspects of stress responses in yeast. Two signal transduction pathways have been discussed, as well as stress-activated transcription factors. Although incomplete, there is currently a good understanding of signalling mechanisms in yeast. This study focused on two mechanisms, namely the HOG pathway and the Ras-cAMP cascade. The HOG pathway is an example of a MAP kinase signal transduction pathway and functions in the transmission of information regarding increases in osmolarity. The Ras-cAMP pathway is a nutrient sensing pathway that uses cAMP as a second messenger to transmit information concerning nutrient availability. The signalling pathways have different targets and result in changes in the transcription of genes by modifying transcriptional activation.

Currently, three types of positive *cis*-acting transcriptional control elements that are activated by stress conditions, have been identified in the yeast *S. cerevisiae*, namely HSEs, STREs and AREs. These control elements seem to have overlapping, but distinct functions. It has been shown that a heat shock gene in yeast can either be under HSE or STRE control, or may be controlled by both these elements. The *SSA4* gene, encoding a heat inducible form of the Hsp70p, is exclusively under HSE control, while the *HSP12* gene is exclusively under STRE control. However, the *SSA3* gene

which encodes another Hsp70p is under both STRE and HSE control. Another interesting phenomenon is that the presence of STREs in gene promoters does not necessarily imply complete co-regulation (Winderickx *et al.*, 1996). This is true for some of the genes encoding enzymes in trehalose biosynthesis, that are less sensitive to down-regulation by glucose than other STRE-controlled genes.

Considering the evidence currently available, it can be concluded that stress proteins encoded by HSE-regulated genes are necessary for growth of yeast under moderate stress, while products of STRE-activated genes seem to be important for survival under more severe stress. ARE-controlled genes may function mainly during oxidative stress and in response to toxic chemicals. It appears that the STRE elements are an integration point of parallel pathways that function through different sets of transcription factors. The recent identification of some specific transcription factors that bind the STRE elements partly clarifies the complicated pathways that transmit the different types of stress signals to this element. However, this field still needs extensive analysis before the full mechanism of stress regulation will be understood.

The protective role of trehalose has been of great importance to several industries that produce or utilise yeast. The review focused on the brewing and baking industries where the protective function of trehalose is of specific importance. However, since trehalose on its own is not sufficient to confer stress resistance, other factors in combination or independently from trehalose have to be considered.

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CHAPTER2

Regulation of the *TPS1* gene expression in *Saccharomyces cerevisiae*

G.G. Bihl, F.F. Bauer, *J. Winderickx, H.J.J. van Vuuren and *J.M. Thevelein

Department of Microbiology, University of Stellenbosch, Stellenbosch 7600, South Africa.

**Laboratorium voor Moleculaire Celbiologie, Katholieke Universiteit Leuven, Kardinaal Mercierlaan 92, B-3001 Leuven-Heverlee, Belgium.*

In the yeast *Saccharomyces cerevisiae,* the *TPS1* gene encodes trehalose-6-phosphate synthase, the first enzyme in the trehalose biosynthetic pathway. Trehalose has been suggested to act as a stress protectant, a role that appears to be linked to the level of the disaccharide in the cell. Since the level of trehalose is partly controlled by the activity of the *TPS1* gene product, we investigated the transcriptional regulation of the *TPS1* gene. Previous work suggested that the gene could be regulated by sequences located within the open reading frame (ORF), possibly through an autoregulatory mechanism. However, no evidence for the presence of *cis*-acting regulatory elements within the ORF or for autoregulation was found. The *TPS1* gene contains six putative STRE elements between -237 nucleotides and -470 upstream of the ATG. This prompted us to investigate the possible regulation of the *TPS1* gene by these STRE elements by means of gel retardation experiments and site directed mutagenesis. Our results showed that the STRE elements in the promoter of the *TPS1* gene are involved in the transcriptional regulation of the gene, in particular STRE1, closest to the ATG.

INTRODUCTION

For many years trehalose was considered to be a carbon and energy reserve due to its accumulation during sporulation, its high amounts in spores and resting cells and its mobilization during spore germination and resumption of growth (Elbein, 1974; Thevelein, 1984). More recently, various authors suggested a role for trehalose as a stress protectant in the yeast *S. cerevisiae* (De Virgilio *et al.*, 1990; Hottiger *et al.*, 1987, 1989, 1992; Van Laere, 1989). This hypothesis was largely based on the positive correlation observed between the trehalose content and thermotolerance, freeze tolerance, desiccation resistance, osmotolerance and exposure to toxic chemicals. The activity of trehalose as a possible stress protectant has been used by the baking (Gelinas *et al.*, 1989; Trivedi and Jakobsen, 1986) and brewing industries to increase the freeze tolerance of the yeast. However, there is a need in both industries to further optimize the freeze tolerance of the yeast and to use the protective function of trehalose to the fullest extent (Van Dijck *et al.*, 1995).

Trehalose is synthesized in two steps: in the first step uridine diphosphate glucose and glucose-6-phosphate are combined to form trehalose-6-phosphate, a reaction catalyzed by trehalose-6-phosphate synthase encoded by the *TPS1* gene (Bell *et al.*, 1992; Vuorio *et al.*, 1993). The second step is catalyzed by trehalose-6-phosphatase encoded by the *TPS2* gene (De Virgilio *et al.*, 1993), where trehalose-6-phosphate is dephosphorylated to form trehalose. The two enzymes form a complex together with two regulatory proteins encoded by the *TPS3* and *TSL1* genes (Vuorio *et al.*, 1993). Trehalose is mobilized upon glucose addition to cells growing under non-fermentative conditions or stationary phase cells. Hydrolysis of trehalose is carried out in the cytoplasm by a neutral trehalase (Thevelein, 1984) that is encoded by the *NTH1* gene (Kopp *et al.*, 1993, 1994). The activity of this enzyme is regulated by cAMP-dependent protein kinase A (cAPK) through a phosphorylation/dephosphorylation mechanism (App and Holzer, 1989; Uno *et al.*, 1983). In addition to the neutral trehalase, the gene encoding an acid trehalase, *ATH1*, has been cloned (Harris and Cotter, 1987; Mittenbühler and

Holzer, 1988). Acid trehalase is found in the vacuole (Destruelle *et al.*, 1995) and the gene is constitutively expressed.

The *tps1* mutants show a pleiotropic phenotype. Besides defects in trehalose accumulation (Neves *et al.*, 1995), *TPS1*-deficient cells show an inability to grow on fermentable sugars such as glucose, fructose, sucrose and mannose. However, growth on non-fermentable carbon sources such as glycerol or slowly fermentable sugars such as galactose, is not affected. The mutant cells loose their ability to sporulate and glucose-induced signalling seems to be absent (Gonzalez *et al.*, 1992; Hohmann *et al.*, 1992). Hohmann and co-workers (1993) showed that growth and signalling defects of the *tps1* deletion mutants are suppressed by a deletion of the *HXK2* gene that encodes hexokinase PII. These observations led to the hypothesis that the trehalose biosynthesis pathway may play a possibly role in controlling the glycolytic flux.

Earlier studies on the regulation of the *TPS1* gene by Winderickx and co-workers (1996) described a discrepancy between *TPS1* mRNA levels when studied by northern blot analysis on the one hand and by β-galactosidase assays in strains carrying a *LacZ* reporter gene fused to the promoter of the *TPS1* gene on the other hand. The expression of the *LacZ* fusion construct was repressed in media containing glucose as the sole carbon source or after addition of glucose to cells growing on a non-fermentable carbon source. However, when northern blot analysis was carried out under the same conditions, the *TPS1* transcript was only transiently repressed after the addition of glucose, followed by transcription of the gene at a lower level. The results of the β-galactosidase assays indicate that this regulation does not seem to occur via the promoter area of the *TPS1* gene, and it was suggested that the gene could be regulated by elements located within the ORF. In addition, the transient repression of the wild type *TPS1* gene could be explained by the existence of an autoregulatory mechanism. Such control mechanisms have already been described for various genes in several eukaryotic organisms (Deckert *et al.*, 1995; Dhand *et al.*, 1994; Perlman *et al.*, 1984; Tata, 1994). For these genes, the gene products regulate the

expression of their respective genes by interacting with either the promoter or internal DNA sequences in the (ORF). Based on the discrepancy mentioned above, it seemed that the *TPS1* gene product may interact with protein binding DNA sequences within the ORF rather than with sequences within the promoter.

The role of trehalose as a stress protectant appears to be related to the amount of the disaccharide in the cell (Hottiger *et al.*, 1994). This amount is partly controlled by the activity of the *TPS1* gene product (De Virgilio *et al.*, 1994). To improve our understanding of the relation between *TPS1* activity, trehalose accumulation and stress resistance, we investigated the mechanisms involved in the transcriptional regulation of the *TPS1* gene. Our results suggest that regulatory elements within the ORF and autoregulation are both unlikely to be control mechanisms for the regulation of the expression of the *TPS1* gene. On the other hand, site-directed mutagenesis of six putative STRE elements in the promoter of the *TPS1* suggested that most of the STRE elements act redundantly, with STRE1 (closest to the ATG), being the most influential.

MATERIALS AND METHODS

Strains. All strains used are isogenic to *S. cerevisiae* W303-1A (*MATa leu2-3/112 ura3-1 trpl-1 his3-11/15 ade2-1 can1-100 GAL SUC2 mal0*), refered to as the wild type (WT) in further discussions (Thomas and Rothstein, 1989). Besides the WT, strains W303 *tps1Δ::TRP1* and W303 *tps1Δ::TRP1hxk2Δ::LEU2* were also used (Hohmann *et al.*, 1993). *Escherichia coli* DH5α was used for bacterial transformations and plasmid propagation.

Media. Yeast cells were grown aerobically in YEP medium (2% peptone, 1% yeast extract) supplemented with either 2% galactose, 2% glucose or 2% glycerol/2% ethanol as carbon source. Synthetic media for yeast transformation contained 0.67% Yeast Nitrogen Base without amino acids and ammonium sulphate (Difco Laboratories, Detroit, MI) and was supplemented with 0.5% ammonium sulphate and either 2% glucose or 2% galactose.

Amino acids were added according to the requirement of each strain. *E. coli* cells were grown in LB medium (1% NaCl, 1% bacto-tryptone and 0.5% yeast extract at 37°C (Bolivar and Backmann, 1979). For solid media, 1.5% agar was added. Selection of bacteria containing plasmids was carried out in the presence of 50 mg/l ampicillin.

Growth conditions. Glucose shift experiments were carried out by growing cells aerobically in YEP containing 2% glycerol/2% ethanol at 30°C to mid-logarithmic phase (OD_{600} of 0.8 to 1.0). A sample was taken (t_0) and glucose was added to the culture to a final concentration of 4%. Subsequent samples of 15 ml were taken at 2, 10, 30, 60 and 180 min after the addition of glucose. For induction of stress responses, 100 ml cultures were grown in YEP containing 2% glucose to OD_{600} of 0.8 to 1.0. A sample was taken and NaCl was added to the remaining culture to a final concentration of 0.7 M (Varela *et al.*, 1992). Subsequent samples were taken 10, 20 and 30 min after the addition of NaCl. For induction of a heat shock response, cells were grown 50 ml cultures as described for the salt stress, but only were used. They were grown at 23°C until an OD_{600} of 0.8 to 1.0 and then shifted to 37°C. Samples were taken before the shift and at 10, 30 and 60 min after the temperature shift.

DNA manipulations and transformation. Plasmid DNA isolation from *E. coli* was carried out as described by Del Sal *et al.* (1988). All other DNA manipulations were carried out according to standard molecular DNA techniques as described by Sambrook *et al.,* (1989). Transformation of the yeast *S. cerevisiae* was carried out according to Ito *et al.* (1983).

Site-directed mutagenesis. Site-directed deletions of the STRE elements in the *TPS1* promoter were constructed by changing the DNA sequence of the STRE elements. This was accomplished by designing primers with altered sequences to mutate the native sequences to sequences that represent different restriction enzyme sites as follows: the STRE element closest to the ATG, (STRE1) was replaced with a *Kpn*I restriction site. STRE2, STRE3, STRE4, STRE5 and STRE6 were replaced by *Bgl*II, *Pst*I, *Spe*1, *Sph*1 and *Sal*I recognition sites, respectively. The positions of the STREs and the introduced restriction sites are shown in Fig. 2. For mutation of the STRE1 element the primers STREF1 and GGSR1 was used to yield a fragment (nt -241 to +43)

and primers STRER1 and GGSF1 were used for another fragment (nt -237 to -800). Both fragments were digested with, in this case *Kpn*I, ligated and the product was amplified with primers GGSF1 and GGSR1. The product containing the mutated STRE element, was digested with *Bam*HI and *Xba*I and cloned into *Bam*HI and *Xba*I sites in pGAG1. All clones were verified by automated sequencing. The same approach was used to mutagenize the other five STRE elements.

Construction of *LacZ*-fusions. To fuse the *TPS1* promoter to the *LacZ* gene, a 843 bp *Xba*I/*Bam*HI fragment comprising the nucleotides between position -800 and +43 of the *TPS1* gene was amplified with PCR by using Pwo DNA polymerase (Boehringer Mannheim, Germany). The fragment was fused inframe with the ORF of the *Lac*Z reporter gene using the *Xba*I/*Bam*HI sites. pHP41 plasmid (Park and Cooper, 1992) was modified for cloning of the mutagenized *TPS1* promoter constructs. A 1.06 kb *Xba*I/*Sma*I fragment was excised from pHP41 and replaced with a 0.24 kb *Pvu*II/*Xba*I fragment from pVAN6, a derivative of pBluescriptII with the *Bam*HI restriction site deleted (G. van der Merwe, personal communication). The resulting plasmid was designated pGAG1 (Fig. 1).

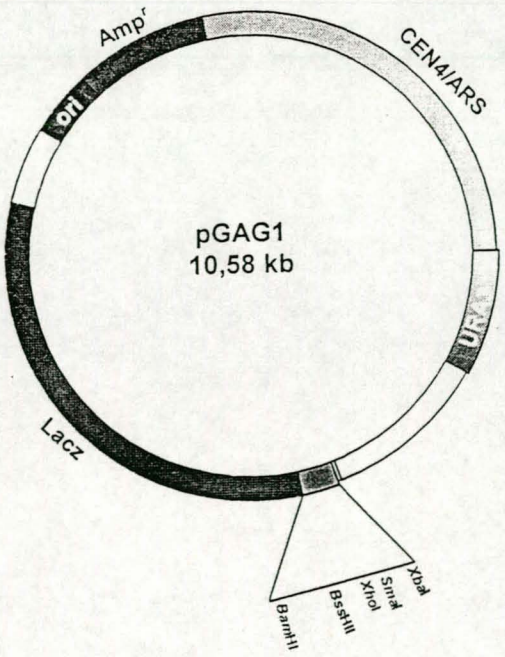

Fig. 1. Plasmid pGAG1 used for cloning of site directed mutagenized *TPS1* promoter fragments.

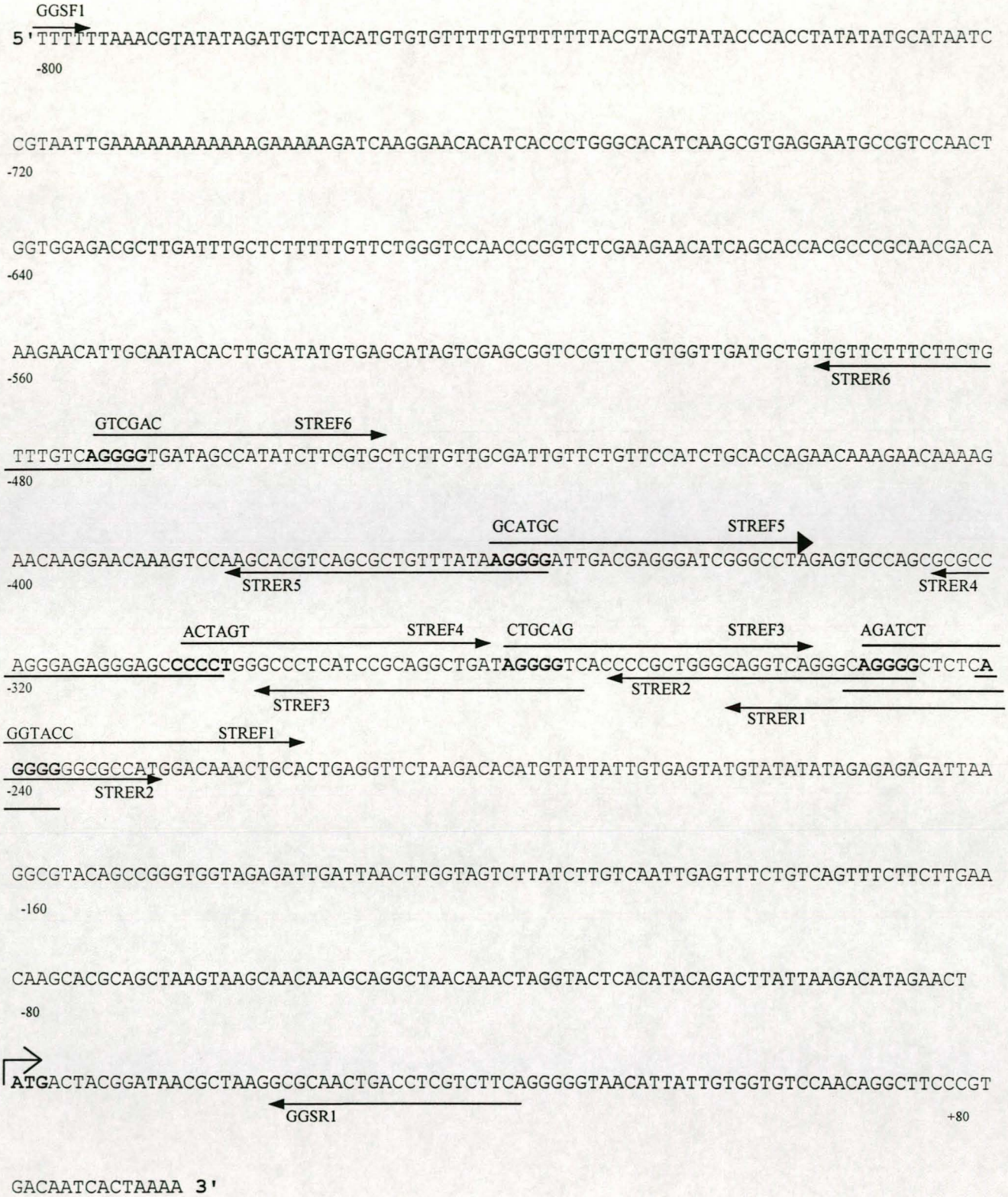

Fig. 2. Promoter sequence of the *TPS1* gene. The ATG is indicated at position +1 and the all STRE elements are in bold, with the sequence of the introduced restriction site above the sequence. The annealing positions of the two extreme primers containing the *Bam*HI (GGSR1) and the *Xba*I (GGSF1) restriction enzyme sites, respectively, are also shown.

β-galactosidase assays. Yeast cells transformed with pGAG1-plasmids were inoculated into 10 ml SC^{-ura} medium and cultures were grown to a cell density of 0.8 to 0.9 at OD_{600}. Assays were performed as previously described by Guarente (1983).

ORF-fragments. Due to the absence of suitable restriction enzymes to study different sections of the *TPS1* ORF, seven 350 bp fragments that represent the entire ORF, as well as a region upstream of the *TPS1* gene, were generated by PCR. These fragments were constructed in such a way that they overlapped with 50 bp (Fig. 3). The DNA sequences and annealing positions of the primers used for amplification are shown in Table 1. These fragments were used as target DNA in gel retardation assays.

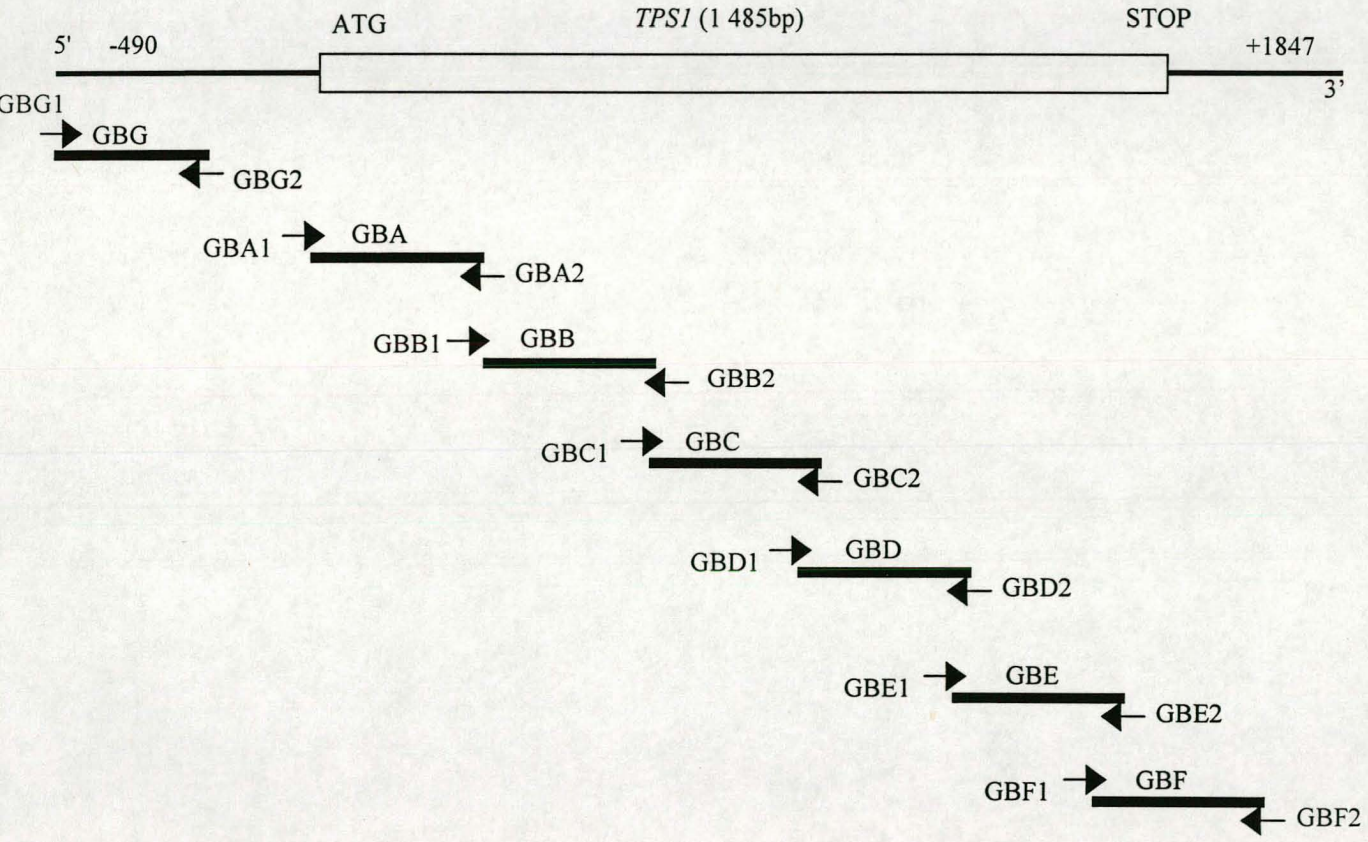

Fig. 3. Schematic representation of the 350 bp PCR - generated fragments used for gel retardation assays all overlap with 50 bps, except for the upstream fragment GBG. The direction of the primers and the name of each fragment are indicated.

Gel retardation experiments. Target DNA was labelled with [α-^{32}P]dATP or [α-^{32}P]dCTP in a standard fill-in reaction using Klenow DNA polymerase (Boehringer Mannheim, Germany). A crude protein extract from the wild type

strain W303 was used and prepared essentially as described by Winkler *et al.* (1988). Before the addition of radioactive labelled fragments (1ng double-stranded oligonucleotide/lane), an excess of poly dI-dC (5 μg) was added to 40 μg of protein extract to exclude non-specific binding. The labelled fragments and protein extract were incubated at room temperature for 20 min in a buffer containing 4 mM spermidine, 0.1 mg/ml BSA, 0.2 mM EDTA, 0.5 mM dithiothreitol, 4 mM $MgCl_2$, 50 mM Tris-HCl (pH 8.0), 50 mM KCl, 20 mM Hepes-KOH and 20% glycerol. Samples were subjected to gel electrophoresis on a 6% polyacrylamide gel (Machler *et al.*, 1993).

ORF truncations of the *TPS1* gene. Different truncations of the *TPS1* ORF were made by Luyten (1996), using a PCR approach. Primers were constructed in such a way that the PCR products lacked a piece at either the 5' or 3' terminus of the coding region (Fig. 4.), leaving the promoter and termination sequences intact. All constructs were subcloned into a multicopy vector (YEp)

RNA isolation and northern blot analysis. Isolation of total RNA from the yeast *S. cerevisiae* was carried out according to De Winde and Grivell (1992). Total RNA was separated on a 1% agarose-formaldehyde gel (50 mM boric acid, 1 mM sodium citrate, 5 mM NaOH, pH7.5, 1% formaldehyde). The RNA was subsequently blotted onto Hybond-N membranes (Amersham) and hybridized with a ^{32}P-labelled *TPS1* fragment. High Prime labeling kit (Boehringer Mannheim, Germany) was used for labeling of the *TPS1* probe. The blots were then analysed using phoshorimager technology (Fuji, BAS-1000; software, PCBAS 2.0). Actin mRNA levels were used as standards.

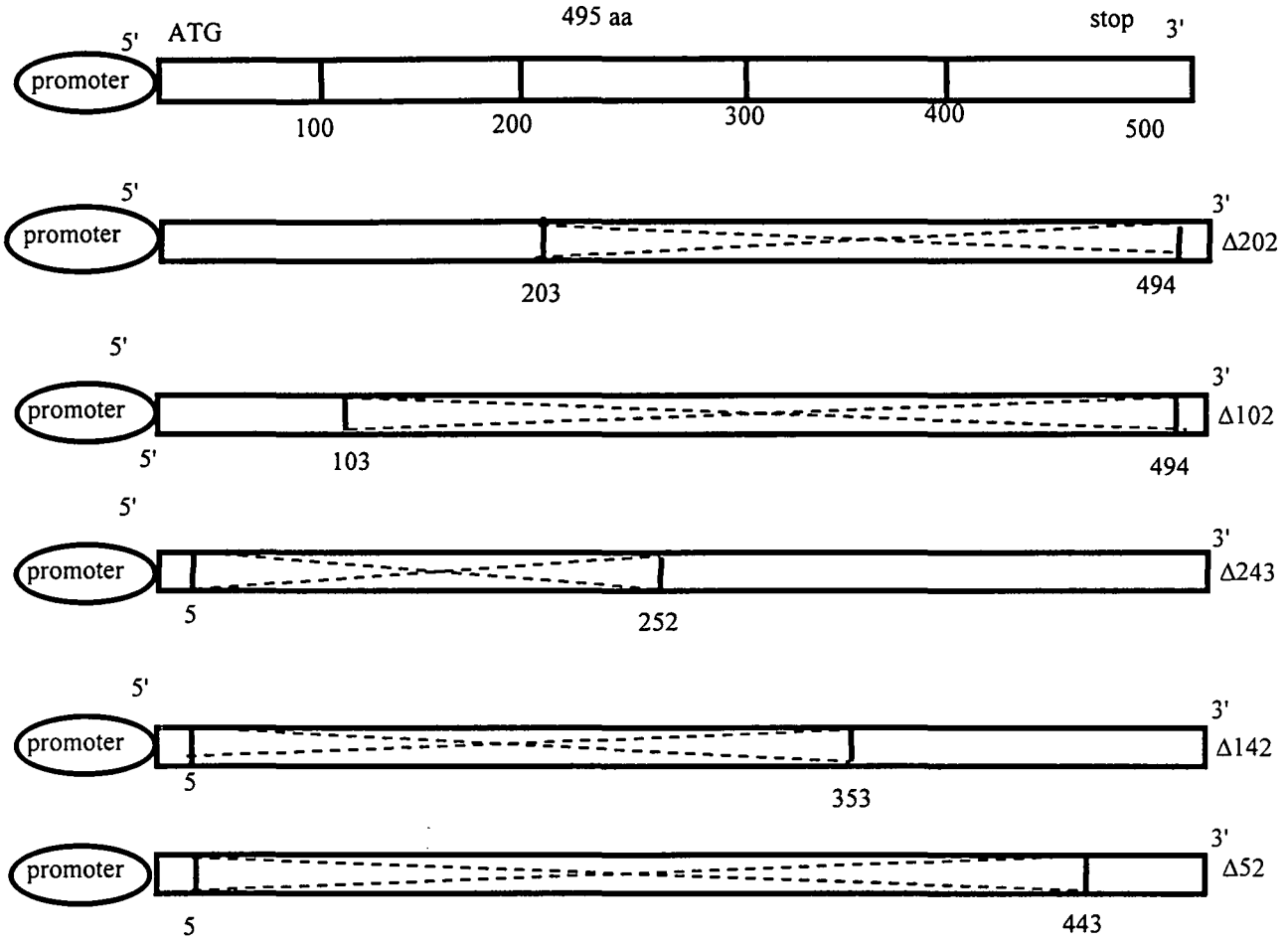

Fig. 4. Different 5' and 3' ORF truncations in the *TPS1* gene. The intact gene is shown at the top and consists of 495 amimo acids (aa). For the deleted constructs, the numbers (Δ202, Δ102, Δ243, Δ142 and Δ52) on the right of each construct indicate the number of amino acids still intact. Deleted fragments are indicated with broken lines.

Table 1. Primers used for PCR amplification of overlapping 350 bp fragments

Primer	Sequence	Annealing position
GBA1	5' ACTATGACTACGGATAA 3'	-3
GBA2	5' GTAATGGAATAACGGCC 3'	+347
GBB1	5' TTACACTACAACGGGTT 3'	+297
GBB2	5' GTGGAACCCGACTAAAT 3 '	+647
GBC1	5' AGACAAGAGATTTTGAA 3'	+597
GBC2	5' TTCTGGATGCTCGTTCA 3'	+947
GBD1	5' GTGCCTCAGAAGTTGCA 3'	+897
GBD2	5' CTCACTCAGGATTAAGG 3'	+1247
BGE1	5' GAATATATTGCTTGCCA 3'	+1197
GBE2	5' AAGAGAGGGCAGAGAAA 3'	+1547
GBF1	5' TGCAAATGAGACGATCG 3'	+1497
GBF2	5' GTTTATGTAGCCAATAT 3'	+1847
GBG1	5' TTCTTTCTTCTGTTTGT 3'	-490
GBG2	5' TACCACCCGGCTGTACG 3'	-140

Synthetic STRE-oligonucleotides. Several oligonucleotides were synthesized (Table 2) to investigate the functionality of the STRE elements in the promoter of the *TPS1* gene. A 23 bp oligonucleotide containing the intact STRE3 element (AGGGG) of the *TPS1* promoter and a 24 bp oligonucleotide containing two STRE elements of the *CTT1* gene were included. The latter served as a positive control for STRE-binding proteins in gel retardation assays. For competition studies, synthetic oligonucleotides with mutated STRE sites of the *TPS1* gene were constructed, and carried either a single or double mutation. The oligonucleotides TPSHUTA and TPSHUTB are complimentary and contained a single mutation in the STRE site changing it from AGGGG to AGGCG. The other two complimentary sequences, TPSHUT1 and TPSMUT2, contained a double mutation with the STRE sites changed from AGGGG to AGCGA.

TABLE 2. Oligonucleotide sequences used in gel retardation assays and competition studies.

Primer	[a]Sequence
TPS1	5' GatcTGAT**AGGGG**TCACCCCGCTGGGCgatc 3'
CTT1	5' gatcAAGGGGATCACCGGTAAGGGGCCA 3'
TPSHUTA	5' gatcGCTGATAGTTGTCACCCCgCTG 3'
TPSHUTB	5' gatcCAGcGGGGTGACAACTATCAGC 3'
TPSMUT2	5' gatcGCTGATAGTTGTCACT**tCgCT**G 3'
TPSHUT1	5' gatcC**AGcGa**AGTGACAACTATCAGC 3'

[a]Nucleotides which differ from the wild-type sequence are given in lower case. STRE-sites are in bold.

RESULTS

Identification of DNA-protein interactions. As mentioned above, previous results suggested the presence of *cis*-acting transcriptional regulatory elements within the ORF of the *TPS1* gene. In addition, *TPS1* the expression pattern suggested that the gene could be autoregulated. We therefore investigated this hypothesis by scanning the *TPS1* ORF for DNA-protein interactions. This was carried out by gel retardation assays using 350 bp PCR-generated fragments which collectively represent the entire ORF and 5' flanking region of the *TPS1* gene (Fig. 3). These fragments overlapped with

50 bp to ensure that the entire ORF was scanned. No specific shift in the electrophoretic mobility of the fragments could be detected in any of the conditions (data not shown), suggesting no specific protein binding sites within the *TPS1* ORF.

Truncations in the *TPS1* ORF and northern blot analysis. The hypothesis of autoregulation was investigated in more detail by truncating the *TPS1* ORF, leaving the promoter region intact (Luyten, 1996). The native Tps1p consists of 495 amino acids (aa), and the smallest deletion construct used was only 52 aa long (Fig. 4).

Expression studies were carried out by transformation of the different truncated *TPS1* constructs into three different *S. cerevisiae* strains; i.e. the wild type strain W303-1A (Thomas and Rothstein, 1989), W303-1A with a disrupted *TPS1* gene (*tps1Δ::TRP1*), and W303-1A with a disruption of both *TPS1* and *HXK2* (*tps1Δ::TRP1hxk2Δ::LEU2*) (Hohmann *et al.,* 1993). In the WT strain W303-1A, northern blot analysis indicated no difference in the expression between the deletion constructs and the native gene (Fig. 5). This suggests that no *cis*-acting element exists within the deleted areas comprising more than 90% of the gene. However, since the wild type gene was present in this strain, we also investigated two additional genetic backgrounds.

The *tps1Δ::TRP1* strain allowed us to study the sole effect of the truncation proteins and ensured that we were not monitoring any artifacts. As mentioned above, the *tps1Δ* leads to pleiotropic phenotypes which include the absence of growth on easy fermentable carbon sources such as glucose. The addition of glucose to the *tps1* mutants leads to an uncontrolled influx of sugar into glycolysis which results in the hyperaccumulation of sugar phosphates, depletion of ATP levels and a drastic drop in free inorganic phosphates (Neves *et al.*, 1995). Due to this metabolic disorder, glycolysis arrests. Since a deletion of the *HXK2* gene suppresses the effect of the *TPS1* deletion (Hohmann *et al.*, 1993), we therefore transformed the constructs into the *tps1Δ::TRP1 hxk2Δ::LEU2* strain to overcome this metabolic problem

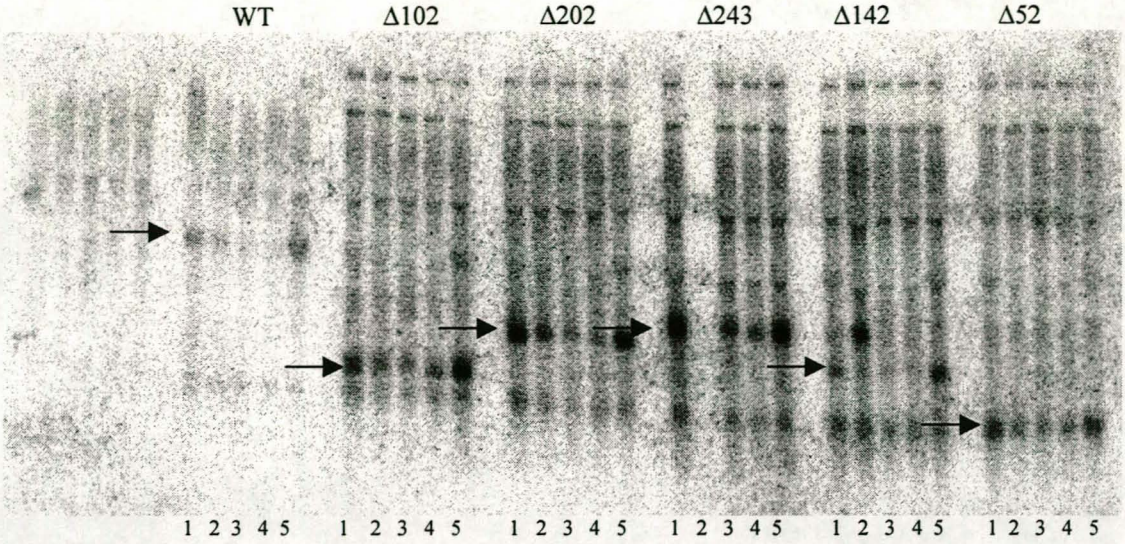

Fig. 5. Northern blot analysis under glucose shift conditions shows the transient expression when *TPS1* truncation constructs (Δ52, Δ142, Δ202, Δ243, Δ102) are transformed into WT strain. The numbers are indicative of the number of amino acids still intact. Cells were grown until exponential phase and mRNA samples were taken at different time intervals. The gel was loaded as follows: lane 5, before addition of glucose; lane 4, 3, 2 and 1 shows the expression 10, 30, 60 and 180 min after glucose addition, respectively.

Northern blot analysis using the *tps1Δ::TRP1* strain showed that the expression of the truncated *TPS1* constructs is completely repressed under glucose shift conditions. This can, however, be explained by the metabolic defects of this strain as described above. Northern blot analysis with the *tps1Δ::TRP1 hxk2Δ::LEU2* strain showed that the construct with the largest truncation *Δ52,* still maintains the transient repression pattern found under glucose shift conditions (Fig. 6). This result suggests that autoregulation is not involved in the regulation of the *TPS1* gene.

Identification of functional STRE elements. The presence of six putative STREs in the promoter of the *TPS1* gene strongly suggests that this gene could be STRE regulated. To study this hypothesis, we performed gel retardation experiments using an oligonucleotide containing the third STRE element of the *TPS1* promoter (Fig. 2) and a crude protein extract from the wild type strain. A STRE-containing oligonucleotide of the STRE-regulated *CTT1* gene (Machler *et al.*, 1993) was used as a positive control. A similar shift was obtained for the *TPS1* and *CTT1* oligonucleotides (Fig. 7).

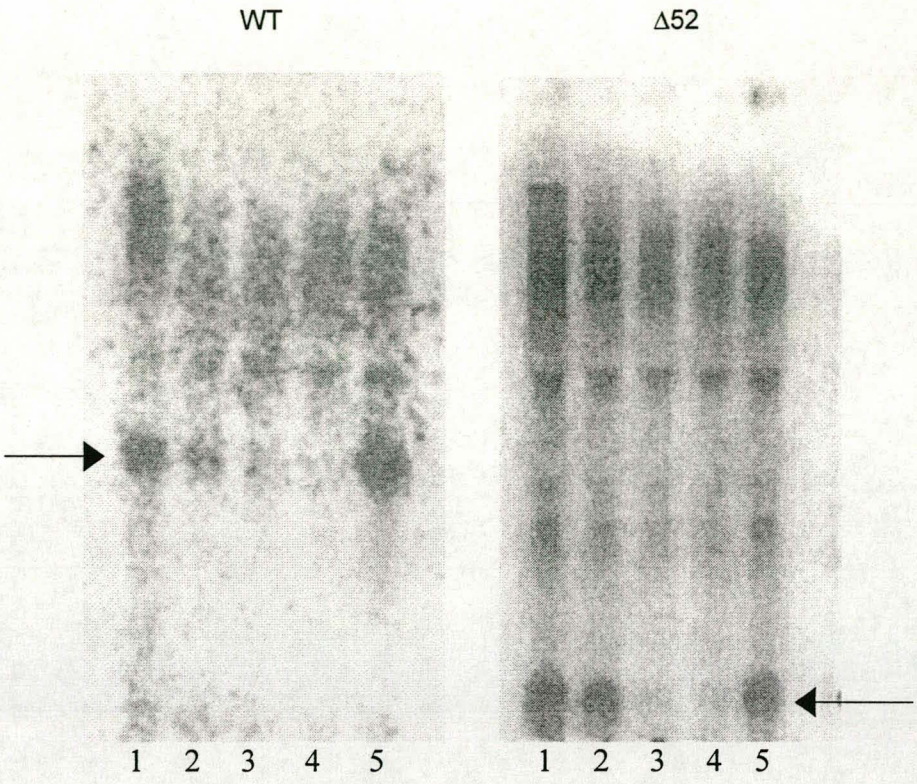

Fig. 6. Northern blot analysis under glucose shift conditions for the *TPS1* truncation construct Δ52, transformed into the W303-1A (*tps1Δhxk2Δ*) strain. Cells were grown until exponential phase and RNA samples were taken at different time intervals. The gel was loaded as follows: lane 5, before addition of glucose; lane 4, 3, 2 and 1 shows the expression, 10, 30, 60 and 180 min after glucose addition, respectively.

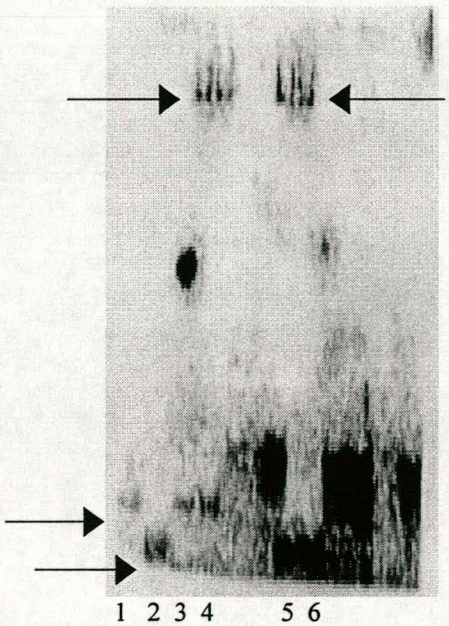

Fig. 7. Gel retardation experiments with ^{32}P-labelled STRE containing oligonucleotides of *TPS1* and *CTT1* for identification of similar DNA-protein interactions. A crude protein extract from stationary phase W303-1A cells was used. Lanes 1 and 2 show the *CTT1* and *TPS1* oligonucleotides without protein extract, lanes 3 and 4 show the *CTT1* oligonucleotide, and lanes 5 and 6 the *TPS1* oligonucleotide with protein extracted from glucose and galactose containing media, respectively.

For competition studies, two sets of oligonucleotides were constructed, containing either a single or a double mutation in the *TPS1* STRE element. Competition experiments were carried out with the different sets of oligonucleotides to verify whether these elements show any specificity for DNA-binding proteins. Excess unlabelled oligonucleotides (60-, 80- and 100-fold) were added to compete with labelled wild type *TPS1* oligonucleotide for specific protein binding. Our results (Fig. 8) showed that the oligonucleotide with the single mutation in the STRE site still competed for binding, although less efficiently than the wild type oligonucleotide, as should be expected. The oligonucleotide with the double mutation did not compete at all, showing that the nucleotides in the putative STRE element are important for binding. Interestingly, the *CTT1* oligonucleotide did not show any significant competition, suggesting that different proteins are involved in the binding of the *TPS1* element.

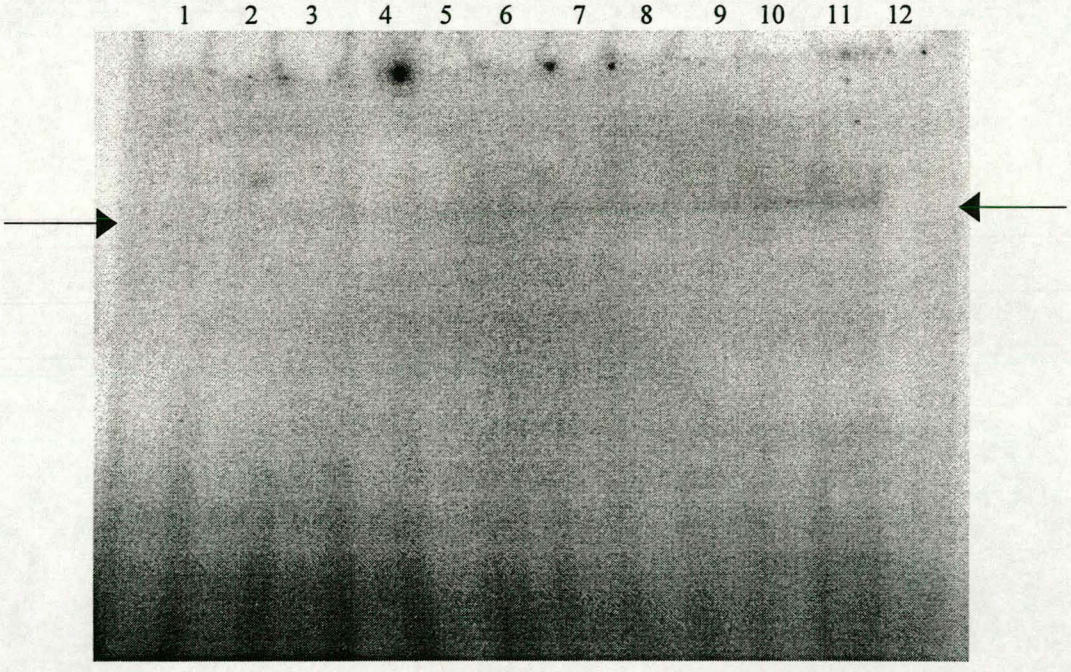

Fig. 8. Competition studies with ^{32}P-labelled oligonucleotides. The gel was loaded as follows: Lane 12- labelled *TPS1* oligonucleotide without protein extract; lane11, labelled *TPS1* oligonucleotide with protein; lanes 10, 9 and 8 is the *CTT1* (control) oligonucleotide with 60-, 80- and 100- fold excess unlabelled oligonucleotide ; lanes 7, 6 and 5 are the *TPS* (HUTA/HUTB) oligonucleotides with 60-, 80- and 100- fold excess unlabelled oligonucleotide and lanes 4, 3 and 2 are the *TPS* (HUTA/HUTB) oligonucleotide with 60-, 80- and 100- fold excess unlabelled oligonucleotide; lane 1 shows the labelled *TPS1* oligonucleotide with 80 - fold excess unlabelled oligonucleotide.

Site directed mutagenesis and β-galactosidase assays. Results obtained for the gel retardation experiments indicated that the putative STRE elements in the *TPS1* promoter can be bound specifically by proteins. We therefore further investigated the possibility of STRE-dependent regulation by performing site directed mutagenesis of the six STRE elements found in a region between nt –235 and – 475 upstream of the ATG. Expression studies were carried out using the 844 bp *Xba*I/*Bam*HI WT promoter fragment or the six mutated constructs fused to a *Lac*Z reporter gene. β-galactosidase assays were carried out under heat shock and salt stress conditions. The results obtained showed induction under both heat shock and salt stress conditions in all cases (Table 4). In all the mutant constructs the β-galactosidase values increased by a factor of 4 to 5 within 1 hour after the stress induction shift, identical to the wild type. The basal level of activity was much lower when STRE1 was mutated, but was less affected by mutations in the other STRE elements. In addition, the mutation in STRE1 led to a significant delay in the onset of the stress-induced transcription, but even in this case the final increase corresponds to a factor of 4 to 5. Apart from the mutated STRE1 element, there was little variation in the relative amount of activity for the other mutated STRE elements. For some STRE mutations, however, differences were observed between the temperature and salt shock response. Whether these differences are relevant, will have to be investigated.

Table 3. β-galactosidase activity measured for mutated STRE elements under heat- and salt shock conditions.

			β-galactosidase activity	
			23°C-37°C	Salt
STRE	pGAG1	T_0:	96	172
		T_{10}:	109	164
		T_{30}:	340	500
		T_{60}:	370	540
	PGAG1-STRE1	T_0:	36	37
		T_{10}:	36	26
		T_{30}:	102	26
		T_{60}:	117	146
	PGAG1-STRE2	T_0:	96	163
		T_{10}:	102	156
		T_{30}:	420	299
		T_{60}:	412	433
	pGAG1-STRE3	T_0:	97	97
		T_{10}:	97	136
		T_{30}:	419	343
		T_{60}:	358	433
	PGAG1-STRE4	T_{10}:	58	164
		T_{10}:	64	164
		T_{30}:	219	475
		T_{60}:	239	541
	PGAG1-STRE5	T_0:	48	188
		T_{10}:	54	119
		T_{30}:	343	503
		T_{60}:	314	517
	PGAG1-STRE6	T_0:	89	148
		T_{10}:	76	192
		T_{30}:	347	450
		T_{60}:	316	509

DISCUSSION

We have studied the regulatory mechanisms of the *TPS1* gene expression and investigated the possible role played by the presence of regulatory elements within the ORF, autoregulation and STRE regulation. These possibilities were investigated by means of gel retardation experiments and expression studies. Contrarily to what the discrepancy between northern blot analysis and β-galactosidase assays under glucose shift conditions had suggested (Winderickx *et al.*, 1996), no evidence for the presence of regulatory elements within the ORF could be found. We investigated autoregulation by constructing different truncations (Luyten, 1996) of the ORF without changing the promoter and termination sequences. The expression studies under glucose shift conditions, particularly in the *tps1Δ::TRP1 hxk2Δ::LEU2* strain, seem to reduce the probability of autoregulation of the *TPS1* gene. Nevertheless, we cannot completely exclude this hypothesis.

A second aspect of our investigation of the transcriptional regulation of the *TPS1* gene, related to the presence of six putative stress response (STRE) *cis*-acting elements in the promoter region of the *TPS1* gene. The STRE element consists of the consensus sequence CCCCT and has been identified in the promoters of several stress-inducible genes in yeast. These include the *CTT1* gene encoding cytosolic catalase T (Machler *et al.*, 1993), the DNA damage-responsive gene *DDR2* (Kobayashi and McEntee, 1993) and the *HSP12* gene encoding a heat shock protein (Varela *et al.*, 1995). Interestingly, reports have shown that all the genes encoding enzymes in the trehalose biosynthetic pathway (*TPS1*, *TPS2*, *TPS3* and *TSL1*) contain STRE elements in their promoters. This is true even for the *NTH1* gene, and it has been reported that expression of the *NTH1* gene is heat stress-inducible (Nwaka *et al.*, 1995), but it is not yet known whether the STREs are functional. For the genes encoding enzymes in trehalose biosynthesis only the STREs in *TPS2* have been reported to be functional and stress-inducible (Gounalaki and Thireos, 1994) .

Typical characteristics of STRE-regulated genes are the induction upon

osmotic stress, heat shock, nutritional starvation, the negative regulation by cAMP-dependent protein kinase A and the repression by glucose. Furthermore, more than one STRE element are present in promoters of stress-inducible genes and each element can have different effects on expression. One example is the stress-regulated *UBI4* gene, coding for polyubiquitin which is required for survival in stationary phase (Finley *et al.*, 1987). At least one STRE appears to participate in heat shock induction of *UBI4* gene (Schüller *et al.*, 1994). Another example is the *HSP12* gene, where five STREs are present in the promoter region, but only the one second most proximal to the ATG allows highly induced levels under stress (Varela *et al.*, 1995). Nevertheless, in most cases a high degree of synergism among different repeats is required for efficient transcriptional activation. Factors binding the STRE elements have been elusive, but recently two zinc finger proteins, Msn2p and Msn4p, have been reported to specifically bind to STRE-containing oligonucleotides (Martinez *et al.,* 1996).

Since trehalose is considered to act as a stress protectant and the *TPS1* gene contains six putative STREs in the promoter, it seemed likely that the gene could be STRE-regulated. However, the regulation by STRE elements alone does not explain the fact that the *TPS1* gene is only transiently repressed in glucose media and is therefore less sensitive to downregulation by glucose than most STRE-controlled genes (Winderickx *et al.*, 1996). As mentioned above, trehalose metabolism has been suggested to play a role in the regulation of yeast glycolysis. The difference in expression between most STRE-regulated genes and *TPS1* could be related to the requirement of low amounts of Tps1p under fermentative growth conditions (Hohmann *et al.*, 1994). The presence of STREs does therefore not necessarily imply perfect co-regulation (Winderickx *et al.*, 1996) and other mechanisms may also be involved in the regulation of the gene.

We have clearly demonstrated in this study that the STRE elements are involved in the regulation of the *TPS1* gene. The gel retardation experiments, competition studies and the β-galactosidase assays with the mutated STRE-

LacZ fusion constructs all showed evidence for the involvement of such a mechanism. Gel retardation assays with the *TPS1* oligonucleotide (intact STRE element) showed that nuclear proteins do bind to the STRE element. Competition studies with oligonucleotide containing single and double mutations in the STRE site showed that the defined STRE consensus sequence is important for the binding of the protein. However, it is puzzling that the *CTT1*-derived oligonucleotide does not seem to compete with the *TPS1* STRE element. This could either suggest a much more favorable sequence context for protein binding in the case of *TPS1*, or that different proteins bind to similar sequences in different genes.

The results of the mutation analysis of the different STRE elements suggest that the element closest to the ATG, STRE1, has the strongest influence on transcription levels. The basal level of activity with STRE1 mutated is significantly lower than the wild type or mutations in the other STRE elements. In addition, there is a delay in the transcriptional activation in the STRE1 mutation construct: The wild type and other mutated STRE constructs were fully induced after 30 min exposure to 0.7 M salt, whereas the STRE1 mutation construct was only induced 60 min thereafter. However, the final induction factor (after 60 min) was identical in all cases. It seems that additional factors could play a role in the regulation of the *TPS1* gene.

Apart from additional mechanisms that could play a role in the regulation of the *TPS1* gene, the next step of investigation would be to combine mutations in different STRE elements and study the effect on transcriptional regulation of the *TPS1* gene. It has been shown that combinations of STREs act cooperatively to allow induction. An example is the *DDR2* gene which encodes a DNA damage-responsive gene. In this case, two copies of the STREs activate transcription synergistically, rather than independently, upon heat shock treatment (Kobayashi and McEntee, 1993). Interestingly, they have also shown that the STREs are dependent on the flanking sequences in mediating a heat or stress response. Their findings suggested that the stress inducibility of the STREs depends on a number of factors. In addition, the

STREs seem to be very specific, since mutated STREs do not confer stress inducibility.

Understanding how the *TPS1* gene is regulated could help to clarify the specific functions of the gene product in some fundamental processes in yeast, e.g. glucose signalling. This could also lead to the elucidation of the link between trehalose accumulation and other stress protectants in yeast.

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