WEAK ACID EFFECTS ON GENE ACTIVATION IN YEAST

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ABSTRACT

Weak lipophilic acids have been commonly used as antifungal agents to preserve low pH foods. In low pH cultures weak acids rapidly enter the cell, dissociating to cause intracellular acidification. The plasma membrane H^+-ATPase maintains pH homeostasis by the active extrusion of protons, but this is energetically expensive causing reduced growth rates and biomass yield. All cells show rapid responses when exposed to unfavourable environmental conditions which appear to increase their ability to grow under moderately stressful conditions, or survive conditions of more extreme stress. Yeasts, especially *Zygosaccharomyces bailii*, and to a lesser extent, *Saccharomyces cerevisiae*, can adapt to growth in weak acids at low pH. In *S. cerevisiae*, weak acids elicit strong induction of two plasma membrane proteins, Hsp30 and Pdr12 which play a major role in this adaptive response.

Discrete gene promoter elements responsive to stress have been identified in *S. cerevisiae*. Investigations carried out for this thesis were aimed at characterising the effects of weak acids on the expression of stress gene promoters and stress promoter elements.

Research described in chapter 3 revealed that weak acids can both inhibit the heat shock activation of stress gene promoters and stress promoter elements in low pH cultures or act as chemical inducers of these same sequences in the absence of heat shock, possibly by a mechanism involving intracellular pH.

Studies on the *HSP30* promoter and stress-responsive transcription factor mutants (chapter 4) demonstrated that none of the previously characterised stress signalling pathways are involved in the stress-induced activation of this gene. This is proof for the existence of a novel stress response pathway.
Chapter 5 outlined a strategy to isolate homologues of *S. cerevisiae HSP30* in other yeast species. Although the approach used was unsuccessful *Candida albicans* and *Hansenula polymorpha* were shown to contain DNA sequences that hybridized to *HSP30* at high stringency.
ACKNOWLEDGEMENTS

I would like to thank my supervisor, Dr Peter Piper, whose intelligence, guidance and scientific insight structured my research. Thanks also to all members of Lab G18, past and present, especially Richard Braley and Barry Panaretou for their helpful advice and friendship. I would also like to thank my mother, father and Maria for their continued support throughout the years. Finally I would like to thank the BBSRC for funding this project and extending the stipend due to a serious illness.
ABBREVIATIONS

A absorbance
AR analytical reagent
ATP adenosine-5'-triphosphate
bp base pairs
cAMP cyclic adenosine monophosphate
Ci(μCi) curies (microcuries)
DEPC diethylpyrocarbonate
dH₂O deionised plus 1 x distilled water
EDTA ethylenediamine tetra acetic acid
Fig Figure
5-FOA 5-fluoro-orotic acid
HSE heat shock element
HSF heat shock transcription factor
Hsp heat shock protein
kb kilobase
kDa kilodaltons
lacZ β-galactosidase
MES 2-(N-morpholino) ethane sulphonic acid
OD optical density
ONPG O-nitrophenyl-β-D-galactoside
PDRE pleiotropic drug resistance element
PDSE post-diauxic shift element
pHi intracellular pH
RNase ribonuclease
STRE stress response element
yARE yeast AP-1 responsive element
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CHAPTER 1

1. INTRODUCTION

1.1 The stress response of yeast

The survival of living cells is dependent on their ability to sense changes in the environment and to respond appropriately to the new situation. Cells can be challenged by variations in temperature, pH, the availability of nutrients, osmolarity or by the presence of pro-oxidants or cytotoxic compounds (Ruis & Schuller, 1995). Exposure to many adverse environmental conditions evokes rapid molecular responses which assist survival. These responses are commonly designated stress responses. They can either increase the ability of cells for growth under moderately stressful conditions or enhance survival under conditions of even more severe stress. Stress responses lead to the acquisition of increased stress tolerance by enhancing the repair of molecular damage and establishing mechanisms that limit stress-induced damage.

A large number of cellular responses to different chemical or physical stress conditions have been described in S. cerevisiae (reviewed by Hohmann & Mager, 1997). These responses include the immediate production of compounds which protect cellular components and the activation of signalling pathways which increase the expression of a wide variety of protective genes (Lindquist, 1986; Mager & Moradas-Ferreira, 1993; Mager & de Kruijff, 1995). A fundamental aspect of the yeast stress response is the phenomenon of acquired resistance whereby cells pre-exposed to a comparatively mild non-damaging stress can withstand a more severe form of the same stress. For example, a mild heat shock induces the highly conserved heat shock response which increases the capacity for the cell to withstand potentially lethal temperatures (Coote et al., 1991; Piper, 1993). The same has been described for pre-treatment with sorbate (Bills et al., 1982; Holyoak et al., 1996), 0.7 M NaCl (Varela et al., 1992) and hydrogen peroxide (Collinson & Dawes, 1992; Jamieson, 1992). Consequently, mild stress conditions probably trigger the appropriate cellular responses to prepare cells for more severe exposure to the same stress. Sometimes, pre-exposure to a mild stress condition will induce resistance not only to this stress, but also tolerance against the severe stress caused by other agents. For example, a mild treatment with either sorbate (Coote et al., 1991), NaCl (Varela et al., 1992) or ethanol (Piper et al., 1994) increases thermotolerance in the absence of heat stress. This cross protection may
partly reflect the inherent overlap between certain stress responses, whereby exposure to a particular stress may lead to tolerance against another type of stress.

In addition to specific stress responses, *S. cerevisiae* also has what has been termed the "general stress response", primarily triggered by low protein kinase A levels (Thevelein, 1994; Siderius & Mager, 1997). Some enzymes already present in non-stressed cells, such as protein kinases and enzymes involved in trehalose metabolism, are activated by the stress of nutrient limitation and provide an immediate stress response (Ruis & Schüller, 1995). The general stress response switches cells to a more quiescent state in which they are much more resistant to practically all stresses. The stress response of yeast is summarised in Fig. 1.1. The effect of each stress response at the level of gene expression is complex but generally the expression of most genes is lowered or switched off under stress conditions while the induction of a small number of specific stress genes is enhanced (section 1.2). Miller *et al.* (1982) found that more than 80 of 500 *S. cerevisiae* proteins examined were induced by a temperature upshift from 23°C to 37°C, while the synthesis of a further 300 proteins was reduced. This suggests that while the majority of genes possess stress-sensitive transcriptional repression, stress genes contain positive control elements which enhance their activation upon stress exposure (section 1.3). This thesis attempts to investigate in greater detail the effects of a weak acid preservative stress on stress gene activation in *S. cerevisiae* (section 1.4).
Fig. 1.1 Different stress conditions are sensed by the yeast cell and trigger both specific and general molecular responses. These stress responses result in changes at the level of enzyme activities and gene expression and lead to the acquisition of stress tolerance (modified from Siderius & Mager, 1997).
1.2 Stress proteins

When cells from a wide variety of species are exposed to an upshift in ambient temperature, they respond by synthesising a small number of highly conserved proteins, the heat-shock proteins (Hsps). Hsps have been implicated in all major growth related processes such as cell division, DNA synthesis, transcription, translation, protein folding and transport (molecular chaperones) and membrane function (Craig et al., 1993; Mager & Moradas Ferreira, 1993). This response is universal and has been called the heat shock response (reviewed by Lindquist, 1986; Nagao et al., 1990; Piper, 1997). An almost identical response is induced by ethanol exposure (Piper et al., 1994), while the responses to osmostress (reviewed by Serrano et al., 1997) or pro-oxidants (Jamieson, 1992; Jamieson et al., 1994) are quite distinct from each other and from this heat shock response, although these mechanisms cause a transient increase in the synthesis of at least some of the Hsps. As shown in this thesis, the response to weak acid exposure is yet another stress response (section 1.4.2).

The heat shock response was first reported as a dramatic increase in transcriptional activity induced by a brief heat treatment of Drosophila larvae (seen as a new set of puffs on the salivary gland polytene chromosomes; Ritossa, 1962). Tissières et al. (1974) proved the existence of heat shock proteins and soon afterwards the heat shock genes of Drosophila were among the first eukaryotic genes to be cloned (Livak et al., 1978; Craig et al., 1979; Voellmy et al., 1981). Similar findings with chicken fibroblasts (Kelley & Schlesinger, 1978), E. coli (Lemaux et al., 1978), yeast (McAlister & Finkelstein, 1980), plants (Barnett et al., 1980) and in many other organisms suggested that the heat shock response represents an evolutionarily conserved genetic system which might be beneficial for the living cell. Although Lindquist (1981) revealed that in Drosophila a sudden heat shock both specifically represses the translation of pre-existing mRNAs and induces the synthesis of mRNAs encoding Hsps, a translational control mechanism is not as prevalent in yeast.

The range of Hsps synthesised in yeast upon a stress challenge is similar to that produced in other cells. The major Hsps can be classified into five families according to their average apparent molecular mass, hsp100 (hsp104 in yeast; section 1.2.1), hsp90 (hsp82 in yeast; section 1.2.2), hsp70 (DnaK in E. coli; section 1.2.3), hsp60 (the chaperonin or groEL-family; section 1.2.4) and small-size Hsps (hsp12, hsp26 and hsp30 in yeast; sections 1.2.5 and 1.4.2.1). Several proteins homologous to Hsps are synthesised constitutively, such as hsc82 (Parsell & Lindquist, 1993), reflecting the important cellular roles performed by these proteins under normal growth conditions. In
addition, the rate of synthesis of several other proteins such as ubiquitin (section 1.2.6), some glycolytic enzymes and catalase (section 1.2.7) are strongly induced upon stress exposure and should therefore be considered as heat shock proteins (see Table 1.1 for summary). In *S. cerevisiae*, heat shock also induces the secretory glycoprotein Hsp150 which has no known function (Russo *et al.*, 1992).

1.2.1 Hsp104

Yeast Hsp104 is a member of the heat shock protein family with a molecular mass greater than 100 kDa (Parsell & Lindquist, 1993; Piper, 1997). The *HSP104* gene was first isolated by Sanchez & Lindquist (1990) and sequenced by Parsell *et al.* (1991). Hsp104 is thought to play an important role in tolerance to extreme stress conditions including severe heat stress and ethanol (Sanchez & Lindquist, 1990; Sanchez *et al.*, 1992; Lindquist & Kim, 1996). Hsp104 is not expressed during normal growth on fermentable carbon sources, but constitutively synthesised in respiring cells and strongly induced during heat shock, transition to stationary phase and early in sporulation (Sanchez *et al.*, 1992). Cells carrying mutations in the *HSP104* gene grow at the same rate as wild-type cells between 25-37°C, demonstrating that Hsp104 is dispensable under normal growth conditions. However, within a few minutes exposure to 50°C (after a sublethal pre-treatment at 37°C), mutant cells begin to die at 100 to 1000 times the rate of the wild-type cells, indicating that Hsp104 is essential for induced thermotolerance (Sanchez & Lindquist, 1990). Mutant cells are not sensitive to copper and cadmium and are only slightly more sensitive to arsenite, suggesting that damage by these agents is different to that caused by heat (Sanchez *et al.*, 1992).

As in other eukaryotes, Hsp104 is thought to have a nuclear localisation (Subjeck *et al.*, 1983). Some clues to the possible functions of Hsp104 come from the analysis of its primary structure. The heat inducible members of the Hsp100 family from yeast, plants, animals and bacteria share approximately 60% homology across their entire lengths and even higher similarity in regions of two ATP-binding site consensus elements, suggesting that they share a conserved heat-related function (Parsell & Lindquist, 1993). Site-directed mutagenesis revealed that these binding sites are essential for the stress protective function of Hsp104 (Parsell *et al.*, 1991). Yeast Hsp104 is closely related to the heat-inducible *E. coli* ClpB protein (Squires *et al.*, 1991).
Table 1.1 Heat shock proteins of yeast (from Mager & Moradas Ferreira, 1993)

<table>
<thead>
<tr>
<th>Designation</th>
<th>Cellular localisation</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hsp150</td>
<td>(Secretory)</td>
<td>Unknown</td>
</tr>
<tr>
<td>Hsp104</td>
<td>Nucle(ol)us</td>
<td>Stress tolerance</td>
</tr>
<tr>
<td>Hsp83</td>
<td>Cystol/nucleus</td>
<td>Chaperone</td>
</tr>
<tr>
<td>Hsp70</td>
<td>Cystol?</td>
<td>Chaperone</td>
</tr>
<tr>
<td>Ssa1</td>
<td>Cystol</td>
<td>Chaperone</td>
</tr>
<tr>
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<td>Unknown</td>
<td>Chaperone</td>
</tr>
<tr>
<td>Ssc1</td>
<td>Mitochondria</td>
<td>Chaperone</td>
</tr>
<tr>
<td>Ssd1 (Kar2)</td>
<td>Endoplasmic reticulum</td>
<td>Chaperone</td>
</tr>
<tr>
<td>Hsp60</td>
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<tr>
<td>Hsp12</td>
<td>Cystol</td>
<td>Unknown</td>
</tr>
<tr>
<td>Ubiquitin</td>
<td>Cystol</td>
<td>Protein degradation</td>
</tr>
</tbody>
</table>

Enzymes

| Enolase      | Cystol                | Glycolysis             |
| Glyceraldehyde-3-P-dehydrogenase | Cystol | Glycolysis |
| Phosphoglycerate kinase | Cystol | Glycolysis |
| Catalase (Ctt1) | Cystol | Antioxidative defence |
Parsell et al. (1994) revealed that vegetative cells lacking Hsp104 show enhanced accumulation of heat-induced aggregates of vital cell structures, implying that Hsp104 may act as a catalyst of protein disaggregation or reactivation. The Hsp70 Ssa1 isoform assumes an important role in tolerance to extreme temperatures in the absence of Hsp104, while in cells with low levels of Ssa1, Hsp104 assumes an important role in growth at normal temperatures, suggesting that Hsp70 Ssa1 and Hsp104 have complementary roles (Parsell et al., 1994). Lindquist & Kim (1996) showed that HSP104 could not be deleted in hsfl-m3 cells (which have a nonsense mutation in the HSF1 gene, section 1.3.1) because expression of heat shock factor (and the viability of the strain) requires nonsense suppression mediated by the yeast prion [psi+], which in turn depends upon Hsp104. The self-modifying psi+ factor becomes lost in yeast strains with overexpression or inactivation of HSP104, suggesting a chaperone role for Hsp104 (Chernoff et al., 1995).

1.2.2 Hsp90

Like the Hsp70s, members of the Hsp90 class of Hsps are highly conserved in bacteria, yeasts and mammals and show among eukaryotes at least 50% sequence identity (Craig & Lindquist, 1988). Hsp90s are abundant chaperone proteins, localised primarily in the cytoplasm; a small fraction translocates to the nucleus upon heat shock (Schlessinger, 1990). Hsp90s differ from both Hsp60s (section 1.2.4) and Hsp70s (section 1.2.3) in that they regulate the function of specific, substantially folded proteins. In vertebrates Hsp90s interact with a variety of cellular proteins, including steroid hormone receptors, several kinases and the cytoskeleton proteins actin and tubulin (Lindquist, 1986; Mager & Moradas Ferreira, 1993; Hartl, 1996). Glucocorticoid receptor proteins are kept in the cytoplasm in an inactive conformation through interaction with Hsp90 which serves as a cytoplasmic anchoring protein (Hunt, 1989). In S. pombe Hsp90 participates in cell cycle control by regulating Weel protein tyrosine kinase activity (Parsell et al., 1991). However, in S. cerevisiae, a demonstrable function for Hsp90 has yet to be identified.

S. cerevisiae contains two genes encoding Hsp90: HSP82 and HSC82 (Borkovich, 1989) whose products are 97% identical (Parsell & Lindquist, 1993). HSC82 (heat shock cognate) is constitutively expressed and is only weakly induced upon stress exposure while HSP82 has a low basal level of expression and is strongly activated during heat shock (Borkovich, 1989), transition to stationary phase (Kurtz & Lindquist, 1984) and early in sporulation (Kurtz et al., 1986). The elevation of Hsp90
levels by heat shock appears to reflect a need for higher levels of Hsp90 during high temperature growth. If either of these genes is inactivated, the cell is unable to grow at high temperature while inactivation of both genes renders the cell inviable. The three-dimensional structure of the 27 kDa N-terminal domain of the yeast protein containing three of the four highly-conserved regions of Hsp90 structure and the ATP-binding domain has recently been solved (Prodromou et al., 1997).

1.2.3 Hsp70

Hsp70 proteins are the most highly conserved Hsps among all species. A 50% identity exists between Hsp70 proteins found in higher eukaryotes and the *E. coli* Hsp70 DnaK, while eukaryotic proteins are between 50 and 97% identical (Lindquist & Craig, 1988). The genes encoding Hsp70 in *S. cerevisiae* constitute a multigene family consisting of eight members (Table 1.1) which are subdivided into four subfamilies, SSA, SSB, SSC and SSD (Stress Seventy). SSA (SSA1-4) and SSB (SSB1-2) constitute the cytoplasmic Hsp70s and are thought to be functionally distinct (Boorstein & Craig, 1990 a and b; Parsell & Lindquist, 1993; Mager & Moradas-Ferreira, 1993; Hartl, 1996). SSA gene products are important for protein translocation across the ER and mitochondrial membranes while SSB products are associated with polysome-associated nascent peptides (Hartl, 1996). Three of the four SSA genes are induced by heat shock and the SSA subfamily is indispensable for growth. The SSB genes are repressed by heat shock and mutations in SSB gene products result in a cold-sensitive phenotype (Parsell & Lindquist, 1993; Mager & Moradas-Ferreira, 1993; Hartl, 1996). The SSC and SSD subfamilies (reviewed by Craig et al., 1993) encode two organelle localised proteins: Ssc1 (SSC1) in the matrix of the mitochondrion (Craig et al., 1987; 1989) and Ssd1/Kar2 (KAR2; section 1.3.4) in the lumen of the ER (Normington et al., 1989).

It is likely that Hsp70 proteins induced upon stress exposure perform functions similar to those under normal growth conditions, namely molecular chaperones. Hsp70 induced by heat shock is thought to mainly function in sequestering partially heat-damaged protein until this protein can either be degraded or reactivated. In unstressed cells a major role of the different cytoplasmic forms of the Hsp70 protein is to associate and control the folding states of newly synthesised polypeptide chains for translocation across the ER or mitochondrial membranes (Piper, 1997). Hsp70 proteins of both eukaryotes and prokaryotes possess both ATPase and peptide binding activities which are crucial for their chaperone activity (Ziegelhoffer et al., 1995). Sequence similarity between Hsp70 proteins extends over the entire protein, particularly in conserved N-
terminal regions where ATP binding sites are located. ATPase activity is stimulated by binding to either unfolded proteins, synthetic peptides or a DnaJ homologue, Ydj1 (Ziegelhoffer et al., 1995). The peptide-binding domain is thought to be located near the C-terminus (Hartl, 1996). Hsp70 may also interact with the heat shock factor (section 1.3.1), thereby modulating its transcription activating potency (reviewed by Mager & Moradas Ferreira, 1993).

1.2.4 Hsp60

The highly conserved Hsp60 protein family (chaperonins, Cpn60s) facilitate post-translational assembly of polypeptides at normal temperatures, a cellular function similar to that of Hsp70 (Mager & Moradas Ferreira, 1993). Hsp60s play a crucial role in binding to unfolded proteins, catalysing ATP-dependent folding of these proteins and assisting their assembly into higher-order protein structures (reviewed by Craig et al., 1993; Parsell & Lindquist, 1993; Piper, 1997). Yeast Hsp60 (encoded by the essential nuclear MIF4 gene) was first identified as a mitochondrial protein (Cheng et al., 1989), showing 54% homology to the heat-inducible groEL of E. coli, a protein involved in bacteriophage head assembly (McMullin & Hallberg, 1988). Hsp60 is assembled into a typical chaperonin structure of two stacked heptameric rings (reviewed by Ellis & van der Vies, 1991). Mutations in the MIF4 gene were first identified as conditional-lethal mutations which prevented the correct folding and assembly of F1-ATPase, cytochrome b2 and the Rieske FeS protein of complex III (Cheng et al., 1989). Although Hsp60 clearly functions in the folding and assembly of mitochondrial proteins (Ostermann et al., 1989; Koll et al., 1992), its role in the translocation of proteins passing from the matrix into the inner membrane space remains to be resolved. At high temperatures, Hsp60 associates with a wide variety of proteins in yeast mitochondria. This association presumably prevents protein aggregation and promotes refolding when cells are returned to lower temperatures (Parsell & Lindquist, 1993). Hsp60 chaperonins have also been implicated in the cytoplasmic protein-folding machinery but, unlike mitochondrial Hsp60 of S. cerevisiae, they are not heat-inducible. In yeast, this cytoplasmic complex is comprised of the essential Tcp1 protein (tailless complex polypeptide) which plays an important role in the biogenesis of tubulin and actin (Hartl, 1996).
1.2.5 Small Hsps: Hsp12 and Hsp26

Yeast cells contain two major small Hsps: Hsp12 (Praekelt & Meacock, 1990) and Hsp26 (Bentley et al., 1992) for which demonstrable functions have not been shown (Petko & Lindquist, 1986; Susek & Lindquist, 1989). Although both of these proteins are synthesised under many stress conditions (Praekelt & Meacock, 1990; Bentley et al., 1992; Parsell & Lindquist, 1993; Mager & Moradas Ferreira, 1993; Varela et al., 1995), loss of both Hsp12 and Hsp26 in S. cerevisiae produces no apparent phenotype. However, an Hsp12 homologue of Schizosaccharomyces pombe suppressed a mutational defect in the cdc4 gene (late septation), suggesting that Hsp12 could play a role in the formation of the F-actin contractile ring at cytokinesis (Jang et al., 1996). The small Hsps represent a very diverse group of Hsps, which nevertheless display conserved structural features (Lindquist & Craig, 1988) and share the ability to form high molecular weight polymeric aggregates called heat shock granules (Tuite et al., 1990; Bentley et al., 1992). Hsp26 shows a significant sequence similarity to α-crystallin proteins, particularly with respect to a highly conserved hydrophobic domain located at the C-terminus (Tuite et al., 1990). Although Jakob et al., (1993) have recently demonstrated that mammalian small Hsps and α-crystallin molecules exhibit chaperone activity, a function has yet to be assigned to hsp26. Unlike Hsp26, Hsp12 does not show homology to α-crystallin (Praekelt & Meacock, 1990), but shares 47% identity to the N-terminal region of Wh11, a 7.8 kDa polypeptide encoded by a gene differentially expressed in the budding phase and hyphal-forming cells of Candida albicans (Srikantha & Soll, 1993). A universal property of the small Hsps may also be their developmental regulation, such that HSP12 and HSP26 are strongly stress-induced while expression is dramatically increased following transition of cells to stationary phase and upon induction of sporulation. The heat shock, weak acid and low pH-induced expression of HSP12 and HSP26 are investigated in Chapter 3. Hsp30 (Régnacq & Boucherie, 1992; Panaretou & Piper, 1992) is another small Hsp, which is found in the plasma membrane.

1.2.6 Ubiquitin

Conjugation of ubiquitin (Ub) to short-lived or damaged proteins mediates their selective degradation (Finley et al., 1987). Ubiquitin, a highly conserved 76 amino acid protein, acts by becoming covalently attached to the free amino acid groups of target proteins through its C-terminal glycine residue, an attachment catalysed by the ubiquitin
ligases (reviewed by Finley & Chau, 1991). Proteins targeted by ubiquitination are then
degraded by the proteosome (proteinase YscE) (reviewed by Heinemeyer et al., 1991).
In yeast, Ub is encoded by four genes (*UBII-4*). *UBII-3* code for hybrid proteins in
which Ub is fused to unrelated amino acid sequences. Polyubiquitin, a protein encoded
by the *UBI4* gene in yeast (containing five tandem Ub repeats), is thought to be a heat
shock protein since it displays a strongly enhanced rate of synthesis under stress
conditions (Finley et al., 1987). Also, production of the Ubc4/5 Ub conjugating
enzymes is heat shock-inducible (Seufert & Jentsch, 1990). This probably indicates a
much greater requirement for turnover of unfolded and non-functional proteins in cells
recovering from heat shock and other forms of stress. In several cases, it has been
shown that the proteolytic signal takes the form of a multi-Ub chain in which successive
Ub molecules are linked tandemly at various lysine residues, a process strongly
dependent on the presence of Ubc4/5 (Arnason & Ellison, 1994).

Strains carrying deletions in *UBI4*, or *UBC4* plus *UBC5* display a considerably
reduced resistance to starvation, an increased sensitivity to high temperatures, amino
acid analogues and alkylating agents, and a block on the sporulation of a/α ubi4/ubi4
diploid cells (Finley et al., 1987; Tanaka et al., 1988; Treger et al., 1988; Fraser et al.,
1991). This reveals the importance of ubiquitination in the cellular response to stress.
Also, the proteosome is important in the stress response since deletion of a gene
encoding a subunit of this protease causes sensitivity to stress conditions and
accumulation of ubiquitin-protein conjugates (Heinemeyer et al., 1991).

### 1.2.7 Other proteins involved in the yeast stress response

Apart from the classical Hsps, proteins of unstressed cells of yeast and other
organisms play a part in the stress response. Some of these exhibit significantly
increased levels of expression following a stress treatment (Mager & Moradas-Ferreira,
1993). Heat shock increases the activities of at least two enzymes important for
protection against oxidative damage. These are cytoplasmic catalase T (encoded by
*CTT1*) and the mitochondrial manganese superoxide dismutase, MnSOD (encoded by
*SOD2*) (Wieser et al., 1991; Costa et al., 1993). Heat shock-induced transcriptional
activation of *CTT1* and *SOD2* is controlled by the stress response element, STRE
(section 1.3.2). Strains carrying a deletion in *CTT1* show reduced thermotolerance in
both proliferating and stationary cells except when PKA levels are high (Wieser et al.,
1991). Loss of *SOD2* also renders the cell more sensitive to the lethal effects of heat
(Davidson et al., 1996).
The effects of these antioxidant defence enzymes on thermotolerance are thought to reflect the more severe oxidative damage to cellular proteins, nucleic acids and lipids caused by reactive oxygen species at higher temperatures, especially in respiratory cultures (Moradas-Ferreira et al., 1996).

Several enzymes of the glycolytic pathway are induced upon heat treatment of yeast cells. One of the genes encoding glyceraldehyde-3-phosphate dehydrogenase (Lindquist & Craig, 1988), enolase (Iida & Yahara, 1985) and phosphoglycerate kinase (Piper et al., 1986) are induced following a heat shock. Heat stress imposes large demands for energy (ATP) generation by the cell (Findley et al., 1983). Increased glycolytic flux may assist stressed cells to restore intracellular ATP levels, although these enzymes are present in such large amounts in unstressed cells, they are not limiting for glycolytic flux. A further consequence of heat shock and other stress challenges on yeast cells is the transient dissipation of the electrochemical pH gradient across the plasma membrane, leading to a decrease in intracellular pH, activation of plasma membrane H⁺-ATPase (section 1.4.1.1) and induction of a 30 kDa plasma membrane heat shock protein (section 1.4.2.1).

It is well known that glycogen and trehalose accumulate in yeast under nutrient starvation and entry to stationary phase, while high levels of trehalose are found in heat shocked cells (reviewed by François et al., 1997). In yeast, trehalose is a stress-protectant rather than a reserve carbohydrate since it is one of the most effective substances known for in vitro preservation of membrane structures and enzyme activities during desiccation, freezing or heating (Hottiger et al., 1994). Heat shock causes the rapid accumulation of a large cytoplasmic pool of trehalose (up to 100-fold) (Hottiger et al., 1987). There is a good correlation between trehalose levels and thermotolerance in stationary phase and nonfermentative yeast cultures, although no such correlation exists in fermentative yeasts (De Virgilio et al., 1994; van Dijck et al., 1995). The mechanism of heat-induction of trehalose is readily reversible since the trehalose accumulated with heat shock is rapidly mobilised with a subsequent temperature downshift (Neves & François, 1992). The genes involved in glycogen and trehalose metabolism exhibit stress regulation, being induced by nutrient starvation, temperature, osmotic and oxidative stresses (Winderickx et al., 1996; Parrou et al., 1997). Almost all genes encoding the enzymes involved in the metabolism of these two reserve carbohydrates contain single and multiple copies of the STRE (section 1.3.2).
Recently, Parrou et al., (1997) demonstrated that the stress activation of the genes encoding glycogen synthase (GSY2) and trehalose-6-phosphate synthase (TPSI) is dependent on STREs, although the levels of transcription varies considerably. Mobilisation of trehalose with temperature-downshift is defective in strains with low Hsp70 levels (Hottiger et al., 1992) and mutants defective in TPSI do not show normal levels of Hsp synthesis with heat shock (Hazell et al., 1995), suggesting that the induction of Hsps and trehalose in the heat shock response may be linked.

Finally, recent evidence suggests that Ca\(^{2+}\)-activated enzymes and signal transduction pathways are strongly stimulated by heat stress (reviewed by Piper, 1997). Weakness of cell walls at high temperatures might be detected by systems responding to plasma membrane stretch, such as ion channels (Kamada et al., 1995), which in turn lead to Ca\(^{2+}\) influx. Cystolic Ca\(^{2+}\) may then activate Ca\(^{2+}\)-regulated enzymes such as phospholipase C (PI-PLC), protein kinase C (PKC) and Ca\(^{2+}\)/calmodulin-dependent protein kinase. Strains that lack PI-PLC progressively lose viability and eventually lyse after a shift to 37°C (Payne & Fitzgerald-Hayes, 1993; Flick & Thorner, 1993; Yoko-o et al., 1993). Mutants defective in the PKC pathway (Kamada et al., 1995) and mutants lacking Ca\(^{2+}\)/calmodulin-dependent protein kinase (Iida et al., 1995) all show impaired acquisition of thermotolerance with heat shock. There is no evidence that induction of Hsps is impaired in these mutants, suggesting that these pathways contribute to a Hsp-independent mechanism of thermotolerance. Also, the stress-activated cell membrane H\(^{+}\)-ATPase possesses potential phosphorylation sites for the Ca\(^{2+}\)/calmodulin-dependent protein kinase (section 1.4.1.1).

1.3 Stress-induced changes in yeast gene expression

The promoter regions of yeast stress genes contain various positive transcriptional control elements that are activated by stress conditions: heat shock elements (HSEs; section 1.3.1), stress response elements (STREs; section 1.3.2), pleiotropic drug resistance elements and AP-1 responsive elements (PDREs and AREs respectively; section 1.3.3) and unfolded protein response elements (UPREs; section 1.3.4). Some stress proteins encoded by HSE-regulated genes are necessary for growth of yeast at high temperatures (37-39°C), products of STRE-activated genes seem to be involved in survival under severe stress, PDRE-controlled genes confer tolerance to a range of cytotoxic compounds, ARE-induced genes mainly function during stresses which generate \( H_2O_2 \) free radicals and in response to heavy metal ions, while UPRE-expressed genes counteract the effects of compounds which cause the
accumulation of unfolded secretory and transmembrane proteins in the endoplasmic reticulum (ER).

1.3.1 Heat shock elements (HSEs)

In prokaryotes, the heat shock response is mediated by a specific heat-induced 32 kDa sigma factor, σ^{32}, which is a product of the rpoH gene (Grossman et al., 1985). This factor binds to the RNA polymerase holoenzyme (RNAP) and directs it to heat inducible promoters located upstream of heat shock genes. The promoters of these heat shock genes are not recognised by the RNAP carrying the σ^{70} subunit, which undertakes most transcription in the cell at normal growth temperatures. At least 13 heat shock promoters are known to be transcribed by RNAPσ^{32}. These differ from regular promoters in their -35 region (consensus sequence, TCTNCCCTTGAA), their -10 region (consensus sequence, CCCCATNTA), and the length of the spacer (13 to 17 nucleotides) that separate these two regions (Cowing et al., 1985). In general, these promoters are recognised by RNAPσ^{32} and not by RNAPσ^{70} in vitro (Zhou et al., 1988). The mechanisms of heat shock-induced transcription in prokaryotes has been reviewed by Mager & De Kruijff, 1995.

In contrast, transcriptional activation of (most but not all) eukaryotic heat-shock genes by elevated temperature and other forms of physiological stress is mediated by the binding of a transcriptional transactivator, heat shock factor (Hsf1), to a short highly conserved DNA sequence, the heat shock element (HSE), (reviewed by Sorger, 1991; Mager & Moradas Ferreira, 1993). The HSF1 gene encoding the 833 amino acid protein, Hsf1, was first isolated from S. cerevisiae, and shown to be essential for viability at all temperatures (Wiederrecht et al., 1988; Sorger & Pelham, 1988). In higher eukaryotes activation of the Hsf1 requires induction of DNA-binding activity (Sorger, 1991). In yeast, however, Hsf1 exists as a trimer which is constitutively bound to HSEs of target genes irrespective of their transcriptional state (Sorger & Pelham, 1987). Hsfs are composed of a DNA-binding domain at their N-terminus, an adjacent cluster of hydrophobic amino acids (leucine zippers) and a distally located heptad repeat near the C-terminus (Mager & De Kruijff, 1995). Hsfs from different species show only limited sequence homology, the similarity being mainly confined to the DNA-binding and trimerisation domains. In S. cerevisiae, Hsf1 becomes highly phosphorylated following heat shock and although this correlates with the transcriptional activity of the factor over a range of temperatures it is not what
determines activity (Flick et al., 1994). Also, under nonstress conditions Hsf1 remains in an inactive conformation, possibly through the interaction of Hsp70 with a conserved heptapeptide element, RXLLKNR, located near the activator region (Jakobsen & Pelham, 1991). It has been proposed that upon stress exposure Hsp70 is either released from the complex or causes a conformational change in the Hsf1 binding domain, thus enabling the factor to change into an active transcription complex (Mager & Moradas Ferreira, 1993). At present, the mechanism by which the Hsf1 stimulates transcription of heat shock genes remains poorly understood.

HSEs, the DNA binding sites for Hsf1 that are essential for the heat shock activation of many heat shock genes, are contiguous repeats of at least three copies of the 5 bp sequence, nGAAn arranged in alternating orientations (n denotes less strongly conserved nucleotides that may be involved in DNA-protein interactions). Each repeat comprises at least one half-turn of the DNA double helix (Sorger, 1991). The distance between HSEs can differ considerably as well as their location from the transcriptional start site. Also, the degree of homology of the bases in each 5 bp unit to the standard nGAAn motif can influence the affinity with which Hsf1 binds to HSEs (Mager & De Kruijff, 1995). Bonner et al., (1994) investigated the interactions between DNA-bound trimers of the yeast Hsf1. They found that Hsf1 can bind DNA with the sequence nGAAnTTCn or with the sequence nTTCnnGAAn, with little preference for either sequence over the other. Therefore, a single Hsf1 multimer can establish contact with an HSE containing a minimum of two 5 bp units. However, (nGAAnTTCn)2 was found to be considerably less active as a HSE than (nTTCnnGAAn)2. This difference was attributed to the fact that (nGAAnTTCn)2 is capable of binding only one Hsf1 trimer while (nTTCnnGAAn)2 is capable of binding two trimers. HSE/Hsf1 has also been implicated in activation of the yeast metallothionein gene (CUP1) in response to glucose starvation (Tamai et al., 1994) and oxidative stress (Lin & Thiele, 1996). Also, Boorstein & Craig, (1990 a) demonstrated that mutations in two overlapping heat shock elements 156 bp upstream of the SSA3 gene reduced the diauxic-shift-induced expression of the promoter by 71% compared to the wild-type sequence. Although the HSE alone exhibited no diauxic shift activation, the element was shown to act positively with a post-diauxic shift upstream activating sequence similar to the general stress response element (section 1.3.2).
1.3.2 Stress response elements (STREs)

It has become apparent that the Hsf1 -HSE cis-trans combination is not the only pathway mediating stress-induced transcription. Smith & Yaffe (1991) proved that the *S. cerevisiae* Hsf1 is not essential for the induction of resistance to severe heat stress. A nonsense mutation in the *HSF* gene (hsfl-m3) causing temperature sensitivity was found to block the induction of the major heat shock proteins at 37°C, but had no effect on the acquisition of thermostolerance at 50°C. This suggests that Hsf1 is needed for growth during moderate stress but is not required for the induction of tolerance against severe stress. In *S. cerevisiae*, Hsf1-independent control elements have been identified in the promoter regions of a DNA damage-responsive gene *DDR2* (Kobayashi & McEntee, 1990; 1993), a gene encoding the cytoplasmic catalase T *CTT1*, (Wieser *et al*., 1991; Marchler *et al*., 1993), a gene encoding a small heat shock protein of unknown function *HSP12* (Varela *et al*., 1995) and genes involved in trehalose synthesis (Winderickx *et al*., 1996). This alternative promoter element is activated by multiple stress conditions including heat shock, low external pH, weak acid preservatives, ethanol, osmotic and oxidative stress and nitrogen starvation (Belazzi *et al*., 1991; Marchler *et al*., 1993; Schüller *et al*., 1994) and was therefore called the general stress response element (STRE). Its core consensus is AGGGG or CCCCT (Kobayashi & McEntee, 1990; Wieser *et al*., 1991). From analysis of promoter sequences for the AGGGG element, many putative STRE-controlled genes activated by multiple stresses have now been identified (Varela *et al*., 1995; Mager & De Kruijff, 1995; Siderius & Mager, 1997) but the STRE sequences in their promoter regions have mostly yet to be proven functional. A few years ago, Boorstein & Craig, (1990 a) revealed that the *SSA3* gene for Hsp70 contains a variant of the STRE element which is activated during diauxic growth and under stationary phase conditions. This post-diauxic shift element (PDSE) (consensus T(A/T)AGGGAT) contains the AGGGA sequence compared to the AGGGG core consensus displayed in STREs. However, in contrast to the STRE, the PDSE is not activated by heat stress.

Kobayashi & McEntee (1993) revealed that the CCCCT element bound a single 140 kDa polypeptide distinct from Hsf1 in yeast crude extracts, but these results were not reproducible. Recently, two zinc finger proteins, Msn2 and Msn4 (Estruch & Carlson, 1993) were shown to bind specifically to STREs (Martínez-Pastor *et al*., 1996; Schmitt & McEntee, 1996). Zinc fingers are a 30 amino acid sequence motif (arranged: Φ-X-Cys-X2,5-Cys-X3-Φ-X5-Φ-X2-His-X2,5-His, where X=any amino acid, Φ=a hydrophobic residue, Cys=cysteine and His=histidine), DNA binding minidomain
folded around a central zinc ion with tetrahedral arrangement of cysteine and histidine residues (reviewed by Klug & Schwabe, 1995). Tandem repetition of structurally similar small finger domains with different DNA recognition sites is widely used in biological systems for modular recognition of specific DNA sequences. Disruption of both the MSN2 and MSN4 genes results in an increased sensitivity to carbon source starvation, heat shock and severe osmotic and oxidative stresses (Martínez-Pastor et al., 1996). Also, Northern analysis indicated that both MSN2 and MSN4 are required for the stress activation of the CTT1, DDR2, HSP12 and TPS2 genes whose induction is mediated via STREs (Martínez-Pastor et al., 1996; Schmitt & McEntee, 1996). In contrast, MSN2 and MSN4 are not required for the activation of the SSA3 gene under identical stress conditions (Martínez-Pastor et al., 1996). This suggests that the PDSE requires an as yet, unidentified DNA-binding protein and does not behave as a functional STRE. Also, Msn2 and Msn4 are specific to the AGGGG motif of the STRE. At present, the factor(s) binding to PDSEs remain to be elucidated. In addition, it is possible that more factors can bind to the STRE and may compete with Msn2 and Msn4 under certain conditions. Recent computer analyses by Böhm et al. (1997) detected a possible 53 yeast C2H2 zinc finger proteins, most of those being of unknown function. The mechanisms by which cells sense stress conditions and transmit signals to stress genes remain poorly understood. It has been proposed that the STRE may function as the element whereby all these signals are integrated with the response leading to general stress resistance (Ruis and Schüller, 1995). Two key pathways have been identified which influence the expression of STRE-controlled genes: the high osmolarity glycerol (HOG) mitogen-activated protein (MAP) kinase pathway (section 1.3.2.1) and the RAS-protein kinase A (PKA) pathway (section 1.3.2.2).

1.3.2.1 The role of the high osmolarity glycerol (HOG) MAP kinase pathway in STRE regulation

When yeast cells are confronted with increases in external osmolarity, they induce the synthesis of glycerol to increase their internal osmolarity (Varela et al., 1992). Osmotic stress inactivates the Sln1 -Ypd1 -Ssk1 two-component membrane-bound osmosensor (Maeda et al., 1994; Posas et al., 1996) which in turn leads to activation of the high osmolarity glycerol (HOG) MAP kinase cascade composed of the Ssk2 and Ssk22 MAP kinase kinase kinases (MAPKKKs), the Pbs2 MAPKK and the Hog1 MAPK (Brewster et al., 1993). Osmotic stress also activates a second osmosensor, Sho1 (Maeda et al., 1995), which activates Pbs2 and Hog1 via the
Stel 1 MAPKK (Posas & Saito, 1997). Although Stel 1 is an integral component of the mating pheromone-responsive MAPK cascade (reviewed in Herskowitz, 1995), there was no detectable cross talk between these two pathways (Posas & Saito, 1997). Ruis & Schüller, (1995) have suggested that two different osmosensors with different concentration dependence and response kinetics may be required.

Recent investigations have revealed that defects in PBS2 and HOG1 almost completely abolish the transcriptional activation of CTT1, DDR2, HSP12 or a STRE-lacZ reporter gene by osmotic stress, showing that STREs are specific targets of the HOG pathway (Schüller et al., 1994; Varela et al., 1995). This group further demonstrated that induction of STREs by other stress factors (section 1.3.2) appears to be HOG pathway independent. HOG1-dependent accumulation of CTT1 transcripts occurred independently of protein synthesis and could be detected rapidly after an increase of tyrosine phosphorylation of Hog1, triggered by high osmolarity. This is consistent with transcriptional activation being triggered directly by Hog1. At present, there is no evidence to suggest that Hog1 interacts directly with the Msn2 or Msn4 proteins. Furthermore, STRE-mediated transcription in msn2 msn4 cells reaches induction levels comparable to that in wild type cells in response to high osmolarity stress. However, elimination of HOG pathway activity in a msn2 msn4 mutant background completely eliminates high osmolarity induction of STRE-dependent transcription, suggesting that additional factors may bind the STRE (Martinez-Pastor et al., 1996).

1.3.2.2 The role of the RAS-protein kinase A (PKA) pathway in STRE regulation

S. cerevisiae cells are able to modulate their metabolic activity, their growth rate and the cell cycle in response to the nutritional conditions (reviewed by De Winde et al., 1997). cAMP has been implicated as an important secondary messenger in transduction of the nutrient signal to various intracellular sites (Matsumoto et al., 1985; Bollag & McCormick, 1991). The nutrient sensing mechanisms of yeasts have not been fully elucidated. It has been demonstrated that the S. cerevisiae Ras1 and Ras2 proteins are activated by glucose, these then activate adenylate cyclase and trigger an increase in cellular cAMP levels. cAMP then activates the PKA (encoded by TPK1, TPK2 and TPK3) by binding to its regulatory subunit (encoded by BCY1). The PKA cascade then phosphorylates a number of target proteins which then trigger a variety of responses at the transcriptional and metabolic level, stimulating cell growth when nutrient status is favourable (Thevelein, 1994). PKA can also be activated in a cAMP-independent manner, provided there is some glucose present (The fermentable-growth medium
induced, FGM pathway; Thevelein, 1994). Low cAMP levels, reflecting nutrient limitation and poor growth conditions, cause an alternative pattern of transcription and metabolism which triggers entry of cells into a resting state (De Winde et al., 1997).

Nutrient limitation activates the general stress response leading to the transcription of a number of S. cerevisiae genes under STRE and PDSE control, particularly SSA3 (Boorstein & Craig, 1990 a), CTT1 (Belazzi et al., 1991) and HSP12 (Varela et al., 1995). Also, Engelberg et al. (1994) demonstrated that the PKA pathway controls the transcription of heat shock genes via a mechanism not involving Hsf1. When the nutrient status is favourable, PKA activity is high, growth rate is maximal and stress gene transcription is repressed, but when PKA activity drops, the transcription of many stress genes is derepressed (Thevelein, 1994). Basal and induced levels of STRE and PDSE-dependent transcription are enhanced in ras2 mutants with low PKA activity and dramatically reduced in bcy1 mutants with high constitutive PKA activity (Boorstein & Craig, 1990 a; Belazzi et al., 1991; Marchler et al., 1993; Varela et al., 1995). Consequently, the STRE and PDS elements are under negative control by PKA. It is thought that PKA does not transmit a stress signal directly to the STRE, but exerts its effects by modulating the expression of STRE-controlled stress proteins under favourable or sub-optimal growth conditions or in stationary phase (Ruis & Schüller, 1995). Also, Schüller et al., (1994) revealed that high osmolarity-induced expression of an STRE-lacZ reporter gene is not significantly affected by the ras2 mutation, suggesting that the PKA and HOG pathways act in parallel.

1.3.3 Pleiotropic drug resistance elements (PDREs) and AP-1 responsive elements (AREs)

Multidrug resistance is a generalised resistance to a broad spectrum of functionally and structurally unrelated drugs. The PDR (pleiotropic drug resistance) network consists of genes influencing the expression of membrane translocator proteins which confer tolerance to a range of cytotoxic compounds, heavy metals or compounds generating superoxide free radicals (reviewed by Balzi and Goffeau, 1994; 1995). The encoded gene products identified so far can be classified into three major classes: membrane transport proteins belonging to either the ATP-binding cassette (ABC) superfamily, or to the major facilitator superfamily (MFS) and factors for transcription regulation.

The ABC proteins function as ATPase driven multidrug transporters and share a common structure comprising of four domains, two hydrophobic (each with six
transmembrane spans) and two hydrophilic (each with a conserved cassette of about 200 amino acids) with ATP binding motifs. *S. cerevisiae* displays induction of the ABC-cassette multidrug transporter Pdr12 (170 kDa) when exposed to sorbic acid at low pH (section 1.4.2.2). This protein is highly homologous to two previously-studied ABC-cassette transporters (Snq2 and Pdr5). Pdr12 has been shown to contribute significantly to weak organic acid adaptation (P.W. Piper, unpublished results). The MFS proteins catalyse the specific uni-, sym- and antiport of sugars, organic acids or drugs. They consist of two-times six transmembrane helical segments separated by a central cytoplasmic loop which does not have the ATP binding cassette of the ABC transporters. The *ATRl* gene is thought to encode an MFS-type aminotriazole and 4-nitroquinoline-N-oxide pump (Kanazawa et al., 1988; Gömpel-Klein & Brendel, 1990).

Many of these genes for membrane proteins are controlled by specific transcription factors. Two PDR loci, *PDR1* and *PDR3* were found to encode homologous transcription regulators containing a Zn2C6 binuclear cluster motif as DNA binding domain (Balzi *et al.*, 1987) and have been shown to control the transcription of the multidrug pump gene *PDR5* (Meyers *et al.*, 1992; Katzmann *et al.*, 1994). A mutation of the *PDR1* locus (*prdl-3*) was shown to cause hyper resistance to more than 20 structurally unrelated inhibitors of both cytoplasmic and mitochondrial functions, since it causes overexpression of several drug transporters (reviewed by Balzi and Goffeau, 1991). Single disruptions in *PDR1* or *PDR3* are generally associated with hypersensitivity to drugs such as cycloheximide, but a double disruption resulted in a more drastic effect, indicating that the two genes are essential in the control of multidrug resistance (Delaveau *et al.*, 1994). Progressive deletions of the *PDR5* promoter and a β-galactosidase reporter gene have demonstrated that the Pdr3 protein binds to the 5'-TCCGCGGA-3' motif ("PDRE-box") which was also shown to bind Pdr1p (Katzmann *et al.*, 1994).

A number of yeast genes induced by hydrogen peroxide or compounds generating superoxide free radicals are controlled by the Yap1 and the homologous Yap2 transcriptional activator proteins, related to the mammalian c-Jun (AP1) regulators (Moye-Rowley *et al.*, 1989), of which Gcn4 is also a member (Hinnebusch, 1988; Pain, 1994; Stephen *et al.*, 1995). These proteins share similar DNA-binding domains and leucine zipper dimerization domains and bind to yAREs (yeast AP1-responsive elements with a 5'-TTA(C/G)TAA-3' motif). Yap1 plays a significant role in the resistance of yeast cells to oxidative stress by inducing expression of a number of antioxidant genes including *TRX2* and *GSH1* (Kuge & Jones, 1994; Grey & Brendel,
1994; Stephen et al., 1995), which are important in maintaining thioredoxin and glutathione levels respectively. Yap1p also mediates the induction of YCF1 which encodes an ABC cassette transporter (Li et al., 1996). Yap1 has also been implicated in the response to the toxic divalent cation Cd²⁺ (Hirata et al., 1994), although induction of HSP12 by cadmium and hydrogen peroxide occurs by a Yap1/Yap2 independent mechanism (Varela et al., 1995). A MAP kinase recognition motif is present in Yap1 (Hirata et al., 1994) while Gounalaki and Thireos (1994) showed that Yap1 was essential for the stress induction of an STRE-/lacZ reporter but did not show any direct STRE/Yap1 interaction. As well as the Yap proteins, other as yet uncharacterised transcription factors seem to be involved in the oxidative and toxic metal ion stress response.

1.3.4 The unfolded protein response element (UPRE)

Accumulation of unfolded secretory and transmembrane proteins in the lumen of the endoplasmic reticulum (ER) triggers the transcriptional induction of certain molecular chaperones localised in the ER. In mammalian cells these are called glucose-regulated proteins (GRPs) since they are also induced by glucose starvation. Yeasts possess an intracellular signalling pathway from the ER to the nucleus, called the unfolded protein response (UPR), induced by tunicamycin, 2-deoxyglucose and 2-mercaptopethanol (Shamu et al., 1994). In S. cerevisiae, this induction is mediated by a single 22 bp unfolded protein response element (UPRE) first identified in the KAR2 promoter (Shamu et al., 1994). Extensive mutational analysis has revealed that the UPRE contains a partial palindrome with a spacer of one nucleotide (CAGCGTG) that is essential for its function. Also the ERN4 gene which encodes a basic leucine zipper protein (Ern4) was found to specifically bind the UPRE and activate transcription of the KAR2 gene (Mori et al., 1996).
Table 1.2 Summary of stress response pathways in yeast

<table>
<thead>
<tr>
<th>Stress&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Tf&lt;sup&gt;b&lt;/sup&gt;</th>
<th>DNA element&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Consensus&lt;sup&gt;d&lt;/sup&gt;</th>
<th>Stress genes&lt;sup&gt;e&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>HS</td>
<td>Hsf1</td>
<td>HSE</td>
<td>nGAAAnTTCn</td>
<td>HSP70</td>
</tr>
<tr>
<td>HS, OX, OS, WA, NS, S</td>
<td>Msn2, Msn4</td>
<td>STRE</td>
<td>CCCCT or AGGGG</td>
<td>CTTI, DDA2, HSP12, HSP104</td>
</tr>
<tr>
<td>CC, HM</td>
<td>Pdr1, Pdr3</td>
<td>PDRE</td>
<td>TCCGCGGA</td>
<td>PDR5, SNQ2</td>
</tr>
<tr>
<td>OX, CD</td>
<td>Yap1</td>
<td>yARE</td>
<td>TTA(C/G)TAA</td>
<td>TRX2, GSH1</td>
</tr>
<tr>
<td>PDS</td>
<td>?</td>
<td>PDSE</td>
<td>T(A/T)AGGGAT</td>
<td>SSA3</td>
</tr>
<tr>
<td>TC, DG, ME</td>
<td>Ern4</td>
<td>UPRE</td>
<td>CAGCGTG</td>
<td>KAR2</td>
</tr>
</tbody>
</table>

(a) stress inducer:- HS= heat shock, OX= oxidative stress, OS= osmotic stress, WA= weak acid stress, NS= nitrogen starvation, S= stationary phase, CC= cytotoxic compounds, HM= heavy metals, CD= cadmium, PDS= post-diauxic shift, TC= tunicamycin, DG= deoxyglucose and ME= 2-mercaptoethanol.

(b) Tf= trans-acting transcription factor (or transcriptional activator), ?=unknown Tf.

(c) DNA element= cis-acting promoter element (upstream activating sequence, UAS).

(d) consensus= (5' → 3') of the DNA element.

(e) examples of stress genes controlled by the various stress response pathways.
1.4 Weak acid preservatives

Yeast, moulds and lactic acid bacteria have been implicated in the fermentative spoilage of foods and beverages of low pH, high sugar content and reduced water activity (A\textsubscript{w}) (Smittle & Flowers, 1982; Fleet, 1992; Neves et al., 1994). The environmental conditions characteristic of such foods inhibit the growth of many bacteria and provide a non-competitive situation promoting the growth of yeasts (Beuchat, 1982). Weak lipophilic acids such as sorbate, benzoate, acetate and sulphur dioxide have been commonly used to preserve low pH foods including mayonnaise, pickles, fruit concentrates, soft drinks and wines (Sofos & Busta, 1981; Restaino et al., 1982; Pilkington et al., 1988). The antifungal action of weak acid preservatives is discussed in greater detail in section 1.4.1. Essentially these compounds cause cell stasis rather than cell death. However, microbial spoilage of these products occasionally does occur despite the maximum permitted level of preservative and good manufacturing practice (Pitt, 1974; Golden & Beuchat, 1992). Exposure to a mild weak acid stress at acid pH evokes a discrete stress response which leads to improved resistance against a more severe weak acid stress (section 1.4.2). When grown in the presence of moderate amounts of benzoate, \textit{Zygosaccharomyces bailii} in particular, as well as several other yeasts including \textit{S. cerevisiae} adapt to tolerate higher levels of the acid. Adaptation of \textit{S. cerevisiae} to growth in the presence of sodium benzoate increased its tolerance from 100 to 175 mg/l and increased the tolerance of \textit{Z. bailii} from 600 to 1300 mg/l (Warth, 1988).

Spoilage of foods and beverages containing preservatives is caused by relatively few species of yeast (Fleet, 1992). The major spoilage organism is the osmotolerant yeast, \textit{Z. bailii} (Thomas & Davenport, 1985), which can become tolerant of preservative concentrations in excess of those legally permitted (Splittstoesser et al., 1978). Unlike bacteria, it is able to acquire resistance to lipophilic acids (section 1.4.3). Neves et al., (1994) found that two strains of \textit{Z. bailii} tolerated 800 mg/l of sorbate at pH values less than the pK of the preservative. This organism has become a universal problem to the food industry in food products which rely on acidic and/or osmophilic conditions for preservation. It is apparent that resistance to one of the common preservatives is often associated with resistance to one or more of the others. In particular, species resistant to benzoate are often resistant to sorbate and also appear to tolerate acetate (Warth, 1985).
1.4.1 Mode of action of weak acid preservatives

Although benzoate and other weak acid type preservatives have been used to preserve acidic foods, beverages and pharmaceuticals for many years, there is uncertainty about which biochemical events are important in inhibiting cell growth under conditions of food preservation. Preservatives may accumulate on the cytoplasmic membrane, thus interfering with substrate transport and oxidative phosphorylation (Freese & Levin, 1978). This may account for their effectiveness in preventing the growth of many sensitive bacteria and yeasts under some conditions, but it is not a likely cause of growth inhibition under conditions such as anaerobic fermentation and nutrient rich foods (Warth, 1985).

The antimicrobial effect of weak acids is greatly pH dependant. It is increased by lowering the pH of the suspending medium, being less effective as pH increases (Macris, 1975; Eklund, 1983). In aqueous solutions, weak acids are present as undissociated weak acid and the weak acid ion, the percentage distribution of these forms at any pH being according to the Henderson-Hasselbalch equation:

\[
pH = pK + \log \frac{[\text{base}]}{[\text{acid}]}
\]

The differences in antimicrobial activity between weak acid preservatives can be explained by considering their dissociation constants (pK) (Sofos & Busta, 1981). Consequently, at low external pH (< 5.0) weak acids exist predominantly in an undissociated molecular form (Beuchat, 1981). Eklund (1983) investigated the antimicrobial effects of dissociated and undissociated sorbate (pK\textsubscript{a}= 4.74) and found that the inhibitory action of the undissociated acid was 10-600 times greater than that of the dissociated acid. Nevertheless, it was found that the anion caused more than 50% of the growth inhibition at pHs above 6.0. Also, Burlini et al. (1993) demonstrated that weak acids elicit a set of metabolic effects on sugar metabolism at medium pH values of 6.8, preventing the glucose-induced switch from a gluconeogenic to a glycolytic state. Undissociated weak lipophilic acids are highly soluble in the lipid component of the plasma membrane and as a consequence, cells in acid cultures are generally very permeable to the undissociated form of the preservative (Warth, 1985). Weak acids continue to enter the cell until the concentrations inside and outside the cell reach
equilibrium. Once inside the cell, weak acids generally encounter a higher pH and dissociate. The release of protons reduces intracellular pH (pH\textsubscript{i}) and causes acidification of the cell interior while the membrane-impermeant anion accumulates intracellularly (Krebs \textit{et al.}, 1983). This fall in pH\textsubscript{i} is a major cause of growth inhibition by weak acids (Brown & Booth, 1991).

Yeasts usually maintain their pH\textsubscript{i} around neutrality (Serrano, 1991) which is essential for optimal activity of many important cellular processes (Busa & Nuccitelli, 1984) including activity of a number of enzymes (Sofos & Busta, 1981), the efficiency of contractile elements and the conductivity of ion channels (Madshus, 1988). Changes in pH\textsubscript{i} also seem to be important in controlling the cell cycle (Anand & Prasad, 1989). In \textit{S. cerevisiae} pH\textsubscript{i} declines more than 1 unit with 2-10 mM benzoate, causing an accumulation of glucose 6-phosphate and fructose 6-phosphate and a decrease in intermediates beyond 6-phosphofructo-1-kinase (PFK1) that reduces glycolytic flux (Krebs \textit{et al.}, 1983; François \textit{et al.}, 1986, 1988). These effects are indicative of an inhibition of PFK1. At pH 5.0, hexokinase is also inhibited but to a lesser extent than PFK1. The subsequent inhibition of glycolysis is thought to cause a fall in ATP concentration which restricts growth (Warth, 1991 a). Weak acid preservatives characteristically cause extended lag phase and cell stasis rather than cell death (Restaino \textit{et al.}, 1982). Derivatives of hydroxycinnamic acids were also shown to have a similar mode of action as weak acids (Stead, 1995). In contrast, inhibition of yeast growth by the more membrane-disruptive medium chain fatty acid decanoate was found to be a result of rapid cell death (Stratford & Anslow, 1996). Although the method of cell entry of decanoate is pH dependent, its mode of action differs from that of the more water-soluble sorbate and benzoate. To counteract the decrease in pH\textsubscript{i}, yeasts have a well-developed system for maintaining pH\textsubscript{i} homeostasis, dependent upon the proton-translocating plasma membrane H\textsuperscript{+}-ATPase (section 1.4.1.1).

1.4.1.1 Plasma-membrane H\textsuperscript{+}-ATPase

The most important system participating in pH homeostasis is the H\textsuperscript{+}-translocating ATPase of the yeast plasma membrane which mediates growth responses through the modulation of pH\textsubscript{i} (Serrano, 1986). The H\textsuperscript{+}-ATPase of \textit{S. cerevisiae} is a member of the (E-P) family of cation-translocating ATPases found in a number of yeast and fungal species, plants and protozoa (Serrano, 1991; Sigler & Höfer, 1991). Cyrklaff \textit{et al.}, (1995) indicated that the H\textsuperscript{+}-ATPase of \textit{Neurospora crassa} comprises six 100
kDa ATPase monomers arranged in a symmetrical ring. The activity of the H⁺-ATPase generates a proton gradient that couples ATP hydrolysis to the extrusion of protons across the membrane, resulting in the establishment of a transmembrane proton electrochemical gradient which drives the secondary transport of nutrients (Serrano, 1988). Two isogenes PMA1 (Serrano et al., 1986) and PMA2 (Schlesser et al., 1988) showing 90% homology at the amino acid level have been reported to encode H⁺-ATPase activity in S. cerevisiae. The PMA1 gene is essential for growth and is expressed at high levels, while PMA2 is not required for yeast growth and expression cannot be detected under normal growth conditions (Serrano, 1991). The H⁺-ATPase accounts for about 50% of the plasma membrane protein of exponentially growing cells and about 25% in stationary phase cells (R. Serrano, unpublished results). Small decreases in the levels of H⁺-ATPase protein and PMA1 transcripts have been observed at late stationary phase (Eraso et al., 1987), heat shock (Panaretou & Piper, 1992) ethanol (Piper et al., 1994) and weak acid stresses (Piper et al., 1997). The PMA1 promoter contains two upstream activating sequences (ACCCATACA) recognised by the glucose-modulated transcription factor TUF (Capieaux et al., 1989) and is strongly constitutive (Serrano, 1991). Also, mutations in the APAl gene cause a decrease in the level of PMA1 expression (Garcia-Arranz et al., 1994).

Several stresses that act to dissipate the proton motive force across the plasma membrane and/or cause intracellular pH decline have been found to stimulate H⁺-ATPase activity. They include heat shock (Coote et al., 1991), ethanol (Fernanda Rosa & Sá-Correia, 1991), low pH (Eraso & Gancedo, 1987; Carmelo et al., 1996), conditions that reduce pH_{i} (Ramos et al., 1989) and weak lipophilic acids (Viegas & Sá-Correia, 1991, Holyoak et al., 1996). This necessitates increased plasma membrane H⁺-ATPase activity for restoration of homeostasis, which exerts a heavy energy load on the cell. The H⁺-ATPase is estimated to utilise 10-15% of the total ATP produced during optimal yeast growth, while under certain conditions the enzyme may consume between 40-60% of the total cellular ATP for maintenance of pH_{i} homeostasis (Serrano, 1991). The increased plasma membrane H⁺-ATPase activity is a major energy demand of weak acid stress that gives rise to a significant decrease in biomass yield in cultures grown in the presence of weak acids (Viegas & Sá-Correia, 1991, Holyoak et al., 1996, Piper et al., 1997). Stimulation of H⁺-ATPase activity by glucose metabolism results from a combined effect on the K_{m}, V_{max} and pH optimum (Serrano, 1991). Meanwhile, acidification of the culture medium activates the H⁺-ATPase by reducing its K_{m} two-
fold while other kinetic parameters remain unaltered (Eraso & Gancedo, 1987). Deletion of the last 11 amino acids of the carboxyl terminus results in constitutive activation in the absence of glucose, nitrogen starvation and acidification of the external medium (Benito et al., 1992). This suggests that glucose, nitrogen starvation and acid pH activation of the H^+-ATPase is based on the modulation of an inhibitory interaction of the C-terminus with the ATP-binding region of the enzyme. Furthermore, double mutations of the Ser^{911} and Thr^{912} residues (Portillo et al., 1991) abolishes the glucose, nitrogen starvation and acid pH activation of H^+-ATPase and leads to an inactive enzyme (Benito et al., 1992). Also, intragenic suppressor analysis of the Ser^{911}/Thr^{912} mutant demonstrated the importance of functional domains, transmembrane helices, the ATP binding domain and substitutions at the C-terminus in H^+-ATPase activation (Eraso & Portillo, 1994). Phosphorylation of the C-domain is probably important in this regulation, through reactions thought to be catalysed by either casein kinase I or Ca^{2+}/calmodulin-dependent protein kinase (Portillo et al., 1991; Eraso et al., 1994; Estrada et al., 1996). Also, Ulaszewski et al., (1989) demonstrated that cAMP may play an important role in the control H^+-ATPase activity. As yet, the mechanisms of H^+-ATPase activation remain poorly understood.

1.4.2 The weak acid stress response

It has previously been reported that yeasts are able to adapt to growth in the presence of a weak acid stress (Warth, 1989; Verduyn et al., 1992; Holyoak et al., 1996). In our laboratory we have shown that a separate stress response is induced by weak acids at low culture pH in S. cerevisiae, involving strong induction of two plasma membrane proteins, Hsp30 (section 1.4.2.1) and Pdr12 (section 1.4.2.2). Weak acids also cause other changes in stress gene expression (section 1.4.2.3).

1.4.2.1 The role of Hsp30

Régnacq & Boucherie (1992) published the nucleotide sequence of a gene preferentially expressed with entry to stationary phase caused by glucose limitation. The analysis of membrane proteins from stationary phase cells revealed that a strain carrying this gene in a multicopy vector overproduced a protein of 30 kDa. Analysis of the sequence indicated a single ORF of 996 bp that potentially encoded a 322 amino acid polypeptide with a molecular mass of 37,044. This gene was called HSP30 and displayed a perfect match to the YCR21C ORF of S. cerevisiae sequenced by a group
led by H. Feldman as part of the yeast chromosome III sequencing project (Oliver et al., 1992). Further investigations have shown that Hsp30 is induced by heat shock, ethanol and weak acid preservatives (Panaretou & Piper, 1992; Régnacq & Boucherie, 1992; Piper et al., 1994; Piper et al., 1997). Experiments in our laboratory have indicated that the 1.3 kb transcript of the HSP30 gene is also weakly induced by growth under acidic conditions or exposure to 0.5 M NaCl, and more strongly induced by the severe osmostress caused by 2 M sorbitol, 1 M KCl or 1 M NaCl (P.W. Piper, unpublished results). The stresses that induce Hsp30 may act by either dissipating the electrochemical potential gradient maintained at the plasma membrane, membrane disordering or decrease in pH.

Investigations were undertaken to examine the phenotype of HSP30 loss on the stress tolerance of *S. cerevisiae* (Piper et al., 1997). Comparison of the survivability of wild-type and mutant cultures in vegetative growth indicated that loss of HSP30 has no effect on thermotolerance, short-term exposure to 15% (w/v) ethanol, 750 mM potassium sorbate at pH 4.5, 4-nitroquinoline-N-oxide, hydrogen peroxide, ultraviolet light, NaCl, a single freeze-thaw cycle or carbon limitation. In contrast, fermentation analysis revealed that there is a phenotype associated with loss of this protein. Hsp30 inactivation appeared to reduce biomass yields and extend the time interval required for cells to adapt to growth at either low pH, the presence of weak acid stress conditions or severe osmostress. This suggests that the mutant is less efficient at re-establishing the levels of homeostasis that will support subsequent growth in media containing these stress agents. HSP30 is the first gene shown to be both weak acid inducible and assisting adaptation to weak acids.

Experiments were carried out to measure the plasma membrane H\(^+\)-ATPase activity in purified plasma membranes from hsp30 mutant and wild-type sorbate-adapted cells (Fig. 1.2). H\(^+\)-ATPase assays indicated that the loss of Hsp30 results in higher plasma membrane H\(^+\)-ATPase activities in heat shocked or weak acid-stressed cells, revealing that Hsp30 leads to a downregulation of the stress stimulation of H\(^+\)-ATPase. Piper et al. (1997) suggested that the primary role of Hsp30 may be for conserving ATP under conditions of prolonged stress and during diauxic shift. Maintaining a maximal H\(^+\)-ATPase activity over long periods of a weak acid stress (at low pH) might be energetically unfeasible. Even though the immediate response of the cell to these energy demanding stresses is rapid stimulation of H\(^+\)-ATPase, this may have to be followed by a delayed response in which H\(^+\)-ATPase levels are decreased.
and Hsp30 is induced. This may limit extreme \( \text{H}^+ \)-ATPase activity, ensuring that cellular ATP levels do not become too depleted. For the cell to adapt to growth under weak acid stress it must be able to balance the need for \( \text{H}^+ \)-ATPase activity for homeostasis and the need to keep cellular ATP levels adequate for growth.

Braley & Piper (1997) demonstrated that Hsp30 reduces the \( V_{\text{max}} \) of \( \text{H}^+ \)-ATPase in heat shocked cells. In addition, the C-terminal regulatory domain of the \( \text{H}^+ \)-ATPase was shown to be non-essential for the heat shock activation of this enzyme, but essential for Hsp30 to suppress this activation. Also, mutation of the Thr\(^{912} \) residue (a potential phosphorylation site by the Ca\(^{2+} \)/calmodulin-dependent protein kinase), abolished any effect of Hsp30 on \( \text{H}^+ \)-ATPase activity. This suggests that Hsp30 is influencing, possibly enhancing, autoinhibitory interactions of the C-terminal domain with the enzyme active site.

**Fig. 1.2** Plasma membrane \( \text{H}^+ \)-ATPase activity in purified plasma membrane from *hsp30* mutant and wild-type cells that had been grown for 20 hours at pH 4.5 in the presence of 0, 0.5 or 2 mM sorbate. Error bars indicate standard deviation of data obtained from three sets of identically-prepared membrane preparations (from Piper *et al.*, 1997).
1.4.2.2 The role of Pdrl2

Warth (1977) proposed that resistance of *Z. bailii* to weak acids results from induction of an energy requiring system for transport of the preservative anion from cells. Cole & Keenan (1987), studying the effect of weak acids and external pH on the pH\textsubscript{i} of *Z. bailii*, suggested that the loss of benzoate observed after the addition of a pulse of sugar to a suspension of starved cells could be attributed to changes in intracellular pH. More recently, Henriques *et al.* (1997) demonstrated that *S. cerevisiae* cells adapted to growth in the presence of benzoate, were able to extrude [\textsuperscript{14}C] benzoate when a pulse of glucose was given to preloaded cells. Extrusion was dependent upon an energy source and prior growth in the presence of the weak acid while diethylstilbestrol (an inhibitor of ATPases) prevented benzoate extrusion. Even though the extrusion mechanism was active, benzoate re-entered the cells by a simple diffusion mechanism.

Recent investigations in our laboratory have demonstrated that a large membrane protein of approximately 170 kDa is induced in *S. cerevisiae* cells adapted to growth in the presence of 0.75 mM sorbate (P.W. Piper, unpublished results). This protein is so strongly induced in sorbate adapting cells that its levels approach those of the most abundant plasma membrane protein, H\textsuperscript{+}-ATPase. Evidence suggests that a similar-sized protein is also present in sorbate-adapted cells of *Z. bailii*. Peptide sequencing of the 170 kDa protein from *S. cerevisiae* and subsequent database analysis revealed a perfect match to a large ORF encoding Pdr12, a protein highly homologous to two previously-studied ABC-cassette transporters Snq2 (Servos *et al.*, 1993) and Pdr5 (Leppert *et al.*, 1990). Northern analysis indicated that the *PDR12* gene shows significant induction in response to severe osmostress, low pH and ethanol but was even more strongly induced by the addition of 1 mM or 9 mM sorbate. In the deletion mutant (Δpdr12) sorbate did not induce a membrane protein of 170 kDa. Also, growth of the wild-type and Δpdr12 deletion strains on sorbate plates revealed an increased sensitivity of Δpdr12 to sorbate at pH 4.5. Continuing the experiments of Henriques *et al.* (1997), our laboratory showed that the Δpdr12 cells extruded 30% less of their accumulated [\textsuperscript{14}C] benzoate compared to the isogenic wild-type after a pulse of glucose (P.W. Piper, unpublished results). Therefore, Pdr12 has been identified as the major weak acid-induced ABC-cassette multidrug transporter of *S. cerevisiae* which confers both sorbate resistance and much of the capacity of the cells for benzoate extrusion. Fig. 1.3 shows a schematic model of Pdr12 action.
Fig. 1.3 Schematic model of how Pdr12 action may help acidified yeast cultures counteract the inhibitory effects of the weak organic acids that are not highly membrane-disruptive. In both unadapted cells (A) and cells which have adapted to growth in the presence of the weak acid (B) the protonated form of the acid (XCOOH) is freely permeable to the plasma membrane and readily enters the cell by diffusion. In unadapted cells (A) the concentration of XCOOH inside and outside the cell should be the same. However, the higher pH environment of the cytoplasm will cause a substantial fraction of the acid to dissociate to the anion (XCOO-), a form which is relatively membrane-impermeant and accumulates inside the cell. This dissociation also releases protons, resulting in a cytoplasmic acidification that inhibits many metabolic processes.

At the plasma membrane, cells maintain an electrochemical potential difference (ZΔpH), maintained largely by the H+-ATPase (Pma1)-catalysed proton extrusion which is essential for many aspects of homeostasis. The weak acid influx in (A) will act to dissipate the ΔpH component, though not the charge (Z) component of this gradient. The extent to which weak acid-induced cytoplasmic acidification can be counteracted in (A) by increased Pma1 activity may be severely limited, since the high level of additional proton extrusion needed will necessitate greater increases to this electrostatic charge across the plasma membrane than can be generated by H+-ATPase action.

In weak acid adapted cells (B) the proposed Pdr12-catalysed anion extrusion will both lower cytoplasmic weak acid levels and, by moving a charge compensating for the charge on a Pma1-extruded proton, enable greater catalysed proton extrusion than would otherwise be possible. The latter process, though energetically expensive, will assist weak acid-stressed cells elevate their intracellular pH to the point where substantial metabolic activity and growth can resume. As weak acids become more liposoluble, thereby increasing their capacity to cause the membrane structure disruption that increases the permeability of membrane ions, this protective mechanism becomes increasingly futile.
1.4.2.3 Other effects of weak acid stress on gene expression

Cheng and Piper (1994) demonstrated that weak acid preservatives exert pronounced effects on the heat shock response and thermotolerance in *S. cerevisiae*. These effects are strongly influenced by culture pH. In low pH cultures, weak acids inhibit the induction of heat shock proteins and thermotolerance by sublethal heat shock, causing strong induction of respiratory deficient petites among the survivors of heat treatment. This reveals that heat increases the damage caused by intermitochondrial accumulation of sorbate (Cole, 1987). Measurements of HSE-*lacZ* activity revealed that weak acids prevent the heat shock induction of the HSE at low pH (Cheng & Piper, 1994). This indicates that the weak acid inhibition of the heat shock response occurs through a block to the heat induction of the HSE sequence of particular heat shock gene promoters. Even though weak acids inhibit heat induction of the heat shock response in acidified yeast cultures, they can also act as chemical inducers of certain stress genes. Northern blot analysis has shown that some stress genes, including *CTT1*, *DDR2*, and *HSP104*, (Schüller et al., 1994), *HSP12* (Martinez-Pastor et al., 1996) and *HSP26* (Piper, unpublished results; Carmelo & Sá-Correia, 1997) are activated by weak acids and growth at low pH. These stress genes have all been shown to contain functional STRE sequences in their promoter regions, a motif which has been implicated in the general stress response of yeast (Kobayashi & McEntee, 1993; Marchler et al., 1993). Varela et al. (1995) analysed the promoters of known stress genes for the presence of STRE-type sequences and found a good correlation in various genes involved in osmotic and oxidative stress, heat shock and ubiquitination. At present, an in depth investigation of the weak acid-induced activation of these genes has not been carried out. Most of these genes, including *HSP30* and *PDR12*, are induced by multiple stresses which suggests that weak acids may be involved in a cross protection mechanism. This may be connected to the observation that in cultures of pH values above 5.5, sorbate acts as a strong chemical inducer of thermotolerance in the absence of sublethal heat treatment (Cheng & Piper, 1994).

1.4.3 Resistance of yeast to weak acid preservatives

Microbial resistance and tolerance to antibiotics are well established and the mechanisms have been widely studied. In contrast, the mechanisms of weak acid resistance in yeasts are, as yet, only partly resolved. Some microorganisms are capable of utilising subinhibitory concentrations of weak acids in their metabolism by the induction of degradative enzymes (Thakur & Singh, 1994). Pronk et al. (1994) reported
that *S. cerevisiae* strain CBS 8066 co-metabolised 5-10mM propionate in a glucose-limited chemostat. Degradation of sorbate has been noted in a few strains of lactic acid bacteria (Lukas, 1964) and among various moulds (Kurogochi et al., 1975; Finol et al., 1982). Mammals are known to metabolise sorbate under normal conditions (Deuel et al., 1954) but no such mechanism has been found in yeasts (Holyoak et al., 1996). This data suggests that metabolism of weak acids is not involved in the resistance of *Z. bailii*.

Exposure to a mild weak acid stress evokes a stress response which leads to improved resistance against a more severe stress (Bills et al., 1982). In *S. cerevisiae*, this adaptation has been explained in terms of induction of the plasma membrane stress proteins Hsp30 and Pdr12 (section 1.4.2), which modulate H⁺-ATPase activity and actively extrude the preservative, respectively (Piper et al., 1997; P.W. Piper, unpublished results). The intrinsic weak acid resistance of *Z. bailii* (Warth, 1985) (section 1.4), may be due to the decreased permeability of this yeast to preservative, since the weak acid resistance of different yeast species is correlated to rates of uptake of benzoate into their cells (Warth, 1989). Golden et al., (1994) demonstrated that the cellular fatty acid composition of sorbate grown *Z. rouxii* cells, contains a higher percentage of oleate (C₁₈:1) than cells not supplemented with the preservative. Various studies have shown that ethanol (Sajbidor & Greco, 1992; Margarida Beleiras Couto & Huis in't Veld, 1995) and osmostress (Hosono, 1992) also increase the relative oleate (C₁₈:1) levels in the plasma membrane of yeasts. Changes in the fatty acid profile of plasma membranes may alter membrane permeability and fluidity, which may in turn contribute to sorbate tolerance. At present, there is no evidence to support this hypothesis. In the presence of sorbate, *Z. bailii* (Cole & Keenan, 1987) and *Z. rouxii* (Golden and Beuchat, 1992 a and b) exhibited a decrease in protoplast volume and cell size, an increase in plasmolysis and alterations in cellular morphology.

Cole & Keenan (1987) also suggested that resistance in *Z. bailii* may reflect a greater ability to maintain a tolerable intracellular pH value. At a pH₃ of approximately 4.9-5.0, *Z. bailii* NCYC563 was capable of exponential growth. In contrast, Krebs et al., (1983) demonstrated that fermentation is inhibited in *S. cerevisiae* at similar intracellular pH values. Recent investigations have shown that heat shock protein induction was not inhibited in *Z. bailii* by 5 mM sorbate at pH 4.5 (P.W. Piper, unpublished results), unlike the equivalent response in *S. cerevisiae* (Cheng & Piper, 1994). This may reflect a greater ability of *Z. bailii* to either tolerate chronic
intracellular pH drops or maintain pH$_i$ during weak acid stress. It was also demonstrated that the rapid pH$_i$ decline caused by weak acids is a possible trigger for the rapid accumulation of the osmoprotectant trehalose in low pH $S$. $cerevisiae$ cultures. This accumulation is enhanced in a $pfk1$ $S$. $cerevisiae$ mutant, suggesting that glycolysis is inhibited at the phosphfructokinase step. In contrast, this sorbate-induced trehalose activation is not apparent in low pH $Z$. $bailii$ cultures, indicating that the PFK of $Z$. $bailii$ may be less sensitive to pH$_i$ depression than the corresponding enzyme in $S$. $cerevisiae$ (P.W. Piper, unpublished results).

Warth (1991 b) investigated the effects of benzoate on glycolytic metabolite levels, energy production and pH$_i$ of $Z$. $bailii$ and found that benzoate levels up to 4 mM stimulated fermentation, and only low levels of the acid were accumulated in the presence of an energy supply. Near the minimum inhibitory concentration (10mM), fermentation was inhibited, ATP levels declined and benzoate was accumulated to relatively high levels (127 mM). Intracellular pH was reduced but not greatly (from 7.0 to 6.3). Analysis of glycolytic intermediates indicated that glycolysis was limited at the pyruvate kinase and glyceraldehyde dehydrogenase-phosphoglycerate kinase steps and not at PFK. The drop in pH$_i$ was not responsible for the resistance of $Z$. $bailii$ because it is able to maintain a higher pH$_i$ than $S$. $cerevisiae$. Results suggest that the primary action of weak acids in $Z$. $bailii$ is to cause a general energy loss as a result of high ATP usage rather than lowered pH$_i$. Warth (1991 a and b) found that the patterns of metabolite levels between $Z$. $bailii$ and $S$. $cerevisiae$ were distinctly different. In $S$. $cerevisiae$, 0.4 mM benzoate stimulated fermentation rates, but from 0.5 to 1 mM benzoate, the fermentation rate, ATP level and pH$_i$ declined in parallel, while the accumulation of benzoate greatly increased. At higher acid levels the pH$_i$ decreased to approximately 5.9 which is sufficient to inhibit fermentation and respiration.

This data suggests that $Z$. $bailii$ tolerates higher levels of weak acids compared to $S$. $cerevisiae$, is less permeable to lipophilic acids accumulating these acids to a lesser extent, and at equivalent amounts of acid, maintains a higher pH$_i$. Resistance could be due to physiological differences between species, notably alterations in cell permeability to the acid, changes in plasma membrane H$^+$-ATPase activity, induction of a weak acid extrusion pump, the ability to maintain pH$_i$, changes in enzyme structure as well as induction or suppression of genes which effect susceptibility to weak acids. $Z$. $bailii$ remains a major problem to the food and drinks industry.
1.5 Aims of this study

Previous studies have shown that *S. cerevisiae* can adapt to growth in low pH cultures in the presence of weak acid preservatives. Studies in our laboratory have shown that the plasma membrane proteins Hsp30 and Pdr12 play a major role in this adaptive response (section 1.4.2). Also, Cheng & Piper (1994) have demonstrated that weak acids can block the induction of the major heat shock proteins at low pH. At present, the mechanisms by which weak acids influence the expression of stress genes is poorly understood. Therefore one of the main aims of this thesis was to characterise the effects of weak acid preservatives on the expression of stress gene promoters and stress gene promoter elements. Three studies were conducted:-

(1) Investigation of pH-dependent weak acid effects on stress gene promoter and stress gene promoter element-*lacZ* expression, involving both the well characterised HSE and STREs (section 1.3) and the *HSP12, HSP26* and *HSP30* promoters (chapter 3).

(2) Investigation of putative stress control elements on the stress activation of the *HSP30* gene by both site-directed mutagenesis of potential transcriptional control regions and the analysis of various mutant *S. cerevisiae* strains (chapter 4).

(3) Since the Hsp30 plasma membrane protein has been implicated in the adaptive response to weak acid preservatives, negatively regulating H⁺-ATPase activity (section 1.4.2.1) a search was therefore conducted for *HSP30* homologues in the yeast species *Hansenula polymorpha, Z. bailii, Candida albicans* and *Schizosaccharomyces pombe* (chapter 5).
## 2. MATERIALS AND METHODS

### 2.1 Materials

<table>
<thead>
<tr>
<th>Materials</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Standard reagents:</strong></td>
<td>AR grade supplied by Sigma and BDH.</td>
</tr>
<tr>
<td><strong>Microbiological media:</strong></td>
<td>Supplied by Difco. Ampicillin and auxotrophic requirements supplied by Sigma.</td>
</tr>
<tr>
<td><strong>Electrophoresis reagents:</strong></td>
<td>40% acrylogel 2.6 solution (40% w/v solution of acrylamide 'electran' and NN'-methylene bis acrylamide 'electran' in deionised water, final ratio 37:1) and ammonium persulphate supplied by BDH. TEMED and agarose supplied by Sigma.</td>
</tr>
<tr>
<td><strong>Restriction enzymes:</strong></td>
<td>Supplied by Promega, Cetus and Pharmacia.</td>
</tr>
<tr>
<td><strong>Radiolabelled nucleotides:</strong></td>
<td>(α-^{32}P)-5' Cytosine triphosphate (3000Ci/mmol; 10mCi/ml) and (α-^{35}S)-5'-Adenosine triphosphate (3000Ci/mmol; 10mCi/ml) supplied by Amersham.</td>
</tr>
<tr>
<td><strong>Other reagents:</strong></td>
<td>SDS (biochemical grade) was supplied by BDH. All buffer salts (Tris and MES), DEPC, 5-FOA and DNase-free RNase supplied by Sigma.</td>
</tr>
</tbody>
</table>

Sources of any other materials are stated where appropriate in the text.
2.2 Yeast strains

The strains of yeast used in this study are given in Table 2.1. All strains are *S. cerevisiae* unless otherwise stated.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>BJ2168</td>
<td>MATα leu2-3,112 trp1 ura3-52 prb1-1122 pep4-3 prc407 gal2</td>
<td>gift of H. R. B. Pelham</td>
</tr>
<tr>
<td>KT3</td>
<td>MATα leu2-3,112 trp1 prb1-1122 pep4-3 prc407 gal2 hsp30:: URA3</td>
<td>Piper et al. (1997)</td>
</tr>
<tr>
<td>W303-1A</td>
<td>MATα SUC2 ade2 can1 his3 leu2 trp1 ura3</td>
<td>Martínez-Pastor et al. (1996)</td>
</tr>
<tr>
<td>Wmsn2msn4</td>
<td>MATα SUC2 ade2 can1 msn2-3::HIS3 leu2 msn4-1::TRP1 ura3</td>
<td>Martínez-Pastor et al. (1996)</td>
</tr>
<tr>
<td>MYY290</td>
<td>MATα leu2 his3 ura3</td>
<td>gift of M. P. Yaffe</td>
</tr>
<tr>
<td>MYY385</td>
<td>MATα leu2 his3 ura3 hsfl-m3</td>
<td>gift of M. P. Yaffe</td>
</tr>
<tr>
<td>PMY1.1</td>
<td>MATα leu2 ura3 his3</td>
<td>gift of P. Meacock</td>
</tr>
<tr>
<td>FY1679-28C</td>
<td>MATα ura3-52 trp-Δ63 leu2Δ1 his3Δ200 GAL2</td>
<td>Delaveau et al. (1994)</td>
</tr>
<tr>
<td>YMM16</td>
<td>MATα ura3-52 trp-Δ63 leu2Δ1 his3Δ200 GAL2 yap1ΔhisG</td>
<td>Delaveau et al. (1994)</td>
</tr>
<tr>
<td>FY1679-28C/TDEC</td>
<td>MATα ura3-52 pdr1::TRP1 leu2Δ1 pdr3::HIS3 GAL2</td>
<td>Delaveau et al. (1994)</td>
</tr>
<tr>
<td>NCYC 563 (Z. bailii)</td>
<td>Prototrophic</td>
<td>NCYC Norwich</td>
</tr>
<tr>
<td><em>C. albicans</em></td>
<td>Prototrophic</td>
<td>gift of A. Brown</td>
</tr>
<tr>
<td>972 (<em>S. pombe</em>)</td>
<td>h- PMA1+ ade7-413</td>
<td>gift of A. Goffeau</td>
</tr>
<tr>
<td><em>H. polymorpha</em></td>
<td>Prototrophic</td>
<td>gift of E. Berardi</td>
</tr>
</tbody>
</table>
2.3 *E. coli* strains

The strains of *E. coli* used in this study are given in Table 2.2.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>JM109</td>
<td>F' traD36 proAB+ lacIq lacZΔM15/recA1 endA1 gyrA96 (Nalr) thi hsdR17 supE44relA1 Δ(lac-proAB) mcrA</td>
<td>Clontech Laboratories, Inc</td>
</tr>
<tr>
<td>BMH 71-18  mutS</td>
<td>F' proAB+ lacIq lacZΔM15 thi supE Δ(lac-proAB) [mutS::Tn10]</td>
<td>Clontech Laboratories, Inc</td>
</tr>
<tr>
<td>XL1-Blue</td>
<td>F' proAB+ lacIq lacZΔM15 hsdR17 supE44relA1</td>
<td>Stratagene®</td>
</tr>
</tbody>
</table>

*E. coli* strains were maintained as frozen stocks in 2x YT plus 15% glycerol at -70°C

2.4 Plasmids

The plasmids used in this study are given in Table 2.3.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>BluescriptM13-</td>
<td>Short <em>et al.</em> (1988)</td>
</tr>
<tr>
<td>pUC19HSP30</td>
<td>Régnacq &amp; Boucherie, (1992)</td>
</tr>
<tr>
<td>pHSE2</td>
<td>Sorger &amp; Pelham, (1987)</td>
</tr>
<tr>
<td>pGM18/17</td>
<td>Marchler <em>et al.</em> (1993)</td>
</tr>
<tr>
<td>pUP41a</td>
<td>gift of P. Meacock</td>
</tr>
<tr>
<td>pUKC414</td>
<td>gift of M. F. Tuite</td>
</tr>
<tr>
<td>YEplac181/Yiplac204</td>
<td>Gietz &amp; Sugino (1988)</td>
</tr>
<tr>
<td>YEplac505</td>
<td>constructed for this study (chapter 3)</td>
</tr>
</tbody>
</table>
2.5 Yeast methods

2.5.1 Growth media and culture conditions

Yeast cultures were grown in rich media (YEPD) or synthetic defined minimal media (SD). The recipes for these are given below (all % values are w/v):

YEPD: 2% D-glucose, 2% bactopeptone, 1% yeast extract.

SD: 2% D-glucose, 0.67% yeast nitrogen base (without amino acids) plus one or more of the following auxotrophic requirements where appropriate; L-leucine (30 mg/l), L-lysine (30 mg/l), L-tryptophan (20 mg/l), L-histidine (20 mg/l), uracil (20 mg/l) and adenine (20 mg/l).

2% bacto-agar was used for the preparation of solid media. Plates were left on the bench for 2-3 days after pouring to dry. Liquid media cultures were grown at the appropriate temperature with rapid agitation in a media volume 1/5 of the flask volume. Yeast strains were maintained as frozen stocks in 2x YEPD plus 15% glycerol at -70°C. All solutions and glassware were sterilised by autoclaving at 15psi for 20 min.

2.5.2 Monitoring of cell growth

Yeast cell growth in liquid media was monitored by taking OD_{600} readings at appropriate intervals and, where necessary, OD_{600} readings were correlated to cell number by using an Improved Neubauer haemocytometer (Hawksley).

2.5.3 Yeast transformation

Transformation of \textit{S. cerevisiae} strains with plasmid DNA was carried out as described by Gietz & Woods (1994). Integrative vectors were linearised with the appropriate restriction enzyme(s) and integrated into the correct chromosomal locus. Proper integration was determined by Southern analysis (section 2.8.4) or by measuring \textit{lacZ} expression (section 2.6.1) and only single copy integrants were used for further studies.
2.5.4 Assaying for stress tolerance

All stress tolerance experiments were done on cells that were in exponential growth (0.5-1x10^7 cells/ml) at the appropriate temperature in YEPD or SD media. The cultures were subjected to the stated treatments prior to measurement of lacZ activity (chapter 3) or isolation of total RNA (chapter 4).

2.5.5 Selection of ura3 mutants

5-FOA selection (Boeke et al., 1984) was used to select for hsp30:URA3 gene replacement by transformation (chapter 4). 5-FOA medium was prepared (SD medium + 2% agar), autoclaved and cooled to 60°C. To this, 500 mg/l of filter sterilised 5-FOA and 50 mg/l uracil plus other auxotrophic requirements were added. Transformants were incubated at 28°C until ura3^- colonies appeared.

2.6 Biochemical assays

2.6.1 β-galactosidase assay

The method used for the in vitro assay of β-galactosidase is as described by Miller (1972). 1 ml aliquots of cells were centrifuged at 12,000g for 1 minute and resuspended in 0.5 ml of Z buffer (60 mM Na2HPO4, 40 mM NaH2PO4.H2O, 10 mM KCl, 1 mM MgSO4.7H2O, 50 mM β-mercaptoethanol; pH 7.0), 60 µl of chloroform and 40 µl of 0.1% SDS. The sample was vortexed for 10 seconds and pre-incubated at 28°C for 5 minutes. The reaction was started by the addition of 0.2 ml of 4 mg/ml ONPG and incubated at 28°C for 10 minutes. The reaction was stopped by the addition of 0.5 ml of 1 M Na2CO3. The reaction mixture was centrifuged at 12,000g for 1 minute and the OD_{420} of the supernatant was measured. ONPG is converted to galactose and o-nitrophenol in the presence of β-galactosidase. The o-nitrophenol is yellow and can be measured by its absorption at 420 nm.

β-galactosidase (MillerU) was determined by the equation:

\[
\text{β-galactosidase (MillerU) = } \frac{1000 \times \text{OD}_{420}}{\text{OD}_{600} \text{ of assayed culture} \times \text{volume (1.3 ml)} \times \text{time (10 minutes)}}
\]
2.7 Recombinant DNA techniques

2.7.1 Restriction enzyme digests

0.1 to 4 μg DNA (in dH2O or TE buffer) was digested with restriction endonuclease (1 to 5U/μg DNA) with 1/10 volume 10x restriction buffer for 1 hour at 37°C.

2.7.2 Polymerase chain reaction

The polymerase chain reaction (PCR) was used for DNA amplification and site-directed mutagenesis (chapter 4). The technique used is as described in Maniatis et al. (1989). DNA amplification was carried out in Omni-E and OmniGene Thermal Reactors (HYBAID). DNA fragments >1 kb in length were amplified using the Expand™ High Fidelity PCR system (Boehringer Mannheim).

2.7.3 Purification and precipitation of DNA

Plasmid DNA was purified by vortexing with an equal volume of phenol/chloroform/iso-amyl alcohol (25:24:1). The top layer was recovered after centrifugation. Routine precipitation of DNA was achieved by adding 2 volumes of 100% ethanol and 1/10 volume 6M ammonium acetate. The mixture was stored at -20°C for 20 minutes and centrifuged at 12,000g for 20 minutes. The pellet was washed with 70% ethanol to remove precipitated salt and resuspended in dH2O or TE buffer (10 mM Tris/HCl pH 7.0; 1 mM EDTA).

2.7.4 Agarose gel electrophoresis of DNA

DNA fragments between 0.5 and 25 kb were routinely separated using the following protocol. The agarose gel was prepared using 1x TBE electrophoresis buffer (89 mM Tris base, 89 mM boric acid, 2 mM EDTA) and the appropriate concentration of electrophoresis grade agarose by melting in a microwave oven, mixing, cooling to 55°C, pouring into a sealed gel casting platform and inserting the gel comb. Ethidium bromide (0.5 μg/ml) was added to the gel prior to pouring. The DNA samples were electrophoresed (at 1 to 10 V/cm of gel) with an appropriate amount of 10x loading buffer (20% w/v Ficoll 400, 0.1 M EDTA, 1.0% w/v SDS, 0.25% w/v bromphenol blue, 0.25% w/v xylene cyanol) and appropriate DNA molecular weight markers. Gels were viewed using a short wave UV transilluminator and photographed.
2.7.5 Isolation and purification of DNA fragments from agarose gels

DNA fragments were recovered from agarose gels using the Geneclean II Kit (Bio 101, Inc) or by electrophoresis onto NA-45 paper (Amersham) as described in Ausubel et al. (1995).

2.7.6 Dephosphorylation of 5' end of DNA and oligonucleotides

An appropriate amount of DNA or oligonucleotide was dephosphorylated using the Calf Intestine Alkaline Phosphatase (CIP) kit (Boehringer Mannheim).

2.7.7 Phosphorylation of 5' end of DNA and oligonucleotides

An appropriate amount of DNA or oligonucleotide was incubated with 5 mM ATP, 10 U T4 polynucleotide kinase (PNK) and 1/10 volume 10x reaction buffer (1x reaction buffer; 70 mM Tris/HCl pH 7.6, 10 mM MgCl₂, 5 mM dithiothreitol) for 30 minutes at 37°C. The PNK was inactivated at 70°C for 10 minutes.

2.7.8 Ligation of fragments of foreign DNA to plasmid vectors

Ligations were routinely carried out as described in Maniatis et al. (1989).

2.7.9 Site-directed mutagenesis

In vitro site-directed mutagenesis of the HSP30 promoter (chapter 4) was carried out by the Quikchange™ site-directed mutagenesis kit (Stratagene®) and the Transformer™ site-directed mutagenesis kit (Clontech Laboratories, Inc). The design of the mutagenic primers, reaction conditions and PCR cycling parameters were as described by Clontech Laboratories, Inc (catalogue # K1600-1) and Stratagene® (catalogue # 200518).

2.7.10 E. coli growth media and culture conditions

E. coli strains were grown in LB (1% bactopeptone, 0.5% yeast extract, 1% NaCl) at 37°C. Cultures for plasmid transformation and selection were grown in LB plus ampicillin to a final concentration of 50 mg/l.

2.7.10.1 Preparation of competent E. coli

Competent E. coli cells were prepared according to a calcium chloride technique as described in Ausubel et al. (1995). The competent cells were resuspended in ice cold 0.1 M CaCl₂/15% glycerol and stored in 0.2 ml aliquots at -70°C.
2.7.10.2 *E. coli* transformation

Competent *E. coli* cells were thawed on ice and 200 µl pipetted into a chilled eppendorf. An appropriate amount of plasmid DNA was added and the cells were incubated on ice for 20 minutes. The cells were heat-shocked at 42°C for 90 seconds and placed on ice for 5 minutes. The cells were then resuspended in 800 µl of SOC medium (0.5% w/v yeast extract, 2% w/v tryptone, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 20 mM MgSO₄, 20 mM D-glucose) and incubated at 37°C for 45 minutes. 200 µl aliquots of cells were then plated on LB plus ampicillin to a final concentration of 50 mg/l and incubated at 37°C overnight.

2.7.10.3 High efficiency transformation by electroporation

A 50 ml culture of *E. coli* in LB was grown to an OD₆₀₀ of 0.5-0.6. The cells were pelleted at 6000 rpm for 3 minutes and washed three times in 10 ml sterile dH₂O, three times in 1 ml sterile dH₂O, resuspended in 0.5 ml of sterile dH₂O and stored on ice. 50 µl aliquots and an appropriate amount of plasmid DNA (<5 µl) were added to a Flowgen cuvette and electroporated (at 2500 Volts, 0.25mF, Resistance 201Ω, Time 5 msecs) in an Easyject+ electroporator (Flowgen). Immediately after electroporation 1 ml of LB was added and the cuvette was placed on ice. 100 µl aliquots of cells were plated on LB plus ampicillin to a final concentration of 50 mg/l and incubated at 37°C overnight.

2.7.10.4 *E. coli* miniprep

Small scale preparations of plasmid DNA were obtained by the alkaline lysis method as described in Maniatis *et al.* (1989). Rapid plasmid DNA isolation was carried out using the Wizard™ Miniprep DNA purification system (Promega).

2.7.10.5 *E. coli* maxiprep

*E. coli* cells were grown overnight at 37°C in 500 ml LB plus ampicillin to a final concentration of 50 mg/l and harvested by centrifugation at 4000 rpm for 15 minutes. Large scale preparations of plasmid DNA were obtained by the alkaline lysis method as described in Maniatis *et al.* (1989). Subsequent purification was carried out using equilibrium centrifugation in CsCl-ethidium bromide gradients as described in Maniatis *et al.* (1989). Rapid large scale plasmid DNA isolation was carried out using the Wizard™ Maxiprep DNA purification system (Promega).
2.8 Procedures for nucleic acid analysis

2.8.1 Isolation of yeast genomic DNA

Yeast genomic DNA was isolated by a modified technique described in Ausubel et al. (1995). A 50 ml culture of yeast in YEPD was grown overnight at 28°C to stationary phase and harvested by centrifugation at 1,200g for 5 minutes. The pellet was resuspended in 1 ml of breaking buffer (2% w/v Triton X-100, 1% w/v SDS, 100 mM NaCl, 100 mM Tris/HCl pH 8, 1 mM EDTA), 1 g of acid washed glass beads (BDH, 40 mesh) and 1 ml of phenol/chloroform/iso-amyl alcohol. The mixture was transferred to a 30 ml siliconised corex tube and vortexed vigorously for 5 minutes. 1 ml of TE was added, vortexed briefly and centrifuged at 1,200g for 5 minutes. 0.4 ml aliquots of the aqueous phase were transferred to 1.5 ml eppendorfs and the DNA precipitated with 1 ml of 100% ethanol at 12,000g for 5 minutes. The pellet was resuspended in 0.4 ml of TER (10 mM Tris.Cl, 1 mM EDTA, 500 μg/ml RNase; pH 8.0) and incubated at 37°C for 30 minutes. The DNA was phenol/chloroform/iso-amyl alcohol extracted twice, precipitated and resuspended in 200 μl of TE.

2.8.2 Isolation of yeast RNA

Yeast RNA was isolated as previously described by Piper (1994). All solutions, glassware and plasticware were treated with DEPC to deactivate RNase.

2.8.3 Quantitation of DNA and RNA

DNA and RNA samples were quantitated as described in Ausubel et al. (1995). An A$_{260}$ of 1.0 was taken to indicate 50 μg/ml double stranded DNA, 37 μg/ml single stranded DNA or 40 μg/ml RNA. The ratio of A at 260 and 280nm was used to indicate nucleic acid purity.

2.8.4 Southern analysis

Following electrophoresis the DNA was denatured and agarose gels were blotted by capillary transfer of DNA as described in Maniatis et al. (1989). Blotting was carried out overnight with Hybond-N nylon transfer membrane (Amersham) in 20x SSC (3M NaCl, 0.3M sodium citrate ; pH 7.0). Membranes were wrapped in 3MM Whatman filter paper and vacuum dried for 2 hours at 80°C.
Colony hybridizations were carried out by the following method. Yeast and *E. coli* cells were transferred onto Hybond NA-45 nitro-cellulose filters (Amersham) using forceps. The original plates were stored at 4°C. The filters were soaked for 5 minutes (cell side up) in three metal trays lined with Whatman 3MM filter paper saturated with denaturation solution (0.5 M NaOH, 1.5 M NaCl), neutralisation solution (0.5 M Tris-HCl pH 7.5, 1.5 M NaCl) and 2x SSC (0.3 M NaCl, 30 mM sodium citrate; pH 7.0). The filters were wrapped in 3MM Whatman filter paper, vacuum dried at 80°C for 2 hours and boiled for 10 min in dH₂O.

### 2.8.5 Northern analysis

Yeast RNA was denatured by treating samples with a combination of glyoxal and dimethyl sulfoxide (DMSO), subjected to electrophoresis and blotted according to the methods of Maniatis *et al.* (1989). Membranes were wrapped in 3MM Whatman filter paper and vacuum dried for 2 hours at 80°C. The blots were washed for 30 minutes at 65°C in 20 mM Tris/HCl pH 8 prior to hybridization.

### 2.8.6 In vitro labelling of single stranded DNA probes

DNA probes were prepared as described by Feinberg & Vogelstein (1984). Double stranded DNA was first denatured by boiling 100 ng of DNA in 10 μl of sterile dH₂O for 10 minutes. The DNA was cooled rapidly on ice and 12.5 μl of oligonucleotide labelling mix (25 μl nucleotide stock: 100 μM dGTP, dATP, dTTP, 250 mM Tris.Cl, pH 8.0, 25 mM MgCl₂; 25 μL 1 M Hepes pH 6.6; 7 μL pd(N)₆ sodium salt 100 ODU/ml [Pharmacia]), 20 μCi of (α-³²P)-5'-cytosine triphosphate and 5 units of Klenow were added. The probe was centrifuged at 12,000g for 10 sec and incubated at 37°C for 1-2 hours. After reannealing, the reaction was quenched by adding 50 μl of 20mM EDTA. The probe was denatured with 100 μl of 0.2M NaOH for 5 minutes at 37°C.

### 2.8.7 Hybridization analysis of DNA probes to membrane bound nucleic acid

Hybridization analysis was carried out by a modified method described by Maniatis *et al.* (1989). Dried membranes were pre-hybridized in siliconised glass bottles (Hybaid) in hybridization solution (0.125 ml/cm²; 5x SSC, 5x Denhardt's solution, 0.5% (w/v) SDS, 10% dextran sulphate and 100 mg/ml single stranded calf thymus DNA) for at least 30 minutes at the appropriate temperature with constant
agitation. The denatured probe was added to the pre-hybridization buffer and hybridization was carried out overnight at the appropriate temperature. After hybridization, the blot was washed at room temperature for 15 minutes in 2x SSC/0.1% SDS. This was followed by a 15 minute moderate stringency wash at the appropriate temperature in 2x SSC/0.1% SDS or a 15 minute high stringency wash in 65°C 0.1x SSC/0.1% SDS. All washes were carried out in duplicate with constant agitation. Rapid hybridizations were carried out using Quickhyb® hybridization solution (Stratagene®). The membranes were then wrapped in Saranwrap.

2.8.8 Autoradiography

Membranes were placed in direct contact with Fuji RX film in light proof autoradiographic cassettes for the appropriate length of time at -70°C with an intensifying screen. X-ray film was developed according to the manufacturers instructions.

2.8.9 Removal of probe from hybridized membrane

The hybridized probe was removed from membranes by boiling in 0.1% SDS for 10 min followed by cooling to room temperature. The membranes were wrapped in Saranwrap and stored at room temperature.

2.8.10 DNA sequence analysis

Nucleotide sequences were determined using the dideoxy chain-termination method, essentially as described by Sanger et al. (1977) using the T7Sequencing™ kit (Pharmacia Biotech). An acrylamide/urea sequencing gel was prepared as described in Maniatis et al. (1989). The gel was pre-run in 1x TBE buffer at 40W constant power for 30 minutes. The samples were denatured for 4 minutes at 80°C and 3 μl aliquots were electrophoresed at 40W constant power for the appropriate time. After electrophoresis, the gel was soaked in 15% methanol and 5% acetic acid for 20 minutes, washed in dH₂O, transferred to Whatman 3MM paper and wrapped in Saranwrap. The gel was dried for 2 hours under vacuum on a gel drier at 80°C and autoradiographed as described previously.
CHAPTER 3

3. INVESTIGATION OF WEAK ACID EFFECTS ON STRESS GENE PROMOTER AND STRESS PROMOTER ELEMENT-\textit{lacZ} ACTIVATION

3.1 Introduction

In low pH \textit{S. cerevisiae} cultures, weak acid preservatives induce a discrete stress response which probably increases the capacity of the cells for growth under conditions of weak organic acid stress (section 1.4.2). This response leads to strong induction of two plasma membrane proteins, Hsp30 (Piper et al., 1997) and the ABC-cassette multidrug transporter Pdr12 (P.W. Piper, unpublished results). Weak acids and low pH also increase expression of the \textit{HSP12}, \textit{HSP26}, and \textit{CTT1} genes (Varela et al., 1995; Carmelo & Sá-Correia, 1997; Schüller et al., 1994).

In \textit{S. cerevisiae}, stress conditions are thought to activate stress genes by various positive control elements (section 1.3). HSEs (Sorger and Pelham, 1987) bind heat-shock transcription factor (HSF) and are activated by stress conditions which cause the accumulation of abnormal proteins, such as heat shock or treatment with ethanol or arsenite (section 1.3.1). STREs (Kobayashi & McEntee, 1993; Marchler et al., 1993) bind the zinc finger proteins Msn2p and Msn4p (Martínez-Pastor et al., 1996) which mediate transcriptional activation by multiple stress conditions, including heat shock, weak acids, low pH, ethanol, exposure to oxidants, oxidative and osmotic stress and nitrogen starvation (section 1.3.2) (Schüller et al., 1994). PDREs bind the Pdr1p and Pdr3p transcription factors and are activated by a range of cytotoxic compounds (section 1.3.3). AREs bind the Yap1p and Yap2p transcription factors that direct gene activation during oxidative stress and exposure to toxic pro-oxidant compounds (Kuge & Jones, 1994; Hirata et al., 1994), while UPREs bind the Ern4p leucine zipper protein and are activated by compounds which cause the accumulation of unfolded secretory and transmembrane proteins in the ER (section 1.3.4) (Shamu et al., 1994).

At the start of this study, no detailed analysis of weak organic acid effects on stress gene promoters and stress promoter element-\textit{lacZ} activation had been undertaken although it had been shown that the STRE is sorbate-inducible in pH 5.8 cultures (Schüller et al., 1994). The aim of the investigation described in this chapter was to determine the interdependent effects of a weak organic acid, heat shock, and pH on
activation of a lacZ reporter under the control of two stress gene promoters (HSP12 and HSP26) and stress promoter elements (HSE and STRE). It is known from northern blot analysis (P.W. Piper, unpublished results) that transcription of the HSP30 gene is strongly induced by weak acid preservatives at low pH, heat shock and ethanol; also from the study of Régnacq & Boucherie (1992) that this gene is induced by entry into stationary phase. However, no information was available concerning the positive control elements responsible. Therefore, attempts were also made to construct an HSP30 promoter-lacZ fusion to compare stress activation of this fusion to the other fusions under investigation.

3.2 Results

3.2.1 Stress gene promoter and stress promoter element-lacZ vectors

The plasmids shown in Figs. 3.1 a-d were used to insert lacZ genes under stress control into yeast:

Fig. 3.1 (a) pHSE2 (Sorger & Pelham, 1987), an episomal URA3 vector that contains four copies of the (nGAA)n HSE sequence (HSE2 oligonucleotide, 5'-TCGACTCTTA GAAGCTTCTAGAAGCTTCTAGAGGATC-3') inserted within a CYC1 promoter-lacZ gene fusion (HSE-lacZ) in place of the normal CYC1 upstream activating sequences. Transformants of strains with pHSE2 are designated by a pHSE2 suffix to the strain name (e.g. PMY1.1-pHSE2 is PMY1.1 transformed with pHSE2).

![Diagram of pHSE2 vector](diagram.png)
Fig. 3.1 (b) pGM18/17 (Marchler et al., 1993), an integrative URA3 vector that contains a LEU2-lacZ fusion gene driven by 7 copies of the STRE (base pairs -367 to -355 of CTT1; 5'-AATTGGTAAGGGGCCTTACC-3') (orientation of insert copies: →→→→→→←←←←←). pGM18/17 was digested with NcoI and integrated in the URA3 locus. Single copy integrants were used for stress investigations. This lacZ gene under STRE control is referred to as STRE-lacZ and transformants are designated by a pGM18/17 suffix to the strain name.
Fig. 3.1 (c) pUP41a (gift of Dr P. Meacock), a single copy YCp50-based HSP12 promoter-lacZ fusion vector, identical to pUP39a (Praekelt et al., 1994) but for substitution of the THI4 promoter sequences by the -606 to +13 region of HSP12. This lacZ gene under HSP12 promoter control is referred to as HSP12-lacZ and transformants are designated by a pUP41a suffix to the strain.

Fig. 3.1 (d) pUKC414 (gift of M. F. Tuite), a single copy YCp50-based HSP26 promoter-lacZ fusion vector containing the -1600 to +139 region of HSP26 fused to codon 6 of the lacZ open reading frame. This lacZ gene under HSP26 promoter control is referred to as HSP26-lacZ and transformants are designated by a pUKC414 suffix to the strain name.
3.2.2 Investigations of weak acid stress on gene promoter and stress promoter element-lacZ induction

Experiments were carried out to investigate how stress gene promoter and stress promoter element-lacZ fusions were induced by heat shock, changes in medium pH and sorbate. *S. cerevisiae* strain PMY1.1 (Table 2.1) transformed with lacZ fusions (section 3.2.1) was grown to an OD$_{600}$ of 1-1.5 (0.5-1x10$^7$ cells/ml) at 28°C in SD media plus the appropriate auxotrophic requirements and harvested in 1 ml aliquots at 12,000g for 1 minute. The pellets were resuspended in 900 µl of SD medium containing 100 mM of the appropriate buffer (KH$_2$PO$_4$ pH 3.0, 3.5, 4.0, 4.5, 5.0; MES/KOH pH 5.5, 6.0, 6.5) and 100 µl of filter sterilised 20% D-glucose. The cultures were subjected to alternative treatments prior to measurement of lacZ activity: (i) 60 minutes incubation at 25°C; (ii) 60 minutes heat shock at 39°C; (iii) addition of 1 or 9 mM potassium sorbate, followed by 60 minutes incubation at 25°C; and (iv) addition of 1 or 9 mM potassium sorbate, followed by 60 minutes heat shock at 39°C.

3.2.2.1 The effect of heat shock, medium pH and sorbate on HSE-lacZ expression

Fig. 3.2 a and b show the influences of medium pH and sorbate respectively on the basal and heat induced expression of a HSE-lacZ fusion. Basal expression of HSE-lacZ was negligible in the absence of heat stress and was unaffected by either medium pH or sorbate (Fig 3.2 a). Therefore, sorbate and changes in medium pH are not HSE inducers. Heat induced HSE-lacZ expression (in the absence of sorbate) was not greatly affected by the pH of the culture, showing almost maximal lacZ activation across the whole pH range (Fig. 3.2 b). This result differs from Cheng & Piper (1994) who demonstrated that the heat shock expression of HSE-lacZ at pH 4.5 (in the absence of sorbate) was 2-fold lower compared to pH 6.5. No such inhibition of HSE-lacZ activation was evident from our data. Cheng & Piper (1994) used 50 mM sodium acetate to buffer low pH media, a buffer that may also exert weak acid effects on cells. Buffering low pH media with 100 mM KH$_2$PO$_4$ prevented this inhibition of HSE-lacZ induction by heat shock at low pH (section 3.2.2). In contrast, sorbate caused heat induced expression to be dramatically suppressed at low pH. 1 mM sorbate abolished HSE activation below a medium pH of 5.0 while 9 mM sorbate halved HSE-lacZ activation at pH 6.5 and completely abolished expression below pH 6.0. These results show that the inhibitory effects of sorbate on HSE-lacZ expression are greatly pH-dependent.
Fig. 3.2 Influences of medium pH and sorbate on the expression of a HSE-\(\text{lacZ}\) fusion in PMY1.1-pHSE2 maintained at (a) 25°C or (b) heat shocked to 39°C for 1 hour in the absence (■) or the presence of sorbate (●, 1 mM; ▲, 9 mM). Data presented are the mean of at least three independent experiments.

(a) 25°C

(b) heat shocked to 39°C
3.2.2.2 The effect of heat shock, medium pH and sorbate on STRE-lacZ expression

Fig. 3.3 a and b show the influences of medium pH and sorbate respectively on the basal and heat induced expression of a STRE-lacZ fusion. In the absence of heat stress and sorbate slight STRE-lacZ induction occurred below pH 4.5, becoming maximal in the lowest pH tested (pH 3.0; giving a 4-fold induction compared to pH 6.5). In the presence of sorbate, β-galactosidase expression by low pH was inhibited, but sorbate enhanced expression between pH 4.5 and 5.5 (1 mM) and above pH 5.5 (9 mM). Thus the STRE-lacZ fusion responds to changes in both medium pH and sorbate concentration in a pH dependant manner. This may partly explain why sorbate is a chemical inducer of thermotolerance at high pH in the absence of heat (Cheng & Piper, 1994).

Heat induced expression (in the absence of sorbate) was influenced by the pH of the culture, showing a two-fold greater induction at pH 3.0 compared to pH 6.5 (Fig. 3.3 b). pH 6.5 treatment in the presence of heat shock gave STRE-lacZ expression comparable to pH 3.0 treatment in the absence of heat shock, while the combination of heat shock and low medium pH induces the STRE by an amount that is the sum of the inductions with either of these stresses alone. Sorbate caused the heat induced STRE-lacZ expression to be suppressed at low pH, a result identical to the sorbate effect on HSE-lacZ expression (Fig. 3.2 b). Previous work by Cheng & Piper (1994) showed that sorbate selectively inhibited heat induced protein expression in low pH yeast cultures. At pH 5.5 and above in the presence of 1 mM sorbate, STRE-lacZ expression is greater than with a heat shock in the absence of sorbate. With 9 mM sorbate, this was only true at the highest pH tested (pH 6.5). It is clear from this data that, depending on medium pH, sorbate may either inhibit or activate expression of the STRE-lacZ fusion. In view of the correlation’s between STRE activity and stress tolerance (Ruis & Schüller, 1995) the effects of weak acids on stress tolerance are therefore likely to be pH-dependent.
Fig. 3.3 Influences of medium pH and sorbate on the expression of a STRE-lacZ fusion in PMY1.1-pGM18/17 maintained at (a) 25°C or (b) heat shocked to 39°C for 1 hour in the absence (■) or the presence of sorbate (○, 1 mM; ▲, 9 mM). Data presented are the mean of at least three independent experiments.

(a) 25°C

(b) heat shocked to 39°C
3.2.2.3 The effect of heat shock, medium pH and sorbate on \textit{HSP12-lacZ} expression

Fig. 3.4 a and b show the influences of medium pH and sorbate respectively on the basal and heat induced expression of a \textit{HSP12} promoter-\textit{lacZ} fusion. These expression patterns are very similar to that of the STRE-\textit{lacZ} fusion which is not unexpected since \textit{HSP12} is thought to be under STRE control (Varela \textit{et al.}, 1995). β-galactosidase levels are in general about two-fold lower which may result from the use of different expression vectors. As with the STRE-\textit{lacZ}, sorbate suppressed the heat induced expression of \textit{HSP12-lacZ} at low pH (Fig. 3.4 b). In the absence of heat stress and sorbate, expression at pH 3.0 was elevated about 2-fold compared to basal levels of transcription. Also, sorbate slightly raised expression at pH 5.0 (1 mM) and at a pHs above 5.5 (9 mM) in the absence of heat stress (Fig. 3.4 a).

3.2.2.4 The effect of heat shock, medium pH and sorbate on \textit{HSP26-lacZ} expression

Fig. 3.5 a and b show the influences of medium pH and sorbate respectively on the basal and heat induced expression of a \textit{HSP26} promoter-\textit{lacZ} fusion. In the absence of heat stress and sorbate, basal \textit{HSP26-lacZ} expression was unaffected by changes in medium pH. In the presence of 1 mM sorbate, expression was increased 2-fold at pH 3.5, rising to 4-fold at pH 5.0. 9 mM sorbate gave no increased β-galactosidase expression at low pH but resulted in a 4-fold increase at pH 6.0. Thus the \textit{HSP26} promoter shows a small response to sorbate in a pH dependent manner but low medium pH (3.0) alone is not sufficient to increase its activity (Fig. 3.5 a). However, such sorbate induction of \textit{HSP26-lacZ} expression was negligible compared to the heat induced levels seen in Fig. 3.5 b.. Heat shock resulted in a 60-fold increase in expression at pH 6.5 and 80-fold increases at pH 3.0, increases almost an order of magnitude greater than seen with the STRE-\textit{lacZ} or \textit{HSP12-lacZ} fusions. As with the other promoters studied, sorbate blocked heat induced expression of \textit{HSP26-lacZ} at low pH (Fig. 3.5 b).
Fig. 3.4 Influences of medium pH and sorbate on the expression of a HSP12-lacZ fusion in PMY1.1-pUP41a maintained at (a) 25°C or (b) heat shocked to 39°C for 1 hour in the absence (■) or the presence of sorbate (●, 1 mM; ▲, 9 mM). Data presented are the mean of at least three independent experiments.

(a) 25°C

(b) heat shocked to 39°C
Fig. 3.5 Influences of medium pH and sorbate on the expression of a HSP26-lacZ fusion in PMY1.1-pUKC414 maintained at (a) 25°C or (b) heat shocked to 39°C for 1 hour in the absence (■) or the presence of sorbate (○, 1 mM; △, 9 mM). Data presented are the mean of at least three independent experiments.

(a) 25°C

(b) heat shocked to 39°C
3.2.2.5 Construction of an *HSP30* promoter-\(\text{lacZ}\) fusion

An *HSP30* promoter-\(\text{lacZ}\) fusion was constructed to compare its stress activation to that of the other fusions (section 3.2.1). A 4.6 kb *UBI4* promoter-\(\text{lacZ}/\text{ADH}^\dagger\) *HindIII/SalI* fragment of pDP501 (Kirk & Piper, 1994) was ligated into *HindIII/SalI* digested, dephosphorylated YEplac181 (Gietz & Sugino, 1988) to make YEplac501. The -872 to -8 region of the *HSP30* promoter was synthesised by PCR using the oligonucleotides shown in Table 3.1. The PCR product was *HindIII/SpeI* digested and ligated into *HindIII/SpeI* digested, dephosphorylated Yeplac501 creating YEplac505 (Fig. 3.6.). This *lacZ* gene is referred to as *HSP30-lacZ* and transformants are designated by a YEplac505 suffix to the strain.

Table 3.1 Primers used for PCR synthesis of *HSP30* promoter

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence(^a)</th>
<th>Location(^b)</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward</td>
<td>5' -GCCAGCTTGAGTATTACTATTACA-3'</td>
<td>-872 to -855</td>
<td>Cruachem Ltd</td>
</tr>
<tr>
<td>Reverse</td>
<td>3' -AACTAATGATTTTTGTTGATCGG-5'</td>
<td>-27 to -8</td>
<td>Pharmacia Biotech</td>
</tr>
</tbody>
</table>

(a) bold nucleotides indicate: Forward primer *HindIII* site, Reverse primer *SpeI* site.
(b) underlined sequence in *HSP30* promoter region.

3.2.2.6 Stress activation of *HSP30-lacZ* expression

PMY1.1 was transformed to leucine prototrophy with YEplac505 to make PMY1.1-YEplac505 (*HSP30-lacZ*). Basal, heat shock, sorbate and stationary phase \(\beta\)-galactosidase levels were then measured (data not shown). The *HSP30-lacZ* fusion did not respond to heat shock or, at low pH, to the 9mM sorbate concentrations previously shown to strongly increase levels of *HSP30* mRNA. PMY1.1-YEplac505 did however show a 6-fold increase in levels of \(\beta\)-galactosidase expression upon transition to stationary phase. Further analysis of this *HSP30-lacZ* fusion was not pursued, since we were primarily interested in the pH-dependent effects of sorbate and heat shock on this stress promoter. It is possible that the stress-responsive elements lie further upstream than -872 and were not present within the *HSP30-lacZ* fusion. However, STRE-like sequences do exist in the -8 to -872 region and it was decided to pursue the analysis of these by site-directed mutagenesis of the *HSP30* gene on a vector which could then be integrated into a recipient *hsp30* null strain (chapter 4).
Fig. 3.6. YEplac505 (HSP30 promoter-lacZ) vector construction.
3.3 Discussion

3.3.1 STRE sequences are induced by weak acid treatment in low pH cultures

The work in this chapter confirmed that STRE sequences, unlike HSEs, are induced by weak acid (sorbate) treatment at low pH. Schüller et al. (1994) showed that sorbate is a strong STRE inducer in pH 5.8 cultures. However, even with multiple STREs in the vector (as in pGM18/17, section 3.2.1), this induction was only about 4-fold even when maximal (Fig.3.3 a). We were never able to obtain the 20-fold induction reported by Schüller et al., (1994) using the same vector (though integrated into a different recipient yeast strain). In addition, this is the first study to demonstrate the influence of culture pH over the effects of a weak organic acid on STRE activity. Similar induction of STRE sequences occurred at low pH (in the absence of sorbate) and at pH 5.5 with 1 mM sorbate. This suggests that the yeast cell can somehow sense changes in pHi brought about by weak acid treatment or growth at low culture pH, switching on a signalling pathway that activates STRE-controlled genes. Sigler and Höfer (1991) and Thevelein (1991) have outlined a signal transduction pathway in which intracellular acidification stimulates the cAMP-dependent PKA pathway. However, the STRE is negatively regulated by PKA, so that this stimulation of PKA by low pHi should suppress STRE activity. Results outlined in Chapter 4 suggest that PKA is not involved in the stress activation of HSP30, a gene shown to be induced by stresses that lower pHi. Recently, Maeda et al. (1994) and Posas et al. (1996) isolated a two-component membrane-bound osmosensor that switches on the HOG MAP kinase pathway and activates certain STRE-controlled genes in response to osmotic stress (section 1.3.2.1). At present, a pHi sensor has not been isolated in yeast. Changes in pHi also stimulate the H⁺-ATPase-catalysed extrusion of protons to maintain pH homeostasis (section 1.4.1.1). At present, the mechanisms by which reductions in pHi stimulate H⁺-ATPase activation are poorly understood.

Northern blot analysis of heat shock gene mRNAs has shown that the transcripts for HSP30, HSP12 and HSP26 are all induced at 25°C after cells are treated with sorbate (P.W. Piper, unpublished results). Our results show that a HSP12 promoter-lacZ fusion has an almost identical pattern of sorbate induction to the STRE sequences (Fig. 3.4). This is to be expected from the results of Varela et al. (1995) who demonstrated that the stress-inducibility of the HSP12 gene is controlled by five repeats of the STRE. In contrast, the stress-inducibility of the HSP26 gene is more complex. Chen &
Pederson (1993) have implicated HSEs in the heat shock induction of \textit{HSP26} while Martínez-Pastor \textit{et al.} (1996) have presented evidence that STRE elements are involved in carbon source starvation-induced activation of this gene. Our results indicated that the \textit{HSP26} promoter-\textit{lacZ} fusion is only slightly induced by sorbate at low pH, but that this is negligible compared to heat shock activation. Unlike the HSE (Fig. 3.2 b), heat shock activation of the \textit{HSP26} promoter-\textit{lacZ} is influenced by culture pH (Fig. 3.5 b) but not as greatly as the STRE. This data suggests that a combination of HSE and STRE elements may be controlling the stress activation of \textit{HSP26}.

### 3.3.2 Weak acid preservatives inhibit heat induction of stress gene promoter and stress promoter element-\textit{lacZ} fusions in low pH cultures

This study has also shown that weak acid treatment inhibited the heat shock-induced expression of the stress gene promoter and stress promoter element-\textit{lacZ} fusions at low pH (Figs. 3.2-3.5). Cheng & Piper (1994) demonstrated that weak acid preservatives inhibit the heat induction of major heat shock proteins in low pH cultures. Measurements of HSE and STRE-\textit{lacZ} activity (Figs. 3.2 and 3.3 respectively) show this to be due to lack of induction of the HSE and STRE sequences. Our results reveal that heat shock or sorbate alone induce certain heat shock genes and stress promoter elements, but in combination at low pH they act to inhibit these same genes. The inhibitory effects of heat shock and sorbate at low pH may be explained by their effects on the cells ability to maintain pH homeostasis. Weak acids cross membranes only when undissociated, concentrating inside the cells and lowering pH\textsubscript{i} in response to a higher pH on the cystolic side of the cell membrane (section 1.4.1). Heat shock causes membrane disordering and protein denaturation (Casey & Ingledew, 1984; Piper, 1993), causes an inhibition of glycolysis and enhances induction of petites (Neves & François, 1992). This stress increases the permeability of the plasma membrane, resulting in an increased passive proton and weak acid influx that acts to dissipate the electrochemical potential gradient that the cell maintains at this membrane. This is reflected in the decline in pH\textsubscript{i} that is observed both with weak acids (Krebs \textit{et al.}, 1983) and with heat shock (Weitzel \textit{et al.}, 1987; Coote \textit{et al.}, 1991; Coote, 1993). Weak acid and heat stress will adversely affect those vital processes for which a plasma membrane electrochemical gradient is essential, such as nutrient uptake, maintenance of potassium balance and regulation of pH\textsubscript{i} (Serrano, 1991).
Heat shock or sorbate treatment at low pH both stimulate H⁺-ATPase activity (Coote et al., 1991; Viegas & Sá-Correia, 1991; Holyoak et al., 1996) which results in active extrusion of protons for pHᵢ maintenance. The combination of both stresses will more severely effect ability to maintain pHᵢ homeostasis. As a consequence, the reduction in glycolytic flux (Krebs et al., 1983; François et al., 1986) and increase in H⁺-ATPase activity may deplete cellular ATP levels significantly (Cole & Keenan, 1987; Holyoak et al., 1996). Upon entry to yeast cells sorbate is thought to concentrate within mitochondria, causing pronounced disruption of mitochondrial structure (Cole, 1987). Cheng & Piper (1994) demonstrated that heating in the presence of sorbate at low pH causes strong induction of respiratory deficient petites, indicating that this sorbate-induced damage to mitochondria is enhanced considerably by heat. The cells inability to generate ATP or maintain pHᵢ homeostasis by a combination of these stresses may block the heat shock response (Cheng & Piper, 1994) and the heat shock-induced expression of the stress gene promoter and stress promoter element-lacZ fusions shown in this chapter.

3.3.3 An HSP30 promoter-lacZ fusion was not stress-inducible

Hsp30 is induced by a variety of different stress conditions such as entry to stationary phase, heat shock, ethanol, low pH and weak acid preservatives at low pH (Régnacq & Boucherie, 1992; Panaretou & Piper, 1992; Piper et al., 1994). An HSP30 promoter-lacZ fusion constructed for this study (Fig. 3.6) was not stress-inducible under conditions previously shown to strongly increase levels of HSP30 mRNA (P.W. Piper, unpublished results). However, the HSP30 promoter-lacZ fusion displayed a 6-fold increase in levels of expression upon transition to stationary phase (data not shown). We were not able to obtain the 100-fold stationary phase induction of a HSP30 promoter-lacZ fusion (encompassing +17 to -1083 region of the HSP30 promoter) reported by Riou et al. (1997). The HSP30 promoter-lacZ fusion used in our studies contained the -8 to -872 region of the HSP30 promoter. It is therefore possible that a positive transcriptional control element exists in the -872 to -1083 upstream region. This region possesses a single weak match (5/8 bp) to the PDSE at -1074 to -1078 (section 1.3.2 and Table 1.2) while STRE-like sequences do exist in the -8 to -872 region. Analyses of the HSP30 promoter are discussed in greater detail in chapter 4.
3.4 Conclusions

This work has shown that sorbate can both inhibit the heat shock activation of stress gene promoters and stress promoter elements in low pH cultures or act as chemical inducers of these same sequences in the absence of heat shock. A possible reason for the different effects of weak acids in stress gene activation or repression can be explained by the existence of a mechanism in yeast for sensing and maintaining pH\textsubscript{i} homeostasis. In low pH cultures or the presence of sorbate, yeast cells can sense a slight pH\textsubscript{i} decrease, leading to increased H\textsuperscript{+}-ATPase activity for pH maintenance, activation of STRE-controlled genes and induction of the Hsp30 and Pdr12 membrane proteins (section 1.4.2). In contrast, combination of both heat shock and sorbate at low pH may cause a large pH\textsubscript{i} decrease which is incompatible for both growth and the efficient induction of stress genes and stress promoter elements, or that the cell is incapable of maintaining intracellular pH homeostasis.
4. INVESTIGATION OF THE ROLES OF PUTATIVE STRESS-RESPONSIVE PROMOTER ELEMENTS ON STRESS ACTIVATION OF THE HSP30 GENE

4.1 Introduction

In this work we were especially interested in the promoter sequences leading to strong gene induction by sorbate. Two genes very strongly induced by sorbate at pH 4.5 are HSP30 and PDR12 (section 1.4). Fig. 4.1 shows northern blots of HSP30, PRD12, PDR5 and SNQ2 stress activation (P.W. Piper, unpublished results). Indeed, sorbate induces HSP30 as strongly, if not more strongly than either heat or ethanol stress. With PDR12, sorbate induces much more strongly than any other stress tested so far. In chapter 3 the STRE element and also the HSP12 and HSP26 promoters were found to be induced by low pH and sorbate. The HSP12 gene is controlled by multiple STRE elements (Varela et al., 1995) but, although HSP26 has both STRE and HSE consensus sequences in its promoter, it is not known which of these are functional elements. In contrast, the HSE did not respond to sorbate stress at low pH. An HSP30 promoter-lacZ fusion did not respond to sorbate, low pH or heat shock and was only weakly induced upon transition into stationary phase. When we examined the promoter sequences of HSP30 and PDR12, a number of putative stress regulatory elements were revealed (Table 4.1). Both genes contained a number of agreements to the PDSE consensus (section 1.3.2), which controls activation of the SSA3 gene in response to low cAMP levels (Boorstein & Craig, 1990 a). This element is thought to be a variant of the STRE since it contains a similar motif (4/5 bp match) to the STRE consensus. HSP30 also had one 4/5 bp match to the STRE consensus (Table 4.1), while PDR12 had none. Neither promoter had two or more adjacent inverted repeats of the nGAAAn consensus (section 1.3.1), generally thought to be needed for efficient binding of HSF (Sorger, 1991), although Régnacq & Boucherie (1992) demonstrated that the HSP30 promoter did contain two stretches of DNA showing 7/8 bp matches to the HSE consensus (Table 4.1). We therefore surmised that the PDSE may be the stress element directing activation of these two genes in response to weak organic acid stress, since there were no exact matches to other known stress elements present in both the HSP30 and PDR12 promoters.
Fig. 4.1 Northern analysis of total RNA from yeast cells subjected to different stresses. Hybridization to radiolabelled probes specific for the genes indicated to the left of the figure panels was carried out as described in chapter 2. An actin specific probe (ACT1) served as a control for equal RNA loading. About 20 μg total RNA each from unstressed control cells (Lane 1); heat shocked at 40°C for 1 hour (Lane 2); cold shocked at 15°C for 3 hours (Lane 3); osmostressed at 30°C for 1 hour with either 2.0 M sorbitol (Lane 4); 0.5 M or 1.0 M NaCl (Lanes 5 and 6); 0.5 M or 1.0 M KCl (Lanes 7 and 8); and treated at 30°C for 1 hour with 6% (w/v) ethanol (Lane 9) were northern blotted (P.W. Piper, unpublished results).
Table 4.1 Putative regulatory elements in the *HSP30* and *PDR12* promoters

<table>
<thead>
<tr>
<th>Name</th>
<th>Putative element</th>
<th>Consensus</th>
<th>bp match</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>HSP30 gene</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>STRE1</td>
<td>-789 eGGGG -785</td>
<td>AGGGG or CCCCT</td>
<td>4/5</td>
</tr>
<tr>
<td>PDSE1</td>
<td>-664 aAAGGGAT -657</td>
<td>T(T/A)AGGGAT</td>
<td>7/8</td>
</tr>
<tr>
<td>PDSE2</td>
<td>-585 gcAGGGAg -592</td>
<td>&quot;</td>
<td>5/8</td>
</tr>
<tr>
<td>PDSE3</td>
<td>-409 eAAGGGAT -416</td>
<td>&quot;</td>
<td>7/8</td>
</tr>
<tr>
<td>PDSE4</td>
<td>-344 aAAGGGAT -351</td>
<td>&quot;</td>
<td>7/8</td>
</tr>
<tr>
<td>PDSE5</td>
<td>-285 TTAGGGAg -292</td>
<td>&quot;</td>
<td>7/8</td>
</tr>
<tr>
<td>PDSE6</td>
<td>-230 TAAGGGAT -237</td>
<td>&quot;</td>
<td>8/8</td>
</tr>
<tr>
<td>HSE1</td>
<td>-557 cTCTAGAA -550</td>
<td>nTTCnnGAAn</td>
<td>7/8</td>
</tr>
<tr>
<td>HSE2</td>
<td>-319 TTCTAGeA -312</td>
<td>&quot;</td>
<td>7/8</td>
</tr>
<tr>
<td><strong>PDR12 gene</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PDSE1</td>
<td>-622 TAAGGGAc -629</td>
<td>T(T/A)AGGGAT</td>
<td>7/8</td>
</tr>
<tr>
<td>PDSE2</td>
<td>-608 cAAGGGAc -615</td>
<td>&quot;</td>
<td>6/8</td>
</tr>
<tr>
<td>PDSE3</td>
<td>-564 gAAGGGtT -571</td>
<td>&quot;</td>
<td>6/8</td>
</tr>
<tr>
<td>PDSE4</td>
<td>-562 TeAGGGca -555</td>
<td>&quot;</td>
<td>5/8</td>
</tr>
<tr>
<td>PDSE5</td>
<td>-355 gcAGGGGAT -348</td>
<td>&quot;</td>
<td>6/8</td>
</tr>
</tbody>
</table>

The position of each element in the *HSP30* and *PDR12* promoter is indicated together with the consensus sequence and number of base pair matches to the general consensus. Mismatches are indicated in lowercase bold and the designation of the elements in the text is also indicated. The nucleotide sequence of the proximal *HSP30* 5'-flanking region together with the position and orientation of the putative stress-responsive elements are shown in Fig. 4.2.
Fig. 4.2 Nucleotide sequence of the proximal \textit{HSP30} 5'-flanking region. The single STRE and six PDES repeats are shown in bold, and their orientation is depicted with an underlying arrow. The two putative HSEs are indicated by a dotted overline. Nucleotides matching the HSE consensus sequence are underlined. TATA boxes are boxed.

$$\begin{array}{c}
-900 \quad \text{AGCAGCCCTGAAGAAGAAAACCTCAACACGAGTATTACTATTACAATCAA} \\
-850 \quad \text{ACAACCTTTAGGCCGCCTGATACCCGGGGTTGAAGTGGGTGACATTGAGCC} \\
\text{STRE1} \\
-800 \quad \text{GTATTCTTCTCCCCCGTAAGAAAGTTGTGTATCCTTTTACTGCGGTTGTA} \\
\longrightarrow \\
-750 \quad \text{ATAGCTTCTGAAAAACCTAAAAAATGAACGATCGTACATTACTTT} \\
\text{PDSE1} \\
-700 \quad \text{TGCATAAGTAAGAATAACTTTGTGCAGGGTGCCGAAGGGATGGAAGA} \\
\longrightarrow \\
-650 \quad \text{CCGCTGACGCAACCCCTTTTACATACAGTCGATCCATCTGACTTTACTTT} \\
\text{PDSE2} \\
-600 \quad \text{CCTTGCGTCTCCCTGCGATACCGGGGGTTGAAGTGGGTGCATTGAGCC} \\
\text{HSE1} \\
-550 \quad \text{AATTTTCAGGGGTGCGACCTGAGGATGTTCTGCAGAAAGGCCAAAAACGC} \\
-500 \quad \text{ATCGAAACGTGCTTTTGTAAGAATATTGGTGATGCTAAAGTAAGCMAAG} \\
\text{PDSE3} \\
-450 \quad \text{CCATATCCCCGATCCCCGATCCCCGACTTCTATTCCGATCCCTTCCGCCACAT} \\
\text{PDSE4} \\
-400 \quad \text{CCTGCATGTITATTGCAATACCAATTTAGCTCATCTGCTATTCTTCTTTTCTAAGCATTCTCATCGAA} \\
\text{HSE2} \\
-350 \quad \text{CCCTTCTCCTGCTATGGGAAGACAGTTTTTTCTAGCATCTCATCGAA} \\
\text{PDSE5} \\
-300 \quad \text{ACTTTCCTCCTCCTTAATTGGCAAGGAAAGTTTTTCTTTTCTATTACATCGTACAGTTAGA} \\
\text{PDSE6} \\
-250 \quad \text{AAGTATATAATATCAACTCCCCTACCTCATACATATGTTAGTGATCACAATAAAAA} \\
\text{PDSE7} \\
-200 \quad \text{AATCATATATAATTGTGAGGCTTCTCATTACAAAGTGGTGATCACAATAAAAAA} \\
\text{PDSE8} \\
-150 \quad \text{TCACCTCTCTTTTTCTTTTTTCAATTCTGCAATACACAACACAC} \\
-100 \quad \text{AAACAAATATTTAATTATTATTATATAATATTACAAAAACAAAAACAAAA} \\
-50 \quad \text{CAAGTTGGAGACTTTTAATATCTTTTGTATTACTAAAAACAAAAATAATTC} \\
\end{array}$$
In this chapter we try to identify the cis and trans-acting elements responsible for stress activation of the HSP30 gene by the analysis of transcription factor mutants; also by point mutation of putative stress control elements. The relative importance's of the known stress signalling pathways (section 1.3) were investigated by measurement of HSP30 gene activation in S. cerevisiae mutants with either constitutively high or low PKA activity (Belazzi et al., 1991); a temperature-sensitive allele of the HSFI gene, hsfl-m3 (Smith & Yaffe, 1991); or disruptions of the MSN2, MSN4 (Martinez-Pastor et al., 1996), YAP1, PDR1 and PDR3 genes (Delaveau et al., 1994). To test the apparent involvement of the putative STRE and PDSEs in the regulation of the HSP30 gene expression (Table 4.1 and Fig. 4.2), site-directed mutagenesis was used to mutate all seven of these elements, singly and in combination, within plasmid pUC19HSP30 (Fig. 4.3). This vector incorporates a 2.3 kb Clal fragment of the HSP30 gene, inserted into pUC19 at the AccI restriction site (Régnacq & Boucherie, 1992). Both the Clal and AccI restriction sites were destroyed upon ligation. As described later (Fig. 4.7), 1.4 kb Ndel/Kpnl fragments of the pUC19HSP30-derivates, containing the desired mutation(s) were then transplaced into S. cerevisiae strain KT3 (hsp30:URA3) and strains possessing the desired mutation in the HSP30 gene were selected on 5-FOA plates (section 2.5.5), tested and used for stress investigations.

Fig. 4.3 Restriction map of the pUC19HSP30 plasmid

![Restriction map of the pUC19HSP30 plasmid](image-url)
4.2 Results

4.2.1 Site-directed mutagenesis of the STRE1 motif

A mutant allele of *HSP30* harbouring a mutation of the STRE1 consensus was generated using the Clontech Transformer™ Site-directed Mutagenesis kit (Clontech Laboratories, Inc). This method works by simultaneously annealing two oligonucleotide primers to one strand of a denatured double-stranded plasmid. One primer introduces the desired mutation while the second primer mutates a restriction site unique to the plasmid for purposes of selection (Fig. 4.4).

**Fig. 4.4** Strategy for generating specific base changes using the Transformer™ Site-directed Mutagenesis kit (from Clontech, Ltd, PT1130-1).
For the purposes of this study, oligonucleotides to mutate the pUC19 SphI site from GCATGC to GCTTGC and the HSP30 STRE1 consensus from CCCCG to GCGCG were designed (Table 4.2).

**Table 4.2 Primers used for the site-directed mutagenesis of the STRE1 motif**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence(^{a})</th>
<th>Location(^{b})</th>
</tr>
</thead>
<tbody>
<tr>
<td>STRE1(^{M})</td>
<td>5'-GCCGTATTCTTgCgCGTAAGAAAGTTG-3'</td>
<td>-803 to -774</td>
</tr>
<tr>
<td>SphI</td>
<td>5'CGACCTGCAGGCCtTgCAAGCTTGGC-3'</td>
<td>pUC19 MCS</td>
</tr>
</tbody>
</table>

(a) Nucleotides which differ from the wild-type sequence are shown in lowercase bold.
(b) Location (5' → 3') of the sequence in the HSP30 promoter region annealing to the primer.

The two oligonucleotides were simultaneously annealed to one strand of the denatured pUC19HSP30. Using PCR, the DNA was elongated, ligated and a primary selection was carried out by digesting with SphI. The mixture of mutated and unmutated plasmid DNA was then transformed into a mutS E. coli strain defective in mismatch repair (see methods). Transformants were pooled and plasmid DNA was prepared from the mixed bacterial population. The isolated DNA was then subjected to a further SphI restriction digestion. Since the mutated DNA lacks the SphI restriction site, it is resistant to digestion whereas the parental DNA will be linearised, reducing transformation efficiency at least 100-fold. Following a second transformation, plasmid DNA from single E. coli colonies was isolated. Plasmid DNA resistant to digestion by SphI was then sequenced (Fig. 4.5).

From the sequencing gel it can be deduced that the wild-type STRE1 (CCCCCG) has been mutated to form an STRE1\(^{M}\) mutant allele of the HSP30 gene. The mutant pUC19HSP30 displayed only two C bands in the -789 to -785 bp upstream region compared to the four present in the wild-type plasmid (CCCCCG has been mutated to GCGCG). The mutant plasmid, denoted pUC19HSP30-STRE1\(^{M}\) (whereby \(^{M}\) denotes a mutation), was then used as a template for the mutation of the six remaining PDSE motifs.
Fig. 4.5 DNA sequencing of wild-type (A) and mutant (B) pUC19HSP30. ddCTP (Lanes 1, 3, 5 and 7) and ddATP (Lanes 2, 4, 6 and 8) reactions were sufficient to determine the point mutations.
4.2.2 Site-directed mutagenesis of the PDSE motifs

Mutations in the PDSE motifs were introduced using the Quikchange™ Site-directed mutagenesis kit (Stratagene®). This method utilises a double-stranded vector and two synthetic oligonucleotide primers (containing the desired mutation), each complementary to opposite strands of the vector. Mutant DNA is extended by PCR and a mutated plasmid containing staggered nicks is generated. Following temperature cycling the product is treated with DpnI (target sequence: 5'-Gm6ATC-3'), specific for methylated and hemimethylated DNA found in almost all E. coli strains. The parental DNA template is dam methylated and susceptible to DpnI digestion while PCR generated mutant plasmid DNA is unmethylated and resistant to digestion leading to a high mutation efficiency (Fig. 4.6). For the purpose of this study, mutagenic oligonucleotides each complementary to opposite strands of the pUC19HSP30 vector were designed, encompassing mutations of all six PDSE motifs (Table 4.3).

Table 4.3 Primers used for the site-directed mutagenesis of the PDSE motifs.

<table>
<thead>
<tr>
<th>Primera</th>
<th>Sequenceb</th>
<th>Locationc</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDSE1M</td>
<td>5'-GCAGGGTGCCGAATcGctTGGAAACCCGC-3'</td>
<td>-675 to -647</td>
</tr>
<tr>
<td>PDSE2M</td>
<td>5'-CCTTGCGTcagCgaGGCGGATTTGGTGG-3'</td>
<td>-600 to -572</td>
</tr>
<tr>
<td>PDSE3M</td>
<td>5'-CGACTCTTATTCCGAagCgaTCCGCCACATCC-3'</td>
<td>-430 to -399</td>
</tr>
<tr>
<td>PDSE4M</td>
<td>5'-CTTCGTTATTTTCATCAgCgCTcTCTGCTATGGCAAGG-3'</td>
<td>-366 to -335</td>
</tr>
<tr>
<td>PDSE5M</td>
<td>5'-GCATCTCATCGAAAACTTTCCTCagCgagATTGGCCAAAG-3'</td>
<td>-314 to -275</td>
</tr>
<tr>
<td>PDSE6M</td>
<td>5'-GTTAGAAAGTATAATATCcggTCgCTcgcCTCATTACAAGTTG-3'</td>
<td>-256 to -215</td>
</tr>
<tr>
<td>STRElWT</td>
<td>5'-GCCGTAATTCCTCCCCGTAGAAAGTTGATCC-3'</td>
<td>-803 to -767</td>
</tr>
</tbody>
</table>

(a) only the sequence of the HSP30 coding strand mutant oligonucleotide is shown, although this was also used in conjunction with primers of the complementary sequence. The STRElWT oligonucleotide was used to reverse the STRElM of the pUC19HSP30 derivatives to a wild-type STREl.

(b) Nucleotides which differ from the wild-type sequence are shown in lowercase bold.

(c) Location (5' → 3') of the sequence in the HSP30 promoter region
Fig. 4.6 Strategy for generating specific base changes using the Quikchange™ Site-directed Mutagenesis kit (from Stratagene®, catalogue number 200518).

**Step 1**
Plasmid preparation

Gene in plasmid with target site (●) for mutation

**Step 2**
Temperature Cycling

Denature the plasmid and anneal the oligonucleotide primer containing the desired mutation (X)

Mutagenic primers

Using the nonstrand-displacing action of Pfu DNA polymerase, extend and incorporate the mutagenic primers resulting in nicked circular strands

**Step 3**
Digestion

Digest the methylated, nonmutated parental DNA template with DpnI

Mutated plasmid (contains nicked circular strands)

**Step 4**
Transformation

Transform the circular, nicked dsDNA into XL1-Blue supercompetent cells

After transformation, the XL1-Blue supercompetent cells repair the nicks in the mutated plasmid

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The pUC19HSP30-STRE1M vector was used to generate different combinations of mutant alleles of the HSP30 promoter harbouring mutations in the PDSE motifs (Table 4.4.). After PCR extension and DpnI digestion, mutated plasmid DNA was transformed in Epicurian coli® XL1-Blue Supercompetent Cells as described in the Stratagene® instruction manual (catalogue number 200518). Plasmid DNA from single Epicurian coli® colonies were isolated and sequenced. Each mutation was introduced singly, the resulting mutant plasmid being checked by sequencing before being used to generate the next pUC19HSP30 allele.

Table 4.4 The complete mutagenised set of pUC19HSP30 vectors incorporating mutant alleles of the HSP30 gene.

<table>
<thead>
<tr>
<th>Name</th>
<th>Promoter Mutations</th>
</tr>
</thead>
<tbody>
<tr>
<td>a pUC19HSP30-STRE1M</td>
<td>STRE1 only</td>
</tr>
<tr>
<td>b pUC19HSP30-STRE1M PDSE1M</td>
<td>STRE1, PDSE1</td>
</tr>
<tr>
<td>pUC19HSP30-STRE1M PDSE1-2M</td>
<td>STRE1, PDSE1,2</td>
</tr>
<tr>
<td>pUC19HSP30-STRE1M PDSE1-3M</td>
<td>STRE1,PDSE1,2,3</td>
</tr>
<tr>
<td>pUC19HSP30-STRE1M PDSE1-4M</td>
<td>STRE1,PDSE1,2,3,4</td>
</tr>
<tr>
<td>pUC19HSP30-STRE1M PDSE1-5M</td>
<td>STRE1,PDSE1,2,3,4,5</td>
</tr>
<tr>
<td>pUC19HSP30-STRE1M PDSE1-6M</td>
<td>STRE1,PDSE1,2,3,4,5,6</td>
</tr>
<tr>
<td>pUC19HSP30-STRE1WT PDSE1-6M</td>
<td>PDSE1,2,3,4,5,6 only</td>
</tr>
</tbody>
</table>

(a) a set of pUC19HSP30 vectors were also generated with single PDSE motif mutations only, though their expression in yeast was not studied.
(b) also a set of vectors were generated with a range of double mutations of the STRE1 and PDS motifs.
4.2.3 Attempted transplacement of mutant alleles of the \textit{HSP30} gene into the \textit{hsp30::URA3} locus of strain KT3

1.4 kb \textit{NdeI/KpnI} fragments of the pUC19HSP30 mutant vectors (100 ng), incorporating the desired mutations, were integrated into the \textit{HSP30} locus of Chromosome III in \textit{S. cerevisiae} strain KT3. This strain is isogenic to the protease deficient BJ2168 wild-type except for a \textit{URA3} gene fragment insertional inactivation of the \textit{HSP30} gene, at the \textit{HindIII} site (+18) within the \textit{HSP30} open reading frame (Piper \textit{et al.}, 1997). Selection for integration of the mutant \textit{HSP30} gene was carried out after transformation by growth on 5-FOA plates (Section 2.5.5). \textit{URA3} \textsuperscript{+} strains are unable to grow on medium containing the pyrimidine 5-FOA while \textit{ura3} \textsuperscript{−} mutants are resistant (Boeke \textit{et al.}, 1984). This method selected for transplacement of the \textit{URA3} insert by mutant alleles of the \textit{HSP30} gene (Fig. 4.7).

Integration at the \textit{HSP30} locus on Chromosome III was confirmed by Southern analysis. \textit{ura3} \textsuperscript{−} transformants were isolated and genomic DNA samples were prepared. 1 µg samples of genomic DNA were restricted with \textit{PstI} (to cut in \textit{URA3}) and electrophoresed on a 1% agarose gel. The Southern blot was then probed with the 1.1 kb \textit{BamHI} \textit{URA3} fragment from the Ydp11 vector (Fig. 4.8). The hybridization showed that KT3 DNA (Lanes 1 and 2) hybridized to three \textit{URA3 PstI} fragments; two of approximately 7-7.5 kb in length and one of 3.3 kb as previously reported (Talreja, 1995). The former two correspond to two \textit{ura3-52} allele fragments while the latter corresponds to the \textit{URA3} gene fragment inserted within the \textit{HSP30} coding region (Fig. 4.7). In contrast, BJ2168 DNA (Lane 3) displayed only the two larger \textit{PstI} \textit{URA3} fragments. All the \textit{ura3} \textsuperscript{−} transformant DNA's (e.g. Lanes 4 and 5) showed the two larger bands, suggesting that the \textit{hsp30:URA3} gene had been transplaced with mutant alleles of the \textit{HSP30} gene. A set of \textit{S. cerevisiae} strains isogenic with BJ2168 but for single or multiple point mutations in the \textit{HSP30} gene promoter should thus have been constructed. Mutant strains are denoted BJ2168-30 followed by the name of the mutation present in the pUC19HSP30 vector used in construction of the strain (e.g. BJ2168-30-S1\textsuperscript{M} P1\textsuperscript{M} is BJ2168 derived by transformation of KT3 with a fragment having mutations in the STRE1 and PDSE1 of the \textit{HSP30} promoter). KT3 was also transformed with the wild-type pUC19HSP30 fragment (BJ2168-30WT) as a control.
Fig. 4.7 Map of Chromosome integration of mutant alleles of the HSP30 promoter. Diagram (a) shows the hsp30::URA3 locus on Chromosome III of S. cerevisiae strain KT3. (b) shows the 1.4 kb NdeI/Kpnl fragment of pUC19HSP30 vector which replaces the hsp30::URA3 gene by homologous recombination (c). M denotes point mutation.

(a) S. cerevisiae strain KT3 hsp30::URA3

(b) 1.4 kb NdeI/Kpnl fragment of pUC19HSP30

(c) S. cerevisiae strain BJ2168 HSP30M (mutant allele)
Fig. 4.8 Southern blot of *PstI* digested genomic DNA. Lanes 1 and 2 - KT3, Lane 3 - BJ2168 and Lanes 4 and 5 - BJ2168-30-S1M.
4.2.4 Influences of weak acid preservatives, ethanol and heat stress on HSP30 gene expression in BJ2168-30 strains

The strongest inductions of the wild-type HSP30 gene are seen with ethanol, heat shock (Fig. 4.1) and weak acid preservative stresses (Piper et al., 1997). These stresses were therefore employed to study the effect of mutations in the STRE1 and PDSE motifs on the stress expression of the HSP30 gene. YEPD cultures of wild-type BJ2168, BJ2168-30WT and mutant BJ2168-30 strains were grown to mid-exponential phase (OD$_{600}$ 1.5-2.0), centrifuged and resuspended in YEPD only (basal HSP30 expression), YEPD+7% ethanol and YEPD pH 4.5+9mM sorbate and incubated at 28°C for 1 hour with constant shaking. A further sample was resuspended in YEPD only and incubated at 39°C for 1 hour. Total RNA was extracted and electrophoresed on a 1% agarose gel. Northern blots were then probed with sequences of the 2 kb PstI/BamHI fragment of pUC19HSP30 and a PCR generated fragment of the PDA1 gene as an internal standard (Wenzel et al., 1995) (northerns not shown). The wild-type BJ2168 showed typical stress induced levels of HSP30 mRNA. HSP30 gene expression was activated by a heat shock of 39°C, 7% ethanol and 9mM sorbate pH 4.5 as previously reported (Panaretou & Piper, 1992; Régnacq & Boucherie, 1992; Piper et al., 1994; Piper et al., 1997). In contrast, none of the BJ2168-30 mutant strains showed detectable HSP30 mRNA either before or after stress exposure. Even the BJ2168-30WT strain (the hsp30::URA3 gene replaced with wild-type HSP30 sequences from pUC19HSP30) did not show either basal or stress induced levels of HSP30 mRNA. This suggests that although the Southern blots indicate replacement of the hsp30::URA3 gene (Fig. 4.8), the transplacements have not occurred in the proper manner and that a transcriptionally active HSP30 gene has not been integrated in the genome.

Gene replacement by homologous recombination is generally assumed to occur at the ends of the DNA fragment used in transformation (Fig. 4.7), double-stranded termini being particularly active in recombination. Part of the evidence for this is that integrative vectors transform yeast cells much more efficiently when linearised to provide double-stranded breaks with homology to chromosomal target sequences (Struhl, 1983). It was to be assumed therefore that the NdeI/KpnI fragments from pUC19HSP30 derivatives would recombine at their termini to give the precise gene replacement shown in Fig. 4.7, and the Southern blot analysis indicated that this had indeed happened (Fig. 4.8). However, this should also have led to restoration of normal HSP30 expression, at least in the BJ2168-30WT transformant. When we did not see this (results not shown) we decided to investigate if the desired mutations were indeed
present in some of the BJ2168-30 transformants. The \textit{HSP30} promoter of BJ2168-30-S1\textsuperscript{M} was amplified by PCR (using the same forward and reverse primers outlined in section 3.2.2.5) and the resultant 870 bp fragment was sequenced (results not shown). Sequencing showed that this transformant did not have the STRE1\textsuperscript{M} mutation (GCGCG) in the \textit{HSP30} promoter sequence, but instead possessed the wild-type sequence (CCCCG). It was clear at this stage of the investigation that the mutant \textit{KpnI/NdeI} fragments had not integrated correctly in the transformants. Rather than expend time and effort characterising what had gone wrong with the integration, we decided instead to stick to the original objective of analysing \textit{HSP30} expression. The already constructed \textit{HSP30} alleles with mutant promoter sequences were therefore transferred to a well-characterised integration vector that would allow integration at the \textit{TRP1} locus.

4.2.5 Construction of \textit{YIplac 204}-derived vectors containing mutant alleles of the \textit{HSP30} gene

Since gene replacement did not give a functional \textit{HSP30} gene it was decided to employ an integrative vector to place mutant \textit{HSP30} alleles into the \textit{TRP1} locus of the genome. Unfortunately, the pUC19HSP30 vector does not possess a yeast selectable marker. To overcome this problem the 2.4 kb \textit{NdeI} fragments of pUC19HSP30 derivatives, incorporating the entire \textit{HSP30} gene open reading frame and 963 bp upstream of the start codon, were ligated into \textit{NdeI} digested, dephosphorylated \textit{YIplac204} (Gietz & Sugino, 1988) to make the \textit{YIplac204-30} series of plasmids listed in Table 4.5. An outline of \textit{YIplac204-30} construction is shown in Fig. 4.9.

\textbf{Table 4.5} The \textit{YIplac204-30} series of vectors incorporating different promoter mutations within the \textit{HSP30} gene.

<table>
<thead>
<tr>
<th>Name</th>
<th>Promoter mutations</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{YIplac204-30-STRE1\textsuperscript{WT}}</td>
<td>none</td>
</tr>
<tr>
<td>\textit{YIplac204-30-STRE1\textsuperscript{M}}</td>
<td>STRE1 only</td>
</tr>
<tr>
<td>\textit{YIplac204-30-STRE1\textsuperscript{M} PDSE1-6\textsuperscript{M}}</td>
<td>STRE1, PDSE1,2,3,4,5,6</td>
</tr>
<tr>
<td>\textit{YIplac204-30-STRE1\textsuperscript{WT} PDSE1-6\textsuperscript{M}}</td>
<td>PDSE1,2,3,4,5,6</td>
</tr>
</tbody>
</table>
Fig. 4.9 YIplac204-30 construction.
4.2.6 Integration of mutant alleles of the *HSP30* gene in the *TRPI* locus

100 ng of each of the YIplac204-30 series of vectors (Table 4.5) were digested with *EcoRV*, which cuts once in the *TRPI* gene (Fig. 4.9), and integrated into strain KT3 by transformation to tryptophan prototrophy. This method targeted the integration of mutant alleles of the *HSP30* gene into the *TRPI* locus (Fig. 4.10). Correct integration and determination of plasmid copy number was determined by Southern analysis. *TRPI*\(^+\) transformants were isolated and genomic DNA samples were prepared. 1 µg samples of genomic DNA were restricted with *PstI* (to cut at -644 upstream of the *HSP30* open reading frame) and electrophoresed on a 1% agarose gel. The Southern blot was then probed with the 2 kb *PstI/BamHI* fragment of pUC19HSP30 (Fig. 4.11). Hybridization to KT3 DNA (Lane 1) revealed a single band of 3.3 kb in length derived from the *hsp30::URA3* allele as previously reported (Talreja, 1995). In contrast, the *TRPI*\(^+\) transformants (Lanes 3-7) displayed two bands of 3.3 and 2.4 kb respectively. The former corresponds to the *hsp30::URA3* gene while the latter corresponds to the integrated YIplac204-30 vector. Since the 2.4 kb fragment comes from entirely within the *HSP30* cassette we cannot assume that it has integrated in the *TRPI* locus. However, digestion of YIplac204-30 by *EcoRV* (cuts in *TRPI*) suggests that the linearised plasmid is likely to recombine in this region. This Southern blot was used to identify single copy integrants (Lanes 3, 5, 6 and 7), these being used for further studies. A set of KT3 strains with the YIplac204-30 derivatives in Table 4.5 were constructed, these being designated by a YIplac204-30 suffix to the strain name (e.g. KT3-YIplac204-30-S1\(^M\) is KT3 transformed with YIplac204-30-S1\(^M\)).
Fig. 4.10 Map of integration of YIplac204-30 derivatives in the TRP1 locus.

Fig. 4.11 Southern blot of PstI digested genomic DNA. Lane 1- KT3, Lane 3 to 7- TRP1+ transformants obtained with EcoRV-cut Yiplac204-30 vectors.
4.2.7 Influences of weak acid preservatives, ethanol, nitrogen starvation and heat stress on mutant promoter *HSP30* genes in KT3-YIplac204-30 strains

In order to obtain direct evidence as to whether the putative STRE and PDSE elements are regulating stress activation of the *HSP30* gene, a detailed analysis of the mutant alleles was performed using different stress treatments. YEPD cultures of wild-type BJ2168, KT3-YIplac204-30WT, KT3-YIplac204-30-S1M, KT3-YIplac204-30-S1M P1-6M and KT3-YIplac204-30-S1WT P1-6M were grown to mid-exponential phase (OD<sub>600</sub> 1.5-2.0), centrifuged and resuspended in YEPD only (basal *HSP30* expression), YEPD+7% ethanol, YEPD pH 4.5+9mM sorbate and STMD (nitrogen starvation medium (0.17% yeast nitrogen base without amino acids and ammonium sulphate, 2% D-glucose and limiting amounts of auxotrophic requirements (1 mg/l for tryptophan, 5 mg/l all others))). The cultures were incubated at 28°C for 1 hour with constant shaking. A further sample was resuspended in YEPD only and incubated at 39°C for 1 hour. Total RNA extraction, northern blots and hybridizations were carried out as described previously. The northern blots were probed with *HSP30* and *PDA1* sequences (Fig. 4.12).

Northern analysis showed that the 1.3 kb *HSP30* transcript (B) is strongly induced in both BJ2168 (Lane 12) and KT3-YIplac204-30WT (Lane 13) in response to a 9 mM sorbate stress at pH 4.5 (Fig. 4.12). The *TRP1* integrated YIplac204 *HSP30* of the latter transformant, comprising the nucleotides between -963 and +1228 (relative to the translation start site) demonstrated identical stress induction to the endogenous *HSP30* gene of BJ2168. In the KT3 transformants this is derived from the introduced *HSP30* allele since untransformed KT3 (Lane 11) displays only a weak induction of a larger transcript derived from the *hsp30::URA3* allele, a transcript which is absent in its parental BJ2168 (A). Exposure to a severe sorbate or ethanol stress also caused weak induction of this larger transcript in KT3 and KT3-derived strains. These results suggest that the proximal 963 bp of the *HSP30* 5'-flanking region are necessary and sufficient to confer wild-type regulation to this gene.

The wild-type and mutant alleles of the *HSP30* gene are not strongly induced in exponential growth phase (Lanes 1-5) or conditions of nitrogen starvation (Lanes 6-10). *HSP30* expression is not therefore induced by nitrogen starvation as reported for other STRE controlled genes (Belazzi et al., 1991). In contrast, all the strains tested displayed essentially wild-type levels of induction of the *HSP30* transcript in response to 9 mM sorbate at pH 4.5 (Lanes 11-16), 7% ethanol (Lanes 17-21) and a 39°C heat shock (Lanes 22-25).
Probes:

- HSP30
- PDA1

Basal vs. starvation

- 9 mM sorbate pH 4.5
- 7% ethanol
**Fig. 4.12** Stress-induced expression of *HSP30* sequences in RNA from KT3 (Lane 11), BJ2168 (Lanes 1, 6, 12 and 17), KT3-Ylplac204-30<sup>WT</sup> (Lanes 2, 7, 13, 18 and 22), KT3-Ylplac204-30-S1<sup>M</sup> (Lanes 3, 8, 14, 19 and 23), KT3-Ylplac204-30-S1<sup>M</sup> P1-6<sup>M</sup> (Lanes 4, 9, 15, 20 and 24) and KT3-Ylplac204-30-S1<sup>WT</sup> P1-6<sup>M</sup> (Lanes 5, 10, 16, 21 and 25). Strains were grown to mid-exponential phase, centrifuged and resuspended in YEPD (basal-Lanes 1-5), STMD (nitrogen starvation medium-Lanes 6-10), YEPD pH 4.5+ 9 mM sorbate (Lanes 11-16), YEPD+7% ethanol (Lanes 17-21) and YEPD 39°C (heat shock-Lanes 22-25) for 1 hour with constant shaking. 10 μg total RNA samples were analysed in each lane, the northern blots were probed with *HSP30* and *PDA1* sequences.
The presence of mutations in the STRE1 or PDSEs of the HSP30 promoter (KT3-YIplac204-30-S1M, KT3-YIplac204-30-S1M P1-6M and KT3-YIplac204-30-S1WT P1-6M) did not therefore significantly effect the stress induction of HSP30. This indicates that the putative STRE and PDSEs are not essential for sorbate, ethanol and heat shock-induced expression of HSP30. The lack of nitrogen starvation induction of HSP30 is also an indication that stress activation of this gene does not use STRE sequences.

4.2.8 Comparison of the stress-induced expression of HSP30 and HSP12

A detailed analysis of the HSP12 promoter revealed that five repeats of the AGGGG STRE motif are essential to confer wild type levels of stress activation to a reporter gene upon osmostress, heat shock and transition into stationary phase (Varela et al., 1995). Data from section 4.2.7 suggest that the HSP30 gene is not under STRE or PDSE control. Experiments were therefore carried out to compare the stress-induced expression of HSP30 and HSP12. 10 μg samples of total RNA were prepared from BJ2168 grown exponentially or stressed for either 1 hour with 9 mM sorbate at pH 4.5, 7% ethanol, nitrogen starved in STMD or given a 39°C heat shock. The samples were electrophoresed, blotted and probed with HSP30, HSP12 and PDA1 sequences (Fig. 4.13).

Northern analysis (Fig. 4.13) showed that both the HSP30 and HSP12 transcripts are not strongly expressed in the exponential growth phase (Lane 1). HSP30 is strongly sorbate-inducible while HSP12 is expressed only moderately (Lane 2). Both transcripts are significantly induced in response to an ethanol stress (Lane 3), HSP12 is strongly induced by nitrogen starvation whereas HSP30 mRNA levels were essentially unchanged from basal expression (Lane 4). HSP12 is strongly heat shock-inducible while HSP30 was only moderately induced (Lane 5). Although the genes share some similarities in their stress activation, the differences in sorbate, nitrogen starvation and heat shock-induced expression suggest that HSP30 is not under the same controls as HSP12.
**Fig. 4.13** Comparison of stress-induced expression of *HSP30* and *HSP12*. Cultures from BJ2168 were grown to mid-exponential phase, centrifuged and resuspended in YEPD (basal-Lane 1), YEPD pH 4.5+ 9 mM sorbate (Lane 2), YEPD+7% ethanol (Lane 3), STMD (nitrogen starvation medium-Lane 4) and YEPD 39°C (heat shock-Lane 5) for 1 hour with constant shaking. 10 μg total RNA samples were electrophoresed, Northern blotted and probed with *HSP30*, *HSP12* and *PDA1* sequences.
4.2.9 Effects of the *hsfl-m3* and *msn2 msn4* double mutations on the stress-induced expression of *HSP30* and *HSP12*

The Msn2 and Msn4 zinc finger proteins are transcriptional activators (Estruch & Carlson, 1993) that have recently been shown to be needed for strong stress activation of several STRE-regulated yeast genes, such as *CTT1*, *DDR2* and *HSP12* (Martinez-Pastor et al., 1996). STRE activation is severely reduced in *msn2 msn4* mutants and the cells are hypersensitive to several stresses. The transcript levels of *HSP30* and *HSP12* in the wild-type and isogenic *msn2 msn4* double mutant strains were analysed after heat shock and sorbate stress (Fig. 4.14). Normal levels of sorbate induction of *HSP30* were displayed in the *msn2 msn4* double mutant while sorbate induction of *HSP12* was completely abolished (Lane 4). Heat shock expression of *HSP12* was severely reduced in the mutant while the moderate heat induction of *HSP30* was similar in both strains (Lane 6). This suggests that Msn2 and Msn4 are not required for the sorbate and heat induction of *HSP30*. In contrast, the stress activation of *HSP12* is severely impaired or abolished. Also, an STRE-*lacZ* reporter gene lacked heat-shock, sorbate and low pH activation in the double mutant (data not shown). From this, also the data from promoter mutations (section 4.2.7), it can be concluded that the stress induction of *HSP30* is not due to STREs.

Smith & Yaffe (1991) demonstrated that heat shock activation of *SSA1*, encoding a major 70-kDa Hsp, is almost completely abolished in the *hsfl-m3* temperature sensitive mutant (*hsfl-m3* is a nonsense mutation in the *HSFl* gene, causing lack of heat-inducibility of HSF-regulated genes). Also, deletion analysis and site-directed mutagenesis indicated that a HSE element is essential for the heat-inducible expression of the *SSA1* gene (Park & Craig, 1989). To test the relative importance of the two putative HSE sequences in *HSP30* (HSE1, -557 to -550; HSE2, -319 to -312 bp upstream of the coding region), heat activation’s of *HSP30* and *HSP12* were measured in wild-type and isogenic *hsfl-m3* mutant cells (Fig. 4.14). Northern analysis showed that the *HSP30* gene is moderately inducible in response to heat shock, while *HSP12* is more strongly activated (Lane 9). Upon a shift to 39°C similar levels of the *HSP30* and *HSP12* mRNA accumulated in both *hsfl-m3* and congenic wild-type cells (Lanes 9 and 10 respectively). Since *HSP12* is under STRE (not HSE) control, this induction of *HSP12* in *hsfl-m3* cells is not unexpected. However, the lack of an effect of *hsfl-m3* on *HSP30* expression is also evidence that the putative HSE elements are not involved in the activation of *HSP30* gene expression in heat shocked cells.
**Fig. 4.14** Effect of the *hsf1-m3* and *msn2 msn4* double mutations on the stress-induced expression of *HSP30* and *HSP12*. Cultures from isogenic wild type (W303-1A) (Lanes 1,3 and 5) and *msn2 msn4* double mutant cells (Lanes 2,4 and 6) were grown to mid-exponential phase, centrifuged and resuspended in YEPD (basal-Lanes 1 and 2), YEPD pH 4.5+ 9 mM sorbate (Lanes 3 and 4) and YEPD 39°C (heat shock-Lanes 5 and 6) for 1 hour with constant shaking. Also, cultures from isogenic wild-type (MYY290) (Lanes 7 and 9), and *hsf1-m3* mutant cells (Lanes 8 and 10) were grown to mid-exponential phase at 23°C, centrifuged and resuspended in YEPD (basal-Lanes 7 and 8) and YEPD 39°C (heat shock-Lanes 9 and 10) for 1 hour with constant shaking. 10 μg total RNA samples were electrophoresed, northern blotted and probed with *HSP30*, *HSP12* and *PDA1* sequences.
4.2.10 Effects of the yap1 and pdr1 pdr3 double mutations on the sorbate-induced expression of HSP30

Transcription factors encoded by YAPI (Moye-Rowley et al., 1989), PDR1 (Balzi et al., 1987) and PDR3 (Delaveau et al., 1994) are thought to control the expression of several genes responsive to oxidative stress, cadmium and other toxic compounds (section 1.3.3). Recent work has shown that the ABC-cassette multidrug transporter Pdr12 is strongly sorbate induced at the level of transcription, but that this is independent of either the Yap1, Pdr1 or Pdr3 transcription factors (P.W. Piper, unpublished results). It was therefore interesting to investigate the sorbate-induced activation of HSP30 in wild-type, yap1 and pdr1 pdr3 double mutant cells. As shown in Fig. 4.15, HSP30 mRNA was strongly induced by 9 mM sorbate in pH 4.5 cultures. In contrast, the addition of 1 mM sorbate only slightly increased transcript levels from those of basal expression. Both isogenic mutants displayed normal induction of HSP30 by 9 mM sorbate, indicating that this induction is not under the control of Yap1, Pdr1 or Pdr3. Additional evidence that HSP30 is not subject to PDR type controls is that its promoter region does not contain a close match to the 5'-TCCCGGGA-3' motif (PDRE-box) found in the promoter of PDR-responsive genes (Balzi & Goffeau, 1995).
**Fig. 4.15** Northern analysis of total RNA from wild-type (FC1679-28C) and isogenic Δyap1 and Δpdr1 Δpdr3 strains grown in pH 4.5 YEPD medium and treated with 0, 1 or 9 mM sorbate for 1 hour. Six identical blots were prepared, each containing the same RNA samples of 10μg per lane and electrophoresed through a 1% agarose formaldehyde gel. Hybridization to radiolabelled probes specific for the genes indicated to the left of the figure panels was carried out as described in chapter 2. An actin specific probe (ACT1) served as a control for equal RNA loading.
4.3 Discussion

4.3.1 The putative STRE, PDSEs and Msn2 are not essential for the induction of \textit{HSP30} gene expression in stressed cells

Hsp30 is strongly induced by heat shock, weak acids, ethanol, severe osmostress and entry to stationary phase (Régnaq & Boucherie, 1992; Panaretou & Piper, 1992; Piper et al., 1994; Piper et al., 1997). Prior to this study, there was no data present on the stress regulation of the yeast \textit{HSP30} gene. The wild-type expression of the plasmid-based \textit{HSP30} gene (comprising the nucleotides -963 to +1228) integrated in the \textit{TRP1} locus (Fig. 4.12) suggests that the stress-responsive cis-acting elements are located in the 963 bp proximal to the \textit{HSP30} ORF.

Several stress responsive genes such as \textit{CTT1} (Bissinger et al., 1989), \textit{SOD2} (Flattery-O'Brien et al., 1997), \textit{SSA3} (Werner-Washburne et al., 1989), \textit{HSP12} (Varela et al., 1995), \textit{GSY2} and \textit{TPS1} (Parrou et al., 1997) and \textit{DDR2} (Kobayashi & McEntee, 1993) are regulated either via the STRE element or the similar PDSE (Ruis & Schüller, 1995), and both elements have been shown to be under negative cAMP control (Boorstein & Craig, 1990 a; Marchler et al., 1993). In contrast, no information is currently available on yeast genes under positive cAMP control. Analysis of the \textit{HSP30} promoter revealed a number of putative STRE and PDSE regulatory elements which may be responsible for the increased expression observed in stressed cells (Table 4.1). Our study shows that the introduction of point mutations in at least two key consensus residues in each of these putative elements has no effect on the overall stress inducibility of the \textit{HSP30} gene (Fig. 4.12). These results demonstrate that the putative STRE and PDSEs are unlikely to contribute to the stress inducibility of \textit{HSP30} and are therefore non-functional. In contrast, site-directed mutagenesis of potential STRE and PDSEs in \textit{SSA3} (Hsp70) (Boorstein & Craig, 1990 a), \textit{HSP12} (Varela et al., 1995) and \textit{SOD2} (Flattery-O'Brien et al., 1997) abolished the stress activation of these genes.

Further evidence to support the STRE and PDSE-independent expression of \textit{HSP30} was gained by comparing the stress-induction of this gene with the well-characterised STRE-controlled \textit{HSP12} gene (Praekelt & Meacock, 1990; Varela et al., 1995) in \textit{S. cerevisiae} strain BJ2168 exposed to diverse stresses (Fig. 4.13) and \textit{msn2 msn4} double mutant cells (Fig. 4.14). It has been shown that the homologous and functionally redundant zinc-finger proteins Msn2 and Msn4 bind specifically to STRE-containing oligonucleotides. These proteins are important factors for the stress-induced activation of STRE-dependent promoters such as \textit{CTT1}, \textit{DDR2} and \textit{HSP12} but
not SSA3 (Martínez-Pastor et al., 1996). The sorbate-induced activation of \textit{HSP12} was much lower than previously reported for STRE-controlled genes (Schüller et al., 1994; Martínez-Pastor et al., 1996). This result agrees with the weak levels of sorbate induction shown with the \textit{HSP12-lacZ} fusion (Fig. 3.4 a). Analysis of \textit{HSP12} expression confirmed that stress-induced activation is defective in the \textit{msn2 msn4} double mutant, as reported for other STRE-regulated genes (Martínez-Pastor et al., 1996). However, \textit{HSP30} was induced at the same level in wild-type and mutant cells (Fig. 4.14), suggesting that this gene is controlled by STRE-independent mechanism(s).

Site-directed mutagenesis of putative \textit{HSP30} control elements, comparison with \textit{HSP12}, and expression in the \textit{msn2 msn4} double mutant has confirmed that Msn2/Msn4-regulated elements are not essential for the induction of \textit{HSP30} gene expression in stressed cells.

Transcription of \textit{HSP12} is strongly derepressed when cells are transferred to a medium containing limiting amounts of nitrogen (STMD) (Fig.4.13). This derepression is a consequence of the effects of low PKA on STRE activity as described in section 1.3.2.2. However, the results described in this chapter indicate that \textit{HSP30} is not derepressed by nitrogen starvation (Fig. 4.12 and 4.13), unlike STRE-controlled genes (Boorstein & Craig, 1990 a; Bissinger et al., 1989; Marchler et al., 1993; Varela et al., 1995). Régnacq & Boucherie (1992) and Riou et al. (1997) showed that \textit{HSP30} is preferentially expressed during entry to stationary phase under conditions where glucose becomes limiting. This increase in \textit{HSP30} expression might be influenced by the increasing ethanol levels in batch fermentation. It would appear not to reflect the fall in PKA with glucose exhaustion and might be influenced by catabolite derepression systems, something which could be investigated using the appropriate mutants. Since STRE and PDSEs are under negative cAMP control, mutations in the RAS-cAMP pathway should affect the expression of \textit{HSP30} if these elements are functional. Belazzi et al. (1991) reported that heat-induced expression of \textit{CTT1} is enhanced by a \textit{ras2} mutation and is almost entirely abolished by an \textit{sral-13} mutation. The \textit{sral-13} mutation is an allele of \textit{bcyl} and is one of a number of suppressors of the growth defect of \textit{ras2} mutants on non-fermentable carbon sources (Cannon et al., 1986; 1990). Northern analysis of these same mutants revealed that the sorbate inducibility of \textit{HSP30} was unaffected by the \textit{ras2} or \textit{sral-13} mutations (results not shown). This result implies that sorbate-induced expression of \textit{HSP30} is independent of PKA control, further evidence that this gene is not subject to STRE or PDS control.
4.3.2 Hsf1, Yap1, Pdr1, and Pdr3 are not essential for the induction of HSP30 gene expression in stressed cells

HSE sequences have been implicated in the regulation of several heat-induced genes (Mager & Moradas-Ferreira, 1993) and also play an important role in the glucose or nutrient starvation induction in some of these same genes (Boorstein & Craig, 1990a; Tamai et al., 1994). The results reported in this chapter demonstrate that the two putative HSE elements in the HSP30 promoter (Table 4.1), are not responsible for the heat shock regulation of this gene (Fig. 4.14). This is hardly surprising since these two motifs are not exact matches to the nGAAnnTTCn consensus shown to be essential for binding of the yeast Hsf1 transactivator (Mager & De Kruijff, 1995).

The Yap1 transcriptional regulator plays an important role in the resistance of yeast cells to oxidative stresses by inducing expression of a number of antioxidant genes (section 1.3.3). Yap1 also mediates the stationary-phase induction of GLR1, encoding glutathione reductase (Grant et al., 1996). The stress regulation of TPS2 is mediated by STRE elements and also requires the function of Yap1 (Gounalaki & Thireos, 1994). However, our results indicated that the sorbate-induced expression of HSP30 does not require the Yap1 protein (Fig. 4.15). This is not surprising since the HSP30 promoter does not contain any putative Yap1 binding sites (yeast API-responsive elements ((yAREs) with a 5'-TTA(C/G)TAA-3' motif) (Moye-Rowley et al., 1989). The Pdr1 and Pdr3 transcriptional regulators have been shown to control the transcription of several genes encoding plasma membrane ABC-cassette multidrug transporters (Meyers et al., 1992; Katzmann et al., 1994; section 1.3.3). The HSP30 promoter region does not contain any putative Pdr1/Pdr3 binding sites (pleiotropic drug resistance elements ((PDREs) with a 5'-TCCCGGA-3' motif) (Katzmann et al., 1994). Our study also demonstrated that the sorbate-induced expression of HSP30 does not require the Pdr1 and Pdr3 proteins.
4.4 Conclusions

The results in this chapter indicate that the stress-induced activation of the *HSP30* gene is not controlled by any of the known yeast stress response pathways. Site-directed mutagenesis and analysis of mutants defective in stress transcriptional activator proteins has eliminated the Hsf1 /HSE, Msn2 /Ms4 /STRE, Yap1 /yARE, Pdr1 /Pdr3 /PDRE and unknown transcription factor/PDSE stress response pathways as potential regulators of *HSP30* activation. This work has also shown that induction of the *HSP30* gene appears to operate via a cAMP-independent mechanism. Although the *HSP30* gene shares some similarities to other stress genes in its stress activation, only *PDR12* is induced as strongly by a weak acid stress. Based on the results presented in this chapter it seems unlikely that the putative PDSEs present in the *PDR12* promoter (Table 4.1) control the stress activation of this gene, although this will have to be determined experimentally. A possible trigger for all the different stresses that activate *HSP30* may be their common effect of disrupting the electrochemical gradient at the plasma membrane and the decrease in intracellular pH. This study has indicated the existence of a novel stress response pathway in *HSP30* expression but its mechanism remains to be elucidated.
CHAPTER 5

5. A SEARCH FOR HSP30 GENE HOMOLOGUES IN OTHER YEASTS

5.1 Introduction

The (E-P) type H^+-ATPase has been found in the plasma membranes of diverse fungi, plants and protozoa (Serrano, 1991). The activity of this enzyme generates a proton gradient that couples ATP hydrolysis to the extrusion of protons across the membrane, resulting in the establishment of a transmembrane proton electrochemical gradient which drives the secondary transport of nutrients (section 1.4.1.1). In *S. cerevisiae*, Hsp30 has been implicated in the downregulation of H^+-ATPase activity to limit excessive ATP usage under conditions of prolonged stress such as weak acid treatment and during diauxic shift (section 1.4.2.1). In the sequence of Chromosome II of *S. cerevisiae*, Aljinovic & Pohl (1995) identified an open reading frame YBR0507 (YR02) that possessed a considerable homology to HSP30 (27%). Hydropathy analysis suggested that the putative gene product exhibits a similar hydrophobicity profile to HSP30, although its function is unknown. Also at positions 151-161 there is a pattern weakly homologous to the prokaryotic membrane lipoprotein lipid attachment site consensus (Hayashi & Wu, 1990). Since heat shock proteins are amongst the most highly conserved proteins (Craig *et al.*, 1993; Mager & Moradas-Ferreira, 1993) it seems likely that a protein of similar function to *S. cerevisiae* Hsp30 may be present in other organisms, especially those with a H^+-ATPase. The presence of an Hsp30-type protein in the plasma membrane of *Z. bailii* could contribute to this organism’s inherent resistance to weak acid preservatives (section 1.4). The aim of the investigation described in this chapter was to search for genes homologous to *S. cerevisiae* HSP30 in *Z. bailii* (weak acid tolerant), *C. albicans* (a medically important pathogen), *H. polymorpha* (a methylotrophic yeast that grows up to 50°C) and the fission yeast *S. pombe*. Southern analysis was used to identify sequences hybridizing to a HSP30 gene probe and one hybridizing sequence from *C. albicans* was cloned into pBluescript M13- and its DNA sequence determined to look for possible homology to *S. cerevisiae* HSP30.
5.2 Results

5.2.1 Analysis of low stringency hybridization of a *S. cerevisiae* HSP30 gene probe to genomic DNA of different yeast species

Genomic DNA was isolated from *Z. bailii*, *C. albicans*, *H. polymorpha* and *S. pombe* as described in section 2.8.1. Approximately 1 µg samples of each DNA were digested with the restriction enzymes *BamHI*, *EcoRV* and *HindIII* and electrophoresed on a 1% agarose gel. The Southern blot was then probed with an *S. cerevisiae* HSP30 gene fragment as described in sections 2.8.6 and 2.8.7. Increased stringency washes were carried out from 42 to 65°C, the blot being autoradiographed after each wash. Figure 5.1 shows a Southern blot of restriction enzyme digests probed with an *S. cerevisiae* HSP30 gene fragment washed at high stringency (65°C in 0.1xSSC/0.1% SDS). The *S. cerevisiae* HSP30 gene fragment did not hybridize to *Z. bailii* (Lanes 1, 2 and 3) or *S. pombe* (Lanes 10, 11 and 12) sequences (Fig. 5.1). Even at low stringency there was no hybridization to these DNA’s (results not shown). In contrast, the HSP30 probe hybridized to *C. albicans* (Lanes 4, 5 and 6) and *H. polymorpha* (Lanes 7, 8 and 9) sequences at high stringency (Fig. 5.1). The *EcoRV* and *BamHI* digested genomic DNA samples hybridized to what was apparently a single band approximately 5 kb in length, while the *HindIII* samples annealed with a single band of approximately 1-1.7 kb (Lanes 4 and 7). The *HindIII* digested *C. albicans* DNA also gave a signal at 5 kb (Lane 4) suggesting that a part of the *C. albicans* HSP30 hybridizing sequence may lie within this larger band.
**Fig. 5.1** Southern blot of restriction enzyme digested genomic DNA. 1 μg samples of DNA from *Z. bailii* (Lanes 1, 2 and 3), *C. albicans* (Lanes 4, 5 and 6), *H. polymorpha* (Lanes 7, 8 and 9) and *S. pombe* (Lanes 10, 11 and 12) were digested with *HindIII* (Lanes 1, 4, 7 and 10), *EcoRV* (Lanes 2, 5, 8 and 11) and *BamHI* (Lanes 3, 6, 9 and 12). The Southern blot was probed with an *S. cerevisiae HSP30* gene fragment, washed at 65°C in 0.1xSSC/0.1% SDS and the X-ray film was exposed for 4 days.
5.2.2 Cloning of the *C. albicans* and *H. polymorpha* genomic DNA fragments hybridizing to the *S. cerevisiae HSP30* gene

Section 5.2.1 revealed that *C. albicans* and *H. polymorpha* genomic DNA gave a *HindIII* fragment 1-1.7 kb in length that hybridized to an *S. cerevisiae HSP30* gene fragment. Since this indicated only a single hybridizing sequence in each genome, *HindIII* fragments of the DNA of these two yeasts were ligated into pBluescript, in order to isolate these as possible *HSP30* homologues. 1 μg samples of *C. albicans* and *H. polymorpha* genomic DNA were *HindIII* digested and electrophoresed on 1% agarose. The DNA fragments between 1 and 2 kb in length were excised and purified as described in section 2.7.5. These fragments were then ligated into *HindIII* digested, dephosphorylated pBlueScript M13- vector and transformed in *E. coli* strain XL1-Blue. Approximately 1000 colonies from each batch of mixed pBlueScript vectors were colony hybridized (section 2.8.4) with an *HSP30* probe. 3 positive colonies from the *C. albicans* batch and 1 positive from *H. polymorpha* were restreaked onto fresh ampicillin plates and colony hybridized once again. Only one colony containing a pBlueScript M13- with a *C. albicans* insert rehybridized to an *HSP30* probe (pBlueScript-CA1). It was therefore decided to proceed with a sequencing strategy for pBlueScript-CA1.

5.2.3 Restriction mapping and Southern hybridization to the insert fragment of pBlueScript-CA1

The pBlueScript-CA1 clone contains a *C. albicans* genomic DNA insert approximately 1.6 kb in length (determined from electrophoresis of *HindIII* digested pBlueScript-CA1, results not shown). In order to save time when sequencing the entire 1.6 kb it was decided to restriction map the clone and determine which sequences hybridized to the *HSP30* probe. If only a small fragment hybridized to *HSP30* then a different cloning and sequencing strategy could be employed to reduce the workload. 1 μg samples of pBlueScript-CA1 were restriction digested (with restriction enzymes from the pBlueScript M13- polycloning site) and electrophoresed on 1% agarose (Fig. 5.2 a). From this an approximate restriction map of pBlueScript-CA1 could be deduced (Fig. 5.2 b). The fragments in Fig. 5.2 a were Southern blotted and probed with the *S. cerevisiae HSP30* gene fragment, washed at 42°C in 2xSSC/0.1% SDS and the X-ray film was exposed for 24 hours (Fig. 5.2 c).
Fig. 5.2 (a) Electrophoresis of restriction digests of pBluescript-CA1. λ HindIII (Lane 1), AccI (Lane 2), ApaI (Lane 3), EagI (Lane 4), HincII (Lane 5), SacII (Lane 6), SpeI (Lane 7), HindIII/BsaI (Lane 8), HindIII/SalI (Lane 9) and BsaI/BstXI (Lane 10). (b) Restriction map of pBluescript-CA1 1.6 kb fragment insert (c) Southern blot of restriction digested pBluescript-CA1 (Lanes 1-10 correspond to the gel in (a) blotted and probed with a HSP30 fragment.
From this data it can be deduced that the 1.6 kb *C. albicans* insert of pBluescript-CA1 does not possess *ApaI*, *EagI*, *SacII* or *SpeI* sites (Fig. 5.2 a) although the *EagI* and *SacII* digest (Lanes 4 and 6 respectively) are only partial. There was a particularly strong hybridization signal with a 1.1 kb fragment from the *HindIII/SalI* digest (Fig. 5.2 c, Lane 9). Since there was no hybridization signal in the 0.5 kb band it can be deduced that the annealing sequences lie within the 1.1 kb fragment. In order to reduce the amount of work required to determine these sequences we decided to generate a smaller plasmid lacking the 0.5 kb *SalI* fragment (pBluescript-CA2). To construct pBluescript-CA2, pBluescript-CA1 was *SalI* digested and electrophoresed on a 1% agarose gel. The 4 kb fragment was excised, purified (section 2.7.5) and religated to form pBluescript-CA2 (containing the 1.1 kb hybridizing fragment). The regions at the ends of the pBluescript-CA2 insert could be sequenced using the T7 and T3 primers.

### 5.2.4 Sequencing strategy of the 1.1 kb *C. albicans* genomic DNA fragment

DNA sequence analysis of both strands of the 1.1 kb insert of pBluescript-CA2 was carried out as described in section 2.8.10 using bacteriophage T3 and T7 primers. Sequences of approximately 200-250 nucleotide from the cloning site were determined, new primers being designed corresponding to the end of the readable part of the sequence gel, enabling the sequence analysis to continue further into the insert (Table 5.1). This was repeated until the sequences of both strands overlapped. Figure 5.3 shows an outline of the sequencing strategy for the whole fragment, and the extent of the sequence readable from each primer. Approximately 61% of the 1.1 kb was sequenced in both strands.
Fig. 5.3 Outline of the strategy for sequencing the *C. albicans* genomic DNA insert of pBluescript-CA2. The length of each arrow shows the amount of sequence determined from each primer (Table 5.1).

Table 5.1 Primers used in the DNA sequence analysis of pBluescript-CA2

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Locationa</th>
</tr>
</thead>
<tbody>
<tr>
<td>CA-T3A</td>
<td>5'-CCGTTCAATTATGCCGGATC-3'</td>
<td>185 to 205</td>
</tr>
<tr>
<td>CA-T3B</td>
<td>5'-CTGGTTGACAAGTCCATACCAA-3'</td>
<td>453 to 473</td>
</tr>
<tr>
<td>CA-T7A</td>
<td>5'-TTTATATTGCTCTTTAGATAT-3'</td>
<td>983 to 963</td>
</tr>
<tr>
<td>CA-T7B</td>
<td>5'-CGATAATAGTTTGCTTTTTTG-3'</td>
<td>742 to 722</td>
</tr>
</tbody>
</table>

location of primers (5' → 3') along the 1168 bp insert of pBluescript-CA2.
5.2.5 Nucleotide sequence analysis of the 1.1 kb *C. albicans* genomic DNA fragment

Fig. 5.4 shows the nucleotide sequence of the 1.1 kb *HindIII/SalI* *C. albicans* genomic DNA fragment (5' → 3' from the T7 primer). The potential translated polypeptide sequence that reads throughout the 1.1 kb fragment is also indicated. The first 51 nucleotides encode the *NotI, XbaI, SpeI, BamHI, Smal, PstI, EcoRI, EcoRV* and *HindIII* sites of the pBluescript M13- polycloning site (Short *et al.*, 1988). The *SalI* site of the polycloning site could not be identified at the other end of the fragment because of its proximity to the T7 primer. The DNA Star™ program (©1992) was used to identify possible ORFs greater than 50 amino acids in length (Fig. 5.5). The largest ORF started at position 54 on the forward strand (T7 primer) and ran the entire length of the sequenced fragment (Lane 3). Due to the close proximity of the first ATG in this ORF to the *HindIII* polycloning site it cannot be ascertained if this site is an actual start codon or an internal ATG sequence. The polypeptide sequence encoded by this large ORF (analysed using the DNA Star™ program (©1992)) is comprised of 35.3% hydrophobic residues. This compares to 31.9% for the *S. cerevisiae* Hsp30 polypeptide (Régnacq & Boucherie, 1992), suggesting that the putative *C. albicans* ORF may encode a highly hydrophobic protein. Furthermore, the Kyte-Doolittle hydrophobicity profile (Fig. 5.6) (a plot that profiles the local hydrophilicity/hydrophobicity of a protein), indicated a number of potential membrane spanning regions (Kyte & Doolittle, 1982). In such a profile each amino acid is assigned a hydropathy value based on the transfer of free energy of its side chains between vapour and water combined with the preference of the side chain for protein interior (buried) or surface environments. The hydropathy values are summed for N residues and divided by N to produce the average hydropathy value for that region. The value is plotted at the centre of the window of N residues. In Fig. 5.6 a window of 9 amino acids was used. Kyte & Doolittle (1982) concluded that when the hydropathy of a given 19-residue segment averages greater than +1.6 there is a high probability that it will be one of the sequences in a membrane-bound protein that spans the membrane.
Fig. 5.4 Nucleotide sequence of the 1.1 kb C. albicans genomic DNA insert. The potential translated polypeptide sequence that reads throughout the 1.1 kb fragment is also indicated.
Fig. 5.5 Diagram of the putative ORFs present in the 1.1 kb *C. albicans* genomic DNA fragment. Lanes 1, 2 and 3 indicate the possible ORFs in each frame of the forward strand (T7 primer) and lanes 4, 5 and 6 indicate the possible ORFs in each frame of the reverse strand (T3 primer) (< and > indicate the position of the start codon and the direction of the ORF).
Fig. 5.6 Hydropathy plot of the predicted *C. albicans* ORF product. Hydrophobicity was calculated according to the algorithm of Kyte & Doolittle (1982) using a 9-residue window, through the DNA Star™ program (©1992). Hydrophobic regions are above the horizontal line.
5.2.6 Comparison of the *C. albicans* 1.1 kb fragment to the *S. cerevisiae HSP30* gene

Comparison of the 1.1 kb *C. albicans* nucleotide sequence with the *Saccharomyces* Genome Database (Altschul et al., 1990), revealed that this sequence did not display appreciable identity with the *S. cerevisiae HSP30* ORF at either the nucleotide or protein level. This is surprising since the *C. albicans* fragment hybridized to the *HSP30* probe (Fig. 5.2 c). Instead, the search identified a number of *S. cerevisiae* genes sharing weak amino acid similarities to the large ORF of the *C. albicans* fragment (Table 5.2).

**Table 5.2** *S. cerevisiae* genes sharing weak similarities to the *C. albicans* 1.1 kb genomic DNA fragment (from a FASTA search (Pearson & Lipman, 1988) on the *Saccharomyces* Genome Database.

<table>
<thead>
<tr>
<th>ORF</th>
<th>Gene</th>
<th>Location</th>
<th>Description</th>
<th>Identity</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>YOR299W</td>
<td>BUD7</td>
<td>Ch XV</td>
<td>involved in bud-site selection</td>
<td>26.5% in 283 aa overlap</td>
<td>Zahner et al. 1996</td>
</tr>
<tr>
<td>YMR237W</td>
<td>-</td>
<td>Ch XIII</td>
<td>similarity to <em>CHS6</em> ORF YJL099W</td>
<td>25.9% in 421 aa overlap</td>
<td>Bowman et al. 1997</td>
</tr>
<tr>
<td>YJL099W</td>
<td>CHS6</td>
<td>Ch X</td>
<td>chitin biosynthesis protein</td>
<td>25.4% in 393 aa overlap</td>
<td>Klis, 1994</td>
</tr>
</tbody>
</table>

In order to explain the hybridization between the *C. albicans* 1.1 kb fragment and *S. cerevisiae HSP30* (Fig. 5.2 c) it was decided to repeat the restriction mapping and Southern strategy outlined in section 5.2.3 with information gained from the sequence analysis. A comprehensive restriction map of the 1.1 kb fragment was produced using the DNA Star™ program (©1992) (results not shown). Restriction digests were carried out to isolate smaller *C. albicans* fragments that would pinpoint the *HSP30* hybridizing sequences (Fig. 5.7 a). Figure 5.7 b shows the sites of cleavage within the *C. albicans* fragment for the restriction enzymes used to generate the digests in Fig. 5.7 a. This DNA was blotted, then probed with *S. cerevisiae HSP30* sequences, washed at 42°C in 2xSSC/0.1% SDS and the X-ray film was exposed for 24 hours (Fig. 5.7 c). From the Southern blot (Fig. 5.7 c) it can be seen that the smallest *C. albicans* fragment that hybridizes to the *HSP30* probe is a 278 bp *BstXI/BscI* fragment (Lane 6), shown as a shaded box on the restriction map (Fig. 5.7 b). This fragment was compared
to the \textit{HSP30} ORF at the nucleotide and amino acid level using the Martinez/Needleman-Wunsch, Jotsun/Hein (Hein, 1990), Clustal (Higgins & Sharp, 1989), Lipman/Pearson (Pearson & Lipman, 1988) and Wilbur/Lipman (Wilbur & Lipman, 1983) DNA and protein alignment programs of the DNA Star® program (©1992). Minimal sequence similarities were displayed at the protein level although the Martinez/Needleman-Wunsch alignment (Fig. 5.8) showed homology between the \textit{HSP30} ORF between nucleotides +225 to +481 (relative to the ATG codon) and the \textit{C. albicans} BstXI/BscI fragment that would appear to be the cause of the hybridization signals in Figs. 5.2 c and 5.7 c. The Martinez/Needleman-Wunsch DNA alignment (developed by DNA Star® (©1992)) is comprised of two methods that align and optimise the fit between regions of perfect match (minimum match: 9; gap penalty: 1.10; gap length penalty: 0.33). The 91 amino acid polypeptide sequence corresponding to the BstXI/BscI \textit{C. albicans} fragment was also analysed using the FASTA algorithm (Pearson & Lipman, 1988) and showed strongest identity to \textit{CHS6} (32.5% identity in a 62 amino acid overlap; Table 5.2).
Fig. 5.7 (a) Electrophoresis of restriction digests of pBluescript-CA2. λ HindIII (Lane 1), BamHI/SalI (Lane 2), HpaII (Lane 3), Alul (Lane 4), BstXI/XhoI (Lane 5), BstXI/BscI (Lane 6), HindIII/AccI (Lane 7), BstXI (Lane 8), BscI (Lane 9) and Sau3AI (Lane 10). (b) Restriction map of the C. albicans 1.1 kb insert. The 278 bp BstXI/BscI fragment is shown. (c) Southern blot of restriction digested pBluescript-CA2 (Lanes 1-10 correspond to the gel in (a) blotted and probed with a HSP30 fragment.
Fig. 5.8 Martinez/Needleman-Wunsch DNA alignment (DNA Star™ program (©1992)). Minimum match: 9; gap penalty: 1.1; gap length penalty: 0.33 (v indicates HSP30 ORF, +225 to 481; ^ indicates C. albicans BstXI/BscI hybridising fragment, +5 to 265; similarity index: 42.5; gap number: 72; gap length: 112; consensus length: 314).
5.3 Discussion

The phenotype associated with loss of Hsp30 in *S. cerevisiae* is subtle (Piper et al., 1997), so that it would be difficult to isolate Hsp30 homologues from other yeasts by complementation of the null mutant. The work in this chapter sought to isolate possible *S. cerevisiae* HSP30 homologues by low stringency hybridization. Southern analysis (Fig. 5.1) suggested that *C. albicans* and *H. polymorpha* may contain a single sequence that hybridizes to an HSP30 probe. Although no hybridization was obtained to *Z. bailii* and *S. pombe* DNA this does not prove conclusively that these yeast species do not possess HSP30 homologues. Attempts were made to clone the *C. albicans* and *H. polymorpha* sequences into pBluescript M13- (section 5.2.2), leading to cloning and sequencing of the hybridizing sequences from *C. albicans* (sections 5.2.4 and 5.2.5). The *C. albicans* clone possessed a large ORF that is unlikely to arise from noncoding sequences and which consists of 35% hydrophobic residues, even greater than the 31% found in the highly hydrophobic Hsp30. Hydrophobicity analysis (Fig. 5.6) suggested that this encoded a protein with several transmembrane domains. Analysis of the *C. albicans* sequence with the *Saccharomyces* Genomic Database (using the FASTA algorithm (Pearson & Lipman, 1988)) did not reveal any homology to the HSP30 gene although a number of genes showed some sequence similarity (Table 5.2). It appeared that none of these were close homologies to HSP30. The *C. albicans* sequences hybridizing to the HSP30 probe were more precisely mapped (Fig. 5.7 c). A Martinez/Needleman-Wunsch DNA alignment (developed by DNA Star™ (©1992)) demonstrated that a 278 bp BstXI/BseI *C. albicans* fragment possessed a similarity index of 42.5 to the +225 to +481 region of the HSP30 ORF (Fig. 5.8) which may have been sufficient homology to cause the hybridization signal shown in our Southern analyses. It also confirmed that this study had not been fruitful in isolating and identifying a *S. cerevisiae* HSP30 homologue.

An alternative strategy to isolate HSP30 homologues of equivalent function in other yeast species would be to investigate the changes to the protein composition of the plasma membrane of stressed yeast cells (under the same conditions that induce the *S. cerevisiae* Hsp30 protein) as described by Panaretou & Piper (1992). Through in vivo ([^3]H]-leucine) pulse-labelling of plasma membrane proteins synthesised before and during exposure to various stresses, followed by subcellular fractionation, it could be determined if any stress proteins copurify with plasma membranes. Isolation of membrane stress proteins followed by N-terminal polypeptide sequence analysis would be the first step in this investigation. A similar strategy was used to characterise the
ABC-cassette weak acid transporter, Pdr12 (P.W. Piper, unpublished results). The same Southern and cloning strategy outlined in this chapter could then be implemented using a probe specific to the peptide sequence.
CHAPTER 6

6. DISCUSSION

6.1 Introduction

Foods and beverages designed to have a long shelf-life inhibit the growth of most microorganisms by providing environments of either anaerobiosis, low pH, low water activity, food preservatives, high ethanol, high CO₂ levels, or combinations of these factors. However certain yeasts can still spoil these products because they possess physiological attributes that allow growth under these fairly severe physical conditions (section 1.4). While *S. cerevisiae* has been implicated as a food spoilage organism, other more osmotolerant yeasts such as *Z. bailii* appear to be more important as spoilage agents of foodstuffs of low pH and/or low water activity or high preservative levels (section 1.4.3). Both *Z. bailii* and *S. cerevisiae* can adapt to growth in low pH cultures in the presence of weak acid preservatives, but the former yeast is more preservative resistant. The *Zygosaccharomyces* genus is very diverse and unfortunately does not provide a genetically tractable system.

Recent work in our laboratory has characterised a discrete response to weak acid stress in *S. cerevisiae*, a response assisting weak acid adaptation (Piper *et al.*, 1997; P.W. Piper, unpublished results; section 1.4.2). Numerous studies have been carried out on the physiological effects of a weak acid stress on yeast cells (section 1.4) but this stress response has not been studied in great detail at the molecular level. Cheng & Piper (1994) demonstrated that weak acids inhibit the induction of heat shock proteins and thermotolerance in low pH cultures while Schüller *et al.* (1994), Martinez-Pastor *et al.* (1996) and P.W. Piper (unpublished results) have shown that some stress genes are activated by weak acids at low pH (section 1.4.2.3). The mechanisms by which yeast cells sense a weak acid stress and transfer this signal to the transcription machinery has not been elucidated. The main aim of the research described in this thesis was to better understand the effects of weak acid preservatives on the expression of stress gene promoters and stress promoter elements and to identify the signalling pathways involved in this response. Chapter 3 characterised the interdependence of culture pH and weak acids on stress gene promoter and stress promoter element-*lacZ* fusions and revealed that weak acids influence the activation of these sequences probably by a mechanism involving pH_{i} (section 6.2). In chapter 4, the *HSP30* gene (which is
strongly activated by weak acids at low pH) was used as a model to identify the transcription machinery involved in the weak acid stress response. Research described in chapter 4 has shown that this gene is activated by a novel stress response pathway, dissimilar to the previously characterised stress response pathways (section 1.3). Although this thesis provides evidence for this pathway, it yet remains to be elucidated. Section 6.4 of the discussion outlines future work that could reveal the components of this pathway. HSP30 encodes a stress-inducible regulator of the main enzyme of homeostasis maintenance in S. cerevisiae, the plasma membrane H+-ATPase (Piper et al., 1997). A study was therefore undertaken to determine if other yeast species (possessing a H+-ATPase) contained a homologous gene of similar function. Research described in chapter 5 has shown that C. albicans and H. polymorpha contain genomic DNA sequences that hybridize to HSP30 at high stringency (Fig. 5.1). Attempts to clone a HSP30 homologue proved unsuccessful but section 5.3 outlines an alternative strategy that could be used to search for genes of similar function to S. cerevisiae HSP30 in non-Saccharomyces yeasts.

6.2 Intracellular pH is a possible trigger for the weak acid-induced activation of stress gene promoters and stress promoter elements

Yeasts have a well-developed system for maintaining pHj homeostasis, dependent upon the proton-translocating plasma membrane H+-ATPase (Serrano, 1991; section 1.4.1.1). Stresses that cause pHj decline have been found to stimulate H+-ATPase activity although the mechanisms remain poorly understood (Ramos et al., 1989; Coote et al., 1991; Fernanda Rosa & Sá-Correia, 1991; Viegas & Sá-Correia, 1991). The activation of stress genes (Figs. 3.3-3.5 a) and the modulation of H+-ATPase by low pHj suggests that the yeast cell can somehow sense changes in pHj, switching on a signalling pathway(s) whose role may be partly to control pHj homeostasis. This pathway may not be the same for both stress genes and the H+-ATPase since we are dealing with transcriptional regulation on the one hand and modulation of enzyme activity on the other.

This is the first study to demonstrate the strong influence of culture pH over the effects of a weak organic acid on the expression of stress gene promoters and stress promoter elements (chapter 3). The fall of intracellular pH is the major cause of growth inhibition by weak acids (Brown & Booth, 1991; section 1.4.1). Similar induction of
STRE sequences occurred in low pH cultures (pH 3.5) in the absence of sorbate and at pH 5.5 with 1 mM sorbate (Fig. 3.3 a) suggesting that pH$_i$ may be the trigger for the activation of these sequences. Also, defective low pH and sorbate induction of an STRE-lacZ reporter in the msn2 msn4 double mutant (Martinez-Pastor et al., 1996) (results not shown), suggests that the STRE-binding component of the weak acid (low pH$_i$) responsive system is Msn2,4 as with other STRE inducers. However, studies in chapter 4 suggest that this signalling pathway is not involved in the stress activation of HSP30 (section 6.3). The antimicrobial effect of weak acids is greatly pH dependent. It is increased by lowering the pH of the suspending medium, being less effective as pH increases (Eklund, 1983). Results in this thesis suggest that STRE sequences (Fig. 3.3) and the STRE-controlled gene HSP12 (Figs. 3.4, 4.13 and 4.14) are not strongly activated by sorbate. Also the STRE/Msn2,4 general stress response pathway is not specific to weak acids, but is strongly activated by a number of stresses (Schüller et al., 1994).

Our results also show that in combination, heat shock and weak acids act to inhibit the same stress genes in low pH cultures, possibly a lack of response due to inability to maintain pH$_i$ homeostasis (Figs. 3.2-3.5 b). It can therefore be postulated that decreased pH$_i$ caused by a moderate weak acid stress at low pH may trigger a stress signalling pathway(s) that switches on mechanisms to maintain pH$_i$ homeostasis or activate particular stress genes that enhance survival under conditions of an even more severe weak acid stress. In contrast, when pH$_i$ levels decrease to an even greater extent this same signalling pathway may be repressed. This hypothesis is supported by Cheng & Piper (1994) who demonstrated that weak acids inhibited the heat induction of major Hsps in a pH-dependent manner.

A physiological function of Pdr12 is to mediate efflux of water-soluble carboxylate anions (P. W. Piper, unpublished results). Yeast cells grown at pH 7.0 have extremely low Pdr12 levels, although a significant Pdr12 expression is observed at pH 4.5. However, northern analysis (P.W. Piper, unpublished results) has shown that sorbate exposure causes a dramatic induction of PDR12 at both pH values. These results suggest that Pdr12 expression is influenced by both pH$_i$ and the preservative anion. Taken together, these results indicate that a distinct transcriptional machinery is required for mediating the cellular response to weak organic acid stress, a stress
response which induces \textit{PDR12}, \textit{HSP30} and probably other genes through as yet unidentified transcriptional regulators.

To obtain further evidence of whether low pH\textsubscript{i} triggers stress gene expression it would be interesting to measure pH\textsubscript{i} in the various stress conditions outlined in chapter 3. We could better characterise the pH\textsubscript{i} levels that either activate or repress these genes, thereby obtaining evidence for the existence of a pH\textsubscript{i} signalling pathway. A wide range of techniques are currently available for the determination of intracellular pH. These include the use of pH-sensitive electrodes, distribution of radiolabelled compounds (Peña \textit{et al.}, 1994), \textsuperscript{31}P\textsubscript{NMR} (Gillies \textit{et al.}, 1982; although \textsuperscript{31}P\textsubscript{NMR} can only be used down to about pH 6) and various spectroscopic techniques (Haworth \textit{et al.}, 1991; Imai \textit{et al.}, 1994; Imai \& Ohno, 1995). Also, since Hsp30 and Pdr12 expression is strongly activated by weak acid preservatives and other stresses which cause reduction in pH\textsubscript{i}, it would be interesting to characterise the stress-induced activation of \textit{HSP30} and \textit{PDR12} promoter-\textit{lacZ} fusions. An \textit{HSP30} promoter-\textit{lacZ} (-872 to -8 bp upstream of the start codon) constructed for this study unfortunately did not display stress activation comparable to the wild-type promoter, although data from chapter 4 suggest that the stress promoter elements are located in the 963 bp proximal to the \textit{HSP30} ORF.

\section*{6.3 The \textit{S. cerevisiae} Hsp30 gene is regulated by a novel stress response pathway}

Hsp30 is strongly induced by heat shock, ethanol, weak acids, entry to stationary phase and is weakly induced by severe osmostress (section 1.4.2.1). Attempts were therefore made to determine the positive transcriptional control elements responsible for activation of the \textit{HSP30} gene (chapter 4). Our analysis of stress-responsive transcription factor mutants shows that the response to weak acids in \textit{S. cerevisiae} is totally different from all other stress responses studied to date. Our data shows that stress induction of \textit{HSP30} is not due to the heat shock factor (Hsf1) that directs the heat shock response (Fig. 4.14), the Msn2, 4 factors that direct the general stress response (Fig. 4.14), the Yap1 factors that are important in the response to oxidative stress, or the pleiotropic drug resistance system (Fig. 4.15). The \textit{HSP12} gene is activated by a lot of the same stresses that induce the \textit{HSP30} gene (Varela \textit{et al.}, 1995). A detailed analysis of the \textit{HSP12} promoter revealed that five STRE motifs are essential for the stress-induced activation of this gene, a response almost completely abolished in \textit{msn2 msn4} double mutant cells (Varela \textit{et al.}, 1995; Martínez-Pastor \textit{et al.}, 1996). Comparison of \textit{HSP30} and \textit{HSP12} (Figs. 14.12-14.14) indicates that, while these
genes share some similarities in their stress activation, there are major differences in the effects of nitrogen starvation and sorbate. Even though not all STRE-controlled genes are identically regulated, the data nevertheless indicates that HSP30 is not under the same control as HSP12. Study of msn2, 4 cells also indicates that regulation of HSP30 is not directed by the general stress response pathway (STRE/Msn2 , 4 ) (Fig. 4.14). Since none of the previously characterised stress signalling pathways are involved in the stress regulation of HSP30 it was decided to search for putative regulatory elements in the promoter of this gene.

Analysis of the HSP30 promoter revealed a number of putative regulatory elements including a close match to the STRE consensus (STRE1) and six agreements to the post-diauxic shift element (PDSE1-6) consensus (Boorstein & Craig, 1990 a; section 1.3.2 (Table 4.1)). It seems unlikely that the PDSE is a variant of the STRE since the stress-induced expression of SSA3 (controlled by PDSEs) is unaffected by the msn2 msn4 double mutation (Martínez-Pastor et al., 1996). So far the PDSE has only been characterised in SSA3 (Boorstein & Craig, 1990 a), an as yet unknown transcription factor appearing to be involved in its activation. Introduction of point mutations in at least two key consensus residues of each of these putative elements had no effect on the stress regulation of HSP30 (Fig. 4.12), suggesting that PDSEs and STREs are not involved in the regulation of this gene. Instead the data presented in chapter 4 points to stress activation of HSP30, including activation by weak acid stress involving a novel stress signalling pathway and transcriptional activators. HSP30 encodes a stress-inducible regulator of the H+-ATPase and its loss slows, but does not prevent adaptation to low pH growth in the presence of sorbate (Piper et al., 1997). The stress-inducible PDR12 gene, one of several hitherto-unknown genes for ATP-binding (ABC) cassette transporters, encodes the major weak acid efflux pump and its loss causes cells to become extremely sensitive to weak acid stress (P.W. Piper, unpublished results). Preliminary investigations have shown that the stress-induced activation of this gene, as with HSP30, is also not controlled by the other well-characterised stress response pathways (P.W. Piper, unpublished results). Not only is the discovery of a new stress response interesting in its own right, but inhibition of this response may provide a route to novel food preservation strategies since it should prevent weak acid adaptation. Fig. 6.1 outlines a possible model of the putative weak acid stress signalling pathway in S. cerevisiae and section 6.4 outlines the strategies that could be used to unravel this novel stress response.
Fig. 6.1 Model of how a weak acid stress signalling pathway may occur in *S. cerevisiae*. In acidified yeast cultures the protonated form of the weak acid (XCOOH) is freely permeable to the plasma membrane (PM) and readily enters the cell by diffusion. The higher pH$_i$ of the cystol will cause a substantial fraction of the acid to dissociate, releasing protons (H$^+$) and lowering pH$_i$. This reduction in pH$_i$ may be perceived by a pH$_i$ sensor which in turn activates both STRE-controlled genes and an additional stress signalling pathway that leads to the expression of *HSP30* and *PDR12*. The promoter regions of *HSP30* and *PDR12* may possess an element that responds to low pH$_i$ (UAS pH$_i$). This is shown as occurring through activation of an unknown transcription factor (Tf pH$_i$), but could also be a release from transcriptional repression.

low pH$_e$

![Diagram](attachment:image.png)
6.4 Future studies leading to dissection of the novel weak acid response pathway

Research described in this thesis has highlighted the existence of novel weak acid-responsive stress promoter elements (chapter 4). The task for future work must be to identify components that are either involved in the sensing and transduction of the weak acid stress signal to the transcription apparatus, or constitute the transcription factors activated by this stress.

6.4.1 Identification of genes involved in the weak acid stress response pathway

The first step in characterising the weak acid stress pathway would involve the isolation of mutants of *S. cerevisiae* defective in adaptation to weak acid stress. This could be accomplished by mutagenesis of *S. cerevisiae*, plating the survivors onto rich YEPD plates, then isolating colonies that are not able to adapt to growth when replica plated onto pH 4.5 plates containing 1 mM sorbate. The mutants should fall into two categories: (i) those unable to carry out a weak acid response; and (ii) those defective in a key target gene of this response. Those cells which do not show induction of Hsp30 and Pdr12 are likely to be defective in the sensing and response to weak acid stress. These mutant cells could then be transformed with a library of *S. cerevisiae* genomic DNA fragments inserted in a suitable vector and plated onto pH 4.5 plates containing sorbate. Plasmid inserts restoring the capacity for growth under these conditions could be rapidly mapped on the *S. cerevisiae* genome by determination of short flanking DNA sequences of each insert, then BLAST searching the genomic database (Altschul *et al.*, 1990). It should then be possible to identify the ORFs required for the weak acid stress response. The next step would be to use complementation studies to order the mutants into a stress response pathway. By inserting a genomic clone of each ORF in turn into the full series of ORF deletant strains that show no weak acid response, then seeing if the response is restored, it should be straightforward to establish epistasis relationships amongst the genes of interest. Alternatively, the two-hybrid system could be used to detect protein-protein interactions in the weak acid stress regulatory pathway (Chien *et al.*, 1991).
6.4.2 Identification of the cis and trans-acting components of the weak acid stress regulatory pathway acting on the *S. cerevisiae* HSP30 promoter

Both *HSP30* and *PDR12* are very strongly induced by sorbate treatment (Fig. 4.15), an induction that is unaltered in the known mutants defective in stress-responsive transcription (chapter 4). A first step in elucidating the novel stress regulation of *HSP30* would involve the mapping of the 5' upstream noncoding sequences that direct weak acid induction. Firstly we would have to construct an *HSP30-lacZ* fusion gene that is activated like the native *HSP30* gene in response to a weak acid stress. Data from chapter 4 suggests that the 963 bp upstream region of the *HSP30* coding region is sufficient for wild type expression of the gene. A series of progressive 5' deletions of the *HSP30-lacZ* hybrid gene could be generated with exonuclease III (Maniatis *et al.*, 1989) and assayed to identify sequences that control the expression of *HSP30* following a weak acid stress. An alternative strategy would be to create progressively smaller 5' untranslated regions of the gene by PCR amplification, these being ligated into a suitable reporter vector. Either of these methods would allow mapping of the promoter sequences corresponding to the putative weak acid response elements. Data from chapter 4 has also highlighted that the heat shock and ethanol stress-induced activation of *HSP30* is independent of the known stress response pathways. The *HSP30-lacZ* fusion gene should also be assayed to determine whether the cis-acting element causing weak acid induction also activates the gene in response to all these other stresses. A double stranded oligonucleotide corresponding to the weak acid response element could then be ligated into a heterologous promoter to determine if the orientation and position of this cis-acting element is important in the activation of *HSP30*. This oligonucleotide could also be used in gel retardation assays of protein binding.

A strategy similar to that used for characterisation of the heat shock factor (Sorger & Pelham, 1987) could be used to determine the trans-acting components of the weak acid stress response pathway. Using gel retardation and binding competition assays it could be investigated whether these novel stress response elements form specific complexes with yeast stress proteins (possible transcriptional transactivators). Crude extracts from unstressed and (sorbate) stressed cells would be incubated with the labelled putative weak acid response element-containing DNA fragment in the presence or absence of unlabelled competitor DNA and subjected to gel electrophoresis. The presence of excess unlabelled fragment determines the specificity of this complex formation. The DNA-binding proteins could then be purified by means of DNA-affinity chromatography and identified by N-terminal sequencing.
defective mutants (section 6.4.1) could also be investigated to see if they lack binding of a specific protein to the weak acid response element. A similar promoter element may also be present in \textit{PDR12} and the strategy outlined in section 6.4 could also be used to elucidate the stress regulation of this gene.
REFERENCES


