Studies of the heat shock response of the yeast *Saccharomyces cerevisiae*

Thesis submitted for the degree of doctor of philosophy

by

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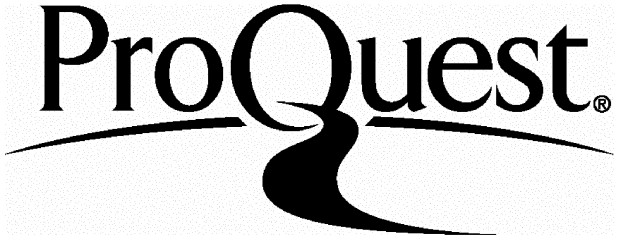

ABSTRACT

Heat shock protein HSP90 has recently been found to be one of the major chaperones of eukaryotic cells. *Saccharomyces cerevisiae* transformants were constructed with 50-150 copies of the homologous heat-inducible gene for HSP90 (*HSP82*) present on a high copy number episomal vector. These transformants were then used to demonstrate: (i) that this gene, normally single-copy in the haploid yeast genome, displays almost normal basal and heat shock-induced levels of expression even at 50-150 copies per cell; (ii) that yeast is an expression host suitable for high level synthesis of HSP90 protein (to 30-40% of total cell protein); and (iii) that increasing cellular levels of HSP90 is not protective against heat killing, causing strain-dependent reductions in growth at 37.5°C and in thermotolerance.

Heat shock protein induction is only one change elicited in yeast by heat shock. Trehalose is also accumulated, while declining intracellular pH stimulates plasma membrane ATPase activity. Recently the trehalose induction was shown to be regulated by levels of HSP70 and, to a lesser extent, HSP104. Another HSP which might contribute to regulation is HSP90, especially as HSP90 forms complexes with heat shock transcription factor and several of the regulatory proteins of eukaryotic cells. This possibility was investigated using isogenic yeast strains with normal, decreased or elevated HSP90. The results show HSP90 levels having a small negative influence over the heat inductions of trehalose and the heat shock element, a minor effect compared with the major regulation exerted by HSP70.

Numerous chemicals are inducers of heat shock proteins, but few agents are known that selectively inhibit expression of these proteins. This thesis provides the first evidence that two widely-used food preservatives (sorbate and benzoate) can act as selective inhibitors of heat shock protein induction in *S. cerevisiae*. Their effects were strongly

dependent on the pH of the culture medium. Below pH5.5 sorbate caused a greatly-increased sensitivity to lethal heat treatment, with strong selection for respiratory-deficient *rho- petites* amongst the surviving cells. In contrast above pH5.5 sorbate acted as a chemical inducer of thermotolerance. In low pH *rho+* cultures sorbate, benzoate, the uncoupler carbonyl cyanide m-chlorophenylhydrazone and the plasma membrane ATPase inhibitor diethylstilboestrol were all shown to act as selective inhibitors of heat shock protein and a heat shock element-*lacZ* fusion induction by heat shock. In low pH cultures the *rho-* mutation was found to confer a higher resistance to growth in the presence of sorbate, as well as a partial restoration of the heat inducibility of heat shock proteins and of heat shock element-*lacZ* fusion in sorbate-treated cultures.

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PUBLICATIONS

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ABBREVIATIONS

ATP	adenosine-5'-triphosphate
bp	base pairs
bis-acrylamide	N,N'-methylene bisacrylamide
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CAPS	3-(cyclohexylamino)-1-propanesulphonic acid
CCCP	carbonyl cyanide m-chlorophenylhydrazone
Ci (μCi)	curies (microcuries)
cpm	counts per minute
DEPC	diethylpyrocarbonate
DES	diethylstilboestrol
dH_2O	deionised plus 1x distilled water
DNAase	deoxyribonuclease
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
Fig.	Figure
h	hour
HEPES	(N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid])
HSC	heat shock cognate
HSE	heat shock element
HSF	heat shock transcription factor
HSP	heat shock protein
IgG	immunoglobulin G
kb	kilobase
KDa	kilodaltons
KHZ	kilohertz
min	minute
MES	2-(N-morpholino)ethanesulphonic acid
NEPHGE	non-equilibrium pH gradient gel electrophoresis
NP40	nonidet P40
N-terminal	amino terminal
ONPG	O-nitrophenyl-β-D-galactoside
PAGE	polyacrylamide gel electrophoresis
PEG	polyethylene glycol

pHi	intracellular pH
PMSF	phenylmethylsulphonylfluoride
PVDF	polyvinylidene
RNAase	ribonuclease
rpm	revolutions per minute
sec	second
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
ss	single strand
TCA	trichloroacetic acid
TEMED	N,N,N',N,-tetramethylethylenediamine
TES	(N-tris[Hydroxymethyl]methyl-2-aminoethane-sulfonic acid)
TLCK	tosyl-lysine chloromethyl ketone
TPCK	tosylamino-2-phenylethyl chloromethyl ketone
Tris	tris(hydroxymethyl)aminomethane
v/v	volume to volume ratio
w/v	weight to volume ratio
4CN	4-chloro-1 napthol

CHAPTER 1

INTRODUCTION

All organisms and cultured cells respond to elevated, supraoptimal temperatures by inducing the synthesis of a small number of proteins called the heat shock proteins (HSPs). HSPs are normally synthesised at much lower rates during non-stress conditions. The heat shock response appears to be universal as it is found in all species that have been examined to date. In this study the role of one specific HSP (HSP90) and its influence in the heat-shock response, as well as the inhibition of the heat-shock response of the yeast *Saccharomyces cerevisiae* were investigated.

S.cerevisiae is the most extensively studied microbial eukaryote. This yeast is used for both classical and molecular genetic analysis, as well as for both traditional and modern biotechnology. Being both unicellular and a eukaryote, yeast can be both handled as easily as bacteria and cultivated on a large scale. However yeast also contains all the organelles and supramolecular structures of mammalian cells, which means that information on molecular processes obtained for yeast is far more likely to be relevant to the cells of higher organisms. It is for these reasons that the heat shock response has been extensively studied in yeast.

1.1 The heat shock response

The heat shock response is homeostatic, serving to repair the damage caused by the heat-shock and allowing cells to survive at potentially-lethal high temperatures. The heat-shock response is a rapid but transient reprogramming of cellular activities to ensure survival during the stress period, to protect essential cell components against heat damage and to permit a rapid resumption of normal cellular activities during the recovery period.

The heat shock response was initially observed in *Drosophila busckii* as a rapid induction of six new polytene chromosome puffs in the salivary glands of young embryos following a heat shock (Ritossa, 1962). The puffs correspond to sites of rapid RNA synthesis on very active genes. Other changes to puffing patterns occur as a consequence of alterations in the titre of ecdysone, the insect's growth and moulting hormone (Ashburner and Bonner, 1979). Ritossa (1962) found that subjecting the larvae to a brief

heat shock treatment dramatically altered the puffing pattern, corresponding to changes in gene activity. This observation was largely ignored until 10 years later when altered gene activity, resulting from a mild heat-shock treatment of *Drosophila* salivary glands, was found to induce the synthesis of a small number of polypeptides (Tissieres *et al.*, 1974). These polypeptides were appropriately named heat-shock proteins (HSPs). From this beginning the heat shock response in *Drosophila* became a model for the investigation of gene structure and regulation.

Some years after the early discoveries in *Drosophila,* investigators found that heat induced the synthesis of similar evolutionarily-conserved HSPs in bacteria (Yamamori *et al.*, 1978; Neidhardt *et al.*, 1984; Watson, 1990), yeast (Miller *et al.*, 1979; McAlister *et al.*, 1979), plants(Kimpel and Key, 1985; Nagao and Key, 1989; Vierling, 1990) and mammalian cells (Kelly and Schlesinger, 1978; Lindquist,1986; Welch *et al.*, 1989).

The heat shock response is induced not just by an increase in temperature, but also by a variety of cytotoxic agents, such as arsenite; heavy metal ions; ethanol and powerful oxidants (Neidhardt *et al.*, 1984; Burdon, 1986) (Table 1.1). In this respect it could be considered a response to several stresses and the proteins it induces as "stress proteins".

The most striking aspect of the response is the remarkable conservation of certain HSP genes in evolution. Not only are some HSP amino acid sequences highly conserved between widely-divergent organisms, but also the regulatory sequences on HSP genes. This conservation would seem to emphasize the essential requirement for HSPs in cell survival (Lindquist, 1986; Burdon, 1986; Bond and Schlesinger, 1987; Schlesinger, 1990). The regulatory cis-acting promoter elements for gene induction by heat have been defined (Mirault *et al.*, 1982; Pelham and Bienz, 1982) and the trans-acting transcription factors that bind to these elements also isolated and characterised (discussed in 1.3.1 and 1.3.2).

Although all the functions of the heat shock response remain unclear, there is increasing evidence that this response provides protection from the toxic effects of intracellular accumulations of aberrant or denatured protein (Craig and Jacobsen, 1986; Lindquist and Craig, 1988; Schlesinger, 1990; Mager and Moradas-Ferreira, 1993). Some HSPs have different isoforms, some of which are produced in non-stressed cells under normal physiological conditions and others which are induced by heat (e.g.HSP70).Some reviews (Lindquist and Craig, 1988; Watson, 1990; Schlesinger,1990; Gething and Sambrook, 1992) have suggested that the major function of these HSPs made under normal

Table 1.1 Inducers of the heat shock response

Inducing agent or treatment	Proposed effect
Heat shock Sodium arsenite Cadmiun Ethanol Methanol Amino acid analogs Various heavy metals Iodoacetamide	Increasing intracellular levels of denatured or aberrant protein.
Return from anoxia Hydrogen peroxide Superoxide ions Other free radicals	Oxygen toxicity, free radical fragmentation of proteins.
Antimycin Rotenone Oligomycin Azide Dinitrophenol Ionophores	Disturbing the processes involved in energy metabolism by inhibiting oxidative phosphorylation, or dissipating the ionic gradients that exist across membranes.

Compiled from data reviewed in Ashburner and Bonner, 1979; Ananthan *et al.*, 1986; Burdon, 1986 and Lindquist, 1986.

physiological conditions is to act as chaperones, aiding the assembly and disassembly of protein complexes and assisting protein translocation across membranes. The term "molecular chaperone" was first used by Laskey *et al.* (1978), to describe the properties of nucleoplasmin, an abundant acidic nuclear protein required for the assembly of nucleosomes from histones and DNA in *Xenopus oocyte* extracts. Molecular chaperones can be defined as proteins whose cellular role is to mediate the folding of other polypeptides and in some instances, their assembly into oligomeric structures, but which themselves are not components of these final structures (Ellis, 1987). However, it is still unclear to what extent HSPs synthesised in stressed cells participate in these events. Stress results in the production of abnormal, misfolded or otherwise damaged proteins. It may be that HSPs function in stress survival and recovery from stress by chaperoning damaged protein. It remains to be seen whether they reinstate the structural integrity of the affected proteins, so that they recover biologically-active conformation, or serve merely to chaperone these proteins until they can be degraded.

1.2 The heat shock proteins

The HSPs of most organisms are classified into four families by virtue of their apparent molecular weight (KDa) on SDS polyacrylamide gels.

(1) HSP90 family: HSPs of between 82KDa and 110KDa (see section 1.2.1).
(2) HSP70 family: HSPs around 70KDa (section 1.2.2).
(3) HSP60 family: HSPs of between 56KDa and 65KDa (section 1.2.3).
(4) Small HSPs: HSPs of between 12KDa and 40KDa (section 1.2.4).

The exact number of HSPs within each of these different classes varies considerably in different organisms and cell types. Some other proteins, such as ubiquitin, have been identified which do not fall within these groups. The four groups of HSPs are discussed individually in the following sections.

1.2.1 The HSP90 family

Although heat shock protein HSP90 is abundant in all unstressed eukaryotic cells, it is considered to be a true heat shock protein since its levels increase with heat shock. Proteins of the HSP90 family are constitutively synthesised in the cytoplasm of most prokaryotes and eukaryotes, where they may constitute up to 1 to 2% of total cell protein. HSP90 genes have been cloned and sequenced from several evolutionarily diverse organisms including

fruit flies, yeasts, chickens, mammals, trypanosomes, bacteria and plants (reviewed in Lindquist and Craig,1988; Watson, 1990; Vierling, 1990). Sequence analysis of these HSP90 proteins demonstrates that they are very highly conserved throughout evolution. The HSP90s of even the most distantly related eukaryotes have 50% identity at the amino acid sequence level (Farrelly and Finkelstein, 1984) and all eukaryotic HSP90s have greater than 40% amino acid identity with the *Escherichia coli* HSP90 protein, the product of the *htpG* gene (Binart *et al.,* 1989).

The number of genes within the HSP90 family varies between organisms. Yeast and humans synthesise two HSP90 proteins, whereas chicken, fruit flies and *E.coli* synthesise only one. Genetic analysis of HSP90 genes has been conducted in both *E.coli* and *S.cerevisiae.* The *E.coli* HSP90 gene, *htpG* encodes a 624-amino-acid, 71.4 KDa protein (Bardwell and Craig, 1987). Its inactivation results in a slightly reduced rate of growth at high temperatures (Bardwell and Craig,1988). The *HtpG* product becomes phosphorylated *in vivo* and is present as a dimer (Spence and Georgopoulos, 1989). *S.cerevisiae* has two HSP90 genes (*HSC82* and *HSP82*) that encode proteins of 97% identical amino acid sequence. *HSC82* is constitutively expressed at a high level and is moderately heat inducible, whereas *HSP82* is expressed at a low level in non-stressed cells and strongly induced upon heat shock (Borkovich *et al.,* 1989). Both proteins are however functionally homologous, since disruption of both *HSC82* and *HSP82* is lethal whereas disruption of just one of these genes has no effect on growth at normal temperatures. The requirement for expression *HSC82* or *HSP82* increases at increased temperatures suggesting that higher levels of HSP90 are necessary for growth at high temperatures (Borkovich *et al.,* 1989).

Humans and mice have at least two functional HSP90 genes, which encode closely related polypeptides. In humans their products are designated HSP90 α and HSP90 β (Simon *et al.,* 1987). HSP90 purified from Hela cells contains almost equal amounts of the HSP90 α and HSP90 β gene products (Lees-Miller and Anderson, 1989a). These α/β forms of HSP90 differ in their phosphorylation states, HSP90 α being phosphorylated within its amino terminus on the two threonine residues in the sequence TQTQDQPM, unlike HSP90β which does not possess this motif (Lees-Miller and Anderson, 1989b). This motif is also absent from the HSP90s of *E.coli, S.cerevisiae, T.cruzei* and *Drosophila* species, suggesting that the α form of HSP90 arose relatively late in evolution, perhaps in parallel with the development of the steroid hormone receptor system (Lees-Miller and Anderson, 1989b). HSP90 genes from plants are not well characterized. Using a *Drosophila HSP83* gene fragment as a probe, the HSP90-related genes from soybean and corn have been isolated, further highlighting the conservation which exists among this group of HSPs

(Nagao and Key, 1989). Mammalian cells also have a HSP90-related protein (GRP94) found exclusively in the lumen of the endoplasmic reticulum (Gething and Sambrook, 1992).

HSPs have also been isolated with molecular weights greater than 90KD, such as the HSP104 involved in thermotolerance in yeast (section 1.6) and the HSP100 from mammalian cells which is a calcium/calmodulin-regulated actin-binding protein (Koyasu *et al.*, 1989).

There is extensive evidence that mammalian HSP90 exists either as a homodimer or in minor proportion in association with diverse other proteins. These HSP90-associated proteins include steroid hormone receptors (Catelli *et al.*, 1985; Sanchez *et al.*, 1985), tyrosine oncogene kinase (Oppermann *et al.*, 1981; Brugge *et al.*, 1981), casein kinaseII, initiation factor eIF2-α kinase and actin (Koyasu *et al.*, 1986). A major tumor-specific transplantation antigen of carcinogen-treated mice is identical with HSP90 and a proportion of patients with the autoimmune disease systemic lupus erythematosus exhibit grossly elevated lymphocyte HSP90 which fluctuates with disease activity (Norton *et al.*, 1988). A strong correlation between HSP90 association and the loss of steroid receptor function suggests that the binding of HSP90 to this receptor plays a role in maintaining the receptor in a non-functional state (Cadepond *et al.*, 1991). HSP90 performs a similar role with tyrosine kinase, at least in the case of the transforming protein of Rous Sarcoma Virus, $pp60^{src}$ (Brugge *et al.*, 1981; Oppermann *et al.*, 1981). As it is initially synthesised, the inactive form of this kinase associates with HSP90 and a 50KD phosphoprotein. The order of events which then follow is unknown, but they involve phosphorylation of the enzyme at a tyrosine residue, its release from HSP90 and insertion into the cell membrane as an active tyrosine kinase. Hence, HSP90 appears to maintain both steroid receptors and the $pp60^{src}$ tyrosine kinase in non-functional states until their activity is required.

HSP90s also associate with another kinase of mammalian cells, namely the heme-controlled eIF2-α kinase (Rose *et al.*, 1987). The eIF2-α kinase is activated under conditions of haem deficiency in mammalian reticulocytes or their cell-free lysates, the resulting phosphorylation of the α subunit of eIF2 leading to a block in protein synthesis. Purified HSP90 stimulates haem-controlled eIF2-α kinase, resulting in inhibition of protein synthesis in the reticulocyte lysate system. It may be that phosphorylation of eIF2-α contributes to the partial inhibition of translation immediately following heat shock, indicating a possible role for HSP90 in the regulation of the heat shock response (see section 1.3.3). In addition, the stimulatory effect of HSP90 on haem-controlled eIF2-α kinase appears to depend on the phosphorylation state of the HSP, since pure HSP90

previously incubated with a phosphatase fails to increase kinase activity (Szyszka *et al.*, 1989).

HSP90 was originally proposed to be involved in the assembly and disassembly of proteins into higher ordered structures. While this hypothesis appears to hold true for the interactions of HSP90 with steroid receptors and tyrosine kinase, it does not account for its interaction with eIF2-α. Later the concept of HSP90 functioning as a docking protein was proposed (Hardesty and Kramer, 1989; Pratt, 1990) in which potentially active molecules, for example steroid receptors, are effectively stored associated with HSP90 in inactive form until their activity required. Release and activation is then promoted by special triggering events such as heat shock, binding of a steroid hormone or phosphorylation. Mammalian HSP90 was shown to possess an ATP binding site and autophosphorylation activity at serine residues (Csernely and Kahn, 1991). This enzymatic activity may have a role in the association of HSP90 with other cellular proteins and aid the identification of a function in both stressed and non-stressed cells. Pure HSP90s of *E.coli, S. cerevisiae*, rat and human, also from the trypanosomatid parasites *Crithidia fasciculata* and *Trypanosoma cruzi* show potent, peptide-stimulated ATPase activity (Nadeau *et al.*, 1993). It has only recently been proven that the HSP90 universally present in eukaryotic cells assists authentic protein folding and therefore represents a major class of chaperone (Wiech *et al.*, 1992). Even so, it is possible that HSP90 works by a completely different mechanism from the HSP70, HSP60 or GroEL chaperones, since its action to catalyse folding of other proteins to functional forms and to prevent protein aggregation *in vitro* does not depend on the presence of nucleoside triphosphates (Wiech *et al.*, 1992). HSP90 also engages in specific interaction with peptidyl prolyl isomerase and heat shock transcription factor (Nadeau *et al.*, 1993). Only with a structure for HSP90 protein can the mechanism of action of this important chaperone be rapidly unravelled.

1.2.2 The HSP70 family

HSP70 is a general name for the most abundant HSPs of molecular weight between 68 and 78KD. These have the most highly conserved protein structure across widely divergent species in both their constitutive and inducible forms. Most of these HSP70 family proteins are synthesised constitutively, providing chaperone functions in the cytosol, the mitochondrion or the lumen of the endoplasmic reticulum (Gething and Sambrook, 1992). Gene disruption studies in yeast have been instrumental in elucidating the function of these different HSP70 family proteins (Craig and Jacobsen, 1986; Lindquist and Craig, 1988). This genetic analysis shows that HSP70 and related genes are essential for growth at all

temperatures, indicating a critical role in normal cellular physiology for the encoded proteins. All HSP70 proteins identified so far possess an ATP-binding site and ATPase activity (Zylicz *et al.*, 1983; Welch and Feramisco,1985; Chappell *et al.*, 1986). The requirement for ATP-binding has been demonstrated by the cyclical interactions of HSP70 with a variety of polypeptides, in which polypeptide release occurs by an ATP-dependent process (Flynn *et al.*, 1989).

In *E.coli*, *dnaK* is the sole HSP70-related gene, whilst most if not all eukaryotes possess multiple genes encoding HSP70-related proteins. *dnaK* encodes a protein of molecular weight 69,000, which possesses weak DNA-independent ATPase activity and is autophosphorylated at threonine (Zylicz *et al.*, 1983). Mutational analysis suggests that the polypeptide is organised into at least two functionally distinct domains regulated by calcium (Cegielska and Georgopoulos, 1989). The highly conserved amino-terminal portion is required for ATPase activity and the carboxyl terminal portion, which is less well conserved, is responsible for autophosphorylation. The ATPase activity of DnaK protein has been implicated in the reactivation of heat-inactivated enzymes (Skowyra *et al.*, 1990). For example, *E.coli* RNA polymerase aggregates at a non-permissive temperature and DnaK is thought to prevent such aggregation by binding directly to the enzyme. *E.coli* strains carrying a *dnaK* deletion are non-viable at high temperatures showing that the gene is essential for cell survival at elevated temperatures (Itikawa and Ryu, 1979). Such mutants also possess defects through a wide range of growth temperatures, indicating that the DnaK protein has important functions in cell metabolism under non-stressed conditions and at low temperatures, as well as during heat shock (Bukau and Walker, 1989).

The HSP70 family of proteins of yeast is the most thoroughly studied HSP family. It consists of at least nine members that differ to varying degrees in sequence and cellular localization (Fig. 1.1). Two members are localized to specific compartments of the cell: the mitochondrion (*SSC1* product), the nucleus (*KAR2* product) and the endoplasmic reticulum (also the *KAR2* product). HSP70-related proteins differ also with regard to the conditions under which they are synthesized (Fig. 1.1). In fact, it was the discovery of the essential function of HSP70 protein made in unstressed cells that provided the data for the chaperone model (Gething and Sambrook, 1992). One of these studies revealed a requirement for HSP70 for the translocation of certain yeast proteins synthesized in the cytoplasm into the mitochondrial matrix (Deshaies *et al.*, 1988). These HSP70 proteins are also needed for import of several proteins into other eukaryotic cell organelles, including the endoplasmic reticulum (Chirico *et al.*, 1988), the chloroplast (Cheng *et al.*, 1989), and the lysosome (Chiang *et al.*, 1989). HSP70-related proteins made under conditions of normal,

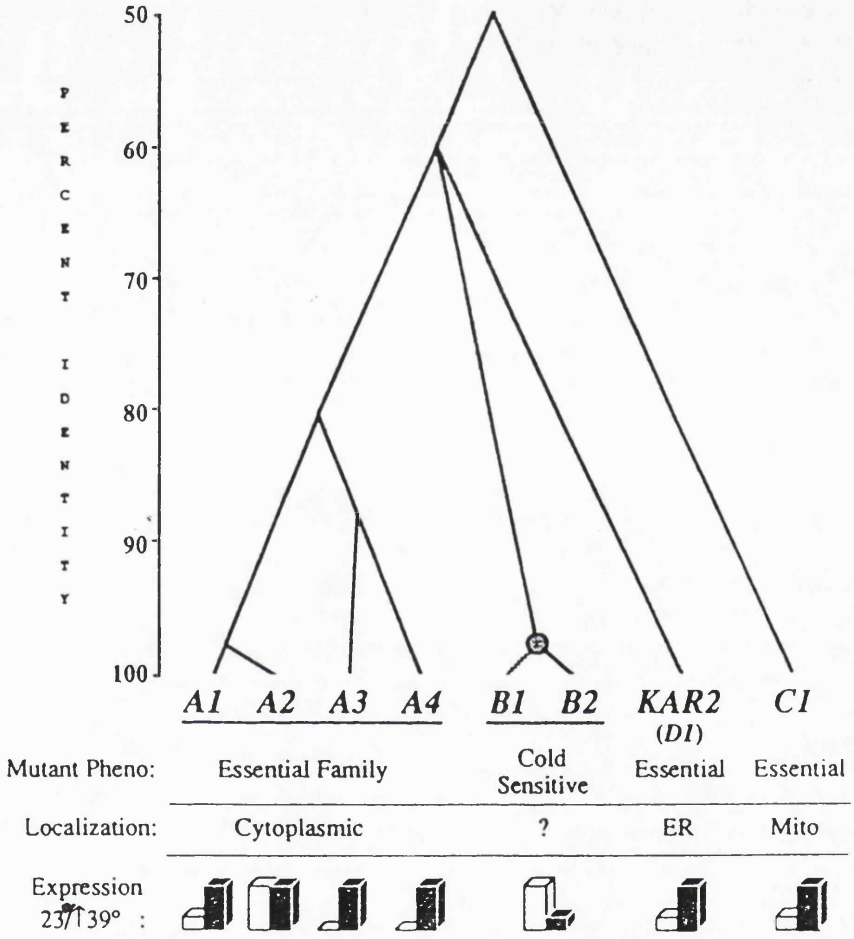


Figure 1.1 The *S. cerevisiae* HSP70 family
Taken from Craig, E. A., Kang, P. J. and Boorstein, W. *Antonie van Leeuwenhoek* **58:** 137-146, 1990.

unstressed growth (e.g. SSA1, SSA2; Fig.1.1) are generally referred to as HSP70-cognates or HSC70 proteins; the HSP70 designation being reserved for the stress-inducible HSP70s (including SSA3, SSA4; Fig.1.1). [HSP70-related proteins form a complex multigene family which is divided into the following groups:*SSA1-SSA4, SSB1* and *SSB2, SSC1, SSD1/KAR2* (Lindquist and Craig, 1988); see Fig.1.1]. The *SSA* gene products are both structurally and functionally complex. Except for *SSA2,* all *S.cerevisiae SSA* genes are heat inducible. However, only *SSA4* and *SSA3* are classic HSP genes being expressed at very low levels under normal conditions and strongly induced upon heat shock (Boorstein and Craig, 1990). The role of the *SSB* gene products remains unknown, although mutations in both *SSB1* and *SSB2* result in a cold sensitive phenotype (Craig and Jacobsen, 1985). *SSC1* is an essential gene (Craig *et al.,* 1987), which encodes a mitochondrial protein (SSC1p) whose sequence is more similar to *E.coli* DnaK than any of the other yeast HSP70 proteins (Craig *et.al,* 1989). *SSD1* and *KAR2* are known to be allelic and *SSD1* is also essential, its product being localised in the endoplasmic reticulum and possessing a similar function to mammalian Bip/GRP78 protein by binding to and, assisting the proper folding of proteins destined for secretion.

Most HSP70 proteins play a role in protein translocation from the cytoplasm into specific cellular compartments (Chirico *et al.,* 1988; Deshaies *et al.,* 1988; Rose *et al.,* 1989; Kang *et al.,* 1990; Scherer *et al.,* 1990). In addition, the mitochondrial product of *SSC1* (SSC1p) possesses chaperone-like qualities, binding partially translocated mitochondrial precursor proteins (Scherer *et al.,* 1990). It is implicated in the completion of protein transport through contact sites of the mitochondrial membrane, maintaining the precursor protein in an unfolded state during import into the mitochondrial matrix (Kang *et al.,* 1990), prior to HSP60-assisted refolding (section 1.2.3).

Like yeast, most eukaryotes possess multiple genes encoding HSP70-related proteins. For example, the nematode *Caenorhabditis elegans* has six distinct HSP70-related genes (Snutch *et al.,* 1988), *Drosophila* has a multigene family of at least nine HSP70 genes, plus one copy of a heat inducible HSP68 gene (Palter *et al.,* 1986). The mammalian HSP70 family is composed of at least three gene subgroups, the 78KD glucose-regulated protein (GRP78), the 70KD heat shock cognate protein (HSC70) and the major stress protein (HSP70). All three proteins binds ATP (Welch and Feramisco, 1985) but function in different intracellular locations (Gething and Sambrook, 1992). GRP78, originally identified as the Bip (binding protein), a protein bound to newly-synthesised immuno-globulin heavy chains of B lymphocytes (Haas and Wabl, 1983), is located in the lumen of the endoplasmic reticulum. GRP78 binds proteins that have been imported into the

endoplasmic reticulum and is postulated to prevent abnormal aggregation of these proteins and to assist their folding into stable macromolecular assemblies (Gething and Sambrook, 1992). HSC70 is a cytoplasmic protein and the first HSP70-related protein to be associated with a biochemical activity, namely the release of clathrin from coated vesicles (Ungewickell,1985; Chappell *et al.*, 1986). HSC70 has also been found to mediate the translocation of proteins across membranes (Chirico *et al.*, 1988; Deshaies *et al.*, 1988). Finally, HSP70 together with HSC70 migrate to the nucleus upon heat shock, where it is proposed they participate in the recovery of nuclei from stress induced damage (Welch and Suhan, 1986).

Not many reports are available for HSP70 genes from plants, although those isolated have been found to occur in multi-gene families. *Arabidopsis* synthesises at least twelve HSP70-related polypeptides, most of which are constitutively expressed (Wu *et al.*, 1988), and the HSP70s of soybean have been resolved into at least ten polypeptides (Mansfield and Key, 1987). In addition to cytoplasmic HSP70 homologues, chloroplast envelope and stromal forms of HSP70 were identified in pea chloroplasts (Marshall *et al.*, 1990). These proteins are present constitutively and show little change with heat stress. Although the role of HSP70 homologues in plant chloroplasts is unknown, there are many processes in which the putative polypeptide chain unfolding or assembly / disassembly function could be required.

It is widely assumed that HSP70s participate in maintaining precursor proteins in a translocation-competent conformation, since HSP70 interacts with partially unfolded proteins, stabilising the loosely folded conformation and thus preventing misfolding or aggregation (Pelham, 1988; Rothman, 1989; Gething and Sambrook,1992). Cytosolic HSP70 was shown to stabilise precursor proteins, preventing the formation of abnormal conformations, while the actual unfolding of such proteins is performed by a membrane-bound import apparatus (Pfanner *et al.*, 1990). In addition, HSP70 and HSP60 (section 1.2.3) have been implicated in the folding of nascent mitochondrial proteins under normal growth conditions (Kang *et al.*, 1990). In stressed cells abnormal proteins are formed and under these conditions the HSP70-polypeptide complex has been shown to be considerably more stable. Normally the formation of an HSP70 complex is transient, suggesting that in stressed cells this increased stability maintains the abnormal protein in a soluble state until it can be presented to the appropriate proteolytic system (Beckmann *et al.*, 1990). As well as being involved in protein folding, HSP70s have been shown to dissociate protein complexes in the presence of ATP, as in the case of clathrin-coated vesicles (Chappell *et al.*, 1986; Ungewickell, 1985) and a lambda bacteriophage DNA replication complex

(Alfano and McMacken, 1989).

The high degree of HSP70 conservation between different species suggests an important role for this protein. The original hypothesis that HSP70s participate in ATP-dependent protein unfolding or assembly / disassembly reactions (Pelham, 1986) and prevent protein denaturation during stress has been largely substantiated (Gething and Sambrook, 1992). In most stressed cells, newly synthesised HSP70 localises to the nucleus and nucleolus, where it is tightly complexed in an insoluble form that is partially solubilised by ATP (Welch and Suhan, 1986). The nucleolus is the site of ribosome assembly and is very sensitive to changes in temperature. Pelham (1986) suggested that HSP70 binds to proteins that are incompletely folded in the pre-ribosome assembly unit and protects them from irreversible denaturation. It has since been proposed that HSP70 is the cellular thermometer in both yeast and *E.coli* promoting expression of other HSPs via the transcription factors HSF and σ^{32} respectively (Craig and Gross, 1991; also section 1.5). The interaction of HSP70 with the heat shock gene transcription factor would allow expression to be related to the functional state of the cell.

1.2.3 The HSP60 family

Members of this family with subunit molecular weights of about60KDahave been found in bacteria (Georgopoulos and Hohn, 1978), plants (Hemmingsen *et al.*, 1988) and mammals (Mizzen *et al.*, 1989). The HSP60 proteins (GroE proteins in bacteria) are not only heat inducible but essential for survival during non-stressful conditions. This family of heat shock proteins has been found to form complexes with newly synthesised polypeptides and to possess ATPase activity. HSP60s play an important role in preventing the aggregation of misfolded proteins (Buchner *et al.*,1991; Clarke *et al.*, 1988) and function in the folding of mitochondrial proteins in eukaryotes (Manning-Krieg *et al.*, 1991). They are therefore a major class of chaperone (Hemmingsen *et al.*, 1988; Gething and Sambrook, 1992).

In eukaryotes HSP60s are localized to cytoplasmic organelles such as the chloroplast and mitochondrion. The HSP60 homologue in the chloroplast is ribulose biphosphate carboxylase-oxygenase heavy chain binding protein, a protein required for assembly of this hexadecameric enzyme complex. The yeast HSP60 protein is encoded by a nuclear gene, *MIF4*. Synthesised in the cytosol as a precursor, the *MIF4* product is translocated into mitochondria where it is proteolytically processed (Reading *et al.*, 1989). Deletions of *MIF4* have shown that HSP60 is essential for cell viability. In addition, conditional-lethal

mif4 mutants show defects in mitochondrial function, demonstrating the essential function of HSP60 as a matrix protein in assembly, processing and sorting events in the mitochondria (Cheng *et al.*, 1989). Yeast HSP60 also acts as a folding catalyst with the HSP60 monomers forming a 14-mer complex, which resembles the *E. coli* GroE in that it comprises two stacked 7-mer rings (McMullin and Hallberg, 1988). This complex binds unfolded proteins at its surface, then catalyses their folding in an ATP-dependent process (Ostermann *et al.*, 1989). HSP60 14-mers are apparently also required for their own assembly because self-assembly of newly synthesised wild type HSP60 subunits does not occur in the HSP60-defective yeast mutant, *mif4* (Cheng *et al.*, 1990).

HSP60 cDNAs from human (Jindal *et al.*, 1989) and chinese hamster (Picketts *et al.*, 1989) sources have been cloned and sequenced. Like yeast HSP60, they are found to encode an N-terminal presequence with the potential of forming a positively charged amphiphilic helix, a structure considered essential for mitochondrial import (Schatz, 1987; Hartl and Neupert, 1990). In addition, human and hamster HSP60s are members of pseudogene families in which all except one are non-functional (Venner *et al.*, 1990). DNA sequence analysis suggests a common evolutionary ancestor, the pseudogenes resulting from gene duplication and gene divergence.

1.2.4 The small HSP family

The small HSPs are a very diverse group showing great variance in both size and number. Their molecular weights range from 12KDa in the yeast *S.cerevisiae* (Praekelt and Meacock, 1990) to 40KDa in the protozoan *S.mansoni* (Nene *et al.*, 1986). The number of small HSPs synthesised does not correlate with the genetic complexity of the organism. Yeast synthesises two small HSPs (HSP12 and HSP26, McAlister *et al.*, 1979; Petko and Lindquist, 1986; Praekelt and Meacock, 1990); higher plants synthesise up to 30(Nagao *et al.*, 1985; Mansfield and Key, 1987); man synthesises only one (HSP28; Arrigo and Welch, 1987) while *Drosophila* synthesises four (HSP22, HSP23, HSP26 and HSP27 ; Tissieres *et al.*, 1974; Ingolia *et al.*, 1982).

Procaryotes also synthesise small HSPs. A 21KDa protein encoded by HSP gene *htrC* from *E.coli* has been shown to possess a similar hydropathy profile and regions of sequence homology to a 17 KDa soybean HSP (Raina and Georgopoulos, 1990). Another procaryote, *Mycoplasma leprae,* synthesises six HSPs, a 18 KDa HSP showing a striking amino acid sequence similarity with eukaryotic HSPs (Nerland *et al.*, 1988).

It is striking that the small HSPs show much greater homology within organisms than between organisms. For example, members of a subgroup of the soybean small HSPs have 90% amino-acid identity with each other, but only 20% amino-acid identity with the small HSPs of *D.melanogaster, C.elegans* and *X.laevis.* Plant small HSPs can be greater than 90% identical at the amino acid level within a gene family, but less than 50% between gene families. Hence, the identity between small HSP families in plants and other eukaryotes is much lower than within the HSP90, HSP70 or HSP60 families (Vierling,1990).

Though divergent in sequence, all small HSPs studied to date assemble into high molecular weight aggregates known as Heat Shock Granules (HSGs). HSGs have molecular weights ranging from 200-800 KD and have been isolated from very diverse organisms including yeast (Tuite *et al.,* 1990), nematodes (Hockertz *et al.,* 1991), tomatoes (Nover *et al.,* 1983), chickens (Collier *et al.,* 1988) and mammalian cells (Arrigo and Welch, 1987). Many small HSPs also share other properties. Both mammalian (Arrigo and Welch, 1987) and *Drosophila* (Rollet and Best-Belpomme, 1986) small HSPs are phosphorylated. Plant small HSPs are both phosphorylated and methylated (Nover and Scharf, 1984). HSGs of *Drosophila* (Kloetzel and Bautz,1983) and tomato cells (Nover and Scharf, 1989) contain RNAs, so HSGs may function as mRNA storage compartments which preserve translationally-inactive mRNA.

Small HSPs are induced by developmental cues at normal physiological temperatures as well as under conditions of stress. This implies that small HSPs may play a role in development as well as in response to stress. In yeast, HSP26 is induced when cells enter stationary phase or undergo sporulation (Kurtz *et al.,* 1986). The four *Drosophila* small HSP genes, *HSP22, HSP23, HSP26* and *HSP27,* which all map in a cluster at Locus 67B, are transcribed in late larval to late pupal stages (Sirotkin and Davidson, 1982) and are induced in tissue culture cells by the addition of the steroid hormone, ecdysterone (Ireland and Berger, 1982). The *Drosophila HSP27* and *HSP26* genes are also expressed in ovarian nurse cells during the middle to late stages of oogenesis (Zimmermann *et al.,* 1983) and deletion analysis of the *Drosophila HSP26* promoter indicates that separate cis-acting elements direct the heat and developmental inductions (Cohen and Meselson, 1985).

It is now known that there are seven genes at Locus 67B in *Drosophila,* four of which are the previously-described small HSP genes. The remaining three genes designated *1* , *2* and *3* are also both developmentally regulated and heat-shock inducible (Ayme and Tissieres, 1985). Locus 67B therefore comprises seven structurally interrelated genes, each

expressed independently during heat shock and normal development. The response of gene *1* in *Drosophila* tissue culture cells to heat shock varies according to the stage of development (Vazquez,1991), suggesting that developmentally regulated factors interact with the heat shock transcription factor (HSF; section 1.3.2) in controlling these genes.

At present, the cellular role of small HSPs is far from clear. A few studies have shown a positive correlation between the cellular levels of small HSPs and the degree of thermotolerance. For example, overexpression of human HSP27 in rodent cells increased thermal resistance acquired by cells after exposure to heat shock (Landry *et al.,* 1989). However, there are exceptions to this correlation. Yeast HSP26 deletion mutants have no detectable phenotype with regard to thermotolerance (Petko and Lindquist, 1986; Praekelt and Meacock, 1990). The three *S.cerevisiae* small HSPs are HSP26, HSP12 and ubiquitin. Both HSP26 and HSP12 are developmentally-induced, being synthesised when cells are entering stationary phase (Kurtz *et al.,* 1986; Praekelt and Meacock, 1990) and HSP26 is also synthesised during sporulation (Kurtz *et al.,*1986). Deletion of both HSP26 and HSP12 shows no obvious phenotype suggesting that these genes are non-essential (Petko and Lindquist, 1986; Praekelt and Meacock, 1990). Analysis of HSP12 expression in mutants affected in cAMP-dependent protein phosphorylation has suggested that HSP12 is induced as a result of a decline in cAMP levels as well as heat shock (Praekelt and Meacock, 1990). This would account for the developmental induction of HSP12, since cAMP levels decline upon entry into stationary phase. *The UBI4* gene is also induced in stationary phase as a result of lowering of cAMP levels (Tanaka *et al.,* 1988). Unlike *HSP12* and *HSP26, UBI4* is essential for resistance to stress (Finley *et al.,* 1987).

Defining the cellular location of small HSPs has not provided clues as to their function. The cellular location of small HSPs is complex, both between and within species, and such studies have proved of little value in identifying a role for these proteins. It could be said that these proteins are members of more than one group since they show such great diversity in many of the aspects studied. However, the homologies in their amino acid sequence and their common induction patterns do suggest a common function.

1.3 Regulation of the heat shock response

The heat shock response is highly conserved in evolution. For example, when HSP70 genes from *Drosophila* are introduced into mammalian cells, yeast or tobacco tissue, they are actively transcribed when the recipient cells are subject to hyperthermia. There are differences in the ways by which heat shock gene expression is regulated in different organisms and even in different cell types within an organism (For reviews see Lindquist, 1986; Bienz and Pelham, 1987; Tanguay, 1988). In *E.coli* and yeast, the heat shock response is controlled primarily at the level of transcription, whereas, in *Drosophila,* mammalian cells and plants, regulation occurs both transcriptionally and translationally. These differences are partly attributable to the variations in mRNA half-life which exists between these organisms. For example, mRNAs have a maximum half life of 1-3 minutes in *E.coli* compared to several hours in *Drosophila* and plants. Hence, in *Drosophila* the translation of pre-existing mRNAs must be blocked to enable the rapid changes in protein synthesis which occurs during heat shock, where in *E.coli,* the relatively shorter mRNA half lives make such a control unnecessary.

1.3.1 Transcriptional control of heat shock gene expression in *E.coli*

In *E.coli,* heat shock results in the rapid and transient expression of at least 17 proteins as a direct result of enhanced gene transcription (Neidhardt *et al.,* 1984). The heat shock-inducible genes occur in unlinked operons and constitute a single regulatory unit under the control of a positive acting protein. This protein, the RNA polymerase sigma factor σ^{32} encoded by the *rpoH* gene, is specifically required for the efficient transcription of heat shock genes (Grossman *et al.,* 1984; Landrick *et al.,* 1984). The rapid and marked increase in heat shock gene expression upon temperature elevation was initially thought to result from an alteration in the activity of σ^{32}. However, Straus *et al.*,(1987), found that the cellular concentration of σ^{32} was directly correlated to transcription from HSP gene promoters. σ^{32} is a very unstable protein that is rapidly degraded during normal growth conditions with a half life of 1 minute (Straus *et al.,* 1987; Tilly *et al.,* 1989). However, it is stabilised for several minutes upon temperature elevation and the rate of σ^{32} synthesis is also rapidly, though transiently, increased almost 10-fold. Hence, both stabilization and increased synthesis contribute to the rapid and transient increase in σ^{32} during heat shock.

σ^{32} participates in a regulatory circuit in which it positively regulates HSP synthesis, while certain of the HSPs it induces (DnaK,DnaJ, and GroE) negatively regulate the function of σ^{32} by enhancing its degradation and repressing its synthesis (Gross *et al.,* 1990). In

addition, a second sigma factor σ^E is involved in the heat shock response. At lethal temperature σ^{32} expression is completely dependent on σ^E, a factor which controls the expression of other genes at high temperatures. Thus, σ^E may be the most important factor of the regulatory network governing heat shock gene expression in *E.coli* (Gross *et al.*, 1990).

1.3.2 Transcriptional control of heat shock gene expression in eukaryotes

Transcriptional regulation also plays an important role in regulating the heat shock response of eukaryotes. An essential trans-activator of HSP genes, heat shock transcription factor (HSF), preexists in sufficient concentration but is largely inactive in unstressed cells. The induction of the heat shock response is a result of the activation of HSF bound to a short highly conserved DNA region of HSP gene promoters known as the heat shock element (HSE). The HSE was originally identified in a *Drosophila HSP70* gene as a 14 bp palindrome CTnGAAnnTTCnAG (Pelham, 1982) localised 5' to the TATA element. Further studies revealed this sequence could be reduced to C--GAA--TTC--G (Pelham, 1985; Bienz, 1985). The evidence has defined the HSE as being composed of tandem repeats of a conserved 5 bp unit, nGAAn, in alternate orientation at each half-turn of the DNA helix (Amin *et al.*, 1988; Xiao and Lis, 1988; Sorger, 1991). In general, three nGAAn units appear to be necessary for transcriptional activation after heat shock, additional units enhancing heat-induced activation, suggesting that multiple nGAAn arrays act in a co-operative fashion (Amin *et al.*, 1987).

Evidence that the HSE is the binding site for HSF comes from *in vitro* transcription experiments with extracts from *Drosophila* cells (Parker and Topol, 1984). Nuclear extracts from heat shocked cells were found to transcribe the *Drosophila HSP70* gene efficiently and HSF bound to the *HSP70* promoter at a region containing two proximal HSEs in extracts from heat shocked cells but not from unstressed cells. Unlike σ^{32}, eukaryotic HSF is constitutively synthesised and requires activation to elicit the heat shock response. In yeast, HSF is constitutively bound to the HSE and has two separate domains directing constitutive and inducible activity respectively, the latter being activated upon heat shock (Sorger *et al.*, 1987; Sorger and Pelham, 1988; Sorger,1990; Sorger, 1991). HSF from *Tetrahymena pyriformis* has been purified and, like yeast HSF, was found bound to the HSE both before and during heat shock (Carmo *et al.*, 1990). In addition, heat increased both the DNA binding affinity and extent of phosphorylation of the *T.pyriformis* HSF, properties which are also found in both yeast and *Drosophila* HSFs.

Although HSF is most commonly induced by heat shock, evidence suggests that HSF is activated either by undergoing a conformational change or indirectly through interactions with unfolded proteins (Mosser *et al.,* 1990). A comparison of HSFs from two yeast species, namely *S.cerevisiae* and *Kluyveromyces lactis,* identified a C-terminal activation domain whose activity is masked at low temperatures (Jakobsen and Pelham, 1991). Regulation of the C-terminal activator domain is dependent on a conserved heptapeptide that is distinct from the activator itself. This element binds either to the structural core of the HSF protein or to another polypeptide, holding the activator domain in an inactive configuration which is disrupted by heat shock.

The mechanism by which HSF activates transcription is unknown, although it has the ability to act at several levels. At one level HSF associates to form trimers. Both *Drosophila* and yeast HSF are trimeric molecules, binding with high affinity to the sites in HSP gene promoters that contain three 5 bp HSEs in alternating orientations (Perisic *et al.,* 1989; Sorger and Nelson, 1989). A second level of interaction is suggested from the analysis of *Drosophila* HSF, where native gel electrophoresis demonstrated that HSF is a hexameric protein (Clos *et al.,* 1990). Thus whether HSF in solution, particularly *in vivo,* exists primarily as a trimer, hexamer or higher order multimer remains to be determined. In addition, longer arrays of 5 bp units which constitute a HSE, display additional cooperation in binding HSF (Xiao *et al.,* 1991). A HSE containing 4, 5 or 6 HSE units in alternating orientation can bind two HSF multimers and tightness of binding increases dramatically with the addition of each 5 bp HSE unit.

1.3.3 Translational control of heat shock gene expression

While heat shock causes considerable changes in transcriptional patterns, effects specifically on translational control are also no less dramatic. On the other hand, it is clear that different organisms achieve rapid shifts in protein synthetic pattern in quite different ways. The best characterised organism in this connection is *Drosophila* where the heat shock response promotes the translation of heat shock mRNAs and specifically represses the translation of pre-existing mRNAs. The mRNAs pre-existing prior to heat shock are not degraded during the stress but are retained and reactivated upon recovery (Lindquist, 1981). Heat shock transcripts possess unusually long 5' untranslated leader sequences that are adenosine rich, have little secondary structure and show conserved sequences in the middle and at the 5' ends which might be recognised by the translational machinery of heat shocked cells (Holmgren *et al.,* 1981). Studies of a fusion of the *adh* gene to the *Drosophila* HSP70 promoter indicated that heat shock mRNAs are distinguished by their

sequence rather than as a result of modification of their secondary structure or compartmentalization of mRNA synthesised before or during heat shock (Klemenz *et al.*, 1985). Deletion analysis identified a region of 26 nucleotides downstream of the transcription start site of the *Drosophila HSP22* gene which is necessary for efficient transcription and also selective translation during heat shock. In yeast the situation might be different from that encountered in *Drosophila.* Yeast is not known to possess a special mechanism for sequestering pre-existing mRNAs from translation. Instead it is thought that most of these mRNAs are simply rapidly degraded when the cell is heat shocked, whilst those that are retained continue to be translated (Lindquist, 1981). In mammalian cells there seems to be no extensive sequestration of pre-existing mRNAs even though, unlike in yeast, the pre-existing mRNAs do not disappear.

Nucleotide sequence analysis reveals that all HSP genes except two do not possess introns. Since introns are present in most, if not all, genes from higher organisms, the absence of introns in heat shock genes may be functionally significant and can be related to the rapid synthesis of the respective gene products upon exposure to stress. Upon heat shock mRNA splicing is inhibited and only the products of genes without introns are synthesised (Yost and Lindquist, 1986). If cells are sublethally heat shocked prior to severe stress, mRNA processing occurs under otherwise restrictive conditions, suggesting a function for HSPs in protecting mRNA splicing from heat-induced disruption (Yost *et al.*, 1990). Ribosomes can also differentiate between heat shock and non-heat shock mRNA (Scott and Pardue, 1981). The mechanism by which such discrimination occurs is unknown but it is thought to involve sequences in the 5' untranslated leader.

1.4 The heat shock response of *S.cerevisiae*

The heat shock response was initially observed in *S.cerevisiae* as a change in the pattern of proteins synthesised when exponentially growing cells were heat shocked from 23°C to 36°C (Miller *et al.*, 1979; McAlister *et al.*, 1979). The response was transient, with maximal induction occurring 20 to 30 minutes after temperature increase and pre-shift protein synthesis patterns being restored within 60 to 90 minutes. Quantitative analysis of 500 proteins identified 80 proteins which were transiently induced at 37°C, 20 of which are classified as major HSPs, and more than 300 proteins transiently repressed at this temperature (Miller *et al.*, 1982). Many yeast HSPs can also be induced by normal developmental cues, for example when vegetative cells enter stationary phase as a result of glucose exhaustion (Boucherie, 1985) and when diploid sporulating cells are starved for nitrogen (Kurtz and Lindquist, 1984).

1.4.1 The *S. cerevisiae* HSPs

HSPs have been identified in *S.cerevisiae* with molecular weights ranging from 104 to 12 KDa.These are listed in Table 1.2, in which the corresponding cellular location and function, where known, are also described.

1.4.2 Regulation of the heat shock response in *S. cerevisiae*

In *S.cerevisiae* the heat shock response is primarily controlled at the transcriptional level (Lindquist, 1981). Genetic experiments have demonstrated that the activity of HSF in budding yeast is negatively regulated and is strongly activated by the deletion of several different regions of the HSF protein (Jakobsen and Pelham,1991). In addition to the standard eukaryotic HSE consensus sequence (nGAAn; section 1.3.2), analysis of a number of *S.cerevisiae* heat shock promoters has revealed the existence of an alternate heat shock induction sequence (AGGGGT) (e.g. at -360 in *CTT1*) and this may have a role in the functioning of many yeast heat shock genes (Marchler *et al.,* 1992). HSF was purified from *S.cerevisiae* according to its HSE binding properties (Sorger and Pelham, 1987). Unlike HSF from *Drosophila,* human and *S.pombe,* HSF from *S.cerevisiae* is bound to DNA both before and after heat shock. Like human HSF, *S.cerevisiae* HSF becomes highly phosphorylated following heat shock, although the 200-fold increase in its activity between 15°C and 39°C is not now thought to reflect this change in phosphorylation state (Sorger, 1991).

In *S.cerevisiae,* the *HSF1* gene encoding HSF is essential for cell viability even in the absence of heat shock, suggesting that the function of HSF at low temperatures may be to promote transcription of heat shock genes at a basal level (Sorger and Pelham, 1988). Analysis of *HSF1* revealed two regions responsible for HSF activity, a N-terminal region which mediates sustained HSF activity and a C-terminal region which is essential for transient increases in activity (Sorger, 1990). These two activation regions may be regulated independently in response to different stimuli (Sorger, 1990; Nieto-Sotelo *et al.,*1990). The exposure of yeast cells to elevated temperatures results in both transient and sustained changes in HSF activity, the transient changes being characterised by the rapid increase in activity that constitutes the classical heat shock response (Miller *et al.,* 1979; McAlister *et al.,* 1979).

Table 1.2 Heat shock proteins of yeast

(modified from table in Mager and Moradas-Ferreira, 1993)

Designation	Cellular localization	Function
HSP150	(Secretory)	Unknown
HSP104	Nucle(ol)us	Stress tolerance
HSP90	Cytosol/nucleus	Chaperone
HSP70		
SSA1	Cytosol	Chaperone
SSA2	Cytosol	Chaperone
SSA3	Cytosol	Chaperone
SSA4	Cytosol	Chaperone
SSB1	Unknown	Unknown
SSB2	Unknown	Unknown
SSC1	Mitochondria	Chaperone
SSD1 (*KAR2*)	Endoplasmic reticulum	Chaperone
HSP60	Mitochondria	Chaperone
HSP30	Plasma membrane	Unknown
HSP26	Cytosol/nucleus	Unknown
HSP12	Cytosol?	Unknown
Ubiquitin	Cytosol	Protein degradation
Enzymes		
Enolase	Cytosol	Glycolysis
Glyceraldehyde 3-phosphate dehydrogenase	Cytosol	Glycolysis
Phosphoglycerate kinase	Cytosol	Glycolysis
Catalase	Cytosol	Antioxidative defense

1.5 The molecular character of the cellular thermometer

Although the transcriptional activators of heat shock genes in both prokaryotes and eukaryotes are now being identified, the molecular mechanism which senses changes in temperature is presently unknown. Recent work suggests that a homeostatic mechanism involving the level of free HSPs in the cell provides a thermometer for detecting and reacting to temperature changes. Under normal growth conditions, HSPs may bind to HSF and repress its activity. These HSPs may include HSP70 and HSP90, binding of HSP90 to HSF having been recently demonstrated(Nadeau *et al.*,1993).During heat shock competition with high levels of thermally damaged proteins for binding of HSPs may cause the dissociation of HSF-HSP complexes, causing either an increase in the DNA-binding affinity of the factor (*Drosophila*) or activation of the C-terminal domain of HSF (yeast). DNA-bound HSF may then direct increased HSP synthesis until levels are sufficiently high to result in the reassociation of HSPs with HSF and the re-establishment of the repressed state. Ensuring overproduction of HSPs will ensure a large free pool of these HSPs and may cause HSF to be inhibited in its action. Strains which synthesise low levels of HSPs might prematurely activate the heat shock response. In yeast, strains carrying deletions of two of the constitutively expressed HSP70 genes (*SSA1* and *SSA2*) express HSPs at high levels, even at 23°C (Craig and Jacobsen, 1984). However, it should be noted that HSP70 is essential for cell viability so reducing the levels of this protein may itself be stressful, causing activation of the heat shock response by a mechanism other than that described above. One of the early events of heat shock must be a dramatic drop in the pool of free HSPs. This is a feasible proposition given the increase in aberrant protein that will ensue, but it has only been reported for levels of free ubiquitin which decrease 75% (Rose and Warms, 1987). Recombinant *Drosophila* HSF produced in *E.coli* will bind to HSEs with high affinity in the absence of heat shock (Clos *et al.*, 1990). In contrast to this, the same HSF will not bind to HSEs if produced in *Xenopus* oocytes. This suggests that HSF may interact with one or more negative regulators found in eukaryotic cells, possibly eukaryotic HSPs. However, in this thesis (Chapter 3) it is demonstrated that the overproduction of HSP90 does not interfere with normal heat shock induction of HSP genes in *S. cerevisiae.*

Aberrant protein seems to be a recurring theme in studies to investigate the heat shock response trigger. Firstly, many of the conditions known to induce HSP genes are thought to cause denaturation of intracellular proteins (Table 1.1). Denatured Lambda repressor can induce the heat shock response in *E.coli* (Parsell and Sauer, 1989) and injecting denatured protein (but not native protein) into *Xenopus oocytes* has the same effect (Ananthan *et al.*, 1986). Also a

mutant mouse cell line which cannot ubiquitinate proteins above a certain temperature exhibits abnormally high synthesis of HSPs at such temperatures (Finley *et al.*, 1984). Finally, there is evidence from biophysical studies of cellular protein denaturation *in vivo* within the temperature range of HSP induction in both bacteria and mammalian cells (Lepock *et al.*, 1988, 1990).

It has been suggested that HSF itself is a cellular thermometer (Hightower, 1991). HSF from unshocked HeLa cells can be induced to bind HSEs *in vitro* by exposing nuclear extracts to elevated temperatures (Larson *et al.*, 1988) and other conditions that promote protein unfolding such as nonionic detergents and increasing concentrations of urea (Mosser *et al.*, 1990). Such results, however, do not distinguish between models where HSF *per se* responds to environment by direct conformational change and models where it is the interaction of HSF with other proteins that is affected.

Heat shock has many effects on the cell besides the build up of aberrant protein. For example, intracellular pH falls and levels of calcium rise. These may also play a role in triggering the heat shock response. Evidence for this is the activation of HSF *in vitro* by decreasing the pH of buffers and by increasing levels of calcium (Mosser *et al.*, 1990). There may even be different induction pathways triggered by different cellular events. Yuzawa has isolated two groups of *E.coli* mutants, one defective in responding to unfolded proteins as inducers but still heat inducible and the other unresponsive to both inducers. This suggests the existence of at least two distinct induction pathways (Hightower, 1991).

Finally, the regulation of the heat shock response in eukaryotes has taken a new twist. Morimoto has described the cloning and characterisation of two murine HSF genes (*HSF1* and *HSF2*) whose products display heat inducible and constitutive HSE binding respectively (Hightower, 1991). This system may be widespread among higher eukaryotes. There are also two human HSFs, HSF1 exhibiting heat inducible HSE binding (Rabindran *et al.*, 1991), while the binding characteristics of HSF2 are unknown (Schuetz *et al.*, 1991). It is well established that HeLa cells have two HSE binding activities, one found in unshocked extracts and the other in stressed cells (Kingston *et al.*, 1987). The HSE protein complex in unstressed cells may correspond to DNA bound to HSF2. Different HSFs may have evolved to respond to different temperature thresholds or to chemical stress signals. This emerging data suggesting the existence of separable induction pathways, make regulation of the response to stress far more complicated than previously thought. Treatment with antibiotics known to inhibit various aspects of

ribosome function has implicated ribosomes as the sensors for both heat and cold shock in prokaryotes (VanBogelen and Neidhardt, 1990). Ribosomes may serve a similar role in eukaryotes, HSPs functioning to alter the translational capacity of the cell.

1.6 Thermotolerance

Pre-treatment of cells to a sub-lethal heat shock induces their temporal resistance to a higher lethal temperature, a phenomenon known as thermotolerance (McAlister and Finkelstein, 1980). This phenomenon is exhibited by a variety of systems including bacteria, *Drosophila,* plant and mammalian cells (Hall, 1983), and is referred to as acquired thermotolerance. We now know that heat shock induces thermotolerance in all the major microbial kingdoms. Elucidation of the mechanism in genetically-tractable organisms such as *E.coli* and yeast promises therefore to lead to real practical benefits for the use and control of quite distantly-related microbes. For example, it may enable the manipulation of strains for altered robustness in industrial bioprocesses or after release into the environment. It will also contribute to the design of food processing procedures for more effective inactivation of food-contaminating microbes. As a phenomenon widespread in industrially-important, food-contaminating and pathogenic microorganisms, thermotolerance acquisition has major implications for microbiological safety (Piper, 1993). Acquired thermotolerance has also been extensively studied in mammalian cells. This is largely because of the upsurge in interest in the use of heat as an adjunctive technique for the treatment of certain cancers in humans.

1.6.1 The role of heat shock protein and other factors in acquired thermotolerance

Individual HSPs have been implicated in acquired thermotolerance, including HSP70 (Angelidis *et al.,* 1991) and HSP104 (Sanchez and Lindquist, 1990). However the importance of HSPs in acquired thermotolerance may have been overestimated. For instance, a *S.cerevisiae* strain carrying a deletion of the gene coding for HSP104 may show defects in acquired thermotolerance, but they only manifest themselves after five minutes at the lethal temperature (DeVirgilio *et al.,* 1991a; Sanchez and Lindquist, 1990; Parsell *et al.,* 1991). Furthermore, the reduction in acquired thermotolerance exhibited by this mutant almost disappears when the time of pre-adaptation at the non lethal temperature is increased from 30 to 60 minutes (DeVirgilio *et al.,* 1991a). This may suggest a minor role for individual HSPs in acquired thermotolerance. All available data lead to the conclusion that, though HSPs may play a minor role in thermotolerance, the mechanisms

that make the greatest contribution are more poorly-understood HSP-independent events associated with heat shock (Piper, 1993). It is obvious that experiments designed to investigate such mechanisms (involving inhibitors of protein synthesis or transcription) have their drawbacks. For instance, the widely employed transcriptional inhibitor 1,10-phenanthroline enhances transcription of certain heat shock genes (Adams and Gross, 1991). These problems can be partly overcome by using the recently discovered *hsf1-m3* mutation which prevents heat activation of HSF and which, even though it causes a general block to heat inducible transcription, has hardly any effect on inducible acquired thermotolerance (Smith and Yaffe, 1991).

One of the most dramatic consequences of heat shock in yeast is a more than 100-fold increase in levels of the non-reducing disaccharide trehalose (Fig.1.2). This has been observed in *S.cerevisiae, S. pombe* and *Neurospora* (Hottiger *et al.*, 1987a; DeVirgilio *et al.*, 1990; Neves *et al.*, 1991). Moreover, thermotolerance increases in parallel to trehalose accumulation and decreases in parallel with the rapid trehalose mobilisation when cells are shifted back to normal temperatures, irrespective of the presence or absence of cycloheximide (DeVirgilio *et al.*, 1990). Accumulation of trehalose also occurs on exposure to other inducers of thermotolerance, such as ethanol and hydrogen peroxide (Attfield, 1987).

It is well known that physiological state has a dramatic effect on both basal and acquired thermotolerance. The level of cAMP-dependent protein kinase (protein kinase A) plays a major role here. Yeast in rapid fermentative growth has high levels of cAMP and low basal thermotolerance. In contrast, quiescent cells have low levels of cAMP and high thermotolerance. Moreover, studies employing mutants of the yeast cAMP system show that low protein kinase A activity correlates with high thermotolerance (Iida,1988), whereas high protein kinase A activity results in low thermotolerance (Tanaka *et al.*, 1989). Heat acquired thermotolerance in rapidly growing cells is also dependent on the activity of this kinase. Compared to its isogenic wild type, a mild heat shock will not induce acquired thermotolerance in a strain (bcy1)with constitutively high protein kinase A activity (Shin *et al.*, 1987). Conversely, cells with constitutively low levels of cAMP are constitutively thermotolerant behaving in exponential growth as if they have already experienced a sub-lethal, pre-adaptive heat shock (Shin *et al.*, 1987).

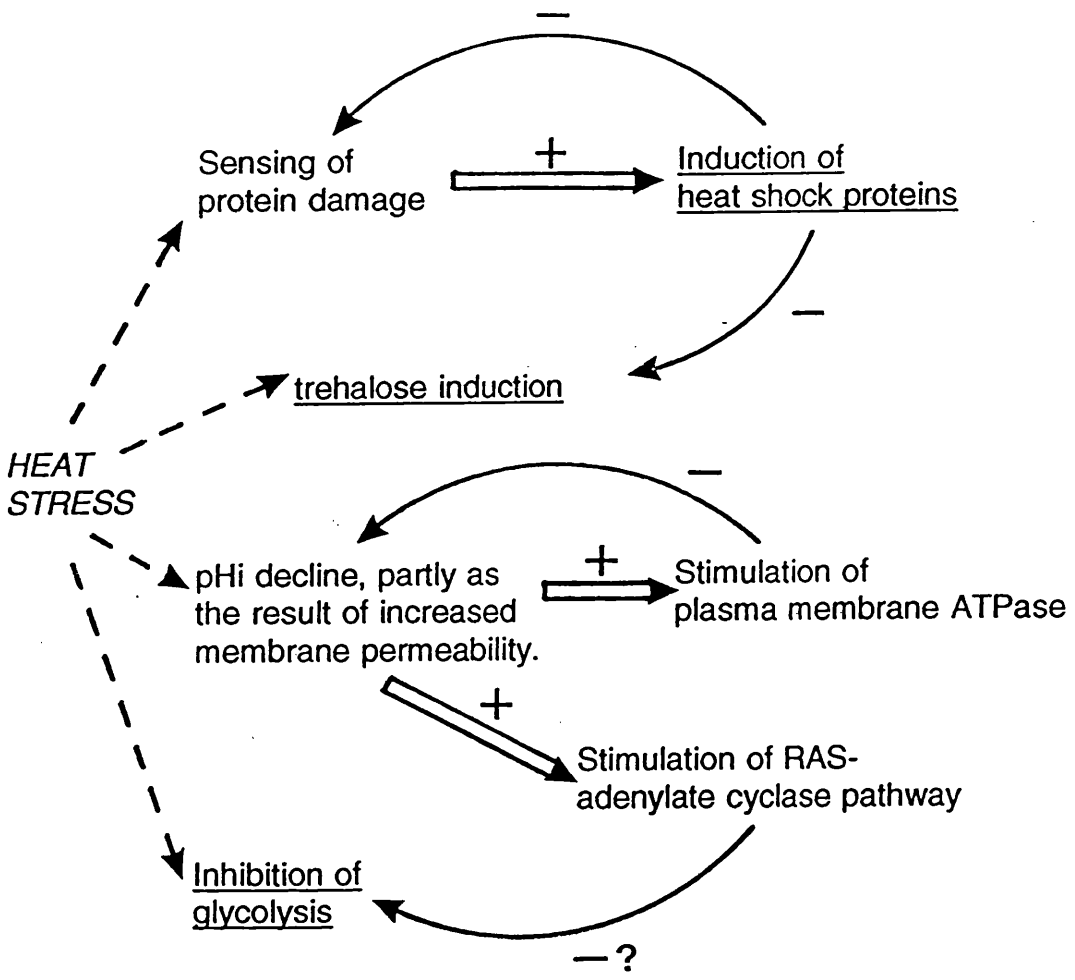


Figure 1.2. Regulatory circuits known, or suspected of operating, in the yeast response to heat stress (from Piper, 1993).

The primary signal sensing protein damage is probably the lack of free, uncomplexed HSP70, as all the available HSP70 binds to the accumulated heat-damaged protein (Craig and Gross, 1991). The same signal may also trigger the trehalose induction with heat shock, since trehalose is also known to be under negative regulation by heat shock proteins during the recovery from heat stress (Hottiger *et al.*, 1992). The increase in plasma membrane ATPase action is rapid and therefore, at least initially, reflects the pHi decline rather than any ATPase activation due to the slower increase in cAMP. Proton extrusion from the cell resulting from this enhanced ATPase action probably helps to counteract the effects of the stress-induced pHi decline (Coote *et al.*, 1991; Panaretou and Piper, 1990).

Many agents can induce thermotolerance in addition to heat. In *S. cerevisiae* these agents include ethanol (Plesset *et al.*, 1982), osmotic stress (Trollmo *et al.*, 1988) and hydrogen peroxide (Collinson and Dawes, 1992). However the physiological overlap between some of these agents and heat shock is unidirectional i.e. heat conditioning does not confer resistance to osmotic (Trollmo *et al.*, 1988) or oxidative stress (Collinson and Dawes, 1992). These phenomena are poorly understood, and understanding stress tolerance remains a future research goal.

1.7 Biochemical and physiological changes that occur in cells experiencing heat stress

Fig.1.2 summarises some of the events known to occur in yeast cells as a result of heat stress. Investigating these changes may yield important information about the mechanisms by which cells recognise stress and acquire stress tolerance. A consequence of heat shock is the abrupt arrest of cell growth and proliferation. However, after a period of time both bacteria and yeast apparently adapt to their new temperature, normal gene expression is re-established, and cells re-acquire their normal proliferative activities (Lindquist, 1986). In contrast, in higher organisms where body temperature is tightly regulated, acclimatisation is not typically observed and cells continue to synthesise HSPs until death.

1.7.1 Changes in cell morphology

Dramatic morphological changes caused by heat stress have been revealed by electron microscopy. One obvious effect is the accumulation of perichromatin granules throughout the nucleus, possibly related to inhibition of precursor mRNA splicing (Yost and Lindquist, 1986). Also, heat shock elicits a loss of the granular components of the nucleolus (representing pre-ribosomes) (Welch and Suhan, 1985), consistent with the inhibition of proper pre-rRNA processing and a cessation of ribosome biogenesis (Bouche *et al.*, 1979). These changes in nuclear and nucleolar morphology appear far less dramatic in cells first made thermotolerant by an incubation at a non-lethal temperature (Pelham, 1984; Welch and Mizzen, 1988). HSP70 has been implicated in limitation of damage to nucleoli as well as enhancing recovery to normal morphology on temperature downshift (Pelham *et al.*, 1985).

On heat shock mitochondria appear swollen with very prominent cristae and enlarged intracristal spaces (Welch and Suhan, 1985). Not surprisingly this is accompanied by a reduction of cellular ATP levels in *Drosophila* (Leenders *et al.*, 1974) and *Tetrahymena*

(Findly *et al.,* 1983). However ATP levels in fermentative yeast do not change on heat shock (J. Dickinson, personal communication), even though glycolysis is inhibited (Neves and Francois, 1992).

1.7.2 Changes in the levels of trehalose in thermotolerance induction with heat shock

A large pool of trehalose accumulates in the cytosol of yeast both after heat shock and during the approach to G1 arrest. This trehalose is thought to act primarily as a stress protectant rather than a storage carbohydrate, its levels generally showing a good correlation with thermotolerance (Wiemken, 1990). *E.coli* cells also accumulate trehalose at stationary phase (though not with heat shock), this trehalose being important for the thermotolerance of stationary phase *E.coli* (Hengge-Aronis *et al.,* 1991)

The trehalose accumulation in yeast at the approach to stationary phase reflects the decline in cAMP activity, loss of protein kinase A activity resulting in inactivation of the cytoplasmic neutral trehalase (Thevelein,1988b;1991). However the rapid trehalose accumulation with a preconditioning heat shock is not controlled by cAMP levels. The accumulation of trehalose during heat shock is partially dependent upon *de novo* protein synthesis (DeVirgilio *et al.,* 1991a; Coote *et al.,* 1992), indicating that heat stress might both stimulate the activity of preexisting trehalose-6-phosphate synthetase as well as increase *de novo* synthesis of this enzyme. Following subsequent temperature shift-down the accumulated trehalose is rapid mobilised, a mobilisation intimately controlled by the levels of certain HSPs synthesised during the heat shock (Fig.1.2 and Hottiger *et al.,* 1992). HSP70 levels act as the major controller of this trehalose mobilisation, with HSP90 and HSP104 levels exerting comparatively more minor effects (Hottiger *et al.,* 1992; also Chapter 5 of this thesis). Free HSP70 levels may therefore modulate the heat induction of trehalose, in much the same way as they acts to regulate the synthesis of HSPs (Fig.1.2 and Craig and Gross, 1991). This finding of a HSP control over trehalose raises the possibility that HSPs might influence thermotolerance indirectly through their effects on trehalose level. In unstressed yeast increased protein kinase A activity activates neutral trehalase, thereby promoting trehalose mobilisation. In heat shocked cells the synthesis of trehalase enzyme is enhanced at 40°C, but actual trehalase activity decreases due apparently to a phosphorylation which is protein kinase A-independent and rapidly reversed as cells are shifted back to 27°C (DeVirgilio *et al.,* 1991b). Also heat shock causes changes in the kinetic properties of the enzymes of trehalose synthesis (Neves and Francois, 1992).

Thermotolerance induction with either declining cAMP or with heat shock of *S. cerevisiae* occurs in parallel with a strong induction of trehalose. However not all inducers of thermotolerance trigger trehalose accumulation. Examples of the latter include the reduction of intracellular pH (pHi) using weak organic acids (Coote *et al.*, 1991), treatment with ethanol or the plant cytokinin N^{6}- (Δ^{2}-isopentenyl) adenine (Coote *et al.*, 1992) and osmostress (which causes only a slight trehalose increase: Singh and Norton, 1991). It appears, therefore, that trehalose induction might be one major route for the yeast cell to acquire thermotolerance, yet not the only way of achieving this objective. Trehalose might contribute to thermostability by influencing hydrogen bonding (H-bonding). Yeast mutants defective in either the trehalose or the HSP inductions of heat shock promise to greatly clarify the relative contributions of HSPs and trehalose to the thermotolerance acquisition with heat shock.

1.7.3 Changes of plasma membrane and intracellular pH (pHi) after heat shock

Heat stress and certain other stress agents such as ethanol act to increase the permeability of membranes. A major effect of increased membrane permeabilisation is increased proton influx, a rapid decrease in the pHi of yeast being an almost immediate effect of mild heat and a number of other stress agents (Leao and VanUden, 1985; Weitzel *et al.*, 1987; Eraso and Gancedo, 1987; Cole and Keenan, 1987). Yeast modifies both the lipid and protein compositions of its membranes in response to heat stress (Watson, 1987; Panaretou and Piper, 1992). As in other organisms, the saturation of membrane lipids increases as part of long-term acclimation to high temperatures. Whole cell ^{3}H and ^{13}C nuclear magnetic resonance (NMR) has shown that a mild, pre-conditioning heat shock influences the susceptibility of yeast lipid bilayers to disordering caused by freeze-thawing. This disordering, manifested as an increased mobility of aliphatic acyl groups, is substantially prevented by prior mild heat shock treatment. This protection would appear to be protein-mediated since it is blocked by the presence of cycloheximide during the heat shock (Komatsu *et al.*, 1990). Heat shock also increases cell viability following subsequent freezing and thawing (Obuchi *et al.*, 1990). The pHi decrease with heat shock stimulates plasma membrane ATPase-catalysed proton extrusion (Fig.1.2), readily demonstrable as an increased acidification of the medium by intact cells in response to heat (Coote *et al.*, 1991). This increased ATPase action and the need for a general restoration of homeostasis, probably represent one of the major energy demands imposed by stress. It is not surprising, therefore, that plasma membrane ATPase action influences not just thermotolerance, but also a number of other stress tolerances (Panaretou and Piper, 1990).

Also inhibition of plasma membrane ATPase hypersensitises yeast to thermal death (Coote *et al.*, 1991). *S.cerevisiae* has recently been found to have a single integral membrane HSP (HSP30; Table 1.2), a protein found predominantly in the plasma membrane fraction (Panaretou and Piper, 1992; Regnacq and Boucherie, 1993). Such a HSP might conceivably act to stabilise or protect plasma membrane components during stress.

Intracellular acidification, as with heat shock, provides a stimulation to the RAS-adenylate cyclase pathway (Fig.1.2; Thevelein, 1991). This occurs irrespective of the catabolite repression status of the yeast, intracellular cAMP increasing approximately twofold with 24°C-36°C (Boutelet *et al.*, 1985; Camonis *et al.*, 1986) or 30°C-42°C (Coote *et al.*, 1992) heat shocks. Small though this cAMP increase is, it may belie a rather greater stimulation to cAMP synthesis since much of the cAMP synthesised by yeast is lost to the culture medium (Smith *et al.*, 1990). It is possible to induce rapid pHi decline in cells in the absence of stress by the addition of weak organic acids such as benzoic acid or sorbic acid (both widely used as food preservatives) at low medium pH values. The undissociated forms of these acids will then readily cross the cell membrane and reduce pHi by dissociating in the higher pH environment of the cytosol. The thermotolerance induced by sorbic acid occurs with practically no induction of either HSPs (Chapter 7 of this thesis) or of trehalose (Coote *et al.*, 1992; DeVirgilio, unpublished). Remarkably sorbic acid treatment also rapidly renders low pH yeast cultures incapable of responding to a heat shock by the normal heat induction of HSPs, despite the fact that the cells still remain competent of protein synthesis (Chapter 7 of this thesis). Ethanol also induces a pHi decline and a rather slow increase in thermotolerance (Coote *et al.*, 1991) yet, unlike weak organic acids, is a strong inducer of HSPs.

1.7.4 Protein phosphorylation and stress

Even though considerable effort has been applied towards understanding cellular and biochemical responses to heat, the underlying processes occurring at the molecular level are still poorly understood. It is well established that protein phosphorylation coupled to signal transduction are major processes in the regulation of cellular functions. Changes in the phosphorylation state of ribosomal, nuclear and nucleolar proteins caused by stress have been reported and are reviewed in section 6.1. Results of preliminary studies designed to detect heat-induced protein phosphorylation events of the cytosolic fraction are presented in chapter 6.

1.8 Aims of this study

This study looked at the role of the heat shock protein HSP90 in *S.cerevisiae* under normal and heat shock conditions and also investigated the effects of weak acid preservatives on the heat shock response and thermotolerance. The specific aims were:

(1) To characterise effects of the expression of multiple gene copies of the *HSP82* gene, as a route to high level production of HSP90 protein for structural and functional investigations (Chapter 3).

(2) The purification of HSP90 and preparation of an HSP90 affinity resin that can be used to identify other proteins selectively binding to HSP90 (Chapter 4).

(3) To determine the influences of differing cellular levels of HSP90 on induction of trehalose, induction of other HSPs by heat shock and kinasing of a specific phospho-protein in extracts from heat shocked cells (Chapter 5, 6).

(4) To identify selective inhibitors of the heat shock response (Chapter 7).

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

Standard Reagents:	AR grade supplied by Sigma, BDH & Fisons
Microbiological media:	supplied by Difco. Auxotrophic requirements supplied by Sigma.
Electrophoresis Reagents:	Acrylamide, bis-acrylamide (Electron grade) and ammonium persulphate supplied by BDH. TEMED supplied by Sigma. Molecular weight markers: i) 29-205 kDa ii) 14.2-66 kDa iii) 14.3-66 kDa (prestained) all supplied by Sigma.
Protease inhibitors:	PMSF, TPCK and Pepstatin A all supplied by Sigma.
Enzymes:	RNase-Free DNaseI supplied by BRL. DNase-Free RNase supplied by Sigma. T4 DNA Ligase supplied by Anglian Biotechnology. Zymolyase-20T supplied by Seikagaku Kogyo Co. Lysozyme supplied by Sigma. Protease V8 from *Staphylococcus aureus* supplied by ICN immunobiologicals.
Restriction enzymes:	supplied by Anglian Biotechnology.
Transfer membranes for protein (Western) blotting:	Genescreen (0.2 μ pore size) supplied by Dupont (NEN research products). PVDF supplied by Millipore (0.4 μ pore size) and Bio-Rad (0.2 μ pore size).
Immunological reagents:	Biotinylated goat anti-rabbit IgG, streptavidin-horse-radish peroxidase conjugate and 4CN all from Bethesda

	Research Laboratories Life Technologies, Inc (BRL).
Reagents for protein assay:	Dye reagent concentrate and BSA standard from Bio-Rad.
Radiolabelled amino acids and nucleotides:	L- [^{35}S]-Methionine (1,000 Ci/mmol; 10μCi/μl), and L-[4,5-^{3}H(N)]-Leucine (60 Ci/mmol; 1μCi/μl) supplied by Dupont (NEN research products). [γ-^{32}P]-5'-Adenosine triphosphate (3,000 Ci/mmol; 10μCi/μl) supplied by Amersham.
Other reagents:	Cycloheximide and Coomassie blue R-250 were supplied by Sigma. SDS (biochemical grade was supplied by BDH. Ecoscint A was supplied by National Diagnostics (Manville, New Jersey, USA). All buffer salts (Tris, MES and CAPS) supplied by Sigma.

2.2 *S.cerevisiae* Strains

The *Saccharomyces cerevisiae* strains used in this study are detailed in Table 2.1.

2.3 *E.coli* Strains

The *E.coli* JM101 (*lac pro thi supE* F'*tra D36 proAB lacZ* M15) was employed for plasmid DNA manipulations and transformations (Messing, 1983).

2.4 Plasmids

All the plasmids used are listed in Table 2.2 and were derived from the yeast:*E.coli* shuttle vector pMA3a (Kingsman *et al.*, 1985).

2.5 Growth media and culture conditions

The recipes for the growth media used in this study were as in Miller (1972) and Sherman *et al.* (1983). 2% bacto-agar was added for the preparation of solid media.

All solutions and glassware were sterilised by autoclaving at 15psi for 20 minutes, or if any were heat labile, by filtration using a Millipore apparatus.

2.5.1 *S.cerevisiae*

Routinely 100ml yeast liquid cultures were grown in 500ml flasks with constant shaking at 25°C.

Media [all concentrations % (w/v)]

YEPD:	2% glucose, 2% bactopeptone, 1% yeast extract
YEPGE:	3% glycerol, 2% ethanol, 2% bactopeptone, 1% yeast extract
Sporulation:	0.3% sodium acetate, 0.04% raffinose
Minimal:	Standard Defined (SD) minimal medium, 2% glucose, 0.67% yeast nitrogen base without amino acids, plus one or more of the following auxotrophic requirements where appropriate; L-leucine (30mg/L), L-lysine (30mg/L L-tryptophan (20mg/L), L-histidine (20mg/L), uracil (20mg/L) and adenine (20mg/L).

Table 2.1 *S. cerevisiae* **strains**

Name	Genotype	Source
W3031A	a*ade2-1, can1-100, his3-12,16, leu2-3,112 trp1-1, ura3-1;*	gift of R. Rothstein.
PMY1	*a leu2-3,112, his4;*	gift of P. Meacock.
MD40-4c	*a leu2-3,112, ura2, his3-15, trp-1;*	gift of A.and S.M. Kingsman. Cheng *et al.*, (1992)
W303leu	*LEU2 his3 ura3 HSP82 HSC82* *LEU2 his3 ura3 HSP82 HSC82*	gift of S. Lindquist. Borkovich *et al.*,(1989)
CLD82	*leu2 his3 ura3 HSP82 hsc82::LEU2* *leu2 his3 ura3 HSP82 hsc82::LEU2*	
PLD82	*leu2 his3 ura3 hsp82::LEU2 HSC82* *leu2 his3 ura3 hsp82::LEU2 HSC82*	
BJ2168	a *leu2, trp1, ura3-52, prb1-1122, pep4-3,*	gift of H.R.B. Pelham. Sorger &Pelham,(1987)
SUB62	a *his3, leu2, lys2,, ura3,*	gift of D. Finley. Finley *et al.*, (1987)
144-3a	*a bar1,his4, leu2, ura3,*	gift of C. DeVirgilio. (unpublished: C. DeVirgilio)
224A-12D	mutant derived from 144-3a which does not accumulate trehalose with heat shock.	

Table 2.2 Plasmids used in this study

Name	Description	Selective marker		Source
		Yeast	*E.coli*	
p82-2B	3.25 kb double *Eco*RI fragment of yeast DNA containing 2u ORI-*STB* and *LEU2d* plus 2.6 kb *Hin*dIII+*Mlu*I fragment containing the *HSP82* gene and part of the pUC8 polylinker.	LEU2	*Amp*	Subcloned in this Lab.
pMA3a	3.25 kb *Eco*RI fragment from yeast containing the 2u origin and *LEU2d* inserted into pBR322.	LEU2	*Amp*	A. J. Kingsman, University of Oxford. Kingsman *et al.*, (1985)
pHSE2	A tandem HSE inserted into the disabled CYC1 promoter of a CYC1 promoter-*lacZ* fusion. Also has URA3 and pBR322 sequences.	URA3	*Amp*	P. Sorger, MRC Cambridge Sorger &Pelham, (1987)

Yeast strains were maintained as frozen stocks in 2xYEPD plus 15% glycerol at -70°C.

2.5.2 Yeast transformation

Yeast transformation with plasmid DNA was achieved by the following modification of the Lithium Acetate Transformation procedure (Ito *et al.*, 1983).
Yeast cells were grown overnight in 100ml YEPD supplemented with 20mg/L adenine at 25°C to mid logarithmic phase($5x10^6$), then harvested and washed once with sterile TE buffer (1mM Na_2EDTA, 10mM Tris-HCl pH8) before resuspension in 5ml LA buffer (0.1M lithium acetate in TE). After incubation at 25°C for 30min the cells were harvested, and resuspended in 0.5ml LA. To 100µl competent cells 2µg plasmid DNA and 100µg ss.carrier DNA were added. After incubation on ice for 10 mins, 1ml 70% aqueous Fison's PEG4000 (polyethylene glycol) was added and incubation continued at 25°C for 45 min. After heat shock for 5min at 42°C, cells were harvested and resuspended in 0.5ml YS(1M sorbitol in 50% YEPD supplemented with 20mg/L adenine). Following incubation at 25°C for 60min they were plated as a 100µl aliquot to each selective plate. Transformant colonies usually appeared after 4-5 days at 28°C and were removed from the agar with a sterile loop and resteaked on selective media.

2.5.3 *E.coli*

E.coli were cultured in 2TY (1.6% bactotryptone, 1% yeast extract, 0.5% NaCl) at 37°C. Cultures for plasmid transformations and selection were grown in 2TY plus ampicillin at a final concentration of 100mg/L.

2.5.4 Monitoring of Cell Growth

Cell growth in liquid media was monitored either :
(1) by using an Improved Neubauer haemocytometer (Hawksley) or
(2) spectrophotometrically via absorbance at 600nm. Approximate cell number was then determined from a standard curve of optical density against cell number which had been calibrated using a haemocytometer.

2.5.5 Genetic techniques

(1) Crossing of haploid strains and isolation of diploids.
The prototrophic selection technique of mixing two suspensions of haploid strains, of

opposite mating-type and different biochemical requirements, on minimal medium (MM) agar plates was used to isolate diploid colonies. The auxotrophy of each diploid relative to its haploid parents was checked by inoculation onto MM plates.

(2) Sporulation
Dense suspensions of diploid cell cultures, previously grown overnight on YEPD plates, were spread on sporulation medium (SPM) and incubated for 3-5 days, which is the optimum time for yielding maximum numbers of viable spores. Efficiency of sporulation was determined by light microscopy.

(3) Tetrad analysis
Ascospore tetrads were microdissected using the De Fonbrune micromanipulator.

2.5.6 Random spore analysis

Following 3-5 days incubation of diploids on sporulation media, asci walls were digested by incubation in 0.5ml sterile water containing zymolyase (10mg/ml) for 1h at room temperature. This digest was vortexed with 0.5ml sterile liquid paraffin and the phases separated by gentle centrifugation. The paraffin phase containing spores was then re-extracted with 0.5ml sterile water and then plated onto YEPD plates. Colonies from germinated spores were analysed by replica plating onto the appropriate selective media.

2.6 Assay for stress tolerance

All stress tolerance experiments were done on cells in exponential growth (0.5-1x10^7 cells/ml) at 25°C on YEPD. Before each experiment *S. cerevisiae* cultures were briefly sonicated for 5sec at 5μ followed by 5sec rest (5x), a treatment which was just sufficient for no cell aggregates to be seen by light microscopy [a microprobe (tuned to 23KHZ) of an MES Soniprep Ultrasonic Disintegrator was used]. The stress was then applied (2.6.1), after which cells were spread on YEPD plates using dilutions designed to give 300 cells per plate. In all of the stress tolerance experiments killing was measured from the ability of the cells to form colonies (including *petites*) when spread on YEPD plates and incubated 2-3 days at 28°C.

2.6.1 Assay for thermotolerance

Both basal thermotolerance (survival after a single step upshift to a lethal temperature) and

acquired thermotolerance (survival at lethal temperature after pre-adapting cells by incubating at a sub-lethal temperature) were assayed. The non acute treatment used for induction of acquired thermotolerance was a rapid shift of part of each culture from 25°C to 38°C; cells being maintained at 38°C for 40min prior to an upshift to 52°C for a variable period. An identical portion of each culture (the cells uninduced for thermotolerance) was immediately transferred from 25°C to 52°C (or 50°C) for variable times. At intervals from 0-15min after the shift to 52°C or 50°C, aliquots were rapidly diluted into 5ml of YEPD (a 10x fold dilution) maintained at room temperature and the cells seeded onto YEPD plates within 20 min of the exposure to the high temperatures.

2.7 Biochemical assays

2.7.1 Glucose assay

Glucose in yeast culture supernatants was determined using the Glu-cinet Glucose Assay Kit (Technicon). 1ml of culture was harvested by centrifugation. 20, 10 or 5µl of supernatant was added to 2.5ml of Glu-cinet reagent and assayed according to the manufacturer's instructions. Volumes used depended on the stage of growth at which the sample was taken in order to stay within the limits of the assay.

2.7.2 Extraction and assay of cAMP

cAMP was extracted and assayed using the Amersham cAMP Assay Kit (based on Tovey *et al.*, 1974). After harvesting 15ml of yeast culture at 4°C, the cell pellet was immediately resuspended in 250 µl of cold 6% (w/v) trichloroacetic acid (TCA). The suspension was kept on ice for 30min with frequent mixing. Cell metabolites were released by freezing in liquid nitrogen, followed by thawing on ice. This process was repeated twice and the extract then centrifuged at 10,000rpm for 10min at 4°C. The supernatant was ether extracted three times and then adjusted to pH 7.5 with 1M Tris. The samples were freeze-dried and the resulting precipitate redissolved in cAMP assay buffer (0.05M Tris-HCl, 4mM EDTA pH 7.5). cAMP concentrations were determined using the Amersham cAMP Assay Kit according to the manufacturer's instructions.

2.7.3 Assay of trehalose

Portions of each culture to be assayed were harvested, washed with ice-cold water and extracted with 500mM ice-cold TCA. Trehalose in the acid extracts was measured by the

anthrone assay (Lillie and Pringle, 1980).

2.7.4 Assay of β-galactosidase

Two methods were used for *in vitro* assay of β–galactosidase in yeast. In the first method, a crude cell extract was prepared and activity was normalized to the amount of protein assayed. In the second method, the cells were permeabilized to allow the substrate to enter the cells, and the activity was normalized to the number of cells assayed. The latter method was particularly suited for observing changes of activity within a single strain and was used for results presented in this thesis.

Cells grown to $1x10^6$-$1x10^7$/ml were harvested by centrifugation and resuspended in 500µl of Z buffer (Miller 1972). 60µl of chloroform and 40µl of 0.1% SDS were added. After 10 sec vortexing, samples were preincubated at 28°C for 5 min. The reaction was started by addition of 0.2 ml ONPG (4mg/ml in Z buffer) and stopped by addition 0.5 ml of Na_2CO_3. Cell debris was removed by centrifuging for 10min and the OD_{420} of the supernatant measured. Units of β–galactosidase per OD_{600} unit of culture were determined from the relationship:

$$\frac{OD_{420}}{OD_{600} \text{ of assayed culture x volume of culture assayed x time (min)}}$$

Where OD_{420} is the optical density of the product, *o*--nitrophenol; OD_{600} was the optical density of the culture at time of assay.

2.8 Recombinant DNA Techniques

2.8.1 Restriction enzyme digests

Routinely, 1-10 µg of DNA was digested with 10U of enzyme in 10 µl of 1x buffer for 2h at 37°C. Restriction buffers, prepared as 10x stocks,were as recommended by BCL:

(1) 100mM Tris-HCl, 50mM $MgCl_2$, 1M NaCl, 10mM 2-Mercaptoethanol (pH8) for *Bam*H1,*Hin*dIII.

(2) 100mM Tris-HCl, 100mM $MgCl_2$, 0.5M NaCl, 10mM DTT (pH 7.5) for *Bgl* II, *Pvu*II.

(3) 0.5M Tris-HCl, 100mM $MgCl_2$, 1M NaCl, 10mM DTT (pH 7.5) for *Eco* R1.

2.8.2 Gel electrophoresis of DNA

DNA restriction fragments were separated by electrophoresis on agarose gels containing 0.1μg/ml ethidium bromide (EtBr). Gels were viewed using a short wave U.V. transilluminator and photographed using Polaroid type 55 positive/negative film. Two types of apparatus were used for electrophoresis:

(1) **Mini-gel** : DNA fragments were separated on 0.8% agarose gels in 0.5xTBE buffer (90mM Tris base, 90mM boric acid, 2mM EDTA), run at a voltage of 1-8 V/cm.

(2) **E-Gel** : DNA fragments were separated on 1% agarose gels in 1x E-gel buffer (0.4M Tris, 0.2M sodium acetate, 20mM EDTA), run at a voltage of 5-20 V/cm.

2.8.3 Recovery of DNA fragments from agarose gels

DNA fragments were recovered from agarose gels by procedure of Young *et al.* (1985).

2.8.4 Ligation and Alkaline phosphatase treatment of plasmid DNA

Ligations were carried out in 10 μl ligation mix (50mM Tris-HCl, 10mM $MgCl_2$, 10mM DTT, 1mM spermidine, 1mM ATP, 100 g/ml BSA) at 15°C overnight. Routinely 100ng of vector DNA was ligated with a four fold excess of fragment DNA, using 0.1U of T4 DNA ligase. Where appropriate the vector DNA was pretreated with Bochringer calf intestinal phosphatase (CIP) to prevent self ligation. The DNA was dissolved in CIP buffer (50mM Tris-HCl pH9.0, 1mM $MgCl_2$, 0.1mM $ZnCl_2$, 10mM spermidine). 0.01U of CIP was added for each pM of DNA termini and the mix incubated at 37°C for 1h. CIP was inactivated by heating 2min at 65°C, followed by recovery of DNA by phenol extraction.

2.8.5 Preparation and transformation of competent *E.coli*

Competent *E.coli* cells were prepared as described in Hanahan (1983) and *E.coli* transformation was as described in Cohen *et al.* (1972).

2.8.6 Rapid isolation of plasmid DNA from *E.coli*

Small scale plasmid DNA preparation from *E.coli* transformants was via the rapid procedure of Birnborm and Doly (1979) as modified by Ish-Horowicz and Burke (1981).

2.8.7 Large scale purification of plasmid DNA

Large scale purification of plasmid DNA was as in Sambrook *et al.* (1989a). Recombinant *E.coli* were grown overnight in 500ml 2TY+AMP at 37°C. The cells were then harvested and resuspended in 8ml ice-cold 10% sucrose, 50mM Tris-HCl (pH 8.0). Lysozyme was then added at a final concentration of 2mg/ml and the cells were incubated for 5min on ice, before the addition of 1.6ml 0.25M EDTA. After a further 15min on ice, 6.4ml of 2% Triton X100, 60mM EDTA, 50mM Tris-HCl (pH 8.0) was added to the cells which were then centrifuged in a Sorvall SS34 rotor at 20,000 rpm for 30min. The lysate was decanted and weighed. Caesium chloride (CsCl) was added to a final concentration of 0.923g/g lysate and EtBr (10mg/ml) was added to a final concentration of 75.4 µl/g lysate. The lysate was transferred to a Beckman ultraclear tube and was centrifuged in a Beckman 70Ti fixed angle rotor at 40,000rpm for 36h at 15°C. The plasmid DNA was collected via a syringe and 1.1mm needle. EtBr was removed by repeated extractions with isopropanol saturated with CsCl. The DNA was then precipitated with isopropanol.

2.8.8 In vitro labelling of DNA probes

DNA probes were prepared by nick translation as described by Rigby *et al.* (1977).

2.9 Procedures for nucleic acid analysis

2.9.1 Isolation of total cellular yeast nucleic acid

Yeast total nucleic acid was isolated by a method based upon that described by Sprague *et al*. (1983) and modified by Chaleff and Tatchell (1985).

30ml of yeast liquid culture were harvested at 4°C in a corex tube and washed with ice cold sterile dH_2O. 1-2 cell volumes of glass beads (BDH 40 mesh) were added to the pellet, followed by 3ml RNA extraction buffer (10mM Tris-HCl pH 7.5, 10mM EDTA, 1% SDS) plus 3ml phenol. After vortexing for 5 min, the two phases were then separated by centrifugation. The aqueous phase was transferred to a fresh tube, made 1.2M ammonium

acetate and precipitated with 2 volumes ethanol. Nucleic acid was pelleted by centrifugation, the pellets washed with cold 70% ethanol and then dried under vacuum before resuspension in TE. For RNA samples, a further ethanol precipitation was carried out using 0.4M sodium acetate. DNA was isolated from total nucleic acid by incubation with 2 μg DNAse-free pancreatic RNase per 100 μg nucleic acid for 1h at 37°C. Following phenol / chloroform extraction, the DNA was recovered by ethanol precipitation.

2.9.2 Determination of nucleic acid concentrations

The nucleic acid was diluted 1:200 in dH_2O and the absorbance at A_{260} was read spectrophotometrically. Nucleic acid concentrations were determined assuming 1 A_{260} unit (1cm light path) = approximately 50 μg/ml DNA or 40 μg/ml RNA.

2.9.3 Plasmid copy number

Plasmid copy number was determined by digesting samples of total yeast DNA with a suitable restriction enzyme, then separating the restriction fragments by agarose gel electrophoresis. Examination of the relative intensity of ethidium bromide-stained plasmid-specific bands to ribosomal bands (present at 100-140 copies/cell) gives an approximate copy number of the plasmid. Plasmid copy number was also assessed by Southern blotting (section 2.9.4) followed by hybridisation with the appropriate nick translated probe (section 2.9.6).

2.9.4 Southern transfer

Following electrophoresis (Section 2.8.2), agarose gels were blotted as described in Sambrook *et al.* (1989b). The membrane used was GeneScreen Hybridization Transfer Membrane (NEN Research products). Capillary blotting was carried out for 16-20h using 20x SSC (3M sodium chloride, 0.3M sodium citrate).

2.9.5 Northern transfer

4.5μl RNA (10-20μg) was denatured in 2μl 5x Northern running buffer (0.2M MOPS, 50mM sodium acetate, 5mM EDTA), 3.5μl formaldehyde, 10μl formamide at 55°C for 15min. The RNA was then loaded onto a denaturing 1.1% agarose gel containing 1x Northern running buffer, 2.2M formaldehyde and electrophoresed at 60V for 4h. The gel was then blotted onto GeneScreen Hybridization Transfer Membrane as described in

Sambrook *et al.* (1989c) for 16-20h using 20x SSC (Section 2.9.4).

2.9.6 Hybridization of membrane bound nucleic acid to nick translated probes

Hybridization of membrane bound nucleic acid to alkali-denatured (0.01M NaOH 5min 37°C) nick translated probes was carried out according to the GeneScreen manual.

Membranes were dried at 80°C for 2-4h and then pre-hybridized in a sealed bag containing 50% formaldehyde, 0.2% polyvinylpyrrolidone, 0.02% BSA, 0.2% ficoll, 50mM Tris-HCl pH 7.5, 1M NaCl, 0.1% sodium pyrophosphate, 1% SDS, 10% dextran sulphate, 100 μg/ml denatured calf thymus DNA. Pre-hybridization took place at 42°C for a minimum of 6h after which the membrane was transferred to a fresh bag and hybridized with the relevant nick translated probe in pre-hybridization buffer without NaCl.

Blots were washed at room temperature for 15min with 2x SSC, followed by a high stringency wash at 65°C in 2x SSC, 1% SDS for 15min and a final low stringency wash at room temperature in 0.1% SSC for 15min. All washes were carried out in duplicate with constant agitation.

2.9.7 Autoradiography and quantification of hybridization signal

Membranes were exposed to Fuji X-Ray film at -70°C in an autographic cassette (Protex). RNA-DNA hybridization signals were quantified by aligning membranes with the corresponding autoradiography, then cutting out the region corresponding to each band plus a non-hybridising region of the blot of similar size prior to counting in toluene based scintillant (Piper *et al.*, 1988).

2.10 Procedures for protein analysis

2.10.1 *In vivo* labelling of proteins

1-2x10^8 cells from exponential YEPD cultures maintained at 25°C were collected by centrifugation (3,000 xg, 5min), resuspended in 10 ml SD medium, recentrifuged as before, resuspended again in 10ml SD medium and incubated for 20min at 25 °C prior to heat shock. During this 20min incubation the cultures were subdivided into 1ml aliquots in glass tubes. To heat shock the cells, aliquots were shifted to the appropriate temperatures

and, at the stated variable times after this upshift, were labelled by the addition of either L-[^{35}S]-methionine (to 10μCi/ml) or [^{3}H]-leucine (to 30μCi/ml). A control aliquot was pulse labelled for 15min at 25°C without any heat shock. Incorporation of radiolabelled amino acid was stopped after the appropriate time by rapidly chilling the aliquots on ice, transferring them to 1.5 ml eppendorf tubes and pelleting the cells by a 30sec microcentrifuge spin (ca. 5,500xg). The supernatant was discarded and the pellets rapidly frozen in dry ice and stored at -70°C.

2.10.2 Extraction of total cell protein

Labelled cell pellets were thawed on ice (if necessary) and two pellet volumes of acid-washed glass beads (BDH 40 mesh) plus sufficient protein extraction buffer to just cover the pellet added. Protein extraction buffer was: 500mM Tris-HCl pH 8.0, 50mM $MgCl_2$, 2mM EDTA, 2% SDS, with the following additions made just before use: 2.5% β-mercaptoethanol, 0.5mg/ml RNAase, 1mM PMSF (phenylmethanesulphonyl floride), 1mM TPCK (toluene-4-sulphonamido-2-phenyl ethyl chloromethyl ketone) and 2μl/ml pepstatin A. After vortexing for 30sec followed by chilling on ice for 30sec (x8), glass beads and unbroken cells were pelleted by a 10sec spin in a microcentrifuge (5,500 xg). The supernatants were transferred to eppendorf tubes precooled on ice, and stored at -70°C. If the samples were to be repeatedly freeze/ thawed the addition of 100μl of 50% glycerol was found to limit fragmentation of proteins during the frozen and thaw process.

2.10.3 Measuring radioactivity incorporated into cellular protein

Radioactivity incorporated into protein was determined by adding 5μl of each sample to 5ml ice-cold 5% (w/v) TCA containing 0.1% bactopeptone. After 30min on ice, precipitated protein was filtered by suction onto 2.5cm glass fibre filters (Whatman GF/C). The filters were washed once with 5ml of cold 5% TCA/bactopeptone and twice with 5ml cold absolute ethanol. Filters were dried (120°C, 15min) and placed into 5ml scintillation vials containing 4ml of Ecoscint A. Filters were counted using a Packard Tris-carb Liquid Scintillation Analyzer using the appropriate standards, channel, and gating settings for the appropriate radioisotope.

Unless otherwise indicated, 100,000 c.p.m of protein extract were routinely used for both one dimensional (section 2.10.6.1) and two dimensional gel electrophoresis (Section 2.10.6.2).

2.10.4 Protein assays

Protein concentrations were determined by the dye-binding assay of Bradford (1976), using the Bio-Rad Protein Assay Kit and BSA as standard, as described in the manufacturer's instructions.

2.10.5 Sub-cellular fractionation

There are several procedures by which one can isolate plasma membrane, mitochondrial and cytosolic fractions (S100 fraction) from yeast. A modified version of the procedure described by Serrano (1978) was used. All steps, including centrifugation runs, were carried out at 4°C.

2.10.5.1 Cell disruption (S100 or the post-ribosomal supernatant preparation)

A 1L culture was harvested by centrifugation (5min, 5,000xg, Sorvall GS3 rotor) and the resulting cell pellet resuspended in homogenisation buffer (HB: 25mM Tris-HCl pH7.0, 6% sorbitol and 1mM EDTA). The following protease inhibitors were added to HB just before use: 1mM PMSF and 1mM TPCK (both from 100mM stocks in ethanol), 0.5 mM TLCK (added fresh) and 2µg/ml pepstatin A (from a 2.5mg/ml stock in methanol). The suspension was divided between two 50ml polycarbonate tubes and the cells were once again pelleted by centrifugation (10min, 5,000xg, Sorvall SS34 rotor).

To each pellet was added two volumes of acid washed glass beads (BDH, 40 mesh), followed by a volume of HB that just covered the cells and glass beads. Cells were ruptured by vortexing on a whirlimixer for 5min. This was just sufficient to disrupt 90% of the cells, as judged by light microscopy. The homogenate was diluted 3x in HB and pH was adjusted to between 5 and 7 with 1M NaOH. Glass beads, cell walls and non ruptured cells were removed by centrifugation (10 min, 530xg, Sorvall SS34 rotor) and discarded. The supernatant was centrifuged for 20 min at 22,000xg (SS34 rotor) to obtain a pellet (P1) composed of plasma membranes and mitochondria. The supernatant from this spin was then centrifuged for 1 hour at 100,000 xg (Beckmann 70 Ti rotor), to give a pellet (P2) and a final supernatant, the soluble or S100 fraction. This S100 fraction was stored at -20°C after the addition of glycerol (to 20%v/v).

2.10.6 Separation of proteins by gel electrophoresis

2.10.6.1 One dimensional slab gel electrophoresis (1D SDS-PAGE)

Protein samples were fractionated by size using the discontinuous electrophoresis system as described by Laemmli (1970). Gels were cast between two glass plates and run on a Studier type slab gel apparatus as described in Hames (1990). Briefly, the Laemmli system is composed of the following: (A) Stacking gel, 125mM Tris-HCl pH 6.8, 0.1% SDS, and 4% polyacrylamide. (B) Resolving gel, 375mM Tris-HCl pH 8.8, 0.1% SDS, and a polyacrylamide concentration to give the best separation of the proteins in question. (C) Electrode buffer pH8.3- 25mM Tris, 192 mM glycine and 0.1% SDS. (D) Protein sample buffer, 125 mM Tris-HCl pH6.8, 10% glycerol (v/v), 5% β-mercaptoethanol (v/v), 2% SDS (w/v) and 0.013% bromophenol blue (w/v). The 30% acrylamide stock used to prepare solutions A and B was composed of 29.2% acrylamide and 0.8% bis-acrylamide. Gels were polymerised by the addition of 0.05% (v/v) of TEMED and 0.7% (w/v) of ammonium persulphate, the latter from a 10% (w/v) stock solution. Samples were prepared for electrophoresis by addition of an equal volume of solution D, incubation for 15min at 40°C, then loading into adjacent gel wells alongside protein size markers of the appropriate size range (see section 2.1).

2.10.6.2 High resolution two-dimensional electrophoresis (2D SDS PAGE)

2D SDS PAGE was performed essentially as described by O'Farrell (1975) and O'Farrell *et al.*, (1977).

Proteins were separated in the first dimension using non-equilibrium gradient (NEPHGE) gels containing 4% acrylamide, 9.5M urea, 2% NP-40 and 2% ampholines pH 3-10. The gel solution was poured into glass tubes (2.5mm by 135mm, sealed at one end with Nescofilm) using a syringe fitted with a piece of fine silicon tubing. Gels were polymerized with 10% ammonium persulphate (10µl/10ml) and TEMED (7µl/10ml).

Protein samples were loaded in protein extraction buffer plus an equal volume of lysis buffer (9.5M urea, 2% NP-40, 1.6% ampholines pH 5-7, 0.4% ampholines pH 3-10, 5% β–mercaptoethanol). The anode solution was 10mM H_3PO_4 and the cathode solution was 2mM NaOH. Gel were run at 400v for 4h.

Following electrophoresis, gels were removed from the glass tubes and equilibrated in 5ml SDS sample buffer (10% glycerol, 5% β–mercaptoethanol, 2.3% SDS, 62.5mM Tris-HCl pH 6.8) for 30min. Equilibrated first dimension gels were then placed onto a 1D slab gel and the proteins were then separated in the second dimension by 1D SDS PAGE (Section 2.10.6.1).

2.10.7 Analysis of gels following electrophoresis

Upon completion of electrophoresis gel plates were separated and the stacking gels discarded. The resolving gels were analysed by one of the following ways:

2.10.7.1 Direct staining

Abundant proteins (1μg or more) were detected by staining with Coomassie Blue R-250. Gels were incubated for 4h at room temperature in 0.1% (w/v) Coomassie blue, 50% (v/v) methanol, 10% (v/v) acetic acid. Destaining was by incubation at room temperature with gentle agitation in 5% (v/v) methanol and 7.5% (v/v) acetic acid; this solution being changed periodically. To visualise proteins that were present in low amounts (10 ng or more) a neutral silver staining procedure was used (Harlow and Lane, 1988a).

2.10.7.2 Gel autoradiography and fluorography

[^{3}H]- or [^{35}S]- labelled proteins were visualised by fluorography. Before drying, gels were soaked in 1M sodium salicylate (pH5-7) for 20 min, which acted as a fluor increasing detection of [^{35}S] or [^{3}H] by >10-fold (Chamberlain, 1979).

Gels were dried onto Whatman 3MM paper under vacuum using a Bio-Rad gel dryer, as described by Harlow and Lane (1988b). Dried gels were placed in direct contact with Fuji RX film in light proof cassettes for the appropriate length of time at -70°C. For [^{32}P]-labelled proteins increased sensitivity was obtained by placing a calcium tungstate intensifying screen against the side of the X-ray film not in contact with the dried gel. Typically, 100,000 cpm of resolved total cellular protein labelled with ^{35}S or ^{3}H could be visualised by an overnight or a 3 day exposure, respectively.

2.10.7.3 Transfer of proteins from gels to membranes

Separated proteins from a gel were blotted onto thin support matrices by electrophoretic

transfer (Western blotting). Both nylon (GeneScreen) and nitrocellulose blotting membrane can be used due to their superior protein binding capacity and its physical strength. Also these membranes do not change size during subsequent processing steps. The membrane was prepared for blotting according to manufacturer's instructions and electrophoretic elution was achieved by complete immersion of a gel-membrane sandwich in a buffer tank with steel plate electrodes followed by electrophoresis at 50V, as described by Harlow and Lane (1988c). After Western blotting, efficiency of transfer was determined by staining the gel with Coomassie blue. Also, prestained markers were run on gels to serve as internal markers for transfer and molecular weight. The transfer buffer was typically of low ionic strength (25mM Tris, 150 mM glycine, pH8.3) and contained 20% methanol to minimise swelling of the gel as blotting progresses.

2.10.7.4 Detection of specific proteins by immunostaining

Proteins immobilised on membranes were visualised by probing with the appropriate anti-sera followed by incubation with the various components of the BRL streptavidin detection system. The procedure described below is a combination of the methods reported by Sambrook *et al.* (1989) and the BRL Products for Immunodetection Applications Guide. Solutions containing immunological reagents or chromogenic substrate were freshly prepared. All incubations were at room temperature with gentle agitation on a platform shaker. Also, incubations of blots with primary antibody, goat anti-rabbit IgG and peroxidase were in heat-sealed plastic bags in a final volume of 0.1ml per cm^2 of membrane. Preliminary experiments were conducted to determine the optimal amounts of primary antibody, while quantities of immunodetection reagents used were as recommended by individual product profiles.

After electrophoretic transfer (see section 2.10.7.3) remaining protein-binding sites on the blot were blocked by 1h incubation in a blocking solution of 5% (w/v) non-fat dried milk in Tris buffered saline (TBS: 50mM Tris-HCL pH7.5, 150mM NaCl), followed by 2h incubation with blocking solution plus an appropriate amount of the primary antibody. The blot was then washed 30min in 3 changes of TBS. This was followed by 1h incubation in blocking solution plus the appropriate quantity of biotinylated goat anti-rabbit IgG. Blots were then washed with TBS as before, followed by 1h incubation with TBS plus the appropriate amount of streptavidin-horseradish peroxidase conjugate. The blots were washed with TBS as before and bound peroxidase detected by incubation with TBS containing 0.01% H_2O_2, and 0.06% 4CN. Within 5-10min the polypeptides to which the primary antibody had bound appeared blue against the white background of the blot. The

stained blots were washed for 10min in 3 changes of dH_2O and stored in the dark.

2.10.7.5 Origin of antisera used in the work described in this thesis

Polyclonal antisera were raised in rabbits as described by Harlow and Lane,(1988d). Essentially, this involved subcutaneous injection of polyacrylamide gel fragments from multiple 1D SDS gel fractionations of the total protein from heat shocked yeast. Antiserum cross-reacting with both isoforms of HSP90 was prepared by myself, and an antiserum cross-reacting with HSP26 was made by J.E. Coleman.

2.11 Peptide mapping by limited proteolysis

The procedure described below is modified from Cleveland *et al.* (1977). Suitable for analysis of a protein band in a gel slice, it involve partial enzymatic proteolysis in the presence of SDS followed by analysis of the cleavage products by 1D SDS-PAGE. The pattern of peptide fragments produced is characteristic of the protein substrate and the proteolytic enzyme and is highly reproducible.

After 1D SDS-PAGE using 1mm thick gels, proteins were visualised by staining with Coomassie Blue R-250. However to avoid possible acid hydrolysis, staining/destaining conditions were not as described in section 2.10.7.1. Instead, proteins were visualised as quickly as possible by staining for 30min in 0.1% (w/v) Coomassie Blue R-250 in methanol : acetic acid : dH_2O (5:1:4 v/v/v) followed by rapid destaining (45min) in methanol : acetic acid : dH_2O (5:1:4 v/v/v). Even under these mild staining conditions there was significant protein hydrolysis at room temperature, this probably being due to the peptide bond between aspartate and proline being particularly susceptible to acid hydrolysis (Matsudaira, 1990). This problem was eliminated by carrying out these staining and destaining steps at 4°C. Bands from SDS-PAGE gels were cut out with a scalpel blade and then soaked for 30 minutes with occasional swirling in 10ml of solution A: 125mM Tris-HCl pH6.8, 0.1% SDS and 1mM EDTA. At this point gel slices could be stored at -20°C.

A second 1mm thick 15% SDS gel was prepared but with a stacking gel which: i) contained 1mM EDTA; and ii) that was twice as long as usual. The sample wells of this gel were filled with solution A: 0.125M Tris-HCl (pH6.8), 0.1% SDS and 1mM EDTA. The bands from the first SDS gel were conveniently digested, without prior elution, by placing them in the sample wells of this second SDS gel followed by overlaying each slice with 20µl of solution A containing 10% (v/v) glycerol, 0.01% (w/v) bromophenol blue and an

appropriate concentration of protease. Electrophoresis was performed in the normal manner with the exception that the current was turned off for 30min when the bromophenol blue dye neared the bottom of the stacking gel. Since each gel slice contained only 1-2 μg of protein, the pattern of peptide fragments generated on the second gel was visualised by silver staining. Altenatively, if the gel slices contained pulse-labelled protein the peptide fragments were visualised by autoradiography.

More than 10 proteases are commonly used for peptide mapping. Endoproteinase from *Staphylococcus aureus* V8 (protease V8) is probably one of the most ideal and was used for the experiments in Chapter 6. It has the additional advantage that the extent of proteolysis can be controlled by manipulation of the digestion buffer as well as by concentration of protease. In phosphate buffer protease V8 cleaves peptide bonds on the carboxyl-terminus of either aspartate or glutamate, whereas in phosphate free buffer the enzyme acts specifically at glutamoyl bonds (Bond, 1989). Phosphate free conditions were used here to promote limited proteolysis. Protease V8 from the manufacturers was resuspended at 5mg/ml (2.6 units/μl) in 125mM Tris-HCl pH6.8 and stored at -20°C.

2.12 Limited N-terminal sequence analysis

Polypeptides which were to be sequenced were prepared by blotting onto PVDF membrane. However, several modifications to the usual SDS-PAGE and Western blotting protocols were found to be required to minimise N-terminal blocking. Collated from a variety of published procedures (Matsudaira, 1987; Flannery *et al.*, 1989 and manufacturers instructions for use of PVDF), these are described below.

1D SDS-PAGE gels were cast as usual except that resolving gels were allowed to polymerise completely by letting the gel stand for 4 days at room temperature. The stacking gel was then cast and allowed to stand for 1 day (also at room temperature) prior to use. Gels were pre- electrophoresed for 2h at 8mA using the standard electrode buffer at the anode and standard buffer plus 50 μM reduced glutathione at the cathode. The cathodic buffer was then discarded and replaced with standard buffer plus 100μM thioglycolic acid. Samples were loaded and electrophoresis carried out as usual.

Electrotransfer to PVDF was performed using the standard Western blotting protocol described in section 2.10.7.3 with the following modifications: i) PVDF was prepared for blotting by immersion in 100% methanol until the membrane became translucent and then

equilibrated in transfer buffer for 5min; and ii) after transfer, the blot was rinsed with dH_2O and stained for 5min in 0.05% (w/v) Coomassie Blue dissolved in 50% methanol, followed by destaining for 10-15min in 50% methanol. The stained blot was rinsed in dH_2O, air dried and stored at -20°C. The appropriate band was cut from the membrane with a clean scalpel blade and trimmed to give a 2x4 mm segment. A limited N-terminal sequence was determined using repeated automated cycles of the Edman degradation using an Applied Biosystems 470A Gas Phase Protein Sequencer equipped with an on-line phenylthiohydantoin amino acid analyser (this was carried out by Dr. Brian Coles, CRC molecular toxicology unit). It was important to determine whether proteins that could not be sequenced were naturally N-terminally blocked or blocked due to electrophoresis or Western blotting procedures. For this reason 10 μg of rat glutathione transferase 3-3 (donated by B. Coles) was treated as described above. This protein is not blocked *in vivo* and gives a high yield (>90%) of the expected N-terminal sequence.

2.13 A general assay for kinase activities associated with the S100 fraction

The soluble (S100) fractions from yeast cultures were isolated as described in section 2.10.5.1. Endogenous ATP was removed in either of two ways:

(A) Dialysis. Visking dialysis tubing (Scientific Industries International Inc, U.K) was pre-treated as described by Findlay (1990). Aliquots of the S100 fractions were dialysed at 8°C against 5 changes of a buffer composed of 10 mM MES-KOH (pH6.0), 50mM KCl, 1mM $MgCl_2$, 1mM DTT and 10% glycerol (referred to as dialysis buffer from now on). Prior to storage at 4°C, PMSF and pepstatin A were added to the dialysed samples, to final concentrations of 1mM and 2μg/ml respectively.

(B) Centrifugal concentration. 2ml aliquots of the S100 fractions were concentrated to 50μl by using Centricon-3 centrifugal microconcentrators (Amicon) as described by manufacturers instructions. To the 50μl retentate was added 900μl of dialysis buffer. This was followed by concentration and addition of dialysis buffer as described before. Addition of protease inhibitors and storage were as for the dialysed samples.

In vitro phosphorylation assays were performed by incubating 10μg (typically 5μl of S100 fraction) for various times at 30°C with 50mM MES-KOH (pH6.0), 6mM $MgCl_2$ and

1mM DTT. Reactions were started by the addition of 10nM ATP and stopped by adding an equal volume of Laemmli gel protein sample buffer. Samples were submitted to 1D SDS-PAGE as described in section 2.10.6.1. and phosphorylated proteins visualised by autoradiography as described in section 2.10.7.2.

CHAPTER 3

INVESTIGATION OF *HSP82* OVEREXPRESSION IN *S. CEREVISIAE* : A ROUTE TO THE LARGE-SCALE PRODUCTION OF HSP90 PROTEIN

3.1 Introduction

A heat shock response is normally induced when cells are suddenly exposed to temperatures close to, or slightly above, the maximum permitting growth. At the molecular level the heat shock response causes strong induction of heat shock proteins, while at the physiological level its most dramatic effect is to increase thermotolerance. Studies on *E. coli* and *S .cerevisiae,* organisms readily amenable to molecular genetic analysis, are proving especially informative in uncovering how individual heat shock proteins function within the cell and influence stress tolerances. The high evolutionary conservation of many of these proteins means that such studies also provide insight into the roles of the stress proteins of higher organisms. For example, *S.cerevisiae* defective in the *UBI4* polyubiquitin gene cannot increase its ubiquitin synthesis in response to heat shock and is killed more rapidly than wild type cells at just above the maximum temperature of growth (Finley *et al.,* 1987). However, upon sudden shift to much higher temperatures, when even the wild type cannot synthesise proteins *de novo,* these *ubi4* mutant cells are killed at the same rate as the wild type. Vertebrates have heat-inducible polyubiquitin genes that, like *UBI4* of *S.cerevisiae,* almost certainly function to increase ubiquitin synthesis in response to stress (Sanchez and Lindquist, 1990).

Proteins that are identical with, or closely related to, heat shock proteins are synthesised during normal growth and development and serve to provide essential functions in the absence of stress (sections 1.2.1, 1.2.2). Thus all cells constitutively synthesise HSP70-related proteins that constitute some of the major "chaperones" in unstressed cells. HSP90 is a major protein in all unstressed eukaryotic cells, although its levels increase with heat shock. HSP90 has been shown recently to be another chaperone (section 1.2.1). It is essential in normal growth, as shown by gene disruption studies of the two *S. cerevisiae* genes for this protein (*HSC82* and *HSP82*). Strains with just one of these genes inactivated (*HSC82,hsp82* or *hsc82,HSP82* strains) are viable, whereas the double disruption mutants (*hsc82,hsp82*) are non-viable (Borkovich *et al.,* 1989). The protein products of *HSC82* and *HSP82* are referred to as HSC90 and HSP90 respectively

throughout this thesis.

It is known that higher HSP90 levels are required for growth of both yeast (Borkovich *et al.*, 1989) and mouse L cells (Bansal *et al.*, 1991) at high growth temperatures. These higher HSP90 levels probably serve to re-establish equilibria of heterodimer formation, compensating for the weakening of noncovalent interactions of HSP90 with its target proteins as temperature increases. There have been no reports of the effects of artificially increasing HSP90 levels. It might be anticipated that HSP90 overexpression would affect stress tolerances, since this protein is induced as thermotolerance increases with the triggering of the heat shock response. Also a heat-resistant mutant Chinese hamster cell line has been shown to overexpress HSP90 (Yahara *et al.*, 1986). This Chapter describes a study in which the homologous yeast HSP90 was overexpressed in three *S. cerevisiae* strains, an investigation conducted with three aims: (i) to determine if a heat shock gene can display essentially normal regulation when present in yeast at high gene copy number; (ii) to gain evidence for the physiological consequences of elevating HSP90 levels; and (iii) to demonstrate that this expression host is capable of high level synthesis of HSP90, a capability which can be exploited to purify HSP90 in quantity for structural investigations on this protein. It was especially interesting to establish whether or not yeast HSP90 fails to be overexpressed from *HSP82* on a high copy number plasmid, since the products of certain other heat shock genes notably *HSP70* appear to be self-regulatory, suppressing their own synthesis and having an important role in recovery from heat shock (DiDomenico *et al.*, 1982; Craig and Gross, 1991).

3.2 Results

3.2.1 Construction of plasmid p82-2B and of p82-2B transformants

Characterization of a gene and its protein product is greatly assisted by the ability to overexpress this gene. In this study *HSP82* was overexpressed from its own promoter on a high copy number yeast episomal plasmid (YEp) vector. To construct this vector a 2.6Kb *Hind*III+*Mlu*I DNA restriction fragment containing the complete *HSP82* gene sequence was subcloned from plasmid pU84 into the high copy number vector, pMA3a. Plasmid pU84 comprises a 5.6Kb *Hind*III fragment of the DNA of *S.cerevisiae* X2180-1B inserted at the single *Hind*III site of vector pUC8. The *HSP82* DNA fragment from pU84 [*HSP82* sequences from the *Hind*III site at -331 relative to the initiation codon to the *Mlu*I site at +2168, 27 nucleotides 3' to the major site of polyadenylation of *HSP82* mRNA (Farrelly and Finkelstein, 1984)] was made blunt-ended, and tailored with *Bam*H1 termini using

*Bam*H1 linkers (as in Sambrook *et al.,* 1989). It was then inserted at the unique *Bam*H1 site of the 2μ*ORI-STB* yeast-*E.coli* shuttle vector pMA3a (Kingsman *et al.,* 1985), thereby generating the plasmid p82-2B shown in Fig.3.1. pMA3a, the parent plasmid, contains sequences from pBR322, the *ORI-STB* region of the yeast 2μ plasmid for autonomous replication and segregation in yeast, also *LEU2d* for plasmid selection and maintenance at high copy levels in *leu2* yeast strains (Kingsman *et al.,* 1985; Piper and Curran, 1990).

Three haploid *cir+ S.cerevisiae* strains were employed for this study (W3031A, PMY1 and MD40-4c; Table 2.1). Each of these strains was transformed to leucine prototrophy using either plasmid pMA3a or p82-2B. Transformants carrying the parent plasmid pMA3a (designated W303-3a, PMY1-3a and MD40-3a respectively) were used for control cultures, cells that were maintaining the sequences of the high copy number vector yet which possessed normal *HSP82* gene and HSP90 protein levels. Transformants obtained using p82-2B (designated W303-82, PMY1-82 and MD40-82 respectively) were subsequently shown to overexpress HSP90 (see 3.2.2), and used in investigations of the physiological consequences of this HSP90 overexpression (see 3.2.5 and 3.2.6).

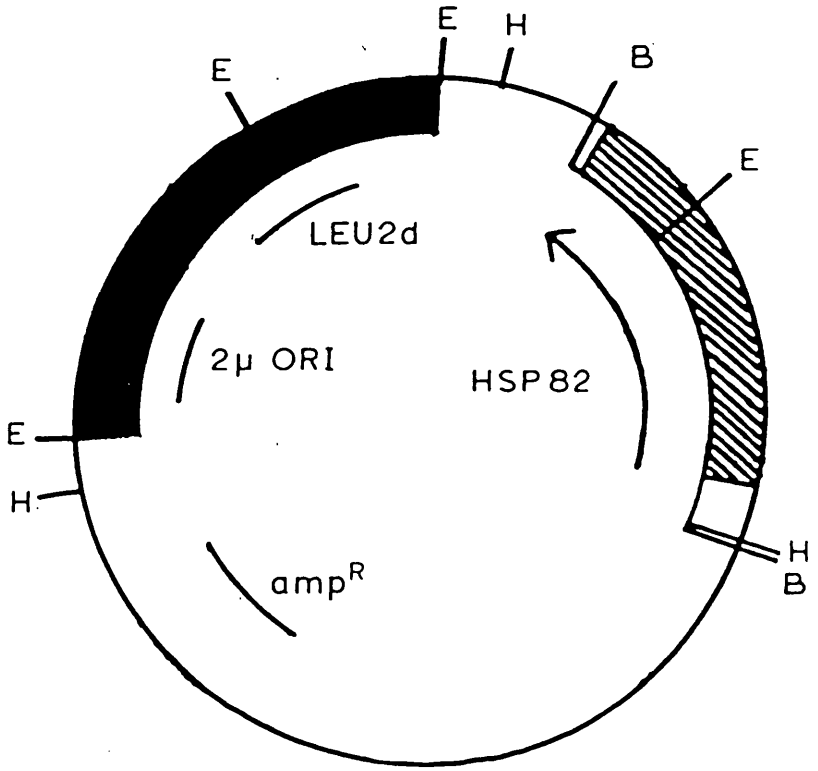


Figure 3.1 The HSP90 expression vector p82-2B.

Thin line, pBR322 sequences; solid line, 3.25 kb double *Eco*RI fragment of yeast DNA containing 2μ *ORI-STB* and *LEU2d* ; open box, 2.6 kb from plasmid pU84 (see section 3.2.1) containing the *HSP82* gene (coding region hatched) and part of the pUC8 polylinker. E, *Eco*RI; H, *Hin*dIII; B, *Bam*HI

3.2.2 Plasmid p82-2B causes moderate HSP90 overexpression in the absence of heat shock stress

The two *S.cerevisiae* genes for HSP90 encode products which are 97% identical at the amino acid sequence level and which appear from genetic analysis to be functionally equivalent (Borkovich *et al.*, 1989). One gene (*HSC82*) displays a high constitutive expression that increases only slightly with heat shock. The other (*HSP82*) displays a 10-fold lower constitutive expression yet is subject to strong activation by heat shock. *HSC82* therefore contributes approximately 90% of the HSP90 present in normal vegetative growth, while *HSP82* is primarily responsible for the increase in HSP90 with heat shock (Borkovich *et al.*, 1989; McDaniel *et al.*, 1989; Gross *et al.*, 1990).

Overexpression of HSP90 due to high *HSP82* gene dosage was investigated in three *leu2-3,112 S.cerevisiae* strains (W303A, PMY1 and MD40-4c). This was due to uncertainty at the commencement of this work as to which genetic background might be most suitable for HSP90 production. Many molecular genetic studies of the yeast heat shock response have been on strains of W303 genetic background (Borkovich *et al.*, 1989; Sanchez and Lindquist, 1990; DeVirgilio *et al.*, 1991a; Parsell *et al.*, 1991; McDaniel *et al.*, 1989; Gross *et al.*, 1990). However there is considerable experience of high level expression of genes carried on *LEU2d-* based vectors in MD40-4c (Kingsman *et al.*, 1985). Also many laboratory strains of yeast grow adequately in shake-flask culture but do not display particularly good growth in fermenter culture. Strain PMY1 has been shown not to suffer from this restriction (D. Pioli, personal communication). While these considerations led us to investigate HSP90 overexpression in all three strains, it was only during investigation of the effects of high *HSP82* gene dosage on high temperature growth and thermotolerance (3.2.6 and 3.2.7) that significant strain differences were observed.

Cultures of these three strains possessing normal or high *HSP82* gene dosage (transformants maintaining either pMA3a or p82-2B respectively) were harvested in exponential growth at 25°C, and their HSP90 levels analysed. Commassie blue-staining of the proteins of these transformants separated on 12.5% gels showed that the maintenance of p82-2B caused HSP90 to be only slightly overexpressed at 25°C in the absence of heat shock (2-5% of total cell protein; data not shown). Western blots (Fig. 3.2) of identical gel fractionations probed with a polyclonal antiserum raised against yeast HSP90 (section 2.10.7.5) showed that the HSP90 levels of 25°C log phase cultures of W303-82, PMY1-82 and MD40-82 were elevated 3 to 7-fold relative to the normal HSP90 levels in the

corresponding control cultures containing plasmid pMA3a (W303-3a, PMY1-3a and MD40-3a; Fig.3.2).

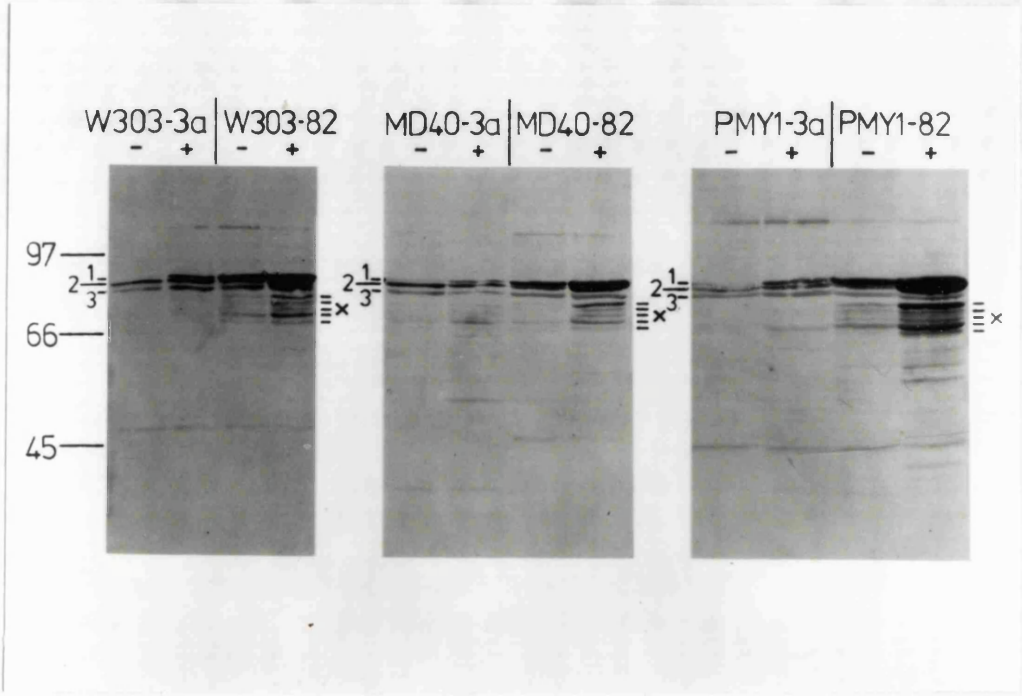


Figure 3.2 Western blot analysis of the levels of HSP90 protein in individual transformants.

Cultures were all initially in exponential growth at 25°C, samples of total cell protein being prepared before (-) or 75min after (+) a 25-39°C heat shock. The polyclonal antiserum used in HSP90 detection crossreacts with both *HSC82* and *HSP82* gene products, the former migrating slightly faster than the latter on SDS polyacrylamide gels (Borkovich *et al.,* 1989). The *HSP82* and *HSC82* products are bands 1 and 2, respectively. Certain lower molecular mass bands cross-reacting with the antiserum (x) are minor, degraded forms of HSP90 that were consistently observed in cells overexpressing HSP90. They were observed even though protease inhibitors were used during the preparation of samples for gel analysis, although as total protein their amount was less than 10% of the amount of intact HSP90. The band (3) detected by this antiserum between the degraded HSP90 (x) and *HSC82* protein (2) is not related to HSP90. The positions of molecular weight markers (phosphorylase *a* , 97kDa; bovine serum albumin, 67kDa and ovalbumin, 43kDa) are indicated to the left of the figure. 10μg of cell protein was loaded in each gel track.

LEU2d containing yeast episomal vectors such as pMA3a and p82-2B are normally maintained at between 50 to 150 copies per cell in *cir* + yeast strains when selection is applied for the *LEU2d* gene on the plasmid (Kingsman *et al.*, 1985; Piper and Curran, 1990). Southern blot analysis of the DNA of W303-82, PMY1-82 and MD40-82 showed that p82-2B was being maintained at around 100 copies per cell in the same cultures of those transformants as were employed for HSP90 protein analysis (data not shown). The low basal expression of *HSP82* at 25°C in the absence of heat shock [10% of *HSC82* expression (Borkovich *et al.*, 1989; McDaniel *et al.*, 1989; Gross *et al.*, 1990)] multiplied by a gene dosage factor of 50-150 would produce a basal HSP90 overproduction of 5 to 15-fold in the absence of heat shock. The western blot analysis (Fig. 3.2 and 3.3b) indicated HSP90 overproduction at 25°C was approximately 2-fold less than this in the absence of heat shock in each of transformants W303-82, PMY1-82 and MD40-82.

3.2.3 Plasmid p82-2B causes an HSP90 overexpression markedly increased by growth at high temperature

These strains possessing normal or high *HSP82* gene dosage (transformants maintaining either pMA3a or p82-2B respectively) were grown to exponential phase at 25°C and 37.5°C and their HSP90 levels analysed. In contrast to the 25°C cultures, log phase cultures growing at 37.5°C showed considerably greater overexpression of HSP90 (approximately 30-fold; Fig. 3.3a). This increased expression at high temperature can be attributed to the appreciable basal expression of genes under HSE control at 37.5°C (Sorger and Pelham, 1987; Kirk and Piper, 1991).

3.2.4 HSP90 overexpression due to p82-2B increased approximately 10-fold in all three expression strains in response to a 25-39°C heat shock

The introduction of approximately 100 extra heat shock genes into cells might be expected to have dramatic effects on the heat shock response. It might, for example, lead to titration-out effects of the available heat shock transcription factor leading to a suppressed heat shock response. Alternatively HSP90 itself might be involved in the regulation of this response. It was important therefore to determine if cells with multiple *HSP82* gene copies display a normal induction of chromosomal heat shock genes (3.2.4.1); also to investigate if these multiple *HSP82* genes display the same level of heat induction as is found when *HSP82* is present as a single copy (3.2.4.2).

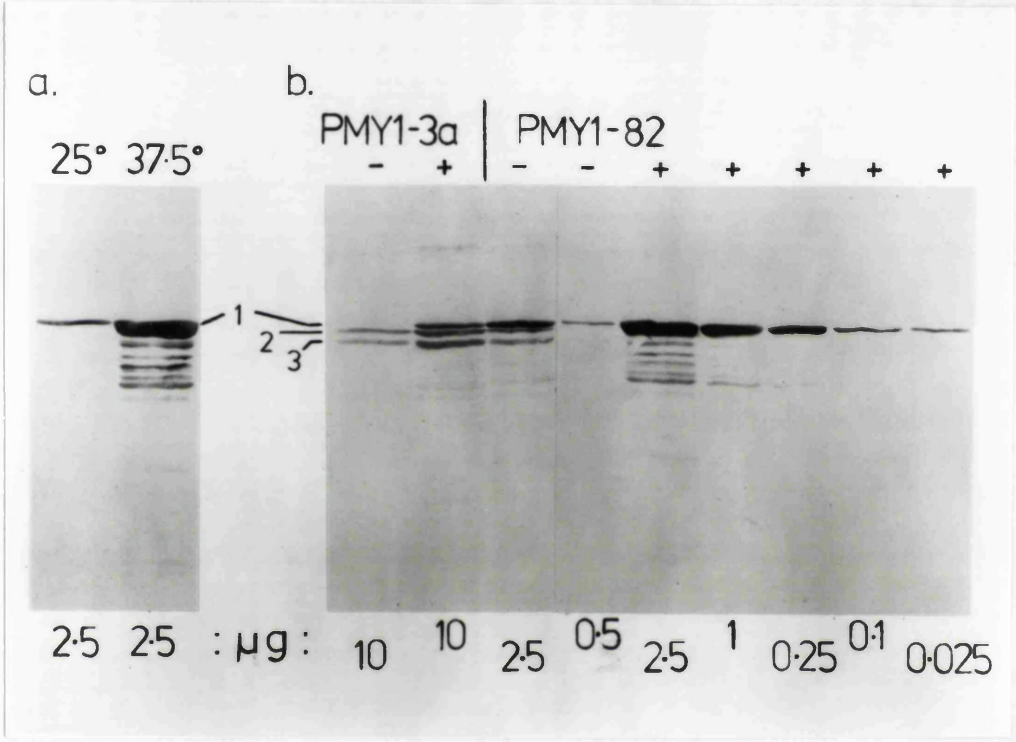


Figure 3.3 Western blot analysis of HSP90 protein levels.
(a) Samples from PMY1-82 maintained in log growth for several generations either at 25°C or at 37.5°C. (b) Comparison of relative HSP90 expression levels through serial dilution of total cell protein samples. The analysis of PMY1-3a and PMY1-82 protein before (-) or 75 min after (+) a 25-39°C heat shock is shown, the amount of protein loaded into each gel track being given at the base of the figure. Bands 1-3 are as indicated in the legend to Fig.3.2.

3.2.4.1 Investigation of the heat shock response of MD40-82 and MD40-3a by protein pulse labelling

The experiments in Figs.3.2 and 3.3b show that the presence of *HSP82* on a high copy number vector allowed strong heat induction of HSP90 in heat shocked cells. The relative overexpression of *HSP82* was also examined by pulse labelling of MD40-82 and MD40-3a with [^{3}H] leucine in the presence and absence of heat shock. Fig.3.4 shows an autoradiograph of a 1D SDS polyacrylamide gel containing the pulse labelled proteins obtained when MD40-82 and MD40-3a were labelled at 25°C and also following heat shock to 39°C. HSP90 labelling in MD40-82 was considerably increased compared with HSP90 synthesis in a transformant with only a single copy of *HSP82* (MD40-3a). Also MD40-82 showed almost normal induction of heat shock proteins other than HSP90. This shows that high *HSP82* gene dosage does not dramatically alter other aspects of the cellular response to heat shock. Fig.3.4 shows the heat induction of HSP104, HSP70, HSP35 and HSP26 in MD40-82 is similar to the induction of these same heat shock proteins in cells isogenic to MD40-82 but for the presence of just a single *HSP82* gene (MD40-3a; Fig.3.4). Similar results were obtained by comparison of the proteins pulse-labelled during heat shock in W303-82 as compared to W303-3a; also PMY1-82 as compared to PMY1-3a (data not shown).

3.2.4.2 Analysis of HSP90 levels by western blotting

To quantitate the extent of HSP90 over-expression, samples of total cell protein from unstressed and heat shocked cultures of W303-82, PMY1-82 and MD40-82 were sequentially diluted, then compared by western blotting. A typical dilution analysis of PMY1-82 samples is shown in Fig.3.3b. It shows the HSP90 of transformants W303-82, PMY1-82 and MD40-82 increasing approximately 10-fold following a 25°C-39°C heat shock, becoming 30-40% of total cell protein 75 min after temperature upshift (Figs.3.2 and 3.3b).

In yeast cells with normal HSP90 gene levels(not overexpressing HSP90) *HSP82* mRNA increases 14-fold with a 25-39°C heat shock (McDaniel *et al.*, 1989; Gross *et al.*, 1990) and total HSP90 protein approximately doubles as the *HSP82* product accumulates to a level approximating to that of the *HSC82* product (see samples from W303-3a, PMY1-3a and MD40-3a in Figs 3.2 and 3.3b). HSP90 measurements for transformants W303-82, PMY1-82 and MD40-82 revealed that the induction ratio of the *HSP82* product with heat

shock was relatively unaffected by an approximate 100-fold increase in *HSP82* gene copy levels (Figs. 3.2 and 3.3b). That this induction ratio has not undergone major change, also that chromosomal heat shock genes are showing normal heat activation (Fig. 3.4), shows that yeast can correctly regulate a large number of artificially-introduced genes subject to HSE regulation.

3.2.5 Effects of HSP90 overexpression on high temperature growth

Reducing the HSP90 of yeast has little effect on growth at 25°C, but causes major reductions in growth at 37.5°C (Borkovich *et al.*, 1989; a study conducted in strains of the same genetic background as W303-3a). Reductions in the HSP90 of mouse L cells also prevent growth at high temperatures (Bansal *et al.*, 1991). In this study the 3 to 7-fold overexpression of HSP90 in W303-82, PMY1-82 and MD40-82 at 25°C (Figs.3.2 and 3.3b) also had minimal effect on growth at this temperature (compare these transformants with W303-3a, PMY1-3a and MD40-3a; Fig.3.5a). However the much higher over-expression of HSP90 in W303-82, PMY1-82 and MD40-82 cultures growing at 37.5°C (Fig.3.3a) was associated with an appreciable reduction in growth rate at this temperature (Fig. 3.5b). This reduction in 37.5°C growth was least marked in cells of W303-3a genetic background, although W303-3a transformants grew less well than transformants of the two other expression strains (MD40-4c and PMY1) at this temperature (Fig.3.5b). Since therefore both underproduction and high overproduction of HSP90 reduce growth at 37.5°C, a temperature close to the maximum for growth of *S. cerevisiae* (Piper *et al.*, 1988), there is probably an optimal HSP90 level for cell proliferation at higher growth temperatures.

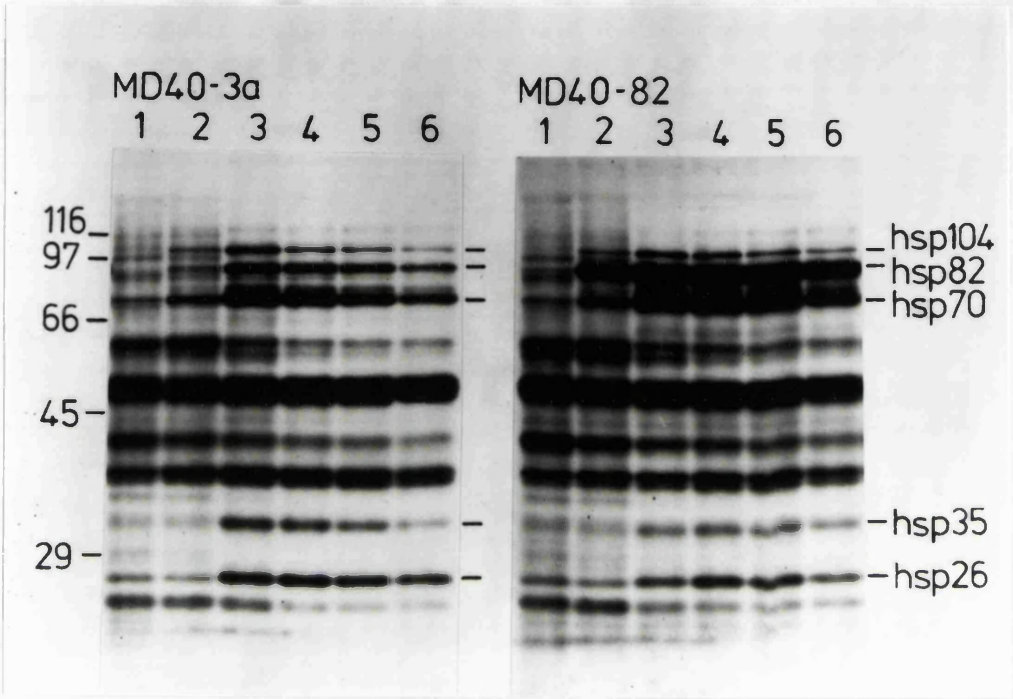


Figure 3.4 [^{3}H] leucine pulse-labelling of the proteins of transformants MD40-3a and MD40-82 before and after heat shock.

Cultures initially in log growth at 25°C were pulse-labelled for 15 min at this temperature (1); and at 0-15 min (2), 15-30 min (3), 30-45 min (4), 45-60 min (5) or 60-75 min (6) following a 25-39°C heat shock. Positions of unlabelled molecular mass markers are indicated on the left in kilodaltons, while strongly-labelled protein bands corresponding to major yeast heat shock proteins (Lindquist, 1986; Lindquist and Craig, 1988; Borkovich *et al.,* 1989; Sanchez and Lindquist, 1990; DeVirgilio *et al.,* 1991a; Parsell *et al.,* 1991) are indicated on the right.

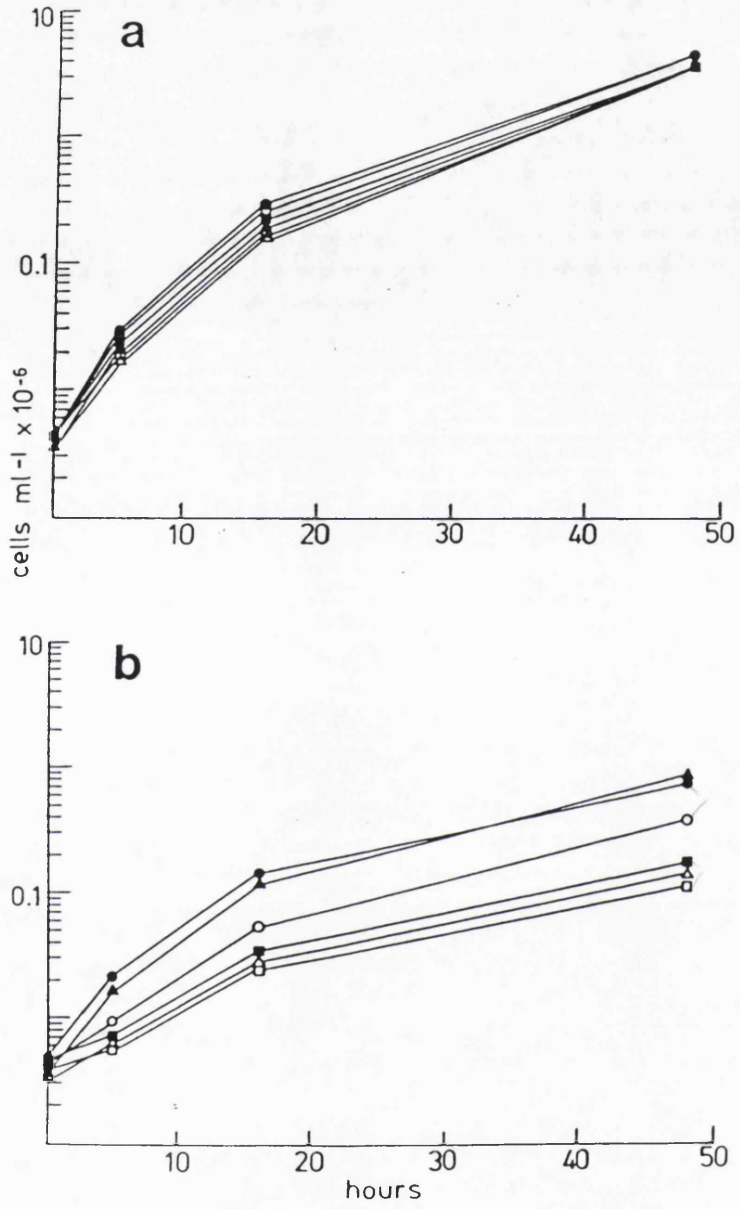


Figure 3.5 Growth at 25°C (a) and 37.5°C (b) of transformants MD40-3a (●), MD40-82 (○), PMY1-3a (▲), PMY1-82 (△), W303-3a (■) and W303-82(□).

3.2.6 Effects of HSP90 overexpression on thermotolerance

As outlined in Chapter 1, HSPs are postulated to protect organisms from the toxic effects of heat and other stresses and to aid in the recovery following stress. Induction of the heat shock response by either an increase in ambient temperature or the action of agents such as ethanol (Plesset *et al.,* 1982) results in elevated tolerance to higher temperatures (thermotolerance) and other stresses (McAlister and Finklestein, 1980). The kinetics of synthesis and degradation of HSPs have been found to parallel thermotolerance induction and decay which has led to the proposal that the heat shock response serves primarily to protect the cell (Lindquist, 1986). Whether the HSPs themselves are involved in the resistance to stress is a contentious issue. There are several lines of evidence which suggest that HSP synthesis is not necessary for elevation of tolerance (reviewed in Lindquist 1986; Piper 1993).

3.2.6.1 Effects of HSP90 overexpression on the thermotolerance of log phase cultures

Thermotolerance can be measured as cell survival during short exposure to high, potentially-lethal temperatures, well above the maximum temperature of growth. It is usually increased by a prior nonacute heat shock, conditions that allow the induction of the heat shock response (Lindquist, 1986; Lindquist and Craig, 1988; Parsell *et al.,* 1991; Panaretou and Piper, 1990). High *HSP82* gene dosage reduced thermotolerance at 52°C in PMY1-82 and MD40-82 initially in log growth at 25°C (compare these transformants with PMY1-3a and MD40-3a; Fig.3.6a,b). 52°C is approximately 10°C above the maximum temperature permitting protein synthesis in *S.cerevisiae* (Piper *et al.,* 1988). Even after prior shift from 25°C to 39°C for 40 min, a sublethal heat shock which will increase thermotolerance and cause appreciable increases in HSP90 (Fig. 3.2 and 3.3b), the presence of multiple *HSP82* genes still caused a reduction in thermotolerance (Fig. 3.6a-c). These effects were however strain-dependent, since in W303-82 the high *HSP82* gene dosage caused only a small thermotolerance reduction (Fig. 3.6c). This was apparently due to recessive mutations in the W303 genetic background, since the diploid strain formed by mating haploid transformants PMY1-82 and W303-82 displayed thermotolerance levels that were practically identical to those observed with its PMY1-82 parent (data not shown).

3.2.6.2 Effects of HSP90 overexpression on the thermotolerance of stationary phase cultures

High *HSP82* gene dosage was also found to cause strain-dependent reductions in the thermotolerance of stationary phase yeast cultures (Fig. 3.6d-f). As with the log phase cultures, the smallest reductions in thermotolerance were those shown by W303-82 as compared to W303-3a (Fig. 3.6f).

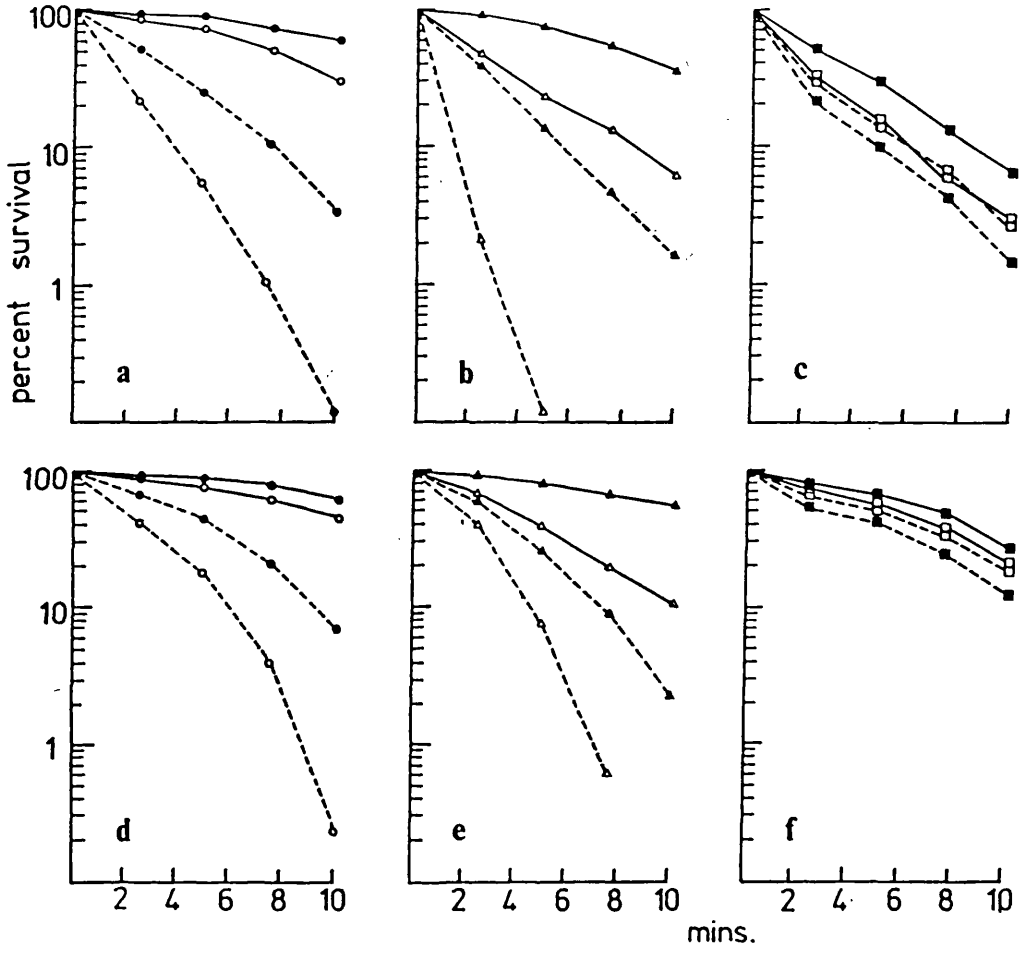


Figure 3.6 Thermotolerance of exponential (a-c) or stationary phase (d-f) 25°C cultures of transformants of strains MD40-4c (a,d), PMY1 (b,e) and W303-1A (c,f) containing either plasmid pMA3a (solid symbols) or P82-2B (open symbols).

Viability was measured at intervals after direct shift to 52°C from 25°C (dashed lines), or a shift to 52°C following a sublethal heat shock (25°C to 39°C for 40 min; continuous lines).

3.3 Discussion

The results reported here describe a study in which the homologous yeast HSP90 was overexpressed in three *S.cerevisiae* strains, an investigation conducted with three aims as stated in section 3.1.

3.3.1 Authentic temperature-regulation of a heat shock gene (*HSP82*) inserted into yeast on a high copy number vector

When regulatable promoters are used to express genes on high copy number yeast expression vectors it is frequently found that regulation of these promoters has become defective. This is due to an insufficiency of key transcription factor molecules now that multiple DNA binding sites for these factors have been introduced on the plasmid. An example of such loss of tight regulation is that shown by the galactose-inducible *GAL1* promoter at moderate copy levels, this resulting from the low cellular abundance of the *GAL4* regulator protein (Johnston and Hopper, 1982; Schultz *et al.*, 1987). In contrast, this study shows that the *HSP82* heat shock gene is still subject to almost normal heat-inducible regulation when present at 50-100 copies per cell on a high copy episomal vector. Cells maintaining plasmid p82-2B showed an approximate 10-fold induction of the *HSP82* product following a 25-39°C heat shock (Figs. 3.2 and 3.3b), an induction level comparable to that seen in cells with the same gene as a single chromosomal copy (Borkovich *et al.*, 1989; McDaniel *et al.*, 1989; Gross *et al.*, 1990).

HSP82 is probably a good model sequence with which to study the effects of gene dosage on heat shock gene expression since its transcription is apparently under the control of a single HSE sequence (McDaniel *et al.*, 1989; Gross *et al.*, 1990). Of all the temperature-regulated systems for controlling gene expression in *S.cerevisiae*, HSE-directed expression is probably the most practicable and versatile (Kirk and Piper, 1991). That HSE-directed expression is still authentically regulated at high gene copy levels further increases the versatility of this expression system. If HSF, or another factor needed for the induction of heat shock genes, was to become limiting in cells with multiple episomal *HSP82* copies, this would result in a pronounced perturbation of the heat shock response. This was not observed, there being no appreciable loss of the inducibility of chromosomal heat shock protein genes in cells with 50-150 episomal *HSP82* gene copies (Fig. 3.4).

3.3.2 Increasing normal cellular levels of HSP90 affects a number of physiological properties

This study did not find that high *HSP82* gene dosage and the resultant increases in HSP90 level elevated thermotolerance. Instead moderate thermotolerance decreases were consistently observed with HSP90 overexpression (Fig. 3.6). This protein in high level is therefore not protective against heat damage, which is perhaps surprising in view of the evidence for protective effects of HSP70 overproduction (Lindquist and Craig, 1988). Also apparent with HSP90 overexpression were reductions in growth at 37.5°C, a temperature close to the maximum for *S.cerevisiae* (Fig. 3.5b). Taken together with a previous study showing that HSP90 underexpression reduces high temperature growth (Borkovich *et al.,* 1989), this indicates that there is an optimum HSP90 level for the growth of yeast at 37.5°C.

3.3.3 Yeast is an expression host suitable for the high level synthesis of HSP90

Log phase cultures of W303-82, PMY1-82 and MD40-82 that have been heat shocked to 39°C for a short period are a suitable starting point for large-scale isolation of substantially-undegraded yeast HSP90. HSP90 isolated from these cultures was greater than 90% intact, as judged by western blotting (Figs. 3.2 and 3.3b). It should also be possible to isolate HSP90 in substantial quantity from stationary cultures of W303-82, PMY1-82 and MD40-82 since, in common with many other heat shock genes of *S.cerevisiae* (Tanaka *et al.,* 1988; Praekelt and Meacock, 1990; Kurtz *et al.,* 1986; Boorstein and Craig, 1990), *HSP82* is induced both by heat shock and as cells undergo the transition from exponential growth to stationary phase (Lindquist and Craig, 1988). However all our attempts to isolate HSP90 from stationary MD40-82, PMY1-82 and W303-82 cultures were unsuccessful, this protein being extensively degraded when the crude cell extracts were analysed by western blotting. In an attempt to avoid this problem the p82-2B expression plasmid was transformed into the multiply protease-deficient strain BJ2168 (Sorger and Pelham, 1987). However the resultant transformants grew poorly and they were therefore not subjected to detailed analysis.

The results described in this Chapter have been published as "Lili Cheng, Karen Hirst and Peter W. Piper (1992) Authentic temperature-regulation of a heat shock gene inserted into yeast on a high copy number vector. Influence of overexpression of HSP90 protein on high temperature growth and thermotolerance. *Biochim. Biophys. Acta 1132,* 26-34".

CHAPTER 4

HSP90 PURIFICATION AND THE PREPARATION OF AN AFFINITY COLUMN FOR THE STUDY OF PROTEINS ASSOCIATING WITH HSP90

4.1 Introduction

As with the other major HSPs, HSP90s are highly conserved throughout evolution and present as multigene families, with members of these families expressed in unstressed cells as well as following heat treatment (Burdon, 1986; Lindquist and Craig, 1988). The strong evolutionary conservation of HSP90s indicates that they must play important functional roles in the cell. However, these roles have not been clearly established, although some HSP90 of mammalian cells exists complexed with the steroid receptor, maintaining the receptor in the inactive state pending agonist binding (Joab *et al.*, 1984; Sanchez *et al.*, 1987). An association of HSP90 with many protein kinases has been observed and mammalian HSP90 is itself phosphorylated (see section 1.2.1). It has only recently been proven that the HSP90 universally present in eukaryotic cells assists authentic protein folding and therefore represents a major class of chaperone (Wiech *et al.*, 1992). It has also been shown that HSP90 has ATPase activity (Nadeau *et al.*, 1993). Even so, it is possible that HSP90 works by a completely different mechanism from the HSP70, HSP60 or GroEL chaperones since ATP is not needed for its chaperone action (Wiech *et al.*, 1992). The field is now set for structural studies on the HSP90 chaperone.

The *S. cerevisiae* HSP90 genes produce proteins with molecular weights of 80,885 (the *HSC82* product; Borkovich *et al.*, 1989) and 81,419 (the *HSP82* product; Farrelly and Finkelstein, 1984). This chapter describes the purification of HSP90 and a preliminary analysis of the HSP90-associating proteins in yeast cell extracts. The strain used for HSP90 preparation (PMY1-82; Chapter 3) contained approximately 100 copies of the *HSP82* gene and overexpressed HSP90 to high levels when heat shocked, facilitating purification of HSP90. Availability of substantial amounts of purified HSP90 should aid the determination of the function(s) of this stress protein.

An HSP90 affinity resin was also prepared to identify the other proteins binding to HSP90. Binding of an effector molecule by a biomacromolecule is the primary step in

most biological functions (e.g. carrier proteins that bind small molecules to transport them in tissues or vascular fluids). Affinity resins present one of the most rapid, efficient and convenient means of detecting specific protein-protein interactions, especially valuable where stability and the amounts of available protein are severely limiting. Since mammalian HSP90 exists either as a homodimer or in heterodimeric association with a number of important regulatory proteins (Lindquist and Craig 1988), it would be interesting to know what kind of proteinare associated with HSP90 in yeast. It is possible that HSP90 forms a complex with a number of unidentified proteins, some of which may be the cohorts or "assistants" (Gething and Sambrook, 1992) of chaperone action. In this study, the affinity support used was Bio-Rad Affi-Gel 15. Affi-Gel 15 is an activated affinity support that offers rapid, high efficiency coupling for all ligands with a primary amino group, including proteins throughout the entire range of pIs. The Affi-Gel 15 is the N-hydroxysuccinimide ester of a derivatized crosslinked agarose gel bead support, and contains a cationic charge in its 15-atom spacer arm which significantly enhances coupling efficiency for acidic proteins at physiological pH. The purified HSP90 was immobilized to Affi-Gel beads (see section 4.2.2) and proteins selectively retained from post ribosomal supernatants (S100) in yeast analysed by elution and gel electrophoresis (see section 4.2.3).

4.2 Results

4.2.1 Purification of HSP90 from heat shocked PMY1-82 cells

For the purification of HSP90, four liters of PMY1-82 cells were grown in minimal dextrose (SD) medium (with histidine but omitting leucine to ensure plasmid maintenance) to log phase at 25°C. These were then heat shocked for 40min at 39°C. The cells were harvested and washed with cold protein extraction buffer (20mM bis-tris propane pH7.4, 1mM dithiothreitol, 1mM EDTA plus proteinase inhibitors, section 2.10.2), and resuspended in 20ml of this buffer. Total cell extracts were prepared by glass bead vortexing and centrifugation at 16K rpm for 20min in a Sorvall SS-34 rotor at 4°C. The large pellet consisting of mitochondria, nuclei and unbroken cells was discarded. The supernatant was treated sequentially with 30%, 40%, 50% and 60% ammonium sulphate at 4°C, the precipitates obtained at each ammonium sulphate level being isolated by centrifugation at 8K rpm (SS-34 rotor) for 20 min. The pellets of protein precipitated by each ammonium sulphate concentration were dissolved in 5-15ml cold protein extraction buffer, and extensively dialysed against four liters of the same buffer. The HSP90 composition of each fraction were analysed by SDS-PAGE (Fig.4.1). This gel indicated that most HSP90 precipitated from 30% to 50% $(NH_4)_2SO_4$. The total

protein precipitating from 30% to 50% $(NH_4)_2SO_4$ treatment was approximately 900mg.

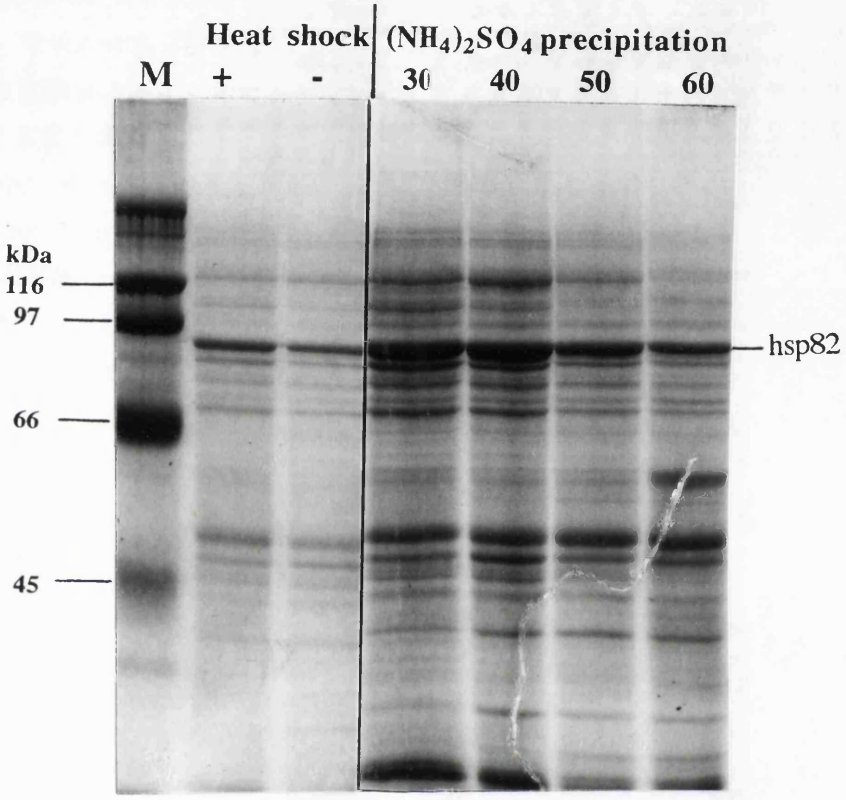


Figure 4.1 Fractionation of heat-shocked PMY1-82 soluble fraction protein by selective ammonium sulphate precipitation

Yeast strain PMY1-82 was grown at 25°C to exponential phase and heat shocked at 39°C for 40min. The soluble protein fraction was treated with 30%, 40%, 50% and 60% $(NH_4)_2SO_4$ at 4°C. The protein precipitating at each step of fractionation was dialysed and analysed for HSP90 level by 10% SDS-PAGE. The total protein of unstressed and heat shocked PMY1-82 was run in parallel tracks. Proteins were stained with Coomassie blue, molecular mass markers being on the left.

The protein precipitating from 30% to 50% $(NH_4)_2SO_4$ was redissolved, centrifuged at 20K rpm for 30min and applied to a Q Sepharose column previously equilibrated with the bis-tris propane buffer. The column was washed with buffer until the A_{280} returned to a base-line value. A number of polypeptides did not bind to the column including 21% of the HSP90. Those proteins which did bind to the column were eluted with a linear gradient of 0-1.0 M KCl in bis-tris propane buffer. One major peak of optical density was observed to elute at approximately 0.3 M KCl (Fig.4.2). This peak, composed of several fractions, contained the vast majority of the HSP90. These fractions were pooled and concentrated by pressure dialysis using a Amicon concentrator with YM10 membrane to yield around 30mg HSP90 in a volume of approximately 5 ml. This was chromatographed on a Sephedex S-200 gel filtration column developed in the same buffer. A single peak of optical density eluted from the column comprising HSP90 with minimal contamination from other proteins (Fig. 4.3). The purified protein was electrophoresed on SDS-PAGE to examine its purity (Fig.4.3). The peak fractions containing HSP90 were pooled and concentrated as before. After this final stage, the yield of purified HSP90 was only around 14mg.

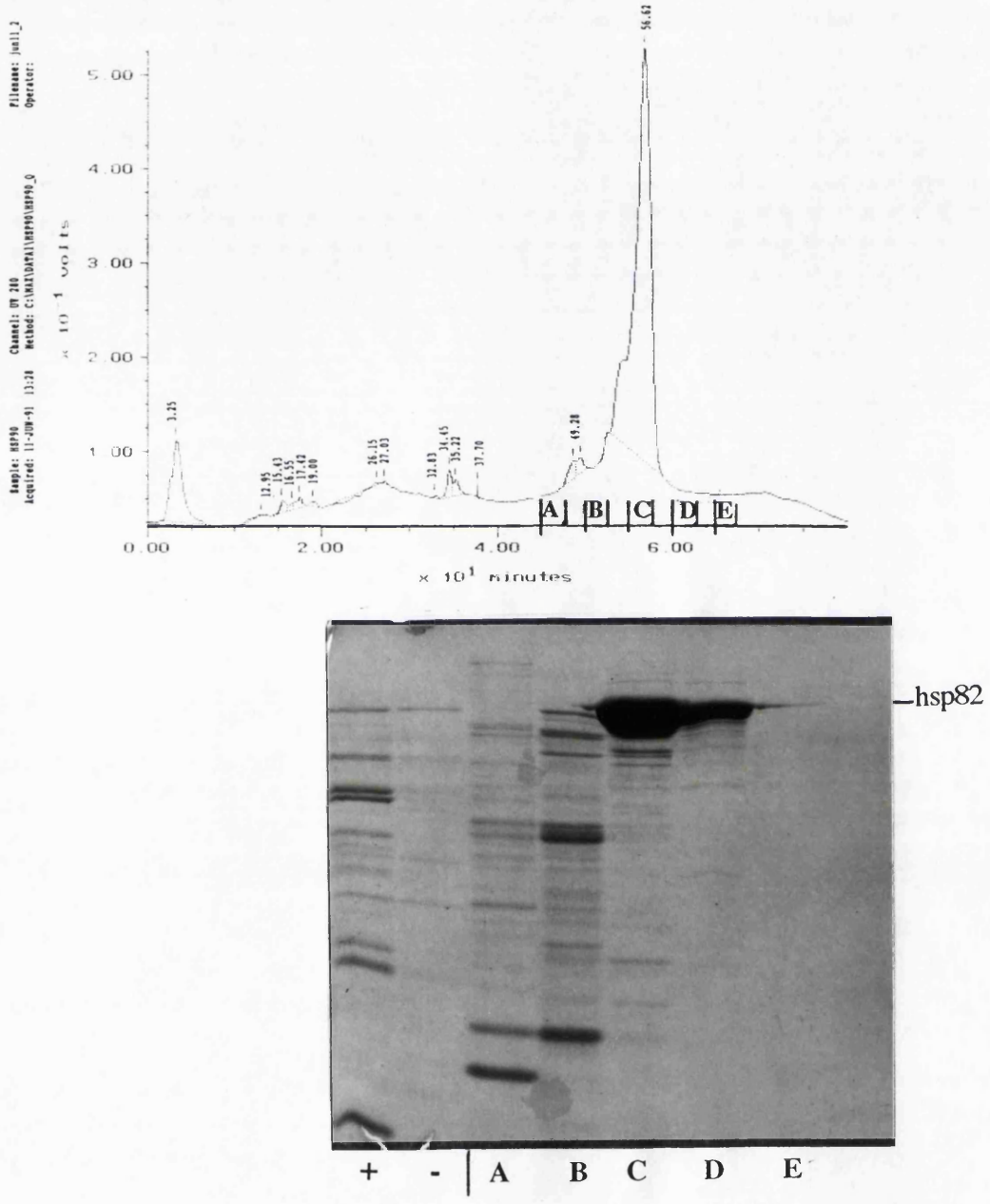


Figure 4.2 Q Sepharose column chromatography of the impure HSP90 after $(NH_4)_2SO_4$ fractionation

Upper diagram: A_{280} elution profile for the column developed with a linear gradient of 0-1.0M KCl in bis-tris propane buffer. The fractions pooled for subsequent S-200 gel filtration are indicated. The flow rate was 120ml/h. Fractions of 4ml were collected.

Lower diagram: analysis of fractions on a 10% SDS-PAGE stained with Coomassie blue. The first two trackes are total protein of unstressed (-) and heat shocked (+) PMY1-82.

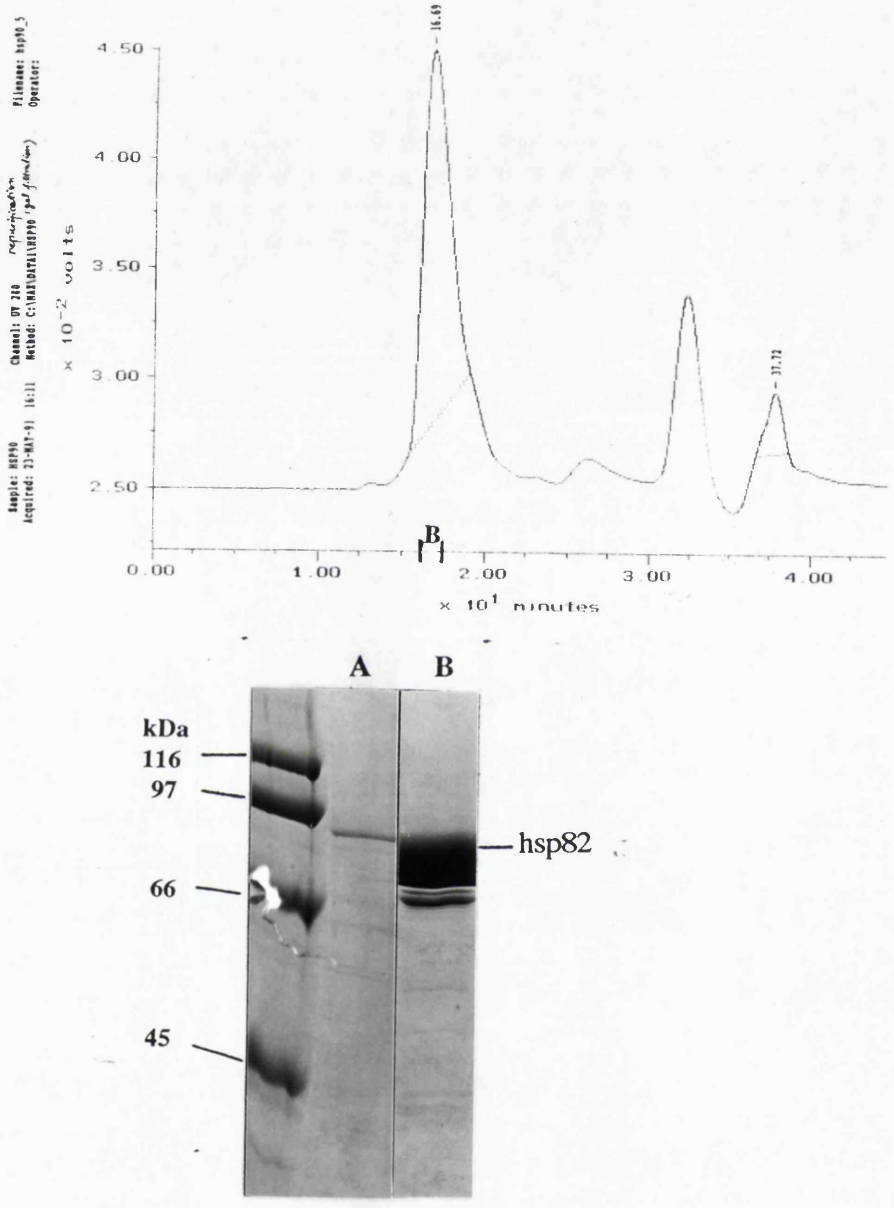


Figure 4.3 Sephadex S-200 gel filtration column chromatography of HSP90 protein.

HSP90-containing fractions from the Q-Sepharose column indicated in Fig.4.2 were concentrated and loaded onto a 60x2.6cm S-200 column. The elution buffer was the same buffer with 0.15M KCl, the flow rate was 90ml/h and fractions of 1ml were collected. The main peak (B) was pure HSP90 according to 10% SDS-PAGE analysis. (A) is a HSP90 marker.

4.2.2 Covalent binding of HSP90 to Affi-Gel-15

Adsorption of purified HSP90 to Affi-Gel beads was essentially as in Nadeau *et al.* (1993). 0.2 ml packed volume of hydroxysuccinimide activated Affi-Gel-15 beads in 50mM $NaHCO_3$ pH8.5 were incubated with 0.1mg purified HSP90 sample. It was important to determine if purified HSP90 binds to Affi-Gel beads under these conditions of coupling. Initially therefore 0.1mg HSP90 was incubated with 0.2ml Affi-Gel-15 beads in 50 mM $NaHCO_3$, pH8.5 for 2h at 4°C. The supernatant was saved and the beads were washed and boiled. All the supernatants were concentrated with centricon microconcentrators. Both concentrated supernatant and boiled sample were loaded on a SDS-PAGE gel and 1µg purified HSP90 was loaded on the same gel as a marker. After Coomassie blue staining the HSP90 band was only apparent in the Affi-Gel sample and not supernatant (data not shown). This confirmed that the Affi-Gel-15 beads had quantitatively bound the HSP90. This HSP90 resin is referred to as HSP90-Affi-Gel below.

4.2.3 Affinity binding of yeast proteins to the HSP90 affinity resin

It is important to determine if the HSP90-Affi-Gel column can selectively retain only HSP90-associated protein. The proteins bound to Affi-Gel which had HSP90 coupled, then treated with β-mercaptoethanol (to block further coupling) can be compared with proteins binding to control resin to which no HSP90 was bound and on which all coupling groups had been blocked with β-mercaptoethanol (referred to as Affi-Gel below).

S. cerevisiae BJ2168 soluble fraction extracts were prepared as in section 2.10.5.1. 0.2 ml of Affi-Gel beads which had been protected with 7mM β-mercaptoethanol were incubated with 10 µl of these BJ2168 protein extracts (5mg/ml) diluted to 0.2ml with 100 mM HEPES, pH7.5 (total volume of 0.4 ml) for 2h at 4°C. The mixture was then centrifuged for 30sec in a microfuge, and the supernatant (0.2 ml) was added to 0.2 ml of either Affi-Gel (control) or HSP90-Affi-Gel beads which had been pre-treated with 7mM β-mercaptoethanol in 0.2 ml of buffer A (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM $MgCl_2$, and 5 mM $CaCl_2$). After incubation for 2h at 4°C, the beads were washed with buffer A plus 0.1% Triton X-100 three times, removing the supernatant each time. Finally, the 0.2 ml of spun beads were mixed with an equal volume of 2x SDS protein sample buffer, boiled for 5 min, respun and the supernatant loaded on a 15% SDS-PAGE gel. After the gel had been run it was subsequently silver stained.

Many proteins were selectively retained on the HSP90-Affi-Gel (Fig.4.4), the pattern of these proteins being totally different from that of total protein extracts (Fig.4.4). The control Affi-Gel column showed virtually no associated protein (Fig.4.4). This demonstrated that the HSP90-Affi-Gel column selectively retained a discrete set of proteins in the yeast postribosomal supernatant fraction.

Since HSP90 is one of the major HSPs, it is interesting to know whether the proteins associating with it are the same in unstressed and heat shocked cells. To investigate this extracts from unstressed and heat shocked BJ2168 cells were incubated separately with HSP90 Affi-Gel beads, and with control Affi-Gel beads. Many proteins were selectively retained by the HSP90 of the HSP90 Affi-Gel column, as revealed by silver-staining of these proteins after gel fractionation (Fig.4.4). The HSP90 associating proteins in unstressed and heat shocked extracts showed no apparent differences (Fig.4.4). Extracts from [^{3}H]-leucine pulse-labelled unstressed and heat shocked cells were also applied to HSP90-Affi-Gel and the retained proteins detected by fluorography were also the same (data not shown).

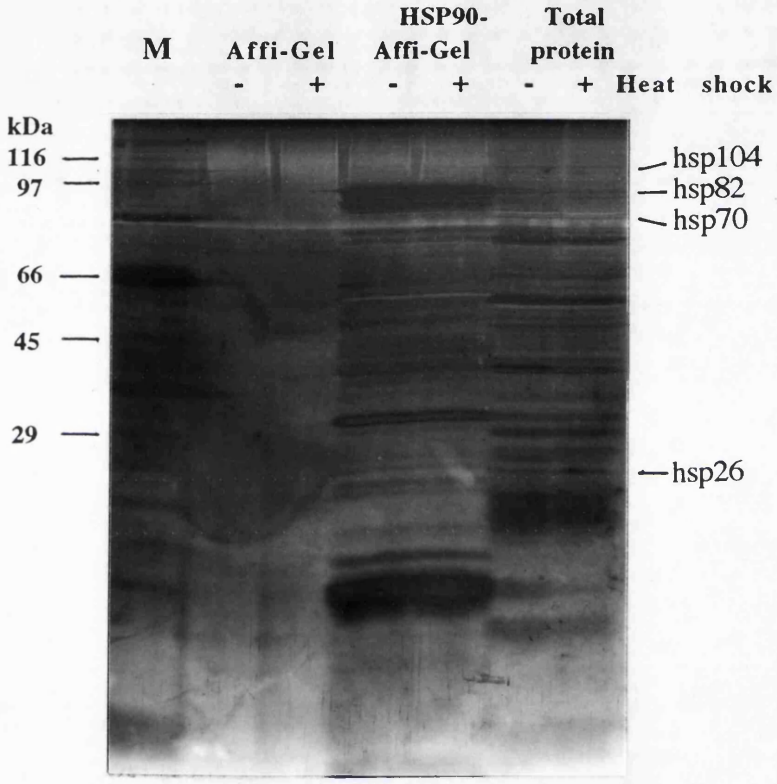


Figure 4.4 Affinity resin chromatography of HSP90-retained proteins in the postribosomal supernatant fraction of unstressed and heat-shocked cells.

HSP90 was coupled to Affi-Gel-15 and the resin washed. Protein extracts from unstressed and heat shocked BJ2168 cells were incubated with the Affi-Gel and HSP90-Affi-Gel resins which were then extensively washed. The beads were mixed with 2x SDS protein sample buffer, boiled, and the eluted protein fractionated by 15% SDS-PAGE, and detected by silver staining. Total protein in the extracts was also loaded, together with molecular weight markers.

Most of the evidence for formation of complexes between HSP90 and other proteins comes from immunoprecipitation studies (Nadeau *et al.*, 1993). As a complementary method, a HSP90-Affi-Gel affinity column was employed here to probe for protein interactions under conditions of physiological ionic strength and pH (150mM NaCl, pH 7.4). It might be thought that different results would be obtained if binding to HSP90 was conducted at different temperatures. To test this samples were applied to the affinity resin at different temperatures: 4°C, 28°C, 39°C and 42°C (equivalent to chill, optimal and heat shock temperatures for yeast). The silver stained band pattern of HSP90-retained proteins showed no effect of temperature, which was unexpected (data not shown).

4.2.4 Experiments investigating the conditions for release of HSP90-Affi-Gel associated proteins

Once protein has bound to the adsorbent it is necessary to wash off non-specifically bound contaminant molecules prior to elution of specifically-retained proteins. This was carried out by several washes with equilibration buffer containing Triton X-100. Elution of bound protein from HSP90-Affi-Gel should occur under any conditions that break the bonds which form the complex. These bonds would be expected to composed of a mixture of weak physical forces such as coulombic salt bridges, hydrogen bonds and Van der Waal's forces. Since many proteins were selectively retained by HSP90-Affi-Gel (Fig.4.4), it is interesting to know the nature of the interactions between HSP90 and these associated proteins. To determine if these interactions were essentially ionic, associated proteins were eluted on ice by sequential addition of different concentrations of NaCl (0.4M, 0.6M, 0.9M and 1.2M) in buffer A, the proteins not removed by 1.2M NaCl being removed by boiling. The result indicated two classes of interaction (Fig. 4.5). Some weakly bound bands were eluted by low salt, but the strongest bands were only seen to be eluted from the affinity resin after boiling. This indicates that the interaction between HSP90 and most of its associated proteins is relatively strong and nonionic, a point that need to be considered when analysing HSP90 function.

Since the ATPase activity of HSP70 has been suggested as a major thermodynamic force in the release and/or folding of HSP70-associated proteins (Gething and Sambrook, 1992), the intrinsic ATPase of HSP90s suggests they too may act as active protein foldases in spite of evidence that their chaperoning role is ATP-independent (Weich *et al.*, 1992). To assess whether or not ATP is involved in the release of HSP90 associated protein from HSP90-Affi-Gel, protein associated with HSP90-Affi-Gel was incubated at

32°C in 0.15M or 1.2M NaCl in buffer A with or without 1mM ATP, before analysis of the HSP90 retained and the released protein (Fig. 4.6). The result shows no effect of ATP on protein release. It may be that the HSP90 ATPase is not involved in this kind of protein release process.

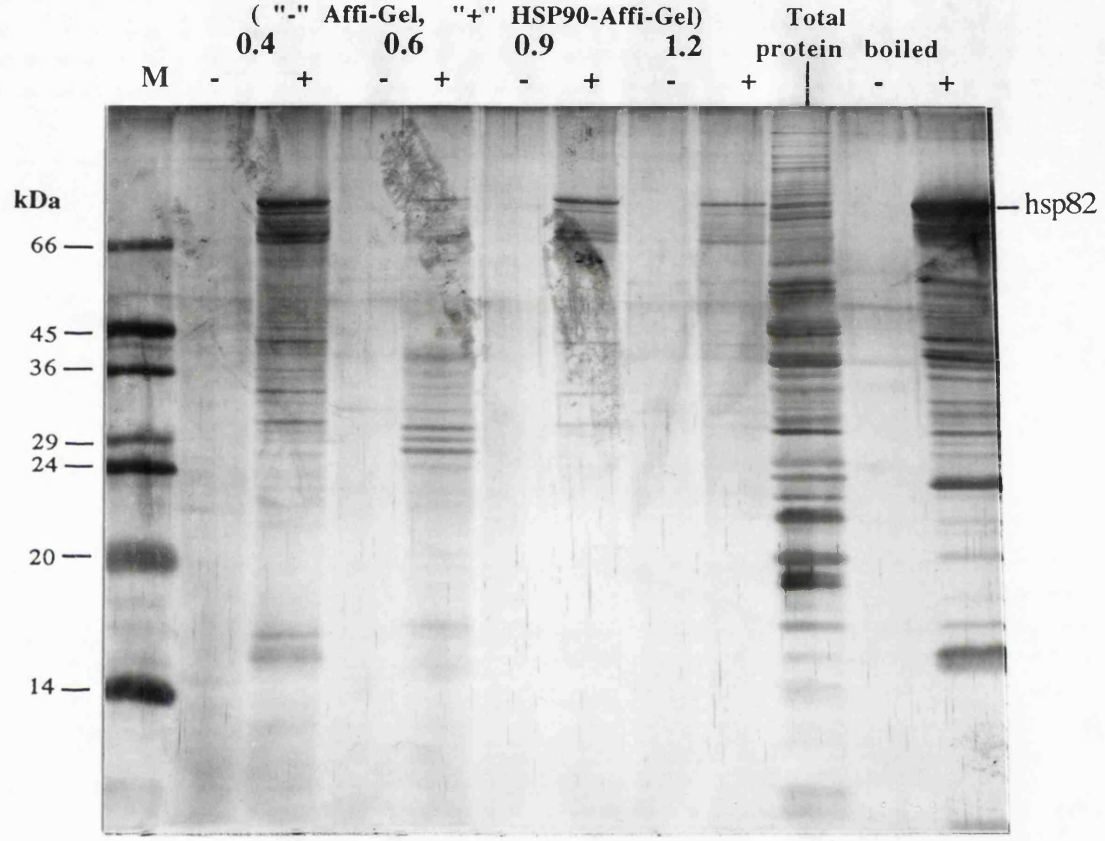


Figure 4.5 Elution of HSP90-associated proteins using different concentrations of NaCl and boiling.

The Affi-Gel coupling of HSP90 was as in Fig.4.4. The HSP90 associated proteins were eluted by sequential addition of 0.4M, 0.6M, 0.9M and 1.2M NaCl, the protein not released by 1.2M NaCl being released by boiling. The eluted samples were concentrated using centricon microconcentrators and analysed on 15% SDS-PAGE. Total BJ2168 protein and total HSP90-retained protein were also analysed. The gel was silver stained.

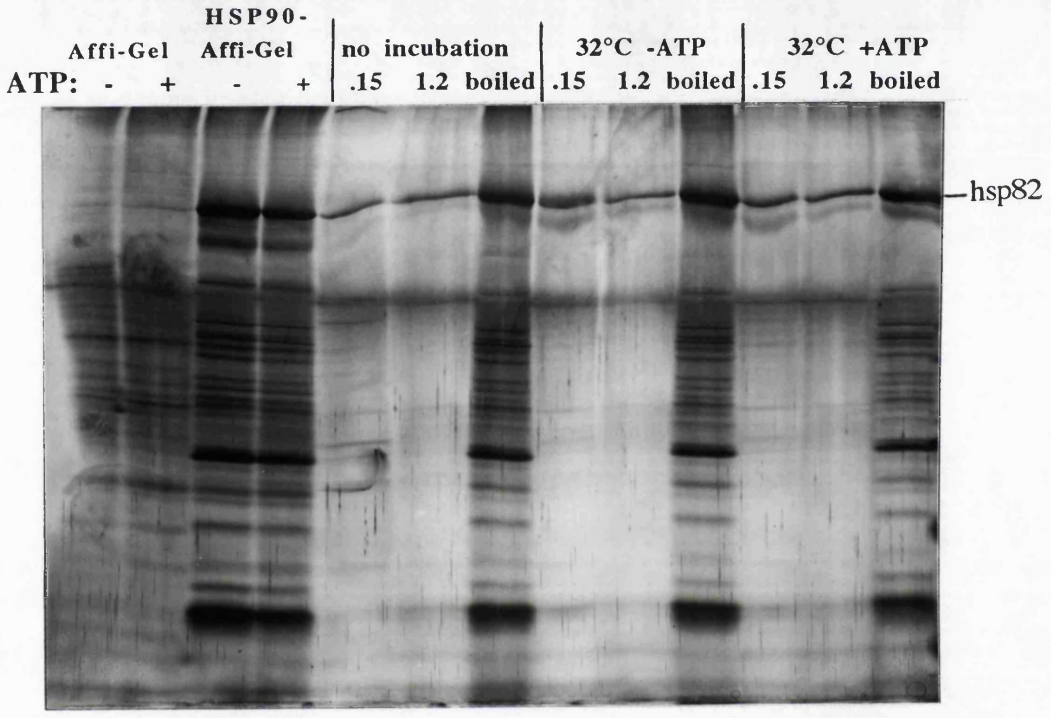


Figure 4.6 Elution of HSP90-associated proteins with or without ATP incubation

The Affi-Gel coupling of HSP90 was as in Fig.4.4. Proteins were eluted by sequential addition of 0.15M, 1.2M NaCl with or without 1mM ATP, the protein still retained in 1.2M NaCl being released by boiling. The eluted samples were concentrated and analysed by 15% SDS-PAGE as in Fig. 4.5.

4.3 Discussion

In order to identity HSP90-associated proteins, the proteins selectively binding to HSP90 Affi-Gel will need to be subjected to partial peptide sequence analysis and these sequences then compared with the current sequence databank. More than 1500 *S.cerevisiae* genes have now been entered into the database. This study showed that HSP90-Affi-Gel selectively retains a number of soluble yeast proteins. Unfortunately the amounts of protein isolated were too low for peptide sequencing. Silver staining is more sensitive than Coomassie blue staining. The procedure used here will need to be scaled up about 20-50 fold to get enough of the HSP90 associated proteins for sequencing. These experiments would have been carried out if time had permitted.

Nadeau *et al.* (1993) found four major protein species retained by immobilized yeast HSP90 as detected by silver staining, ranging in size from 27-29, 52-54 and 62KDa. In this study, the four major HSP90-associated proteins are of different sizes: 15-16, 16-17, 18-19 and 40-42KDa (Fig. 4.4). Since extracts in this study were from a multiply proteinase deficient strain (BJ2168), unlike extracts used by Nadeau *et al.* (1993), proteolysis is unlikely to be the reason that the HSP90 associated proteins seen here were smaller. The molecular weight of one of proteins seen in this study is similar to the 16 KDa yeast calmodulin (CaM). A Western blot of HSP90-retained protein was therefore probed with anti-yeast CaM antibody (gift of Dr M. Stark). While this gave a signal using a sample of total cell protein, it gave no signal with HSP90-retained protein (not shown). This indicates that CaM is not a HSP90-associated protein.

As mentioned in Section 4.1, the strain overexpressing HSP90 with a good yield used in this study was a product of the work in Chapter 3. From four liters of starter culture (about 30g wet weight of cells), 14mg of purified HSP90 was obtained. This should be compared with Nadeau *et al.* (1993) where 1mg of HSP82 was purified from 18g of yeast cells. There is therefore significantly increased final yield of pure HSP90 with *HSP82* gene overexpression, a good example of the advantages of developing a strain overproducing the protein of interest.

At best, affinity chromatography is a powerful technique for protein purification. Its high selectivity can, in principle, allow purification of a single protein of low abundance from a crude mixture of proteins. Secondly, if the affinity of the ligand for the protein is sufficiently high, the technique offers simultaneous concentration from a large volume. In

practice, such single-step affinity chromatography procedures are not common since their successful use requires careful consideration of a number of parameters. Clearly, the proteins associating with HSP90 include many that vary in abundance (Fig. 4.5). Here it has been shown that yeast HSP90 immobilized to Affi-Gel beads selectively retains several proteins in crude cell extracts. This approach is limited in that it gives only qualitative information, is biased toward high abundance partner proteins, could be clouded by nonspecific interactions and could detect secondary interactions with proteins already bound to HSP90. However, it offers a starting point to identify HSP90-associating proteins for further characterization.

CHAPTER 5

THE INFLUENCE OF THE LEVELS OF HSP90 PROTEIN: (1) HSP90 AFFECTS TREHALOSE ACCUMULATION DURING THE YEAST HEAT SHOCK RESPONSE

5.1 Introduction

Heat shock elicits a number of changes in *S. cerevisiae* in addition to the well-characterised induction of heat shock proteins. It triggers the accumulation of a large cytoplasmic pool of the disaccharide trehalose (α-D-glucopyranosyl (1-1) α-D-glucopyranoside), while a decline in intracellular pH stimulates plasma membrane ATPase activity (Fig.1.2). This trehalose accumulation has recently been discovered to be, like HSP synthesis itself, under negative regulation by the levels of certain HSPs, notably HSP70 and, to a lesser extent, HSP104 (Hottiger *et al.,* 1992). Besides regulating trehalose, HSP70 is also a major regulator of HSP synthesis in the heat shock response (Craig and Gross, 1991). HSP90 is another heat shock protein which might participate in the regulation in the stress response, since it forms complexes *in vivo* with several of the regulatory proteins of eukaryotic cells (section 1.2.1) including HSF (Nadeau *et al.,* 1993). This study employed yeast strains with normal, decreased or elevated HSP90 to determine whether HSP90 levels influence the trehalose accumulated in response to heat stress. HSP90 levels were shown to exert a small negative effect on this trehalose accumulation.

To obtain evidence of whether both the trehalose and heat shock protein inductions of the heat shock response are under similar control, the activity of a *lacZ* gene subject to HSE control was measured in the same strains with altered HSP90 levels. A small negative effect of HSP90 level on the heat activation of this sequence was apparent. The results are consistent with a model of the yeast stress response in which both the heat shock protein and trehalose syntheses are controlled mainly by levels of HSP70, the levels of other heat shock proteins such as HSP90 and HSP104 acting as more minor regulators.

Trehalose is accumulated by yeast both when it is heat shocked and when, due to nutrient limitation, it enters the stationary phase of growth (Attfield, 1987; Hottiger *et al.,* 1987a,b;

DeVirgilio *et al.*, 1991b; Hottiger *et al.*, 1989; Thevelein, 1984; VanLaere, 1989; Wiemken, 1990). This trehalose pool is thought to act primarily as a stress protectant rather than as a storage carbohydrate (Wiemken, 1990). The stationary phase trehalose accumulation is in response to declining cAMP levels, low cAMP causing an inactivation of neutral trehalase (Hottiger *et al.*, 1989; Thevelein, 1984; VanLaere, 1989; Wiemken, 1990). The rapid induction of trehalose by heat stress is largely independent of cAMP, since it occurs in mutants that have both high and low cAMP-dependent protein kinase activity (Hottiger *et al.*, 1989; Thevelein, 1984; VanLaere, 1989; Wiemken, 1990). Also it is only partially dependent upon *de novo* protein synthesis (DeVirgilio *et al.*, 1991a,b).

5.2 Results

5.2.1 Levels of heat shock protein HSP90 influence the induction of trehalose

5.2.1.1 Suppression of the normal induction of HSP90 with heat shock results in a small hyperaccumulation of trehalose

For this study three isogenic diploid strains, W303Leu, CLD82 and PLD82 were initially used in investigations of the effects of a 25°C-39°C heat shock on trehalose levels. W303Leu is wild-type with respect to HSP90 genes. CLD82, due to insertional inactivation of both *HSC82* alleles (Table 2.1), has lower HSP90 levels during 25°C growth but exhibits a strong HSP90 induction with heat shock (Borkovich *et al.*, 1989). PLD82, homozygous for an insertional inactivation of *HSP82*(Table 2.1), has almost normal HSP90 during growth but displays very little HSP90 increase with heat shock (Borkovich *et al.*, 1989). Fig. 5.1 shows Western blot analysis and [^{3}H] leucine pulse-labelling of the levels of HSP90 protein in these three strains. Trehalose levels were very low in 25°C cultures of all three strains, but showed the expected large increase with heat shock (Fig.5.2). There was a small, yet reproducible overproduction of trehalose in PLD82 as compared to either W303Leu or CLD82. Since these strains are completely isogenic, this can be directly attributed to the lack of a normal heat induction of HSP90 in PLD82. In all three strains a shift-down to 25°C after 1h at 39°C resulted in the rapid mobilisation of the accumulated trehalose (Fig.5.2). This is in agreement with previous reports (Attfield, 1987; Hottiger *et al.*, 1987a; Hottiger *et al.*, 1987b; Hottiger *et al.*, 1992) and is thought to reflect the activation of trehalase by a mechanism that involves protein phosphorylation (DeVirgilio *et al.*, 1991b).

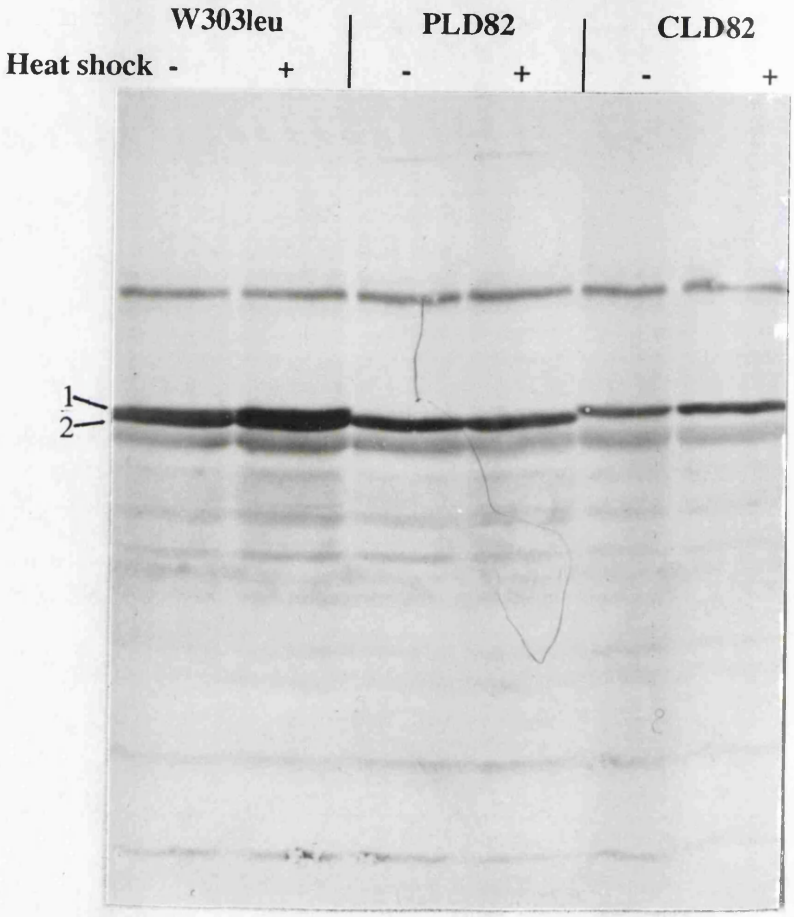
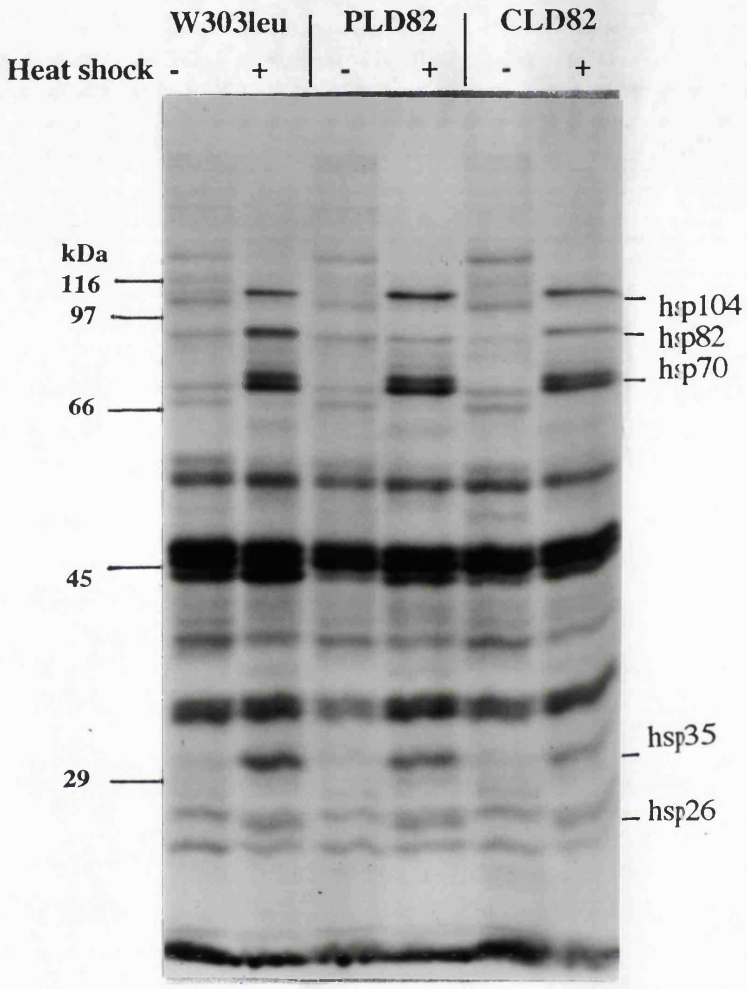


Figure 5.1 Western blot analysis (A) and [^{3}H] leucine pulse-labelling (B) of HSP90 protein in strains W303leu, PLD82 and CLD82.

(A). Cultures were all initially in exponential growth at 25°C, samples of total cell protein being prepared before (-) or 75min after (+) a 25-39°C heat shock. The polyclonal antiserum used in HSP90 detection crossreacts with both *HSC82* and *HSP82* gene products, the former migrating slightly faster than the latter on SDS polyacrylamide gels (Borkovich *et al.*, 1989). The *HSP82* and *HSC82* products are bands 1 and 2, respectively.



(B). [^{3}H] leucine pulse-labelling of the proteins of these three strains for 40min either at 25°C (-) or (0-40min) after a 25-39°C heat shock (+).

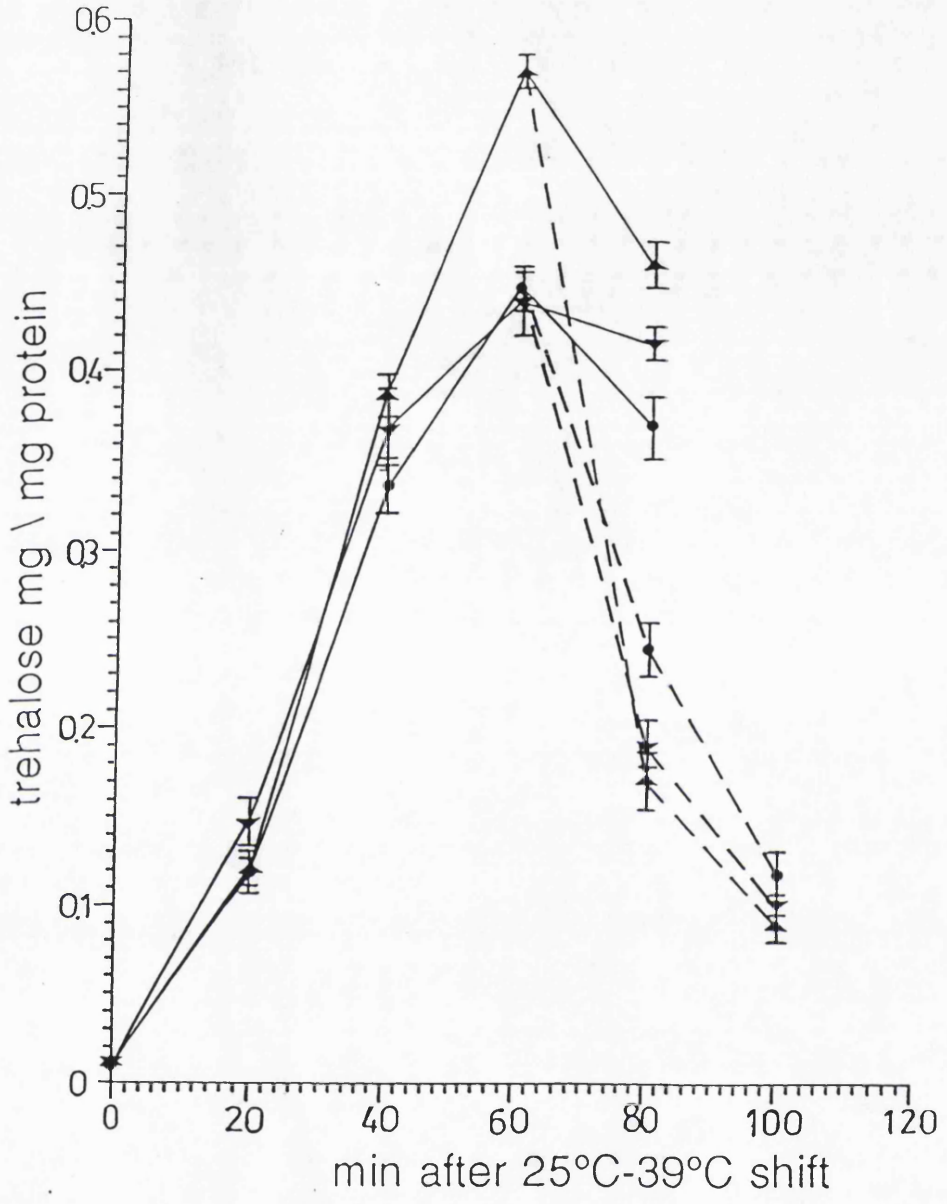


Figure 5.2 Measurement of trehalose induction in strains W303Leu, PLD82 and CLD82 after heat shock.

This experiment was conducted on cultures of W303Leu (●), CLD82(▼) and PLD82 (▲) in the early exponential phase of 25°C growth on YPD medium. At zero time the temperature was raised from 25°C to 39°C and samples taken for trehalose determination at 20min intervals (continuous lines). Part of each culture was heat shocked at 39°C for 60min and then allowed to recover at 25°C (broken lines). Error bars represent SEM for triplicate determinations.

5.2.1.2 Overexpression of HSP90 results in a small reduction in the trehalose accumulated with heat shock

Since substantial reduction in the HSP90 increase in heat shocked yeast leads to a hyperaccumulation of trehalose (Fig.5.2), overexpression of HSP90 might be expected to suppress the trehalose accumulation following heat shock. The experiment in Fig.5.3 showed this to be the case. It employed two transformants of strain W303-1A (W303-3a and W303-82) which are isogenic but for the presence of 50-150 extra episomal *HSP82* gene copies in W303-82 (section 3.2.1). The low basal expression of these multiple *HSP82* gene copies in W303-82 causes a 3 to 7-fold HSP90 overproduction at 25°C in this transformant as compared to W303-3a (section 3.2.2). Also The HSP90 of W303-82 increases 10-fold, to 30-40% of total cell protein, following a temperature upshift to 39°C, while the HSP90 levels of W303-3a are as in wild-type strains (section 3.2.4). At 39°C the trehalose accumulated by W303-82 was approximately 75% of that accumulated by W303-3a (Fig.5.3). This is further evidence of a small negative influence of HSP90 on trehalose, as also seen in the experiments with strains W303Leu, CLD82 and PLD82 (Fig.5.2).

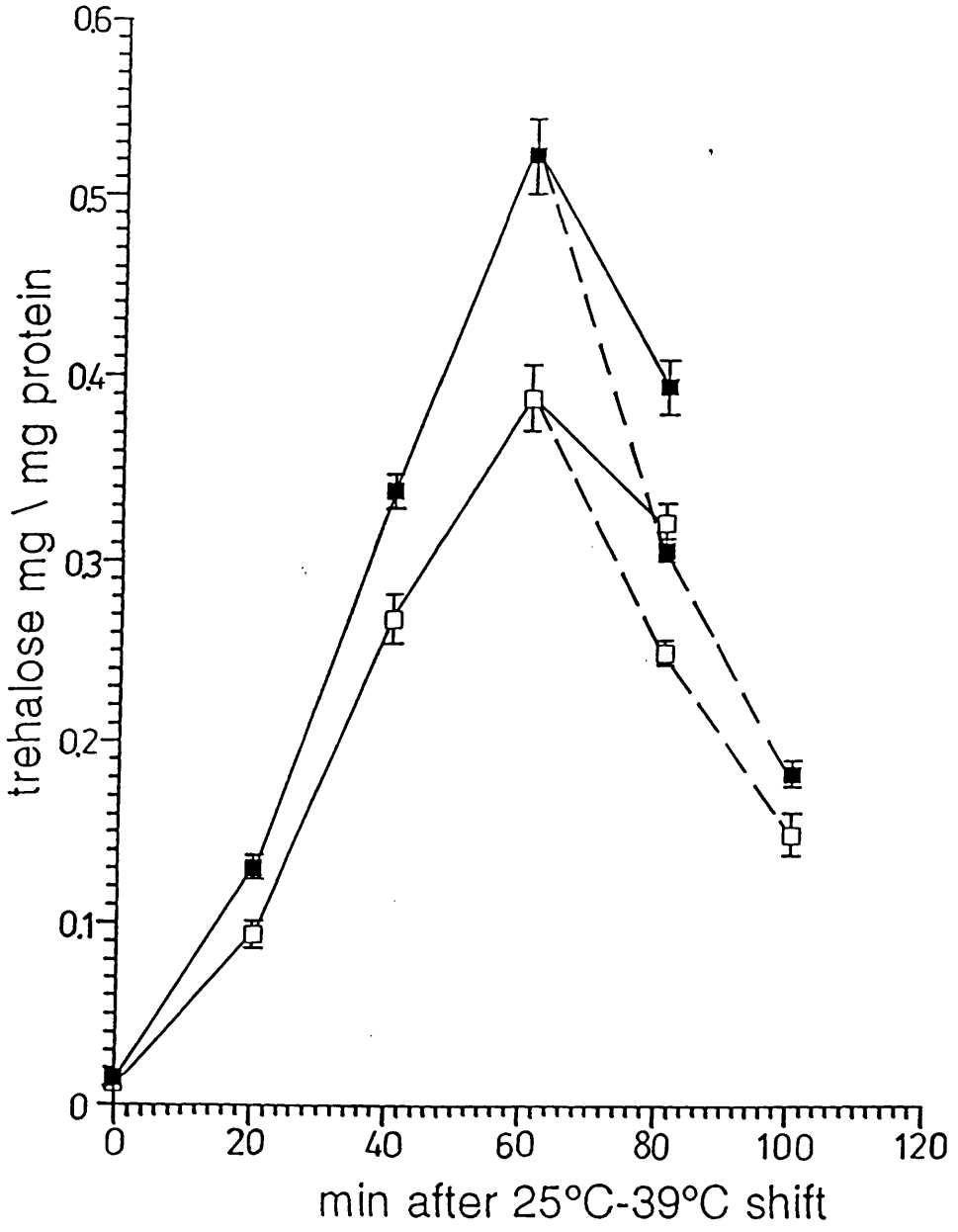


Figure 5.3 Measurement of trehalose induction in strains W303-82 and W303-3a after heat shock.

Cultures of W303-3a (■) and W303-82 (□) in early exponential growth at 25°C on SD medium were subjected to a 25°C to 39°C heat shock at time zero. Samples were taken for trehalose determination at 20min intervals (continuous lines). Part of each culture was heat shocked at 39°C for 60min and then allowed to recover at 25°C (broken lines). Error bars represent SEM for triplicate determinations.

5.2.2 Levels of heat shock protein HSP90 influence the induction from the heat shock element sequence during the heat shock response

5.2.2.1 HSP90 levels exert a small effect on HSE-*lacZ* expression

If the trehalose and HSP inductions of the heat shock response are both under similar control (Fig.1.2), the activity of the HSE promoter element might resemble trehalose in displaying a small dependence on HSP90 levels. The effects of heat shock on the expression of a *lacZ* gene under HSE control inserted into the three isogenic diploid strains, W303Leu, CLD82 and PLD82 (Table.2.1) were therefore investigated. Plasmid pHSE2 is a yeast-*E.coli* shuttle vector that carries the *E.coli lacZ* gene under the control of a HSE-containing yeast *CYC1* promoter (HSE-*lacZ*: Kirk and Piper, 1991). It was transformed into W303Leu, CLD82 and PLD82 by selecting for uracil prototrophy. Single transformants of each strain (W303Leu-pHSE2, CLD82-pHSE2 and PLD82-pHSE2) were then grown and the induction of their β-galactosidase activity determined during the course of a 25°C-39°C heat shock. In results that mirrored the trehalose accumulation by these strains (Fig.5.2), PLD82-pHSE2 showed a slightly greater and more prolonged HSE-*lacZ* expression as compared to either W303Leu-pHSE2 or CLD82-pHSE2 (Fig.5.4). This is the first demonstration of an effect of HSP90 levels on HSE-directed gene expression. The increase in HSE-directed β-galactosidase expression in PLD82-pHSE2 is relatively small (Fig.5.4) and it might therefore be difficult to demonstrate convincingly any similar effect on heat shock gene induction by *in vivo* pulse-labelling of HSPs, or by northern blot analysis of heat-induced transcripts. β-galactosidase produced from HSE-*lacZ* in heat shocked yeast turns over with a halflife of hours rather than minutes (Kirk and Piper, 1991) and, unlike the trehalose, is not rapidly degraded upon temperature shift-down.

HSE-*lacZ* expression was not investigated in W303-3a or W303-82. This was because the several additional heat-inducible (*HSP82*) genes in W303-82 might lead to differences in HSE-directed expression that reflect of the available HSF pool of the cell, rather than any influence of HSP90 on the regulation of the heat shock response. Any such titration effect would however be expected to be small since W303-82 shows almost normal heat induction of its multiple *HSP82* genes (section 3.2.4).

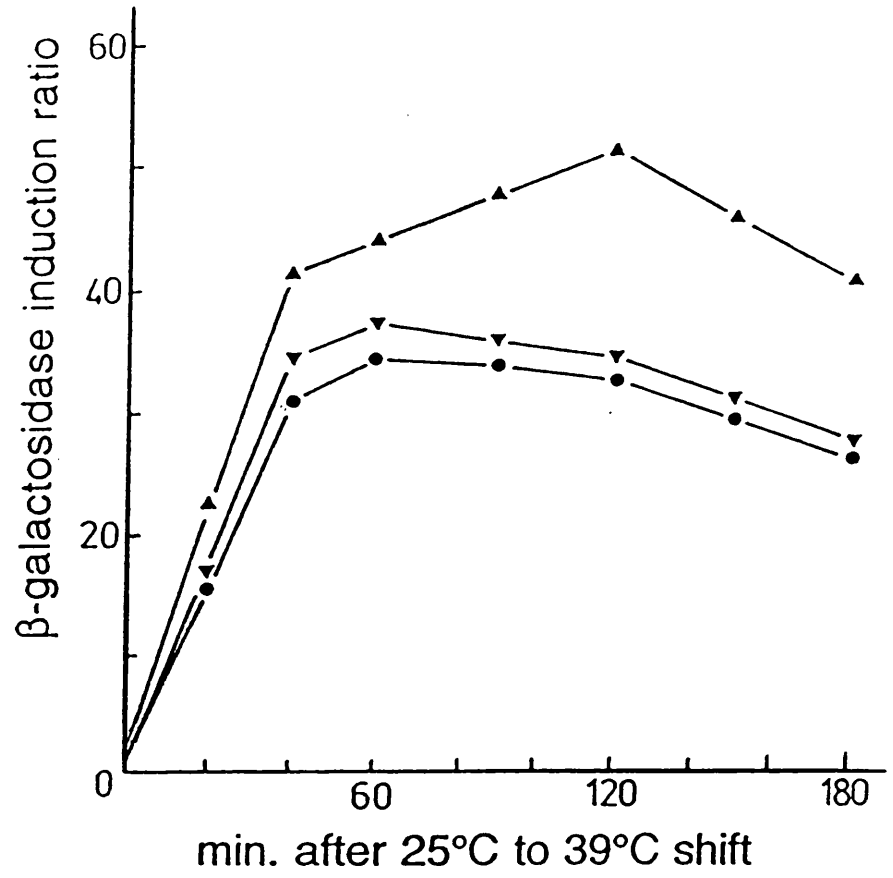


Figure 5.4 Assay of β-galactosidase induction in transformants W303Leu-pHSE2, PLD82-pHSE2 and CLD82-pHSE2 after heat shock.

Transformants W303Leu-pHSE2, CLD82-pHSE2 and PLD82-pHSE2 were grown to exponential phase at 25°C in SD medium (plus adenine, histidine and tryptophan supplements to ensure maintenance of pHSE2). Their β–galactosidase levels were measured at intervals following heat shock to 39°C. HSE-*lacZ* expression is normally switched off 40-60min after 25°C-39°C heat shock with the attenuation of the heat shock response. This was apparent with W303Leu-pHSE2 (●) and CLD82-pHSE2 (▼), but PLD82-pHSE2 (▲) displayed a more sustained HSE-*lacZ* expression.

5.3 Discussion

Many inducible systems have an inbuilt autoregulation, one or more of the induced proteins acting to suppress the induction signal. The heat shock response is typical in this respect, HSP synthesis becoming repressed a short while after it has been induced by a moderate heat shock. This attenuation mechanism is best-understood in *E. coli* , where it reflects heat-activated expression of more than one gene; the products of *dnaK, rpoD* and proteolytic activities being implicated in this process (Craig and Gross, 1991; Tilly *et al.,* 1983; Gottesman, 1989). A temperature-sensitive *dnaK* protein prevents the repression, while overexpressing normal *dnaK* causes lack of a full heat shock response at 43°C (Tilly *et al.,* 1983). The transcription of *E. coli* heat shock genes occurs when the normal σ regulatory subunit (σ^{70}) of the RNA polymerase holoenzyme is replaced by an alternative σ regulatory subunit (σ^{32}, *rpoH* product) (Gottesman, 1989). This σ^{32} exists at low levels under normal conditions, but levels increase quickly after heat shock due to an increased *rpoH* transcription, an enhanced translation of the *rpoH* transcript and a much slower degradation of the normally-unstable σ^{32} protein (section 1.3.1). This is a transient event. Heat-induced proteolysis accelerates degradation of σ^{32} (Gottesman, 1989), while σ^{32}-directs the RNA polymerase to increase expression of *rpoD* , the gene for σ^{70}. Therefore several factors cooperate to allow σ^{70} to compete effectively with σ^{32} for the σ binding site on RNA polymerase, leading to the switch-off the heat shock response (Craig and Gross, 1991; Gottesman, 1989).

In eukaryotic cells inhibitor studies indicate that the switch-off of the stress response is dependent upon heat-induced gene expression and especially levels of HSP70, the eukaryotic homologue of *E. coli dnaK* protein (DiDomenico *et al.,* 1982). Eukaryotes have multiple HSP70-family proteins, some of which (HSP70s) are synthesised in response to heat shock while others (HSC70-related, HSC70 proteins) are made constitutively. Deletion of the genes for cytoplasmic HSC70s of yeast (*SSA1* and *SSA2*) leads to constitutive expression of many heat shock genes (Craig and Jacobsen, 1985; Werner-Washburne *et al.,* 1987). Conversely overproduction of either HSC70 (*SSA1* overexpression) or HSP70 (*SSA4* overexpression) suppresses the normal heat shock induction of the *SSA1* promoter (Stone and Craig, 1990). Yeast HSP70 therefore autoregulates at least one of its own genes at the level of transcription, although the *SSA1* product apparently does not repress other heat shock promoters (Stone and Craig, 1990). Also substantially preventing heat induction of HSP70 with heat shock (as in the yeast *ssa3, ssa4* mutant) does not markedly increase the heat shock expression of other genes

(Werner-Washburne *et al.,* 1987). It appears therefore that cytoplasmic constitutive levels of *SSA* gene products, not the heat-induction of HSP70, are a major influence on heat shock gene expression (Craig and Gross, 1991; Craig and Jacobsen, 1985; Werner-Washburne *et al.,* 1987; Stone and Craig, 1990). Other yeast mutants which constitutively synthesise heat shock proteins include strains defective in degradation of damaged, partially unfolded or aberrant protein by the ubiquitination system for protein turnover [e.g. a *ubc4, ubc5* strain (Seufert and Jentsch, 1990)].

The results in this study indicate HSP90 as a minor negative regulator of heat-induced trehalose accumulation and HSE activity in yeast. HSP104 acts as another minor regulator of trehalose, in contrast to HSP70 which acts as a major negative regulator (Hottiger *et al.,* 1992). Therefore, in the regulation of the yeast heat shock response (Fig.1.2), HSP70 levels probably act as a major controller of both HSP synthesis and of trehalose, while HSP90 and HSP104 (also possibly other heat shock proteins) evidently play more minor roles. The differences in HSP90 level between W303Leu, CLD82 and PLD82 correspond to the HSP90 changes that occur during the normal physiology of the yeast heat shock response (Borkovich *et al.,* 1989; McDaniel *et al.,* 1989; Gross *et al.,* 1990). The prevention of the normal heat induction of HSP90 in PLD82 results in small enhancements to both the induction of trehalose (Fig.5.2) and the induction of HSE activity (Fig.5.4). HSP90 changes during the heat shock response must therefore be considered to influence this stress response, even though HSP70 levels are probably the primary control. The HSE expression measurements in Fig. 5.4 do not provide any clue as to a possible role for the binding of HSP90 to HSF (Nadeau *et al.,* 1993).

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CHAPTER 6

THE INFLUENCE OF THE LEVELS OF HSP90 PROTEIN: (2) HSP90 INFLUENCES KINASING OF A 66KDa PROTEIN IN EXTRACTS FROM HEAT SHOCKED YEAST

6.1 Introduction

It is well established that protein phosphorylation is a major mechanism used in the regulation of cellular functions (for reviews see Hunter, 1987; Kennelly and Krebs, 1991). Involvement of protein phosphorylation in the heat inducible alteration of cell metabolism has been studied, but not in great detail. This chapter describes heat-shock induced changes in the N-linked protein phosphorylation events catalysed by the soluble (post ribosomal supernatant) fraction of *S. cerevisiae.*

There are few reports dealing with the effect of heat shock on protein phosphorylation in yeast, and even fewer with respect to prokaryotes. The sole *E.coli* member of the highly conserved HSP70 family proteins (DnaK) autophosphorylates when incubated with ATP *in vitro* (McCarty and Walker, 1991; Peake *et al.,* 1991). This activity increases dramatically over the range of temperatures physiologically relevant to bacterial growth (McCarty and Walker, 1991). The temperature dependence of this autophosphorylation could be of significance with respect to the role of DnaK as a molecular chaperone helping cells counteract the deleterious effects of heat shock. Alternatively, the extent of DnaK phosphorylation may be involved in the mechanism by which DnaK controls the prokaryotic heat shock response, since it has been proposed that both DnaK and its eukaryotic homolog HSP70 serve as a cellular thermometer directly sensing the temperature of the environment (Craig and Gross, 1991).

Most work concerning the effects of heat shock on protein phosphorylation has been carried out in mammalian systems. Heat shock results in a rapid and reversible dephosphorylation of a single basic protein (S6) of the small ribosome sub-unit. This has been detected in fungi (Richter *et al.,* 1983), *Drosophila* (Glover, 1982; Olsen *et al.,* 1983) and plant cells (Scharf and Nover, 1982). These studies did not determine whether this was due to inactivation of S6 kinase activities or activation of phosphoprotein phosphatases. It has been proposed that S6 phosphorylation is connected with selective

translation of HSP mRNAs in heat shocked cells. However, prolonged incubation at a high non lethal temperature will result in accumulation of HSPs and eventual restoration of a protein synthesis pattern similar to that seen prior to heat shock. S6 does not return to its normal phosphorylated state under these conditions and only a shift back down to normal temperatures will achieve this. Accordingly, changes in phosphorylation of S6 cannot be the main reason for the selective translation of heat shock mRNAs (Scharf and Nover, 1982). Alternatively, S6 dephosphorylation may play a role in heat shock-inducible cell cycle arrest. In metabolically inactive cells S6 phosphorylation is low; entry into the cell cycle being preceded by phosphorylation of S6 (Scharf and Nover, 1982). A protein kinase has been isolated from *S.cerevisiae* which specifically phosphorylates proteins of the large ribosome sub-unit (Pilecki *et al.*, 1992). It remains to be seen whether heat shock stimulates its activity.

Although preferential translation of HSP mRNAs may not be caused by changes in phosphorylation state of ribosomal proteins, phosphorylation of other components of the translational apparatus may be involved. The initiation factors eIF4B and eIF4F are dephosphorylated during heat shock (Legagneux *et al.*, 1990). In contrast heat shock causes the hyperphosphorylation of initiation factor eIF2α in both intact cells and cell lysates (Matts *et al.*, 1992), due to activation of an eIF2α kinase. Similar experiments involving nuclear and nucleolar proteins of chinese hamster ovary cells have also been reported. Heat shock induces the phosphorylation of a 95KDa nucleolar protein. In the non-nucleolar fraction of the nucleus, phosphorylation of a 54KDa protein is induced while a 35kDa protein is rapidly dephosphorylated (Caizergues-Ferrer *et al.*, 1980). These phenomena are proposed to play a role in regulation of transcription and RNA processing during heat shock.

It is well established that HSF becomes highly phosphorylated on heat shock, the heat-inducible activity of HSF closely following the extent of its phosphorylation over a range of temperatures (Sorger and Pelham, 1988). There have been very few reports concerning how this occurs. Whether it involves autophosphorylation or the action of a protein kinase, it is obviously regulated by heat stress. Accordingly, analysis of the HSF activating system will reveal the nature of the cellular components that detect environmental stress, i.e. the so called "cellular thermometer" in the case of heat shock. Certain specific HSPs are almost certainly involved, notably HSP70 (Craig and Gross, 1991). A direct binding of HSP90 to HSF has also recently been demonstrated (Nadeau *et al.*, 1993). Also since HSP90 is known to bind a number of regulatory kinases, it may influence the kinases that phosphorylate HSF. Its levels have a small negative influence on HSF activity (Chapter 5).

The specificity of RNA polymerase II for gene transcription may be altered by heat shock. This might be achieved by changing the phosphorylation state of the carboxyl-terminal repetitive domain (CTD) of the largest RNA polymerase II subunit. Kinases responsible for CTD phosphorylation have been described, and lysates from heat shocked cells possess much higher CTD kinase activities than lysates from unstressed cells (Legagneux *et al.*, 1990).

Reports in the literature concerning heat shock modification to the phosphorylation state of cytosolic proteins are sparse and seem to be limited to studies on HSPs. In higher eukaryotes phosphorylation of HSP26 increases during heat shock (Landry *et al.*, 1988). The yeast homologue does not appear to be phosphorylated (M. Tuite, personal communication). Also, human HSP90 is phosphorylated *in vivo* and can be phosphorylated *in vitro* by both casein kinase II (Lees-Miller and Anderson, 1989a) and a double strand DNA-activated kinase (Lees-Miller and Anderson, 1989b). Human HSP90 also possesses an autophosphorylation activity (Csermely and Kahn, 1991). Again, similar activities have not been reported for the yeast HSP90 homologs. Though the proteins from yeast and mammals are highly homologous, the phosphorylated sequence motif is found in the HSP90 of humans and other mammals but not in yeast HSP90. The physiological significance of the phosphorylation of HSPs is unknown. Changes in the phosphorylation state of cytosolic proteins that exist both prior to and during heat shock (e.g. ribosomal S6 protein) could also be of interest, since they might regulate heat shock inducible events.

This chapter describes a study of a 66KDa protein which is highly phosphorylated *in vitro* in soluble extracts from heat shocked yeast but not unstressed yeast. It is an extension of the work conducted by B. Panaretou in this laboratory (Panaretou, 1993), a study that showed this 66KDa phosphorylation to be acid-labile, and therefore N-linked. The transfer of high energy phosphoryl groups from histidine to aspartate side chains is used by bacterial systems for modifying the activity of the regulator (Stock *et al.*, 1990). So far there is no evidence that it is used in signal transduction in yeast, although a histidine protein kinase has been isolated from yeast (Huang *et al.*, 1991).

The obvious advantage of *in vivo* labelling is that results obtained are likely to be physiologically relevant, but only by *in vitro* labelling of cell free extracts can the factors controlling these events be identified, and the biochemical properties of the reactants established. It has been found that the majority of proteins in the cytosolic (Pilecki *et al.*, 1992) and plasma membrane fractions of yeast cells labelled with $^{32}P_i$ *in vivo* correspond to the proteins phosphorylated in these fractions *in vitro* using [γ^{32}P]-ATP. This is

probably due to the reaction conditions used for *in vitro* studies approximating to physiological conditions. This especially applies to the concentrations of ATP used which rarely exceed 100μM, the approximate physiological concentration of this metabolite in yeast (Weitzel *et al.*, 1987). Indeed, most *in vitro* studies use between 10 and 30 μM ATP (Kolarov *et al.*, 1988; Londesborough and Nuutinen, 1987).

6.2 The conditions used for phosphorylation of cytosolic proteins

Cultures of different strains (see Table 2.1 for genotypes) were grown to mid exponential phase at 25°C in YEPD or standard defined minimal medium (SD) to ensure plasmid maintenance. Cells were harvested immediately (unstressed), or shifted to 40°C for 40 minutes (heat shocked) prior to harvesting. The post-ribosomal supernatant fraction was then prepared from both the heat shocked and unstressed cultures (section 2.10.5.1). Endogenous ATP in the post-ribosomal supernatant was removed by dialysis against a buffer which was at near-physiological pH and contained: i) the most important physiological cations (K^+ and Mg^{2+}) at concentrations similar to those in intact yeast cells; ii) 1mM DTT, since the action of some kinases is dependent on free sulphydryl groups (Pilecki *et al.*, 1992); and iii) 10% glycerol as a stabilising agent to limit proteolysis (North,1989). Kinasing reactions were by incubating 10 μg of protein with 10nM [γ^{32}P]-ATP at 30°C (see section 2.13). At the stated times reactions were terminated by the addition of an equal volume of SDS gel sample buffer, the labelled proteins resolved by 1D SDS-PAGE and the phosphoproteins visualised by autoradiography without acid fixation of the gel (section 2.13). Both short term (5sec) and long term (10min) incubations with labelled ATP were carried out.

6.3 Major phosphoproteins of S100 extracts incubated with [γ-^{32}P]ATP. Evidence that the procedure for preparing S100 extracts affects p66 phosphorylation

Incubation of S100 extracts from both unstressed and heat shocked cells at 25°C with 10 nM ATP resulted in the rapid (within 5sec) phosphorylation of proteins with an M_r of 31 and 32 KDa (see Fig.6.1). They are referred to as p31 and p32 in the ensuing sections. These proteins were sometimes not labelled after a 10min incubation, probably due to the rapid exhaustion of [γ^{32}P]ATP and the removal of ^{32}P from the proteins by phosphoprotein phosphatases.

The proteins phosphorylated *in vitro* in these S100 extracts are significantly affected by

whether the extracts were prepared from cells prior to or after heat shock (Panaretou, 1993). This is shown in the S100 extract phosphorylations in Fig.6.1, which employed 10nM ATP although similar results are obtained at 100μM ATP (Panaretou, 1993). Only the S100 extracts from heat shocked cells extensively phosphorylate a protein of M_r 66KDa (referred to as p66 from now on). This was noted after a 10min incubation with 10nM ATP, but not a 5sec incubation. Phosphorylation of p66 in heat shock extracts is possibly due to phosphorylation of a protein which is only present during heat shock (i.e. a HSP). Alternatively it may represent phosphorylation of a protein present both prior to and during heat shock, but only phosphorylated during heat shock. The second possibility seemed likely as p66 does not co-migrate with any of the major HSPs (B. Panaretou, personal communication, also section 6.4.3). Also, there is no report describing a minor HSP of this M_r in yeast. Although p66 is not an HSP, the possibility remained that its phosphorylation in heat shock extracts requires the presence of HSPs. This is apparently the case since p66 is not phosphorylated in a S100 extract prepared from cells heat shocked in the presence of cycloheximide (B. Panaretou,personal communication).

During the preparation of S100 extracts for *in vitro* phosphorylation, an important step was the removal of endogenous ATP. This can be achieved by dialysis or ultrafiltration. Preliminary experiments involved preparing four 5ml S100 extracts from heat shocked cells of strain BJ2168 according to:

A Dialysis against 5 changes of 6L buffer over 12h, as described in section 2.13.

B Dialysis against 5 changes of 6L buffer over 6h, as described in section 2.13.

C Ultrafiltration by 2h centrifugal concentration as described in section 2.13.

D A combination of B plus C.

Identical *in vitro* phosphorylation reactions using 10nM [γ-ATP] were carried out with all four preparations. The pattern of proteins phosphorylated was identical in each case (not shown), but the time needed to phosphorylate p66 varied. Lengthy dialysis (procedure A above) did not result in the phosphorylation of p66 after a 5sec incubation with ATP, although p66 was phosphorylated after 10min. In contrast, intermediate dialysis (procedure B above) did result in phosphorylation of p66 after 5sec, although the extent of phosphorylation was not as great as that seen after 10min. S100 extracts prepared by centrifugal concentration (procedure C above) gave maximal phosphorylation of p66 after 5sec and the extent of phosphorylation was the same as that seen after 10min. Finally, S100 prepared by steps B plus C resulted in a phosphorylation of p66 that was the same as by procedure A. Since procedure A took a long time and the protein extracts precipitated to some extent during this period, it was abandoned. All subsequent experiments therefore used extracts prepared using intermediate dialysis plus ultrafiltration (procedure D) in order

to yield reproducible kinetics of p66 phosphorylation.

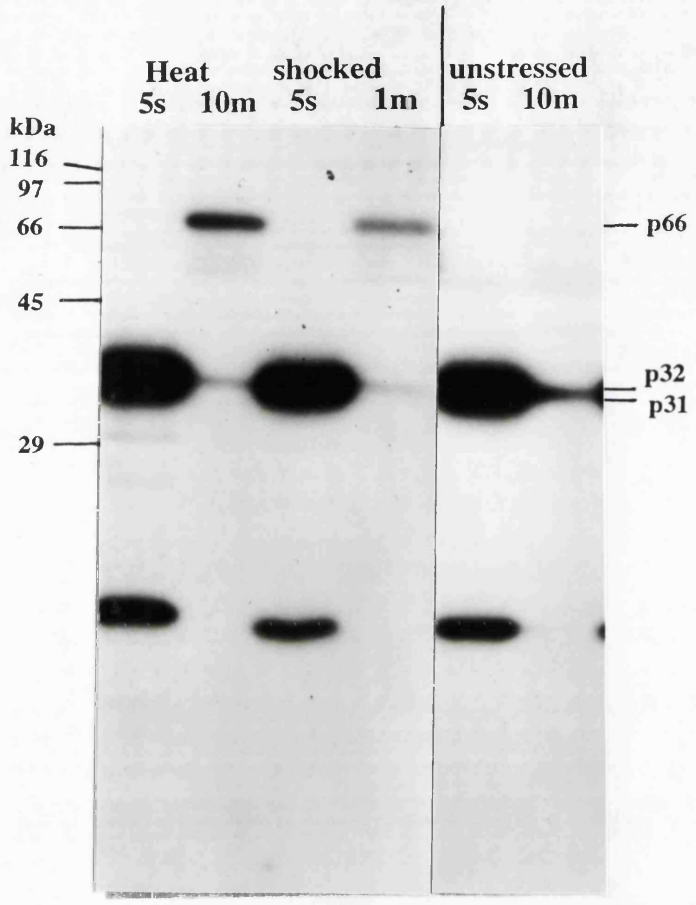


Figure 6.1 Autoradiograph of SDS-PAGE gel with S100 labelled proteins.

Cultures of the protease deficient strain BJ2168 were grown at 20°C to exponential phase in YEPD. Cells were harvested either at 20°C (**Unstressed**) or 40 min after a 25°C-40°C upshift (**Heat shock**). S100 extracts were prepared and incubated with 10nM of [γ^{32}P]-ATP, for the length of time indicated, as described in section 6.2. Each reaction employed 10 μg of protein. Following 15% SDS-PAGE, gels were dried without acid fixation and exposed to film for visualisation of phosphorylated proteins. Molecular masses of markers are indicated on the left. Some of the **p**hosphoproteins are designated by their M_r in kDa e.g.**p66**.

6.4 Results

6.4.1 Analysis of p66 kinasing in S100 extracts from different mutants

6.4.1.1 Different HSP90 levels dramatically affect the phosphorylation of p66

Heat induction of the capacity for S100 extracts to phosphorylate p66 is protein synthesis dependent, phosphorylation of p66 in heat shocked cell extracts appearing to require the presence of HSPs (section 6.3). Accordingly, the best way to determine if a specific HSP is involved is to analyse extracts from strains bearing disruptions in specific HSP genes. Strains of *S. cerevisiae* deleted for most major HSP genes are now available. Such an experiment was carried out with S100 extracts from a strain deleted for two HSP70 genes (*SSA1* and *SSA2*), the result being that p66 was phosphorylated in extracts from both unstressed cells as well as heat shocked cells (B. Panaretou personal communication). This was not surprising given that deletion of these two genes results in high expression of HSPs even at 23°C (Craig and Jacobsen, 1984). Phosphorylation of p66 in extracts from this double deletion mutant indicates that if HSPs are involved in p66 phosphorylation, they are not those encoded by *SSA1* and *SSA2*. HSP90 is another possible regulator of p66 phosphorylation, since it has been shown to modulate the activity of various protein kinases in cells of higher eukaryotes (Section 1.2.1). This section describes experiments using S100 extracts from *S. cerevisiae* strains of altered HSP90 level that show that HSP90 level has striking effects on the phosphorylation of p66.

Initially p66 phosphorylation was investigated in extracts from unstressed and heat shocked cells of the three isogenic diploid strains, W303Leu, PLD82 and CLD82 (Table 2.1). W303Leu is wild-type with respect to HSP90 genes (*HSP82* and *HSC82*). PLD82, homologous for an insertional inactivation of *HSP82*, has almost normal HSP90 during growth but displays very little HSP90 increase with heat shock. CLD82, due to insertional inactivation of both *HSC82* alleles, has lower HSP90 levels during 25°C growth but exhibits a strong HSP90 induction with heat shock. Extracts from unstressed cells of all three strains showed no p66 phosphorylation (Fig.6.2A). In the extracts from heat shocked cells there was a much higher phosphorylation of p66 in CLD82 extracts (Fig. 6.2A) as compared to either W303Leu or PLD82 extracts, (3 times more than W303Leu and 10 times more than PLD82). Since these strains are completely isogenic, this can be attributed to the differences in HSP90 protein level after heat shock to 39°C in these three

strains (quantitated in Borkovich *et al.*, 1989; also Fig. 5.1). Therefore HSP90 levels influence phosphorylation of p66.

If reduction in the HSP90 of heat shocked yeast affects phosphorylation of p66, overexpression of HSP90 may also be expected to affect phosphorylation of p66. The experiment in Fig 6.2B employed two transformants of strain W303-1A (W303-3a and W303-82) which are isogenic but for the presence of 50-150 extra episomal *HSP82* gene copies in W303-82 (section 3.2.1). The basal expression of these multiple *HSP82* gene copies in W303-82 causes a 3 to 7-fold HSP90 overproduction at 25°C in this transformant as compared to W303-3a. Also the HSP90 of W303-82 increases 10 fold, to 30-40% of cell protein, following a temperature upshift to 39°C, while the HSP90 levels of W303-3a are as in wild-type strains (section 3.2.2). W303-3a extracts displayed phosphorylation of p66 only when prepared from heat shocked cells after 10min incubation with 10nM ATP (Fig. 6.2B). In contrast W303-82 extracts displayed extensively phosphorylated p66 when isolated from both unstressed and heat shocked cells, this p66 phosphorylation being considerably greater than with W303-3a extracts (Fig.6.2B). This is further evidence of a striking influence of HSP90 on phosphorylation of p66, also seen in the experiments with extracts from W303Leu, PLD82 and CLD82 (Fig.6.2A).

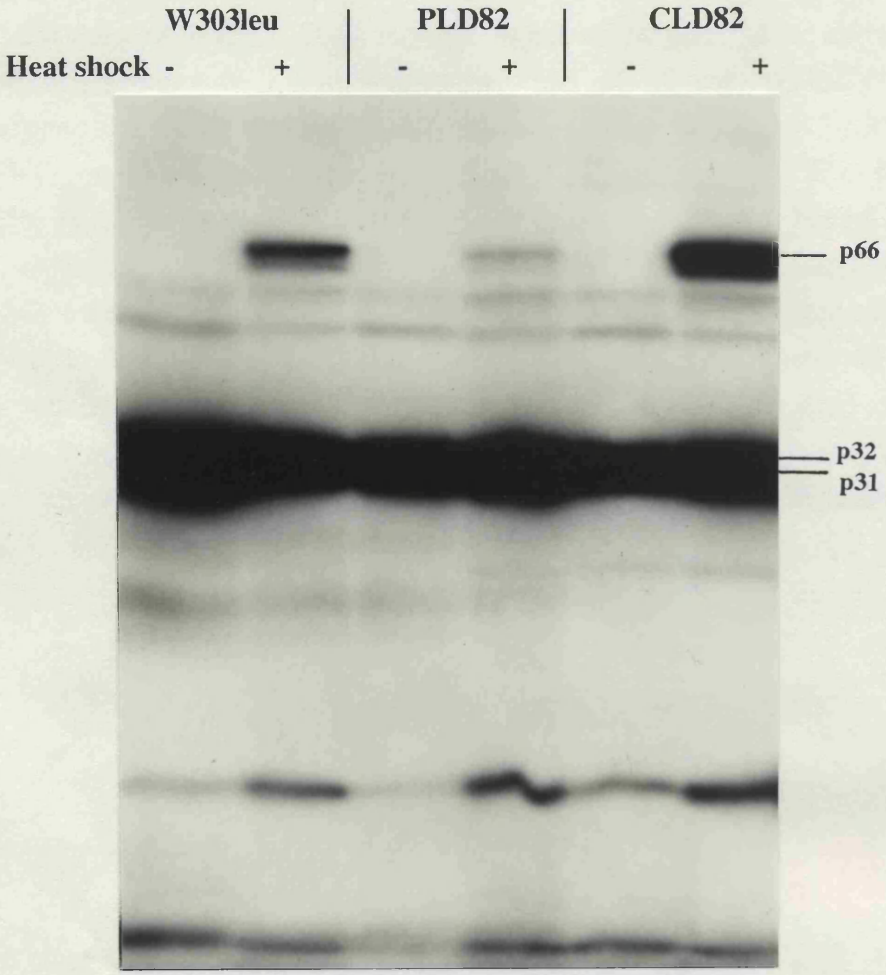
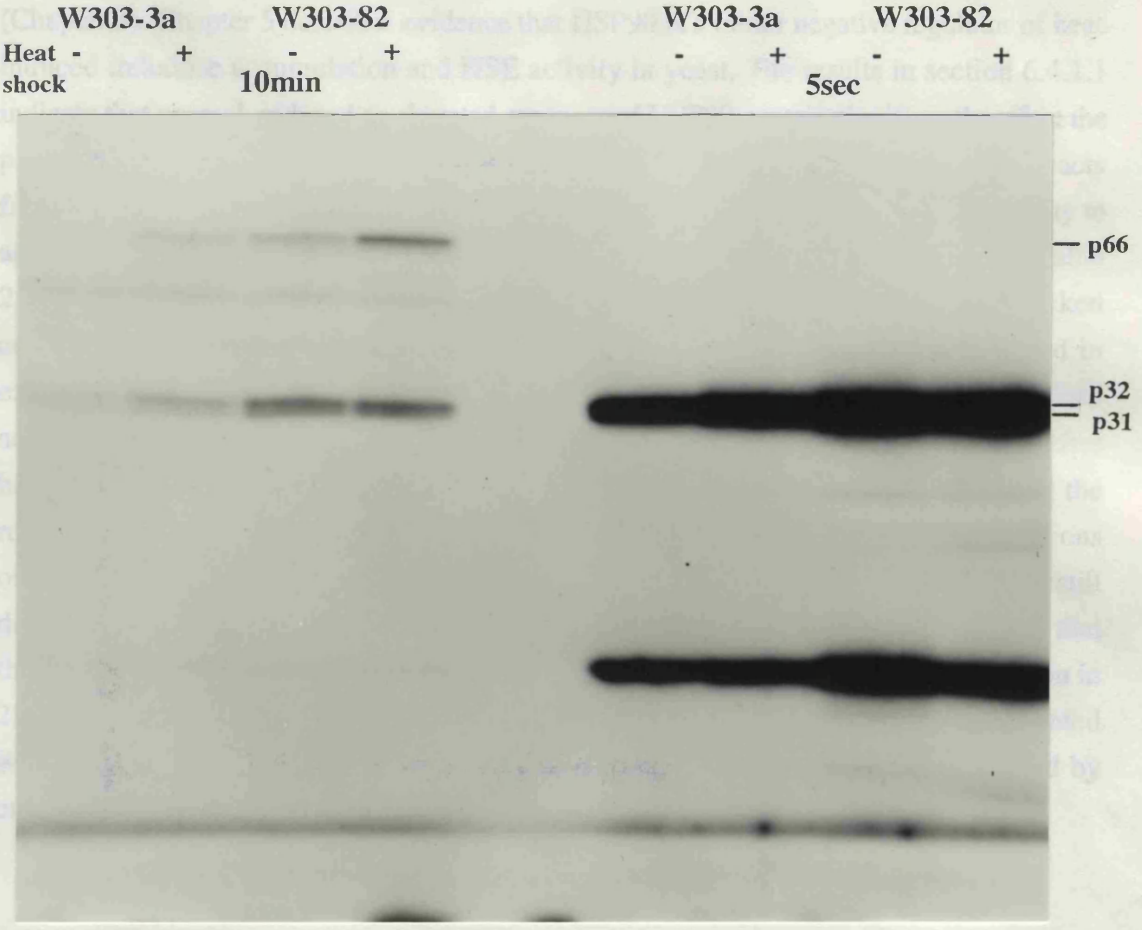


Figure 6.2 Different HSP90 levels affect the phosphorylation of p66.
S100 extracts from unstressed and heat shocked cells of isogenic yeast strains with normal, decreased or elevated HSP90 were subjected to *in vitro* phosphorylation as described in section 6.4.1 and Fig.6.1. Phosphoproteins are visualised as described in Fig.6.1. **A.** Extracts from YEPD medium cultures of W303Leu, PLD82 and CLD82.



B. Extracts from SD medium cultures of W303-3a and W303-82.

6.4.1.2 A mutation that prevents trehalose induction with heat shock also influences the phosphorylation of p66

Changes in HSP90 level can alter both high temperature growth and thermotolerance (Chapter 3). Chapter 5 describes evidence that HSP90 is a minor negative regulator of heat-induced trehalose accumulation and HSE activity in yeast. The results in section 6.4.1.1 indicate that normal, reduced or elevated amounts of HSP90 protein significantly affect the phosphorylation of p66. Therefore the phosphorylation of p66 was investigated in extracts from a mutant isolated by C. DeVirgilio (University of Basel) on the basis of its inability to accumulate trehalose with heat shock (224A-12D) and its wild type parent (144-3a; Table 2.1). Fig.6.3 shows that p66 was phosphorylated normally in extracts from heat shocked cells of the trehalose wild type strain 144-3a, but was only slightly phosphorylated in extracts of mutant strain 224A-12D after 10min incubation. When the trehalose accumulation following heat shock was measured, trehalose dramatically increased after heat shock in strain 144-3a but no trehalose was produced in 224A-12D, confirming the results of C. DeVirgilio (data not shown). The trehalose and heat shock protein inductions of the heat shock response appear to be under similar control (Fig.1.2), yet 224A-12D still displays normal induction of heat shock proteins (not shown). It remains to be shown that the lack of trehalose induction in 224A-12D is related to the lack of p66 phosphorylation in 224A-12D extracts observed here. Had time allowed, it would have been investigated whether these two defects co-segregated during sporulation of the diploid generated by crossing 224A-12D with a wild-type strain.

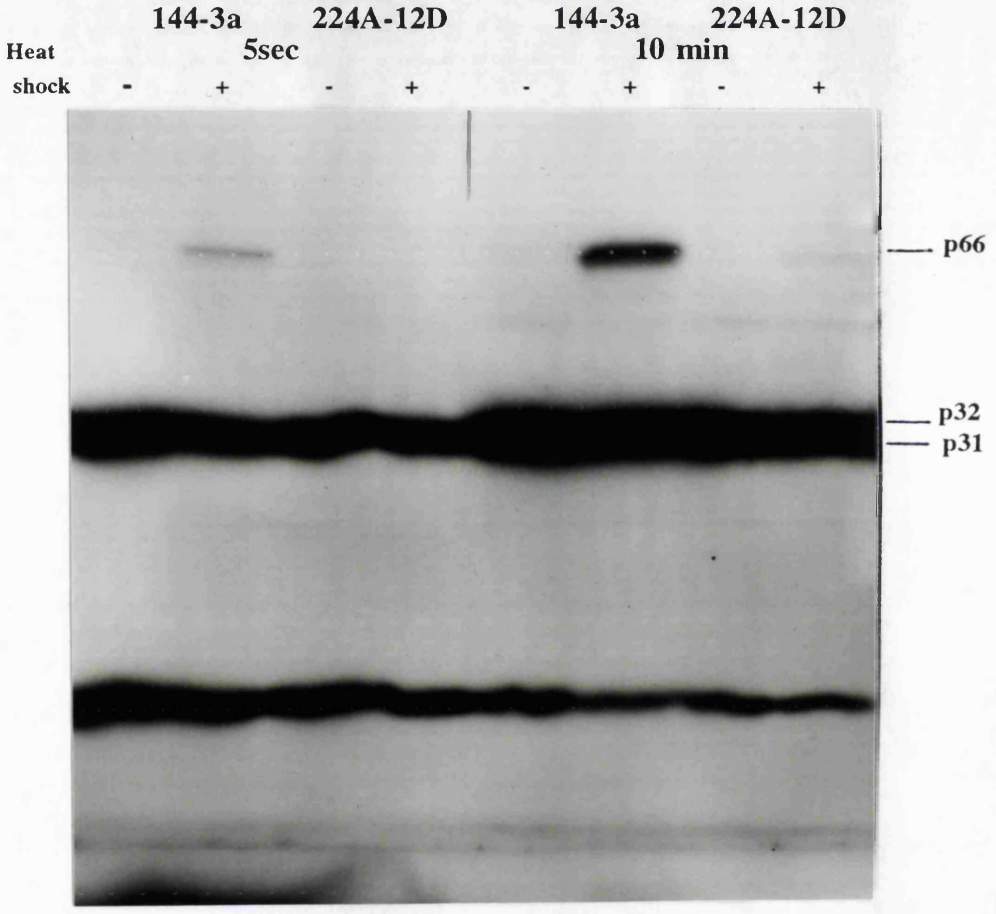


Figure 6.3 Autoradiograph of SDS-PAGE gel with S100 labelled proteins in strains 144-3a and 224A-12D.

S100 extracts from unstressed and heat shocked cells of wild type (144-3a) and trehalose mutant (224A-12D) strains were subjected to *in vitro* phosphorylation as described in section 6.4.1 and Fig.6.1. Phosphoproteins are visualised as described in Fig.6.1.

6.4.2 p66 peptide mapping by limited proteolysis

In vitro phosphorylation of p66 occurred only in S100 extracts from heat shocked cells. Identification of p66, and analysis of any function for this protein or its N-linked phosphorylation would require p66 sequence data. Since over 1000 yeast genes have now been sequenced, there is a good chance of p66 being identified from partial peptide sequence data. Even if database searches did not reveal the identity of p66, the p66 gene could be cloned by screening a genomic library with a degenerate oligonucleotide derived from a partial peptide sequence of p66. Its sequence could be entered into the protein databanks to reveal homology with other proteins, thereby providing a clue as to the function of p66. About one third of sequences present in genomes are related to entries in the current databank (Chothia, 1992). Many proteins are N-terminally blocked and cannot be sequenced directly. The best approach to dealing with this problem is peptide mapping. This consists of breaking down the unknown protein in a specific and controlled manner into a number of peptide fragments, then separating the peptide mixture. The proteinases most commonly used for enzymic cleavage of polypeptide chains are *Staphylococcus aureus* V8 proteinase, trypsin, chymotrypsin, papain and pepsin (Lam and Kasper, 1980). Enzymic hydrolysis has the advantages of being rapid, more specific than most chemical cleavage procedures, and uses buffers that are generally compatible with the subsequent electrophoretic separation of peptides. Digests may therefore be applied directly to gels. Cutting the protein band out of the first SDS-PAGE gel it can then be subjected to limited proteolysis with proteinase V8, followed by separation of the cleavage products by 15% peptide mapping SDS-PAGE (as described in section 2.12).

Soluble extracts from unstressed and heat shocked cells were phosphorylated with 10nM [γ^{32}P]ATP as described previously. The resulting phosphoproteins were resolved by SDS-PAGE, the gels fixed in acid (which largely removes N-linked phosphoryl groups) and stained with Coomassie blue prior to autoradiography. Certain stained polypeptides co-migrated with p31, p32 and p66 and the phosphorylation profiles were not altered during staining and destaining procedures, although much of the label was lost during acid fixation (Fig.6.4). The band that comigrated with p66 was excised and used for proteinase V8 digestion. All proteinase V8 digests of p66 used p66 from 6 adjacent wells of the preparative gel (Fig 6.5) representing a total of ca. 6μg of protein. Electrophoresis on the 15% mapping gel was as usual except that when the Bromphenol Blue tracking dye reached a position close to the bottom of the stacking gel, the electrical current was switched off for 30min. At this point both the sample proteins and the overlaid proteinase

will have migrated down to the stack and will be in close proximity to each other, so switching off the current allows time for the hydrolysis reaction to occur before separation of the resulting peptides is resumed. Having a longer than normal (e.g. 5cm as opposed to 2.5cm) stacking gel helps to ensure complete stacking together of the sample and proteinase.

After the p66 peptide separation the gel was blotted onto polyvinylidene difluoride (PVDF) membrane. Using endoproteinase V8 at the substrate:proteinase ratio given as in section 2.11, p66 gave several peptide fragments after Coomassie blue staining of the blot. From several peptides only one was radiolabelled as detected by autoradiography.

The blot was sent to Dr. Brian Coles (CRC molecular toxicology unit), but unfortunately yielded no sequence data. It was not naturally N-terminally blocked since another blot prepared using the same electrophoresis and Western blotting procedures provided sequence data. If time had permitted, further p66 sequencing attempts would have been carried out after HPLC chromatography purification of peptide fragments.

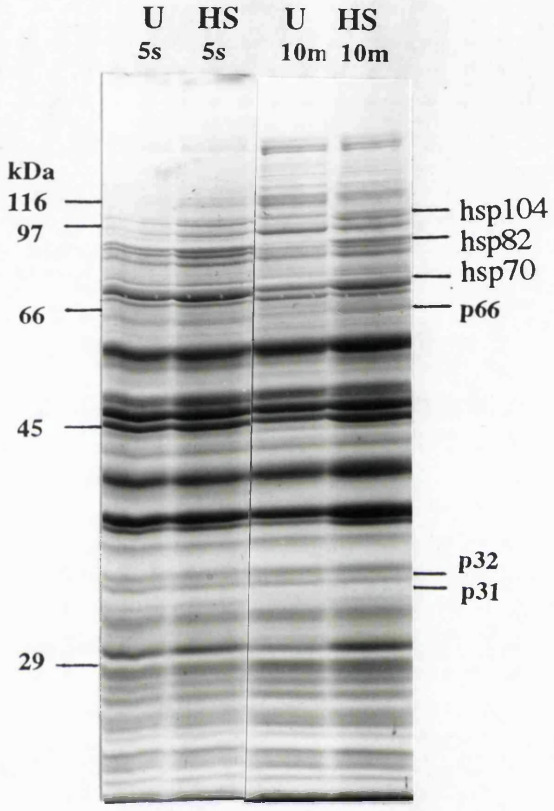


Figure 6.4 Visualising phosphoproteins by Coomassie staining.
S100 extracts from unstressed (**U**) and heat shocked (**HS**) BJ2168 cells were phosphorylated as described in Figure 6.1. Following 12.5% SDS-PAGE and acid fixation, the gels were stained with Coomassie blue, dried and then exposed to film in order to determine which stained polypeptides co-migrate with the p31, p32 and p66 phosphoproteins. Molecular mass markers are indicated on the left, major HSPs and the stained polypeptides migrating with the phosphoproteins are indicated on the right.

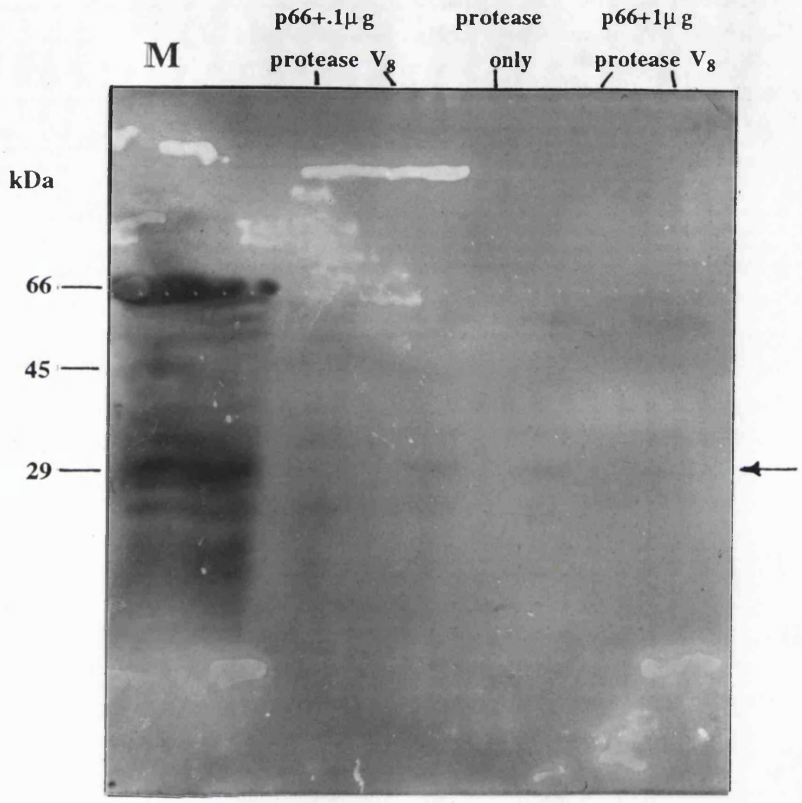


Figure 6.5 p66 peptide mapping by proteolytic cleavage.

S100 extracts from heat shocked cells were phosphorylated as described in Figure 6.1 and resolved by SDS-PAGE as in Fig. 6.4. Gel slices containing p66 were applied to a second SDS gel in the presence of 1μg of protease V8 as described in section 2.11. Incubation in the presence of protease was for 30 min. After the p66 peptide separation the gel was blotted onto polyvinylidene difluoride (PVDF) membrane. Peptide fragments generated were visualised by staining with Coomassie blue. Each lane was loaded with 6 slices containing p66 (ca. 6μg of protein). Position of molecular mass markers is indicated on the left. The ^{32}P labelled peptide fragment is arrowed.

6.4.3 Limited N-terminal sequencing of p66 by 2D gel electrophoresis

In order to determine the correct position of p66 on 2D gels of yeast total protein, a S100 extract from heat shocked cells labeled with [^{32}P]-ATP (section 2.13) was combined with total proteins of heat shocked yeast labelled with ^{3}H-leucine (section 2.10.1), and the combined mixture separated on 2D gels as in Section 2.10.6.2 (Fig. 6.6). The gels were also stained with Coomassie blue. The commonest 2D PAGE method for analysing mixtures of polypeptides is to separate the proteins in the first dimension on the basis of charge by non-equilibrium pH gel electrophoresis (NEPHGE) and in the second dimension on the basis of molecular mass by standard SDS-PAGE (O'Farrell *et al.*, 1977). The two-dimensional gels were fluorographed to reveal both the ^{3}H radiolabeled *in vivo* synthesized proteins and the *in vitro* ^{32}P labeled phosphoproteins. Gels run with 5sec and 10min *in vitro* labelled phosphoproteins readily showed which spot corresponded to p66 arrowed (Fig.6.6B). This gel provided more conclusive evidence that p66 corresponds not to a HSP, but to a protein present both before and during heat shock. The amount of p66 loaded on the 2D gel was not enough to be visualised by Coomassie blue staining and therefore for sequencing, because of the limited amounts of protein that can be loaded onto 2D gels.

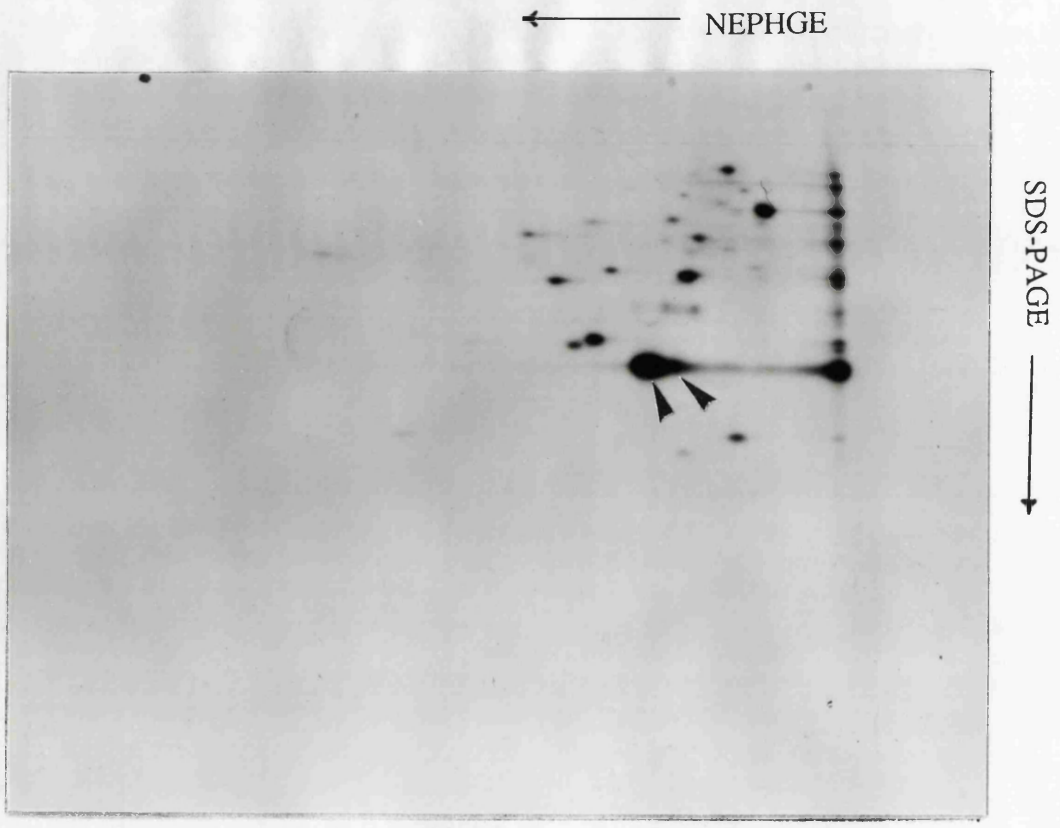
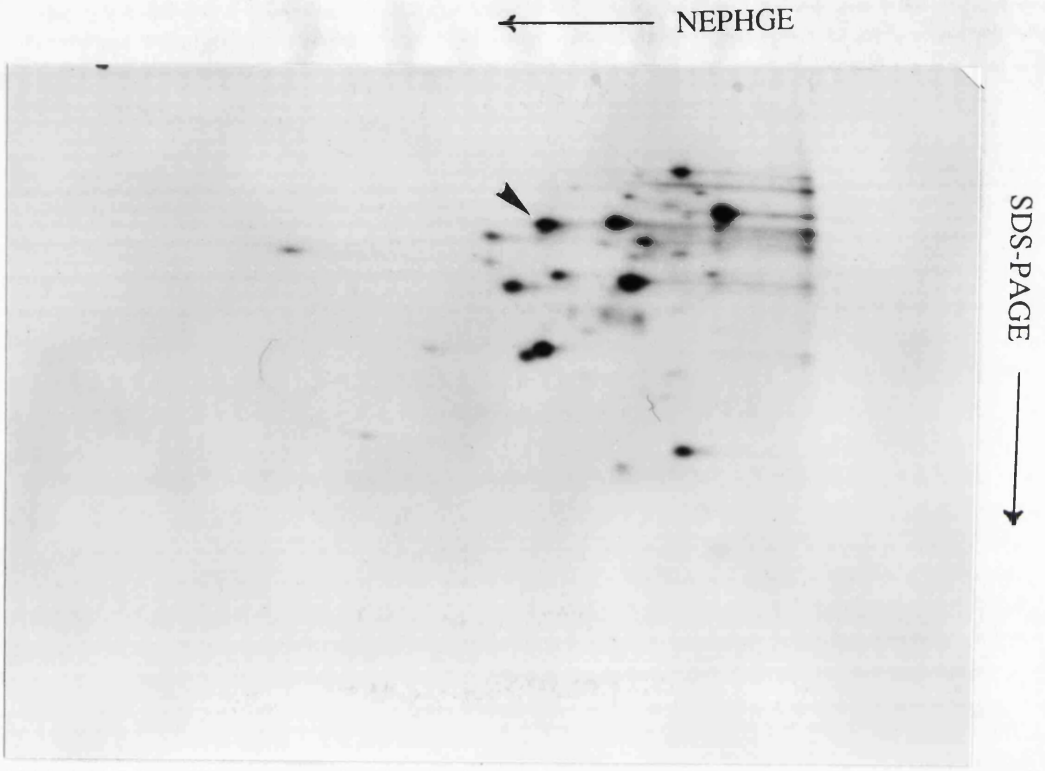


Figure 6.6 2D PAGE of proteins phosphorylated in S100 extracts of heat shocked cells mixed with total protein of pulse labelled cells.

S100 extracts from heat shocked cells were phosphorylated as described in Figure 6.1. These samples phosphorylated (**P**) for either 5sec or 10min were then mixed with samples of total cell protein from cells heat shocked (**HS**) and pulse labelled with [^{3}H] leucine (as described in section 2.10.1). The mixture was resolved in the first dimension (1) by NEPHGE and in the second dimension (2) by 15% SDS-PAGE. The two-dimensional gels were dried without acid fixation and fluorographed to reveal the [^{3}H]-labeled *in vivo* synthesized proteins and *in vitro* labeled phosphoproteins.

A. Sample phosphorylated for 5sec, then mixed with pulse labelled extract.



B. Sample phosphorylated for 10min, then mixed with pulse labelled extract.

6.4.4 Analysis of HSP90-Affi-Gel binding of p66

Section 6.4.1 describes results showing that kinasing of phosphoprotein p66 is related to HSP90 level. p66 might therefore be suspected to be a HSP90-associated protein. Kinased cell extracts containing radiolabelled p66 were therefore bound to HSP90-Affi-Gel (as in section 4.2.3), and the retained and nonretained protein analysed. Initially total heat shocked protein extract was phosphorylated, then the reaction was stopped with 1mM sodium molybdate after 10min and this sample was coupled to HSP90-Affi-Gel resin. An alternative procedure involved binding unlabelled protein extract to the HSP90 Affi-Gel, the associated proteins being released with salt prior to *in vitro* phosphorylation. By both procedures no p66 could be detected in the HSP90-retained protein (data not shown). These experiments indicate that p66 was not present in the HSP90-Affi-Gel associated proteins. Probably HSP90 is indirectly related to the state of p66 phosphorylation, such as influencing the action of the kinase responsible.

6.5 Discussion

The heat shock response has been extensively studied, yet there are few reports concerning the effects of heat on protein phosphorylation, the most common form of regulation by covalent modification. This is reviewed in section 6.1.

Incubation of S100 extracts with 10nM [γ-^{32}P]ATP results in the phosphorylation of three major proteins, p31, p32 and p66 (see section 6.3). p66 is extensively phosphorylated in heat shock extracts only. Though not a HSP itself (Fig.6.5), the phosphorylation of p66 appears to be HSP dependent (Panaretou, 1993). The results in this chapter indicate that HSP90 is probably one HSP involved. Results using isogenic yeast strains with normal, decreased or elevated HSP90 show the HSP90 levels of S100 extracts having significant influence on the *in vitro* phosphorylation of p66 (Figs 6.2A, 6.2B). The difference in HSP90 level between strains W303Leu, PLD82 and CLD82 correspond to the changes in HSP90 that occur during the normal physiology of the yeast heat shock response (Borkovich *et al.*, 1989). Prevention of the normal heat induction of HSP90 in CLD82 increased phosphorylation of p66 (Fig.6.2A). It is possible therefore that HSP90 changes during the heat shock response influence the phosphorylation of this protein *in vivo*. With overexpression of HSP90, the phosphorylation of p66 was dramatically increased (Fig.6.2B), providing further evidence of HSP90 being involved in the control of this protein phosphorylation.

In view of the known association of HSP90 with many protein kinases (Section 1.2.1), it is plausible to suggest that p66 is phosphorylated by a kinase which associates with HSP90, and that this association influences the activity of this kinase. Alternatively HSP90 may bind to p66 rendering it more amenable to phosphorylation. The second interpretation seems not to be the case, since p66 was not found amongst HSP90-associated proteins (section 6.4.4). The control of p66 kinase by HSP90 is suggested from the ability of HSP90 to form complexes with HSF and several of the regulatory proteins of eukaryotic cells (Section 1.2.1). Recently it has been demonstrated that pure *E.coli* HtpG, yeast HSP90, rat and human HSP90 all possess ATPase activity (Nadeau *et al.,* 1993). This may lead to a redefinition of HSP90 as an active rather than a passive chaperone. Mammalian (but not yeast) HSP90 undergoes *in vivo* phosphorylation through the action of such kinases as casein kinase II, the ATPase activity of HSP90s raising the prospect that some of these HSP90 phosphate groups may be due to autophosphorylation. An ATP-dependent interaction of three HSP90s (*E.coli,* yeast, and human) with the homologous forms of cyclophilin indicates that ATP hydrolysis by HSP90 is likely to alter conformation and partner protein recognition (Nadeau *et al.,* 1993). Alternatively cyclophilin may be a cohort of HSP90 action.

Results presented in this chapter suggest that N-linked protein phosphorylation events in the cytosol are likely to be influenced by heat shock and that HSP90 is involved in the control of the responsible kinase(s). The most striking feature of these *in vitro* phosphorylation studies is that only one major new phosphoprotein (p66) is phosphorylated in extracts made after cells are heat shocked and that this phosphorylation is HSP dependent. Peptide mapping and limited sequencing of p66 was tried, but time did not allow this to be repeated. With isolation, cloning, sequencing and expression of the p66 coding sequence, also determining the physiological consequences of a p66 gene disruption, the function of p66 could be determined. Also the phosphorylation sites on this protein could be mutated by site directed mutagenesis to establish if the N-linked phosphorylation studied here has any role in cellular regulation.

CHAPTER 7

INHIBITORS OF THE HEAT SHOCK RESPONSE OF YEAST

7.1 Introduction

A number of chemicals are known to induce the synthesis of HSPs in the absence of heat stress (Ananthan *et al.*, 1985; Lindquist and Craig, 1988; Watson, 1990), but few are known to selectively inhibit expression of these proteins. In this study, weak acid preservatives, uncouplers and diethylstilboestrol were shown to block the heat shock response of *S. cerevisiae* in low pH medium, the first time selective inhibitors of this response have been identified in yeast . The only other reported examples of selective inhibitors of the heat shock response are flavonoids, which cause hypersensitisation to heat and a block to the expression of HSPs in mammalian cells (Hosokawa *et al.*, 1990). The mechanisms of inhibition by weak acid preservatives and flavonoids are probably different, since the flavonoid quercetin is known to be mutagenic (Bjeldanes and Chang, 1977), genotoxic (Macgregor, 1986), and also to inhibit the growth of cultured cells (Kim *et al.*, 1984), glycolysis (Suolinna *et al.*, 1975), macromolecule synthesis (Graziani and Chayoth, 1979), and the activities of protein kinases (Graziani *et al.*, 1981) and ATPases (Kuriki and Racker, 1976).

Sorbate and benzoate are used extensively as food preservatives because they inhibit the growth of fungi and bacteria. It has been clearly established that microbial growth inhibition and penetration of these weak organic acids into cells both increase with medium acidification, being essentially proportional to the concentration of the undissociated acid (Russell, 1991). Following entry to cells of low pH cultures in their undissociated forms, sorbic and benzoic acids subsequently dissociate in the higher pH environment of the cytosol. This releases protons, causing intracellular acidification. In *S.cerevisiae* intracellular pH declines more than 1 unit with 2-10 mM benzoate and glucose fermantation is inhibited (Krebs *et al.*, 1983; Francois *et al.*, 1986). More osmotolerant yeasts, notably *Zygosaccharomyces bailii,* are important causative agents of the spoilage of foods and beverages of low pH. They are able to undergo an adaptation that allows their growth in the presence of preservative concentrations in excess of those legally permitted in foodstuffs (Warth, 1977; 1988; Cole and Keenan, 1986). Such an adaptation to facilitate growth in the presence of weak acid preservatives is not known for bacteria (Russell, 1991).

A mild, sublethal heat stress increases the capacity of microbial cells to survive during a subsequent exposure to higher, potentially-lethal temperatures. This development of the state of induced or "acquired" thermotolerance is widespread amongst food-contaminating and pathogenic organisms and therefore a phenomenon having major implications for microbiological safety (Piper, 1993). A mild heat stress also induces in all cells the highly-conserved heat shock proteins (Lindquist and Craig, 1988; Watson, 1990; Mager and Moradas-Ferreira, 1993). The literature regarding the effects of weak acid preservatives on yeast heat tolerance is confusing. Sorbate has been variously reported either having no effect on (VanUden, 1984), or increasing (Coote *et al.,* 1991), the thermotolerance of *S.cerevisiae.* This study therefore investigated whether it acts as one of the many chemical inducers of the heat shock response. Results in this chapter show that sorbate and benzoate selectively inhibit the induction of heat shock genes by heat in low pH cultures. They also show that the effects of sorbate on thermotolerance are strongly dependent on external pH.

In low pH cultures heating in the presence of sorbic acid caused strong induction of respiratory-deficient *rho- petites*, indicating that heat potentiates the sorbate-induced ultrastructural damage to yeast mitochondria seen in electron micrographs (Cole, 1987). Adaptation of *S. cerevisiae* to fermentative growth at low pH in the presence of sorbate involves a selection for *rho*$^-$ cells, the *rho*$^-$ mutation conferring not just growth in the presence of sorbate but also, remarkably, a partial restoration of the capacity for heat induction of HSPs in the presence of sorbate.

7.2 Results

7.2.1 Sorbate and benzoate inhibit the induction of major heat shock proteins in *S. cerevisiae* cultures of low pH

S. cerevisiae SUB62 cells were transferred to buffered SD media of pH 4.5, 6.5 or 8.5 containing 50mM of the appropriate buffer (sodium acetate pH4.5; MES-KOH pH6.5; TES-KOH pH7.5 or 8.5). Shortly afterwards they were pulse-labelled with [^{3}H] leucine, either at 25°C or after heat shock to 39°C, in the presence and absence of 9mM potassium sorbate. Analysis of the proteins labelled under these conditions by 1D SDS gel electrophoresis (Fig.7.1) revealed that major heat shock proteins had been induced at 39°C in all of the cultures, with the exception of the pH4.5 culture heat shocked in the presence of potassium sorbate. Proteins pulse-labelled in this pH4.5 culture at 39°C appeared similar to those labelled at 25°C (Fig7.1) indicating that the block to heat shock protein expression was not due to a total inhibition of protein synthesis, but rather that sorbate may be acting

to selectively inhibit heat shock gene expression in this pH4.5 sorbate-treated culture. Total inhibition of heat shock protein induction at pH4.5, but not pH7.5, was also obtained in a similar pulse-labelling experiment in which either 3mM or 6mM potassium benzoate was employed in place of sorbate (Fig.7.2). When sorbate or benzoate was used at concentrations of 1mM or less the inhibition of heat shock protein induction in pH 4.5 cultures was only partial (data not shown).

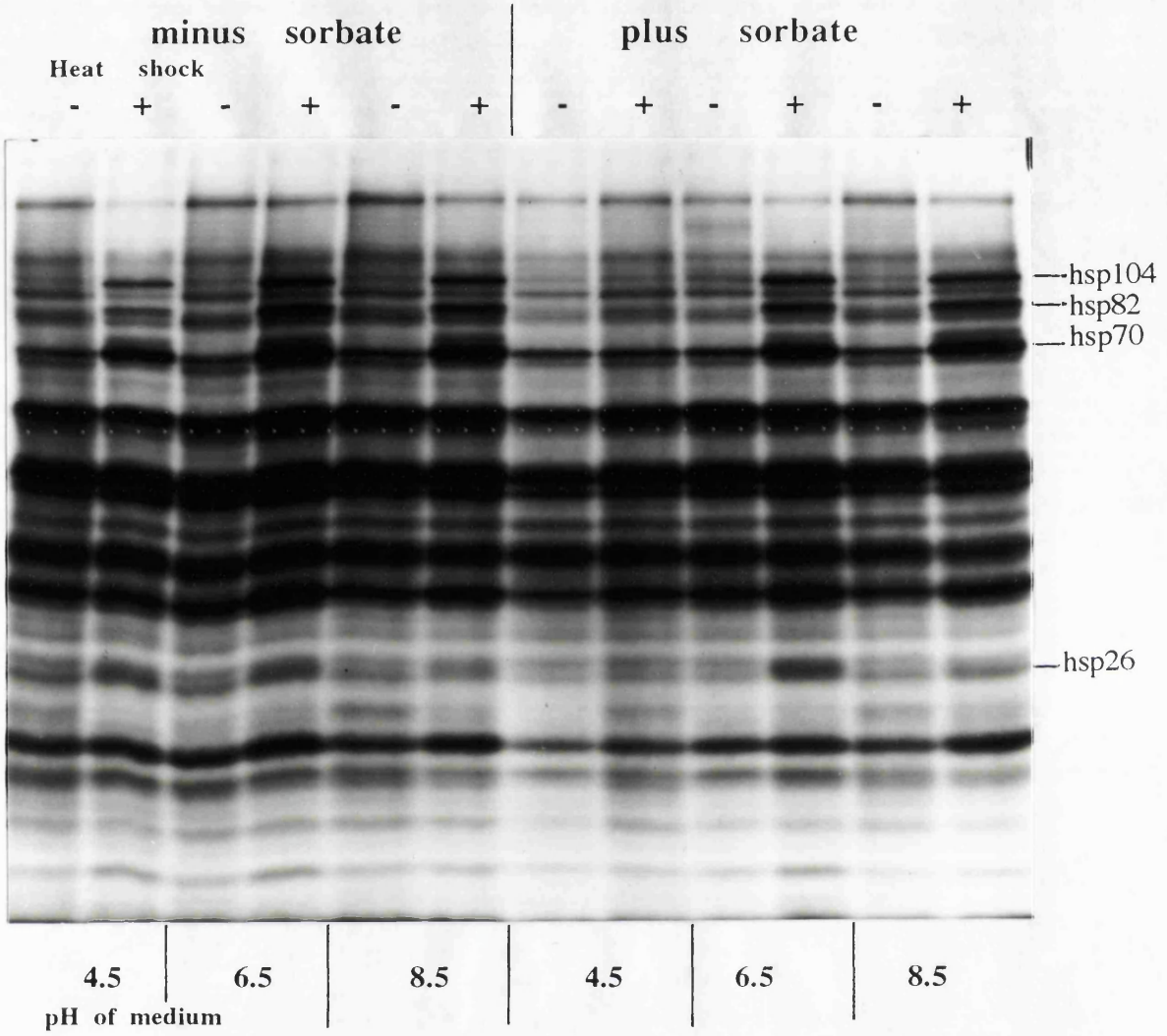


Figure 7.1 Autoradiograph of SDS-PAGE gel with proteins from cells labelled with [^{3}H] leucine for 40min, either at 25°C (-) or immediately after heat shock to 39°C (+) in the presence and absence of 9mM potassium sorbate.

Prior to labelling the cells were resuspended in medium buffered at 4.5, 6.5 or 8.5. The heat shock proteins induced most strongly by heat shock (Mager and Moradas-Ferreira, 1993) are indicated to the right of the figure. This figure shows the sorbate inhibition of the heat shock response in pH 4.5 cultures.

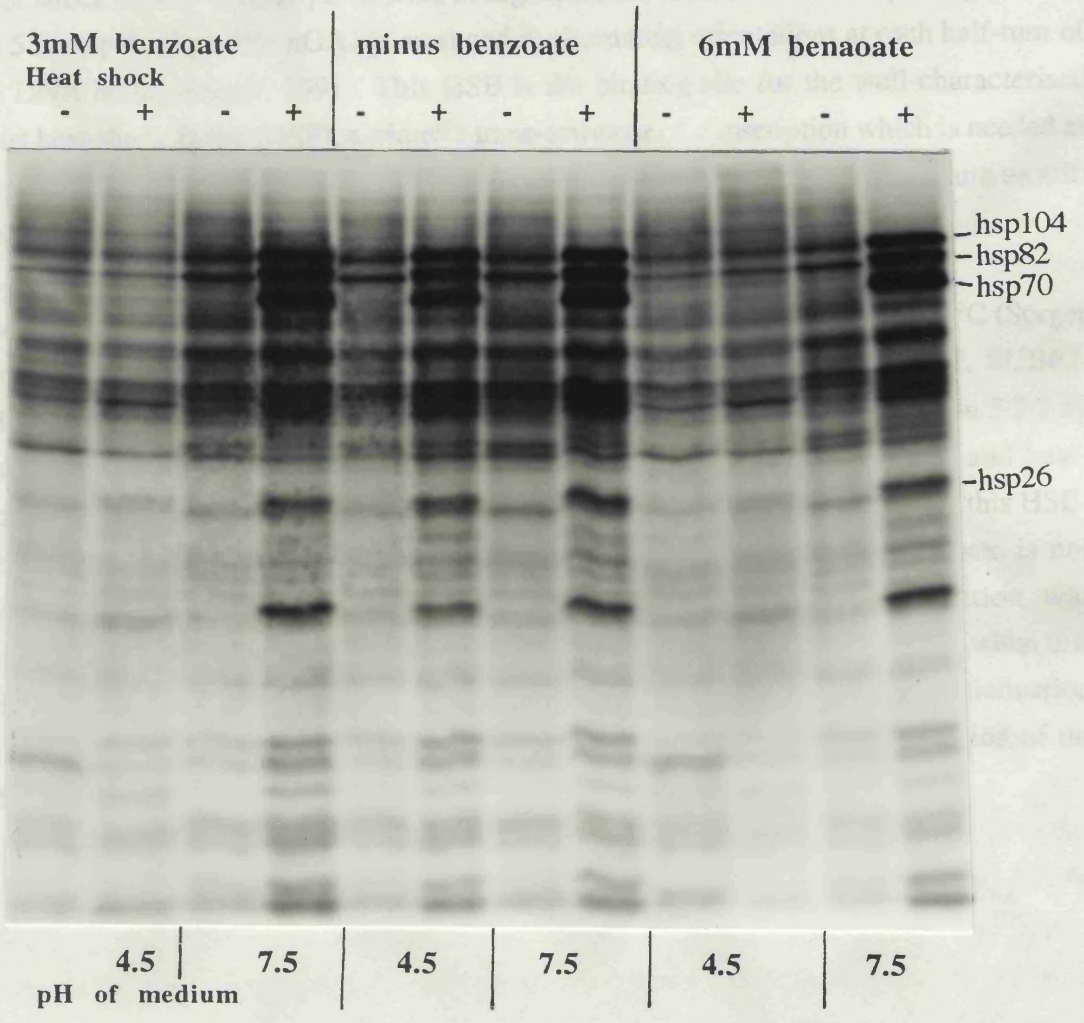


Figure 7.2 Autoradiograph of SDS-PAGE gel with proteins from cells labelled with [^{3}H] leucine for 40min, either at 25°C (-) or immediately after heat shock to 39°C (+) in the presence and absence of benzoate.

Cells had previously been resuspended in medium buffered at pH 4.5 or 7.5. This figure shows the benzoate inhibition of the heat shock response in pH 4.5 cultures.

7.2.2 Effects of sorbate and culture pH on the activity of a heat shock element (HSE)-*lacZ* fusion

Heat induction of most *S. cerevisiae* HSP genes is due to the same promoter sequence [heat shock element (HSE)] as is used in higher eukaryotes, a series of repeating units of the 5 base-pair sequence: nGAAn, arranged in alternating orientations at each half-turn of the DNA helix (Sorger, 1991). This HSE is the binding site for the well-characterised yeast heat shock factor (HSF), a trimeric trans-activator of transcription which is needed at all growth temperatures but which increases its activity in response to temperature upshift (section 1.3.2; Sorger, 1990; 1991).

The optimum temperature for induction of HSE sequences in *S. cerevisiae* is 39°C (Sorger and Pelham, 1987; Kirk and Piper, 1991). A transformant of strain SUB62, SUB62-pHSE2 that carries a *lacZ* gene under HSE control on plasmid pHSE2 (section 5.2.2.1) was used to investigate the influences of medium pH and sorbate on basal and heat-induced HSE activity (Fig. 7.3A). In the absence of heat stress basal activity of this HSE-*lacZ* gene was unaffected by either sorbate or culture pH (Fig. 7.3A). Sorbate is not therefore an inducer of HSE sequences. However, HSE-*LacZ* heat-induction was dependent on the pH of the culture and was dramatically suppressed by sorbate when this pH was below 7.0 (Fig.7.3A). This indicates that the sorbate inhibition of HSP induction in pH4.5 cultures (Fig.7.1; 7.2) is a consequence of a block to the heat induction of the HSE sequences on heat shock gene promoters.

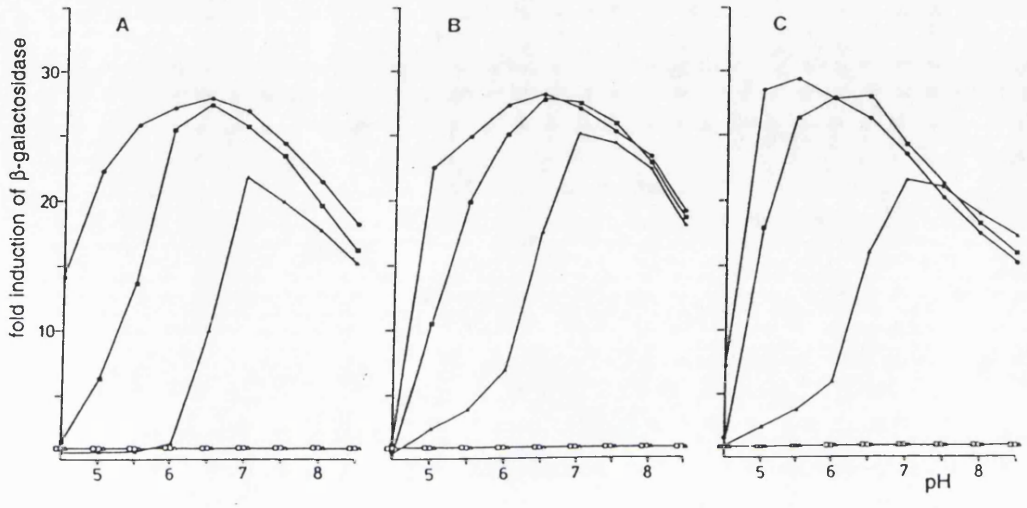


Figure 7.3 Assay of β-galactosidase induction at 25°C or 39°C in cultures of different medium pH. (A) SUB62-pHSE2; (B) a spontaneous *rho⁻ petite* derived from SUB62-pHSE2; (C) the same *rho⁻* mutant adapted to grown for several generations in the presence of 1mM potassium sorbate.

Cells were resuspended in media buffered at different pH values from 4.5 to 8.5, with or without sorbate. After these cultures had been maintained at 25°C or heat shocked to 39°C for 40min β–galactosidase levels were determined as in section 2.7.4. β–galactosidase is given as fold-induction relative to the constant β–galactosidase level of 2.3 Miller units (Miller, 1972) in uninduced 25°C SD medium SUB62-pHSE2 cultures. Basal HSE-*lacZ* expression in the absence of heat shock was unaffected by either mediun pH or sorbic acid, as shown by the low constant β–galactosidase level at 25°C in the absence (○) or the presence of potassium sorbate [1mM (□); 9mM (△)] respectively. *LacZ* expression after HSE induction by heat shock to 39°C showed an appreciable dependence on culture pH even in the absence of sorbate (●). The influences of 1mM (■) or 9mM (▲) sorbate on this heat-induced expression are much more pronounced at low pH values. Also while 9mM sorbate totally inhibited heat induction of HSE-*LacZ* expression in *rho⁺* cells below pH 6.0 (A), this inhibition was only partial in the *rho⁻ petite* (B,C).

7.2.3 Effects of sorbate and external pH on thermotolerance

An increased thermotolerance is one of the major consequence of a mild sublethal heat shock (Piper,1993). To investigate the effects of sorbate and external pH on thermotolerance, cells were transferred to media of different pH for 5 or 60min at 25°C or 60min at 38°C, with or without 9mM potassium sorbate. They were then subjected to a potentially lethal heat treatment (5min 50°C) without change to medium pH or sorbate level (Figs. 7.4; 7.5). In the absence of sorbate, cell survival on both complex medium (YPD medium previously adjusted to pH3.5, 4.5, 5.5, 6.5, 7.5 or 8.5 with HCl/NaOH; Fig.7.4) and chemically-defined medium (SD medium cultures that were diluted with an equal volume of a 100mM solution of the appropriate buffer, sodium acetate pH3.5 or 4.5; MES-KOH pH5.5 or 6.5; TES-KOH pH7.5 or 8.5; Fig7.5) showed that thermotolerance is influenced by the external pH during lethal heat treatment, irrespective of whether the pH preconditioning has been at 25°C or 38°C. This effect of medium pH on thermotolerance is not unexpected, since external pH is known to influence pHi even in unstressed cells (Borst-Pauwells, 1981). The pH of heating affects thermotolerance, tolerance being maximal at a pH approximating to physiological pHi values (6-7) and considerably lower at extremes of pH (Figs.7.4; 7.5). This probably reflects a lowered disturbance to homeostasis when cells are heated at physiological pH values.

Figs. 7.4 and 7.5 also reveal 9mM sorbate exerting strongly pH-dependent effects on thermotolerance. At higher pH values, it caused small increases in thermotolerance whereas at lower pH it had the converse effect, causing thermotolerance reductions. A brief heat treatment at a potentially-lethal temperature (5min 50°C), when applied at low pH in conjunction with sorbate, was found to strongly select for respiratory deficient *petites*. Such *petites* were a substantial fraction of the survivors of pH4.5 cultures heated in the presence of 9mM sorbate (see lower diagrams; Figs. 7.4 & 7.5). It is well-established that brief lethal heating at a potentially lethal temperature, or growth at supraoptimal temperatures (37-39°C), causes *S.cerevisiae* cultures to accumulate a greater proportion of their cells as respiratory-deficient *petites* (VanUden, 1984). This *petite* induction is thought to reflect the sensitivity of the mitochondria to heat damage. Figs.7.4 and 7.5 suggest that this damage is considerably enhanced by the presence of intracellular sorbic acid, an effect which is more pronounced in low pH cultures due to the higher uptake of the acid by the cells of these cultures (see section 7.1).

The experiment in Figs 7.5 B,C was repeated, but changing the procedure such that the cells were transferred to sorbate-free medium of a single, defined pH immediately after the 1h incubation at 25°C or 38°C in media of different pH and prior to the 50°C 5min lethal heat treatment (Fig. 7.6). Survival of the cells heated in the absence of sorbate shows the well-established thermotolerance induction with mild heat (38°C) pretreatment (compare Figs. 7.6D,E with 7.5C,D). A 25°C sorbate pretreatment of cells at higher pH values increased thermotolerance showing that sorbate is a chemical inducer of thermotolerance under these conditions. The same pretreatment at low pH caused relatively little thermotolerance change (Fig. 7.6D). Pretreatment with both mild heat (38°C) and sorbate at higher pH values increased thermotolerance only slightly above the values achievable with heat shock alone, while at lower pH sorbate largely prevented the sublethal heat-induced increases in thermotolerance (Fig. 7.6E). The removal of sorbate prior to lethal heating elimimated the strong selection of *petites* seen in Figs. 7.4 and 7.5, less than 5% of the survivors of lethal heating in the experiment in Fig. 7.6 being *petites* irrespective of the pH of preincubation (not shown).

7.2.4 Effects of the *petite* mutation on HSP induction and the activity of a HSE-*lacZ* fusion

It was observed that *rho- petites* of *S.cerevisiae* strain SUB62 (whether spontaneous or sorbic acid-induced) were capable of fermentative growth at low pH in the presence of concentrations of sorbic acid that totally inhibit the growth of *rho+* cells. On pH4.5 YPD plates *petites* are capable of slow growth in the presence of 4.5mM potassium sorbate, whereas the growth of respiration-competent (*rho+*) cells becomes totally inhibited when sorbic acid exceeds approximately 1mM (data not shown).The small proportion of *petites* in *S. cerevisiae* SUB62 cultures can therefore be selected as the only cells capable of appreciable growth on pH4.5 plates containing 4.5mM sorbate. Adaptation of this *S.cerevisiae* strain to pH4.5 fermentative growth at this concentration of weak acid preservative therefore involves a selective outgrowth of the growth-arrested *rho+* population by the *rho- petites* which are always present as a small proportion of the cells of *S. cerevisiae* cultures.

Millimolar concentrations of sorbate and benzoate inhibit both the growth and heat shock response (Figs. 7.1, 7.2 and 7.3A) of low pH cultures of respiration-competent (*rho+*) cells. We were therefore intrigued to know whether they caused a similar inhibition of heat shock gene expression in the more sorbate-resistant *rho- petites* derived from these *rho+*

cultures. At the lowest pH at which *rho*$^-$ cultures could incorporate labelled amino acid into protein (pH4.5), the presence of 9mM sorbate reduced incorporation of [^{3}H] leucine into protein (Fig. 7.7). However both a spontaneous *petite* and a *petite* induced by heating a SD medium culture in the presence of sorbate (Fig. 7.4B) showed detectable heat induction of heat shock proteins at pH4.5 in the presence of sorbate (Fig. 7.7). Also the *petite* mutation caused a partial restoration of the heat-inducibility of a HSE-*lacZ* fusion in pH5.0, 5.5 or 6.0 cultures heat shocked in the presence of 9mM sorbate (Fig. 7.3B,C), irrespective of whether this *petite* had been cultured on SD medium for several generations in the absence (Fig. 7.3B) or presence (Fig. 7.3C) of 1mM sorbate. It remains to be seen whether this partial recovery of a heat shock response in sorbate-treated low pH cultures of *rho*$^-$ *petites* contribute to the greater capacity of *petites* for fermentative growth in the presence of this preservative.

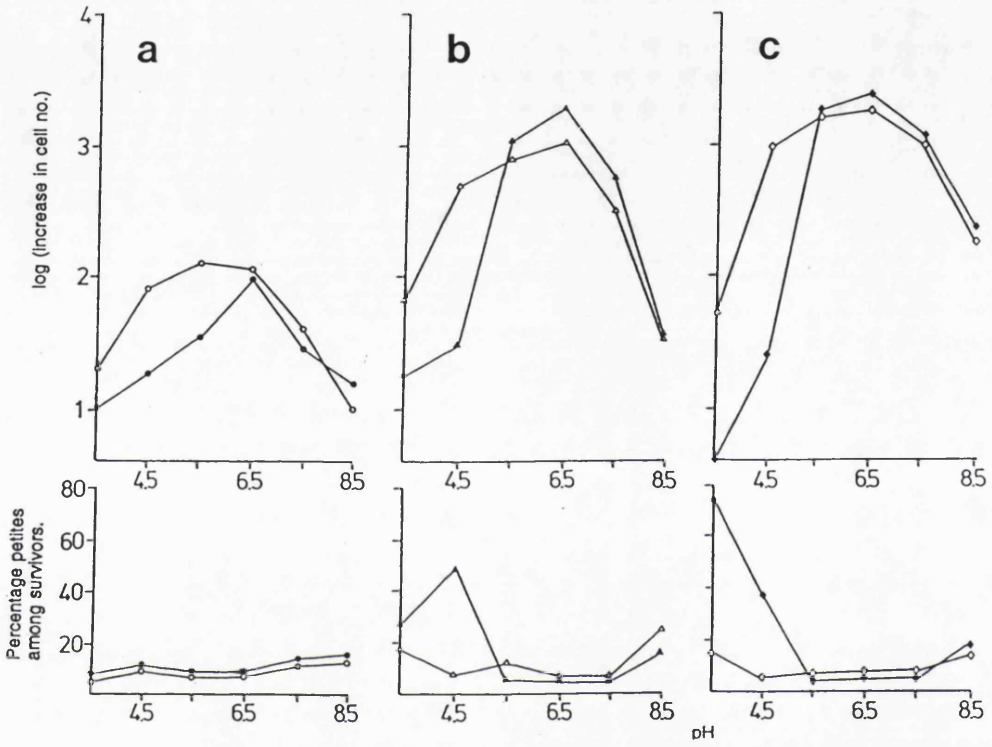


Figure 7.4. Influence of the pH of a preincubation in YPD medium, also the presence and absence of 9mM potassium sorbate, on thermotolerance. Cells were grown to early exponential phase ($5x10^6$ cells ml^{-1}) on pH 6.8 YPD medium at 25°C. They were then centrifuged and resuspended in YPD previously adjusted to pH 3.5, 4.5, 5.5, 6.5, 7.5 or 8.5 either without (open symbols) or with (closed symbols) 9mM sorbic acid. After preincubation in these buffered media for 5 min at 25°C (A), 1h at 25°C (B), or 1h at 38°C (C) cultures were immediately heated 5 min at 50°C and then chilled. Survival is expressed relative to the survival of the original pH 6.8 YPD starter culture given an identical 50°C 5 min heat stress, the percentage of the survivors that were respiratory-deficient *petites* being indicated in the lower diagrams.

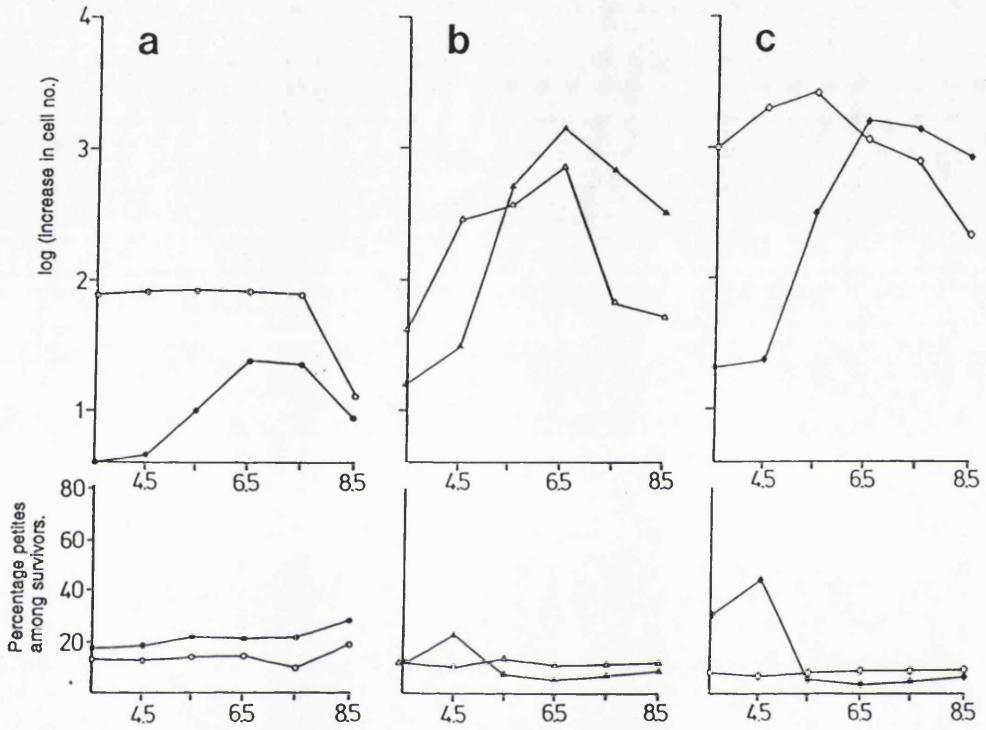


Figure 7.5. Influence of the pH of a preincubation in buffered SD medium, also the presence and absence of 9mM potassium sorbate, on thermotolerance.

SUB62 cells were grown to early exponential phase ($5x10^6$ cells ml^{-1}) on pH 6.8 YPD medium at 25°C. They were then centrifuged and resuspended in buffered 0.5 xSD medium at pH 3.5, 4.5, 5.5, 6.5, 7.5 or 8.5 either without (open symbols) or with (closed symbols) 9mM sorbic acid. After preincubation in these buffered media for 5 min at 25°C (A), 1h at 25°C (B), or 1h at 38°C (C) cultures were immediately heated 5 min at 50°C and then chilled. Survival was measured as ability to form colonies on plates at 28°C, relative to the survival of the original pH 6.8 YPD starter culture given an identical 50°C 5 min heat stress. The percentage of survivors of heat stress in A-C that were respiratory-deficient *petites* in indicated below the thermotolerance data.

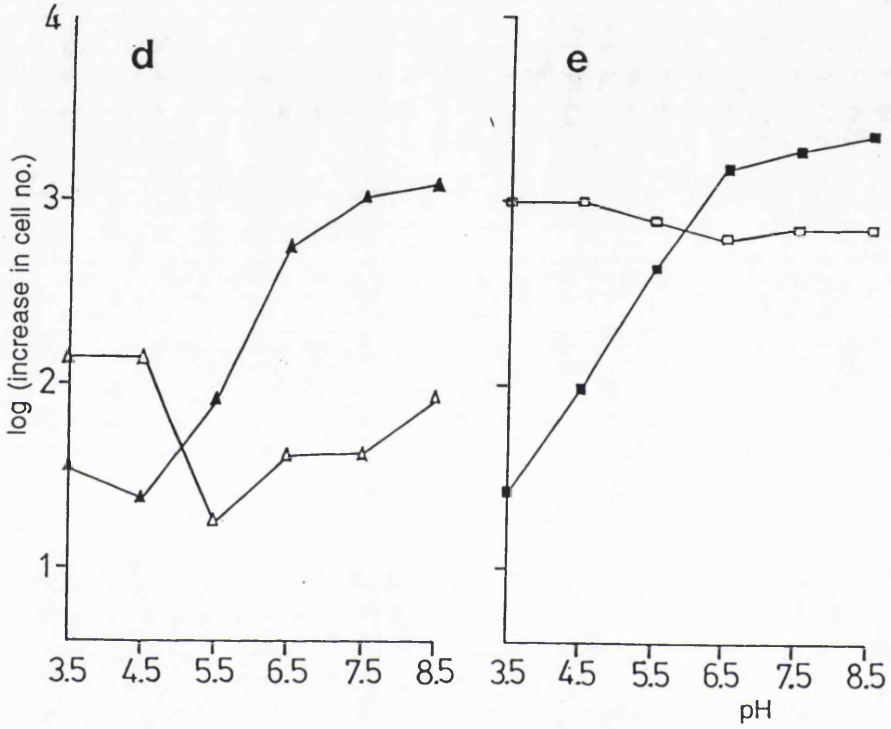


Figure 7.6. Influences of sorbate pretreatment and preincubation pH on thermotolerance, when the subsequent lethal heating was conducted at a single defined pH and in the absence of sorbate.

Cells were grown to early exponential phase ($5x10^6$ cells ml^{-1}) on pH 6.8 YPD medium at 25°C. They were then centrifuged and resuspended in YPD previously adjusted to pH 3.5, 4.5, 5.5, 6.5, 7.5 or 8.5 either without (open symbols) or with (closed symbols) 9mM potassium sorbate. After preincubation in these buffered media for 1h at 25°C (D), or 1h at 38°C (E), cultures were harvested by centrifugation, resuspended in pH6.0 sorbate-free YPD, immediately heated 5 min at 50°C and then chilled. As for Figs. 7.4 and 7.5 survival is indicated relative to the survival of the original pH 6.8 YPD starter culture given an identical heat stress.

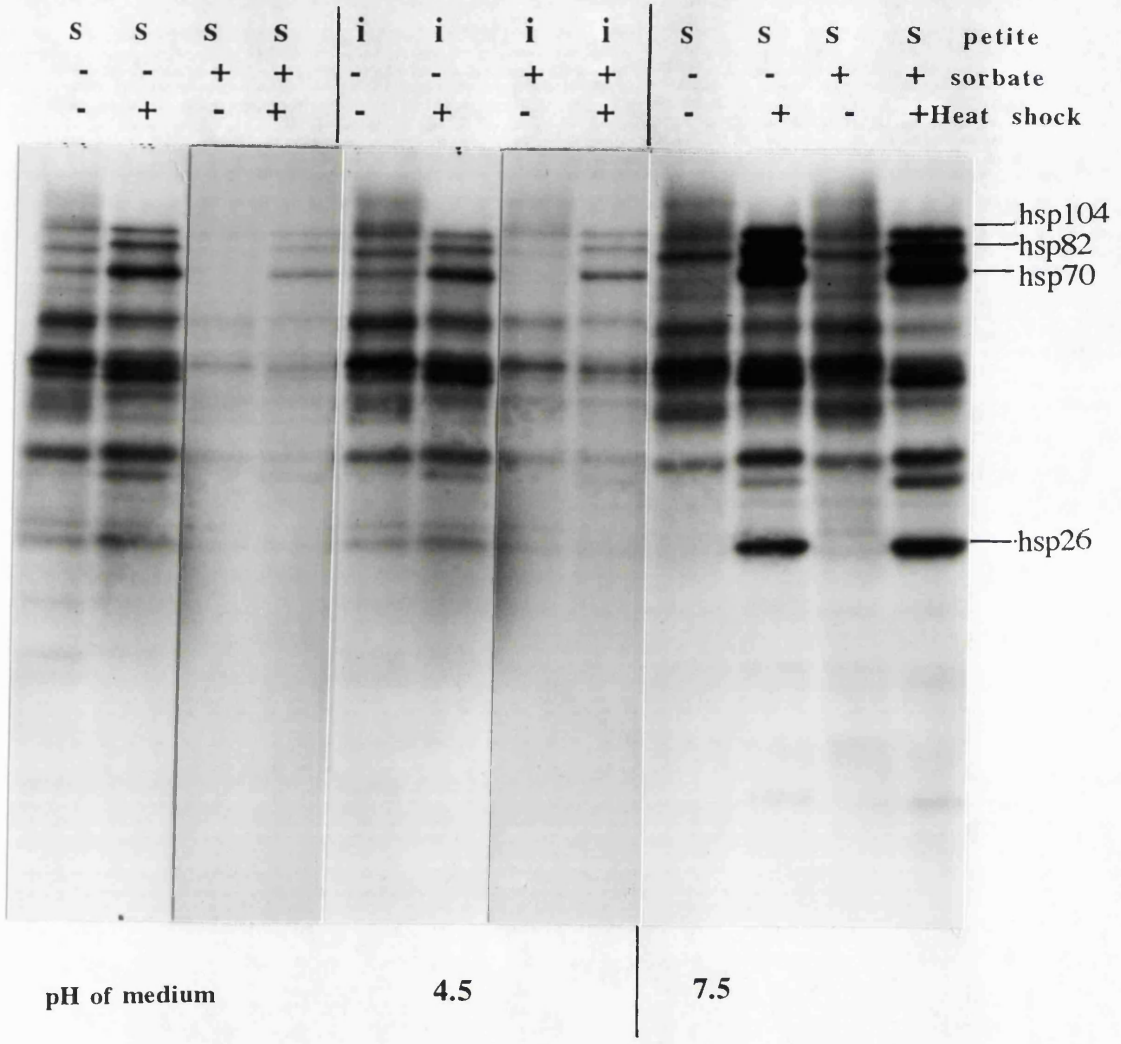


Figure 7.7 SDS-PAGE of extracts of [^{3}H]leucine labelled cells of the *rho-petite* showing the restored capacity for synthesis of HSPs in low pH cultures.

Cells of a spontaneous *petite* derived from strain SUB62 (s) were resuspended in medium buffered at pH4.5 or 7.5; also cells of a *petite* derived by heating SUB62 in the presence of sorbic acid (i) were resuspended in medium buffered at pH4.5. . The cultures were then incubated with [^{3}H]leucine for 40min either at 25°C or immediately after heat shock to 39°C in the presence and absence of 9mM sorbic acid. Major heat shock proteins are indicated to the right figue.

7.2.5 Other inhibitors of HSP induction in yeast

Very few agents have been shown to be selective inhibitors of the heat shock response (see section 7.1) . In mammalian cells flavonoids block HSP expression (Hosokawa *et al.*, 1990). For this reason a flavonoid (quercetin) was tested for its ability to inhibit HSP induction in yeast. By protein pulse-labelling, as in Fig.7.1, the addition of 50μM or 200μM quercetin to pH4.5 and pH7.5 *S. cerevisiae* cultures was found not to influence heat shock protein induction by heat shock (data not shown). It is possible that this result reflects a lack of permeability of yeast cells to quercetin, a point that was not investigated further.

The rapid pHi decline due to weak acid preservatives (see section7.1) is a possible cause of sorbate and benzoate-treated cultures of low pH being unable to mount a response to heat shock (Figs. 7.1, 7.2). Maintenance of pHi in yeast largely occurs through plasma membrane ATPase catalysed proton extrusion and secretion of organic acids (Serrano, 1991). A mutation reducing plasma membrane ATPase activity reduces levels of HSP induction with heat (Panaretou and Piper, 1990). Therefore an inhibitor of plasma membrane ATPase was tested as a possible selective inhibitor of HSP induction. This inhibitor, diethylstilboestrol (DES), is known to hypersensitise yeast to thermal death (Coote *et al.*, 1991). When used at a concentration (50μM) that totally inhibits plasma membrane ATPase action (Coote *et al.*, 1991) it rapidly rendered cells incapable of a heat shock response, the cells displaying no alteration in their protein synthetic pattern with heat shock to 39°C (Fig. 7.8). However diethylstilboestrol differed from sorbic and benzoic acids in that its inhibitory action was independent of external pH (compare Fig. 7.1 with Fig.7.8).

Another method of causing pHi decline in low pH fermentative cultures is to add an uncoupler such as 2,4-dinitrophenol or CCCP. Uncouplers cause membrane depolarisation. They differ from sorbic and benzoic acids in that they are lipophilic in both their dissociated and protonated states so that, unlike these preservatives, they do not show pronounced intracellular concentration when pHi values are higher than the extracellular pH. In pH4.5 cultures 75μM CCCP abolished heat induction of HSPs, while 50μM CCCP caused a much-reduced induction of these proteins (Fig.7.9). This inhibition was not evident at pH7.5 (Fig. 7.9). The concentrations of CCCP which were effective in inhibiting the heat shock response were considerably lower than the concentrations of sorbate or benzoate needed for similar levels of inhibition (data not shown). These results with weak acid preservatives, diethylstilbestrol and CCCP suggest that a substantial drop

in pHi, and possibly depolarisation of the plasma membrane may prevent yeast cultures from mounting a heat shock response.

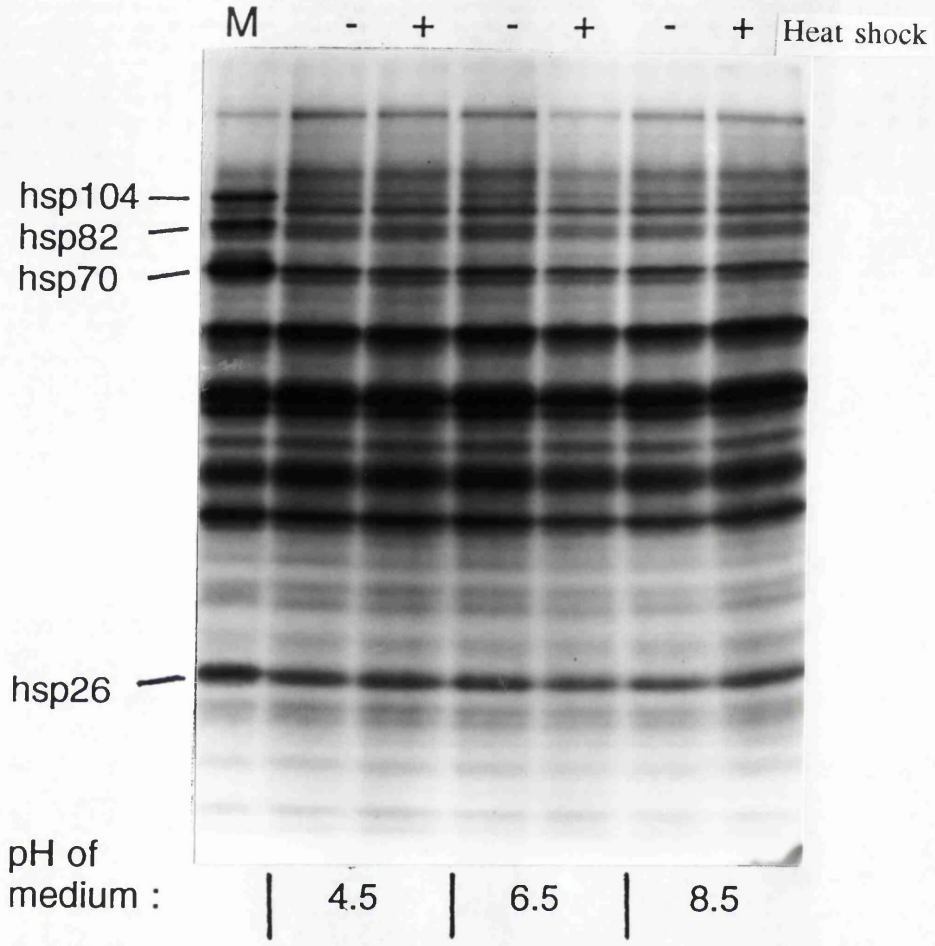


Figure 7.8 SDS-PAGE of extracts of [^{3}H]leucine labelled cells showing effects of diethylstilboeatrol on induction of HSPs in low pH cultures.

SUB62 cells were transferred to SD medium buffered to pH4.5, 6.5 or 8.5. Diethylstilboestrol (50μg^{-1}) was added immediately before addition of labelled leucine. For labellings of the control cells not subject to diethylstilboestrol treatment see Fig. 7.1. M is a marker track containing a sample of the proteins normally labelled in yeast at 39°C.

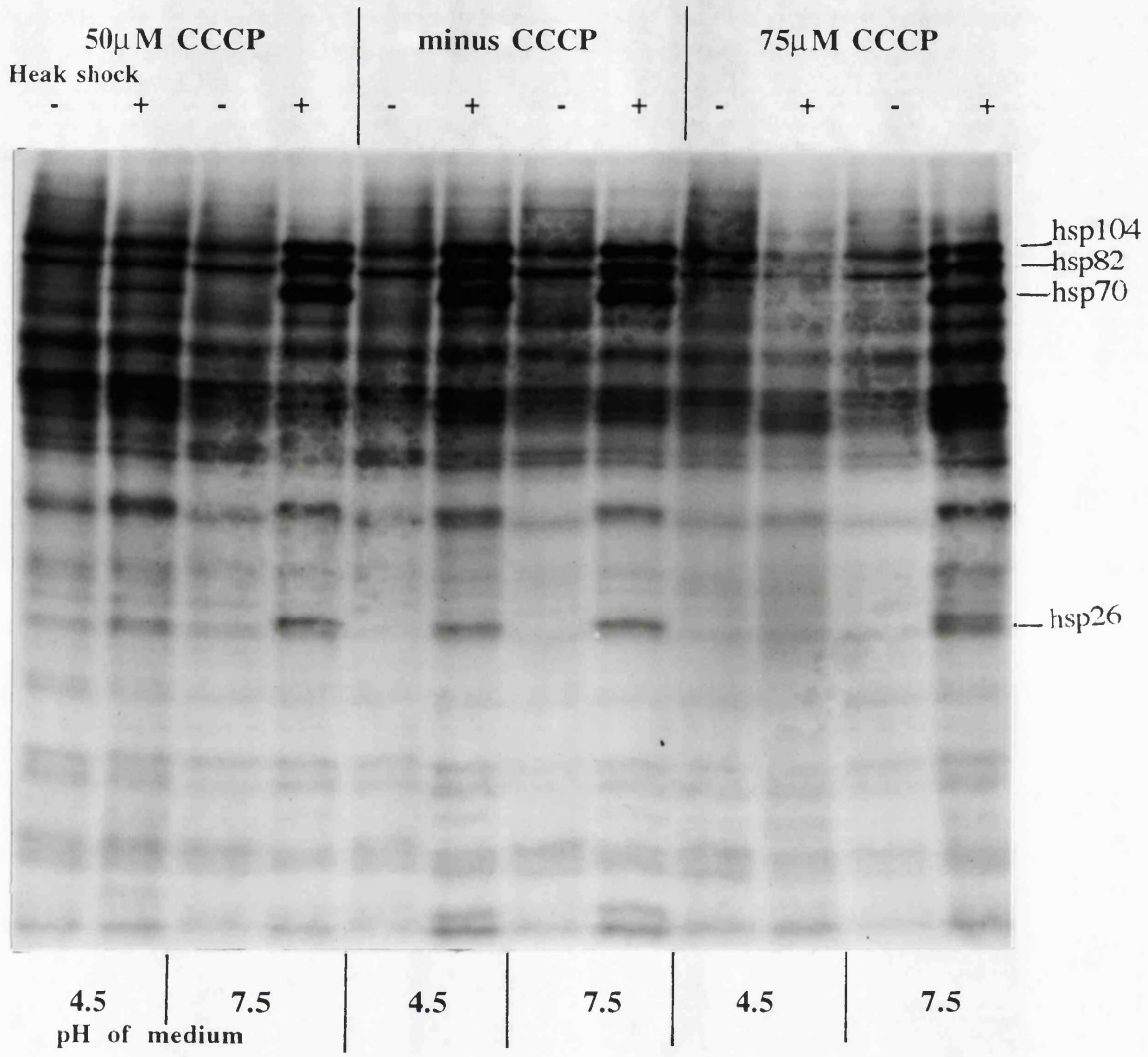


Figure 7.9 SDS-PAGE of extracts of [^{3}H]leucine labelled cells showing effects of an uncoupler (CCCP) on induction of HSPs in low pH cultures.

S. cerevisiae cells were incubated with [^{3}H]leucine for 40min either at 25°C (-) or after heat shock to 39°C (+) in the absence and presence of CCCP. They had previously been resuspended in medium buffered at pH4.5 or 7.5. 75μM CCCP appears to cause a total inhibition of heat shock protein induction, wheres 50μM CCCP produces more partial inhibition.

7.3 Discussion

7.3.1 Weak acid preservatives and uncouplers have dissimilar effects on glycolytic flux

Weak acid preservatives differ from weak acid uncouplers in that they cross membranes readily only when undissociated, while uncouplers are lipophilic in both their protonated and unprotonated forms. Sorbate and benzoate will therefore concentrate inside cells in response to a higher pH on the cytosolic side of the cell membrane (see section 7.1) while uncouplers, in contrast, should depolarise membranes but not concentrate in the cytosol.

Weak acid preservatives and uncouplers also have very different effects on glycolytic flux. In low pH *S. cerevisiae* cultures preservatives inhibit (Krebs *et al.*, 1983; Francois *et al.*, 1986) while uncouplers stimulate glycolysis (see Francois *et al.*, 1986, 1988 for literature). The inhibition of glycolysis by preservatives is associated with increases in glucose-6-phosphate and fructose-6-phosphate and decreases in glycolytic intermediates beyond PFK1, indicating this enzyme as the site of inhibition (Krebs *et al.*, 1983). In yeast, fructose (2,6)-bisphosphate acts as the potent activator of PFK1 and inhibitor of fructose 1,6-bisphosphatase (VanSchaftingen and Hers, 1982; Francois *et al.*, 1984). The enzyme that catalyses its synthesis (6-phosphofructo-2-kinase; PFK2) is inhibited by both benzoate and acid pH *in vitro,* benzoate also acting to decrease fructose (2,6)-bisphosphate levels *in vivo* (Francois *et al.*, 1986). A key regulator of glycolytic flux, PFK2 is activated when glucose is added to yeast cells by a phosphorylation catalysed by cAMP-dependent protein kinase (Francois *et al.*, 1984). cAMP levels increase only slightly with the addition of weak acid preservatives (Francois *et al.*, 1986) but increase dramatically with the addition of uncouplers (Francois *et al.*, 1988). The former cAMP increase is not associated with any fructose (2,6)-bisphosphate increase (Francois *et al.*, 1986), in contrast to the increases in fructose (2,6)-bisphosphate and glycolytic flux with the addition of uncouplers. Instead benzoate causes a disappearance of fructose (2,6)-bisphosphate (Francois *et al.*, 1988), preventing the glucose activation of PFK2. There is, remarkably, no effect on ATP levels indicating that a deficiency in energy supply is not the simple explanation for weak acid-induced growth arrest (Francois *et al.*, 1986).

7.3.2 Similar actions of weak acid preservatives and uncouplers

In this study it has been observed that weak acid preservatives (sorbate and benzoate) and an uncoupler (CCCP) have similar inhibitory effects on the response of low pH yeast cultures to heat shock (Figs.7.1, 7.2, 7.9). It is improbable therefore that these inhibitions are tightly linked to the dramatic, yet opposite, effects of these agents on fructose (2,6)-bisphosphate levels and glycolytic flux. Instead they are more likely to reflect a common action of preservatives and uncouplers.

Both weak acid preservatives and uncouplers will cause a rapid pHi decline in low pH cultures, uncouplers also causing a slower depolarisation of membranes (Thevelein *et al.*, 1987). The electrochemical gradient at the plasma membrane is largely established and maintained in fermentative bacteria, fungi and plants by the action of a proton-translocating ATPase (Kobayashi *et al.*, 1986; Serrano, 1991).This ATPase is essential for a number of vital functions including nutrient uptake, maintenance of potassium balance, and regulation of pHi. Its action also has a strong influence over the thermotolerance of *S. cerevisiae* and *Schizosaccharomyces pombe* (Panaretou and Piper, 1990; Piper, 1993; Coote, 1993), while an inhibition of this ATPase hypersensitises yeast to thermal death (Coote *et al.*, 1991). Previous studies in this laboratory have indicated that plasma membrane ATPase action influences the ability of *S.cerevisiae* cells to synthesise HSPs in response to heat shock. A mutation which reduces ATPase activity (*pma1.1*) results in both a lowered pHi (Ulazewski *et al.*, 1987) and reduced induction of HSPs with heat shock (Panaretou and Piper, 1990). The similarity of uncouplers and weak acid preservatives in preventing a response to heat shock in low pH cultures (Figs.7.1, 7.2 and 7.9) might reflect their common action in causing a rapid decrease in pHi. Consistent with this, the plasma membrane ATPase inhibitor diethylstilboestrol rapidly renders cells incapable of HSP induction by heat shock even though the capacity for protein synthesis is not lost (Fig.7.8). However this effect of diethylstilboestrol appears not to be influenced by extracellular pH (Fig.7.8). Thus while the results in Figs7.1-7.3, 7.8 and 7.9 are largely consistent with lowered pHi preventing a response to heat shock, there may be other explanations for these observed results, indicating a direct inhibition of a stress-signalling pathway by the various chemical agents that have been employed.

7.3.3 Effects of sorbate on thermotolerance

Prior to this study Van Uden had reported that sorbate has no effect on the thermotolerance of *S.cerevisiae,* but shifts the Arrhenius plots and T_{max} of growth to lower temperatures (Van Uden, 1984). It is difficult to assess this study as the conditions, notably pH, of growth were not reported. More recently Coote *et al.* (1991) showed that thermotolerance increases during a 9mM sorbic acid treatment at both pH 4.5 and pH 6.0, this increase occurring over a longer period in the cells exposed to pH 4.5. However a partial thermotolerance increase was also seen in cells exposed to pH 4.5 in the absence of sorbate. In their study Coote *et al.* (1991) conducted lethal heating after resuspending the cells in sorbate-free medium (as in Figure 7.6). Figs.7.4-7.6 show that the pH of lethal heating exerts an appreciable influence on basal (25°C) and sublethal heat-induced thermotolerance in the absence of sorbate. These experiments also show in greater detail the strong influence of medium pH on the manner in which sorbate affects thermotolerance. At low pH 25°C treatment with sorbate alone slightly reduces thermotolerance, while at higher pH a 25°C sorbate treatment acts as powerful inducer of thermotolerance in the absence of sublethal heat (Fig.7.6D). Sorbate at low pH also prevents the usual thermotolerance increase with sublethal heat treatment (Fig.7.6E).

7.3.4 Heating low pH cultures with sorbate selects cytoplasmic *petites*

The strong induction of *petites* by heating in the presence of sorbate (Figs. 7.4, 7.5) indicates that the damage to mitochondria caused by sorbic acid (Cole, 1987) is enhanced considerably by heat. This effect is probably more pronounced in low pH cultures because the acid penetrates cells more effectively in such cultures (see section 7.1). Also it occurs in glucose-grown *S. cerevisiae* in which mitochondrial functions are repressed. This mitochondrial repression is never absolute. On high sugar substrates respiration accounts for only 3-5% (glucose, fructose, maltose) or 29% (galactose) of the sugar catabolised, the remainder being fermented (Lagunas, 1986). However since respiration provides a much higher ATP yield it can provide as much as 34% (glucose), 48% (maltose) or 88% (galactose) of the total ATP yield during aerobic fermentation of these sugars (Lagunas, 1986). Upon entry to the cells of aerobic cultures sorbate concentrates within the respiring mitochondria, causing pronounced disruption of mitochondrial structure (Cole, 1987). The strong *petite* selection in Figures 7.4 and 7.5 indicates that this sorbate accumulation makes the mitochondria hypersensitive to heat inactivation even in glucose-repressed *S. cerevisiae.*

7.3.5 Possible reasons for the higher resistance of *petites* to growth in the presence of sorbate

The higher sorbate resistance of *petites* is unlikely to be due to loss of the residual respiratory activity present in glucose-repressed cells, since ATP levels are not depleted with weak acid-induced growth arrest (Francois *et al.*, 1986). While weak acid preservatives inhibit respiration in *S. cerevisiae* they also cause an inhibition of fermentation (Krebs *et al.*, 1983; Francois *et al.*, 1986; 1988). In the more preservative-tolerant *Z. bailii* long term adaptation to growth in the presence of weak acids involves a switch from a predominantly aerobic to a predominantly anaerobic metabolism. Millimolar amounts of preservative cause almost immediate inhibition of oxidative respiration in *Z. bailii* but, unlike in *S. cerevisiae,* practically no inhibition of fermentation (Cole, 1987; Cole and Keenan, 1987).

It has been proposed that the adaptation of *Z. bailii* to growth in the presence of weak acids involves the induction of an energy-requiring system for extrusion of the acid (Warth 1977, 1988). While such extrusion has not yet been discounted, it would probably be futile in conferring resistance (Cole and Keenan, 1986; 1987). Instead increases in plasma membrane ATPase-catalysed proton extrusion and the secretion of organic acids, as well as a decreased protoplast volume, undoubtedly contribute to maintaining the pHi of *Z. bailii* cells that have adapted to growth in the presence of weak acids (Cole and Keenan, 1987). In addition the PFK1 of *Z. bailii* shows less dramatic inhibition in response to pHi depression (Cole, 1987) as compared to the PFK1 of *S. cerevisiae* (Krebs *et al.*, 1983; Francois *et al.*, 1986). This is possibly another factor allowing the former yeast to maintain glycolytic flux in the presence of pHi-depressing amounts of preservative.

There is no evidence that adaptation of yeasts to growth in the presence of weak acid preservatives might also occur through reduced acid entry, the result of a permeability barrier at the cell membrane. However uptake of certain molecules by *S.cerevisiae* is known to be partly controlled by the functional state of mitochondria. Conversion of cells to *petites,* or treatment with inhibitors affecting mitochondrial function and biogenesis, causes deficiencies in the utilisation of galactose, maltose and α-methyl-D-glucoside that are the result of a reduced uptake of these sugars (Evans and Wilkie, 1976; Mahler and Wilkie, 1978). The mitochondrial genome of *S. cerevisiae* also controls cell permeation by several cytotoxic drugs, *petites* being considerably more resistant to killing by these agents (Mahler and Wilkie, 1978; Cheng, L. and Wilkie, D., unpublished results). It will be interesting to determine if the enhanced resistance of *petites* to growth in the presence of

weak acid preservatives has the same genetic basis. It will also be interesting to determine if this enhanced resistance is associated with the partial recovery of a response to heat shock in preservative-treated *petite* cultures of low pH (Figs. 7.3 and 7.7).

CHAPTER 8

CONCLUSION

8.1 Introduction

The heat shock response is an inducible protective system found in living cells. It simultaneously induces both heat shock proteins and an increased capacity for the cell to withstand potentially lethal temperatures (an acquired thermotolerance). Analysis of heat shock protein function in *S. cerevisiae* by molecular genetic techniques has revealed only a minority of the heat shock proteins of this organism having appreciable influences on thermotolerance (reviewed in Lindquist and Craig, 1988; Watson, 1990; Mager and Moradas-Ferreira, 1993). Instead physiological perturbations and the accumulation of trehalose with heat stress may be more important in the development of thermotolerance during a preconditioning heat shock. This study has shown that overexpression of HSP90 does not increase thermotolerance (Chapter 3).

8.2 Heat shock gene expression and thermotolerance

It has often been assumed that HSP levels must be a major determinant of acquired thermotolerance, since induction of HSPs generally occurs simultaneously with the induction of a greatly-increased thermotolerance during the heat shock response. Actually it is well-documented that HSP levels in yeast cells do not always correlate well with levels of thermotolerance (Watson, 1987; Smith and Yaffe, 1991; Barnes *et al.*, 1990; DeVirgilio *et al.*, 1991b), and induction of thermotolerance in yeast can often occur largely independently of *de novo* protein synthesis, as when it occurs with the osmotic dehydration of osmostress. Targeted HSP gene disruption has shown only a minority of the HSPs of *S. cerevisiae* exerting appreciable effects on basal or heat-acquired thermotolerance, even though rather more of these proteins are essential for proliferation at the highest temperatures of growth. With disruptions of *UBI4, SSA1* plus *SSA2,* or *HSP82,* the growth or viability of yeast at 37-38°C was reduced, yet the extreme temperature (50-52°C) thermotolerance was not similarly affected and was even, in certain instances, increased. For *HSP60* and *KAR2* disruptions, inactivation produced a lethal phenotype (Reading *et al.*, 1989; Rose *et al.*, 1989), preventing a determination of whether the encoded protein contributes to thermotolerance from a gene disruptant. Nevertheless

inactivations of two heat shock genes, *HSP104* and *CTT1*, have been shown to reduce the thermotolerance acquired with heat shock (reviewed in Piper, 1993). This study (Chapter 3) did not find that high *HSP82* gene dosage and the resultant increases in HSP90 level elevated thermotolerance. Instead moderate thermotolerance decreases were consistently observed with HSP90 overexpression (Fig 3.6). This protein in high level is therefore not protective against heat damage, which is perhaps surprising in view of the evidence for protective effects of HSP70 overexpression in *Drosophila* and mammalian cells (Lindquist and Craig, 1988). Also apparent with HSP90 overexpression were strain-dependent reductions in growth at 37.5°C (Fig. 3.5), a temperature close to the maximum for *S.cerevisiae.*

Two separate gene promoter elements have been identified causing gene activation by heat shock in *S. cerevisiae.* Heat inductions of most *S. cerevisiae* HSP genes is due to the same heat shock element sequence (HSE) as is used in higher eukaryotes, a HSE consisting of contiguous arrays of the 5 base-pair sequence nGAAn arranged in alternating orientations at each half-turn of the DNA helix (Sorger, 1991). This HSE is the binding site for the well-characterised yeast heat shock factor, a trimeric *trans*-acting transcriptional activator whose activity is needed at all growth temperatures. This factor has two physically-separable transcriptional activation domains; a N-terminal activator domain mediating the transient heat shock response and a C-terminal activator domain that mediates a sustained HSP expression at elevated temperatures (Sorger, 1991). A subset of the heat-inducible sequences of *S.cerevisiae* appear not to be activated by this factor and HSE sequences, but to use an alternative stress-control element for which the model is the UAS_{-360} element of *CTT1*. This alternative element directs the induction of catalase T by nitrogen starvation, heat shock and osmostress in a heat shock factor and HSE-independent mechanism (Marchler *et al.*, 1993; Bissinger *et al.*, 1989; Belazzi *et al.*, 1991; Weiser *et al.*, 1991). A sequence similar to the UAS_{-360} element of *CTT1* is to be found in the promoters of a number of *S. cerevisiae* HSP genes which also have the HSE sequence (Marchler *et al.*, 1993), raising the possibility that the heat activation of some of these may be under a dual regulation by both this alternative element and heat shock factor bound to the HSE.

8.3 Trehalose induction and heat stress

A large pool of trehalose accumulates in the cytosol of yeast during both heat shock and starvation-induced growth arrest in G1. Trehalose is synthesised from UDP-glucose and glucose-6-phosphate by a two-step reaction catalysed by the trehalose-6-phosphate synthase/trehalose-phosphate phosphatase complex (Neves and Francois,1992; Thevelein,

1988; Francois *et al.*, 1991; Londesborough and Vuurio, 1991; Vandercammen *et al.*, 1989; Thevelein, 1984). It is broken down to glucose by two distinct trehalase enzymes; an acid trehalase confined to the vacuole and a cytoplasmic neutral trehalase which is activated by cAMP-dependent protein kinase catalysed phosphorylation (Neves and Francois,1992; Thevelein, 1988; Francois *et al.*, 1991; Londesborough and Vuurio, 1991; Vandercammen *et al.*, 1989; Thevelein, 1984). The rapid mobilisation of trehalose when heat shocked cells are returned to non-stress temperatures is generally due to an activation of the neutral trehalase (Thevelein,1988; Londesborough and Vuurio, 1991; Vandercammen *et al.*, 1989; Thevelein, 1984). The much slower mobilisation of trehalose in stationary cultures may be due to the acid trehalase (Piper, personal communication), since neutral trehalase is dephosphorylated and inactive.

Trehalose does not accumulate in unstressed vegetative yeast because neutral trehalase is predominantly in its activated (phosphorylated) form and the activity of the trehalose-6-phosphate synthase/trehalose-phosphate phosphatase complex is only 20% of that found at stationary phase (Francois *et al.*, 1991; Francois *et al.*, 1987). When such cells are heat shocked trehalose immediately starts to accumulate, even though there is a lag period of about 10 minutes before any increase in the activities of trehalase or trehalose-6-phosphate synthase/phosphatase (Neves and Francois,1992; Hottiger *et al.*, 1987a). *In vitro* the affinity of both the inactivated and cAMP-dependent protein kinase activated forms of neutral trehalase for Ca^{2+} ions was decreased more than 20-fold at 40°C as compared to 30°C, while the activities of the trehalose-6-phosphate synthase/phosphatase complex were three times more active at 40°C (Neves and Francois,1992). Using cycloheximide (Neves and Francois, 1992; Coote *et al.*, 1992; DeVirgilio *et al.*, 1991a) or a temperature-sensitive mutation (DeVirgilio *et al.*, 1991b) to block protein synthesis during heat shock it has been reported that the trehalose induction with heat stress of *S. cerevisiae* is partially inhibited. Heat stress appears therefore to rapidly stimulate the activity of preexisting trehalose-6-phosphate synthase/phosphatase and also cause a slow increase in *de novo* synthesis of this enzyme complex.

The trehalose accumulated in yeast during heat shock is mobilised very rapidly on subsequent temperature shift-down. This rapid trehalose mobilisation is controlled by the levels of certain HSPs synthesised during the period of heat shock. This regulation was recently shown to occur primarily through levels of HSP70 and, to a lesser extent, HSP104 (Hottiger *et al.*, 1992). This study (Chapter 5) investigated whether HSP90 contributes to regulation since HSP90 forms complexes with heat shock transcription factor (Nadeau *et al.*, 1993) and several regulatory proteins of eukaryotic cells. Using

isogenic yeast strains with normal, decreased or elevated HSP90s showed that the HSP90 level had a small negative influence over the heat inductions of trehalose and the HSE (Figs. 5.2-5.4), a minor effect compared with the major regulation exerted by HSP70. Levels of "free" HSP70 appear therefore to be the major control modulating the heat induction of trehalose, in much the same way as they act to downregulate the synthesis of HSPs, but HSP104 and HSP90 also exert a more minor influence. This recent discovery of HSPs controlling trehalose levels raises the possibility that these HSPs might, in certain circumstances, influence thermotolerance indirectly through their effect on trehalose.

8.4 Heat shock response and thermotolerance effects of weak acids

Both the heat shock response and thermotolerance acquisition can be triggered by a large number of chemical agents (Ananthan *et al.,* 1985; Lindquist and Craig, 1988; Watson, 1990). These are thought to act by causing the accumulation of aberrant protein inside the cell. In contrast very few chemicals selectively inhibit heat shock induction of HSPs. In mammalian cells flavonoids act as selective inhibitors of HSP induction (Hosokawa *et al.,* 1990). Sorbate, benzoate, CCCP and DES (Chapter 7) are the first compounds shown to selectively inhibit heat-induced protein expression in yeast. Not only are sorbate and benzoate not HSP inducers, but at low medium pH values they render yeast incapable of responding to a heat shock by the normal induction of HSPs despite the fact that the treated cells are still competent of protein synthesis (Figs. 7.1, 7.2, 7.8 and 7.9). The inhibitory action of DES however is independent of medium pH (Fig. 7.8). In the presence of sorbate concentrations that, at low pH, totally inhibit the heat shock response and growth of *rho*+ cells, cells of a *rho*- mutant displayed a partial capacity for both growth (not shown) and heat induction of heat shock proteins (Figs. 7.3 and 7.7). This restoration of a response to heat shock in acidified sorbate-treated cultures might contribute to the higher resistance of *petites* to growth in the presence of sorbate.

Weak organic acids preservatives induce a rapid pHi decline in low pH cultures in the absence of stress. At low pH values of medium the undissociated forms of these acids readily cross the cell membrane and reduce pHi by dissociating in the higher pH environment of the cytosol (Cole and Keenan, 1986). In *S. cerevisiae* such weak acids inhibit glycolysis, through a reduction in fructose-2,6-bisphosphate level, and cause transient increases in cAMP and trehalase activity (Francois *et al.,* 1986). Sorbic acid (pKa 4.75) has been variously reported as either having no effect on (Van Uden 1984), or increasing (Coote *et al.,* 1991), the thermotolerance of *S. cerevisiae.* This study has shown that its effects on thermotolerance are actually strongly dependent on medium pH (Figs. 7.4, 7.5 and 7.6).

Sorbic acid acting as a chemical inducer of thermotolerance increases thermotolerance at pH values above 6.5 in the absence of sublethal heat stress. However at lower pH values it greatly decreases thermotolerance (Figs. 7.4, 7.5 and 7.6), preventing the thermotolerance increase with sublethal heat shock and causing a greatly-increased sensitivity to heat killing with strong selection for respiratory-deficient cytoplasmic *petites* amongst the surviving cells (Figs. 7.4 and 7.5). Probably because of the disruption it causes to the mitochondria of low pH cultures (Cole, 1987).

8.5 The contribution of this study to knowledge on HSP90

In this study the effects of altered HSP90 levels were investigated. *S. cerevisiae* transformants were constructed with 50-150 copies of the homologous heat-inducible gene for HSP90 (*HSP82*) present on a high copy number episomal vector. These transformants were then used to demonstrate:

(1) The *HSP82* gene displays essentially normal regulation when present in yeast at high copy numbers; The *HSP82* gene is normally single-copy in the haploid yeast genome, yet even at 50 to 150 copies per cell its expression increased in proportion to the number of *HSP82* gene copies. Therefore each *HSP82* gene was displaying almost normal basal and heat shock-induced levels of expression. Proper regulation of the HSE sequence controlling *HSP82* is therefore not lost at high gene copy levels. In unstressed cultures in exponential growth at 25°C the low basal expression of the multiple *HSP82* gene copies caused a 3 to 7-fold HSP90 overproduction, but HSP90 levels increased 10-fold to 30-40% of total cell protein following temperature upshift to 39°C for 75min. Heat induction of chromosomal genes for other heat shock proteins (e.g. HSP104, HSP70, HSP26) in the same cells was not suppressed relative to cells which were isogenic but for the possession of just a single *HSP82* gene. This is further evidence that yeast can authentically regulate a large number of heat shock genes.

(2) That yeast is an expression host suitable for the high level synthesis of HSP90; Chapter 4 is a good example of the advantages of a strain overproducing the protein of interest. 14mg purified HSP90 was obtained from about 30g wet weight cells using one of these transformants overexpressing HSP90, as compared to Nadeau *et al.* (1993) where 1mg of HSP82 was purified from 18g of yeast cells. There is therefore significantly increased final yield of HSP90 with *HSP82* overproduction.

(3) That increasing normal cellular levels of HSP90 affects a number of physiological properties. HSP90 overproduction was not protective against heat killing, causing strain-

dependent reductions in growth at 37.5°C and in thermotolerance (section 3.2.5 and 3.2.6).

This study also demonstrated a small influence of HSP90 levels on the trehalose and HSE inductions of the yeast heat shock response. Recently the trehalose induction with heat shock was shown to be regulated by levels of HSP70, to a lesser extent, HSP104 (Hottiger *et al.,* 1992). Here HSP90, another major HSP, was shown also to involved in this regulation. The results showed HSP90 levels having a small negative influence over the heat induction of trehalose and the heat shock element, a minor effect compared with the major regulation exerted by HSP70.

Had time allowed, the studies in Chapters 4 and 6 would have been continued to include:

(1) Crystallisation/structure determination of HSP90.

(2) Identification of proteins binding to HSP90-Affi-Gel--Do these proteins have a common HSP90-binding domain?

(3) Identification of p66 (Chapter 6).

(4) Study of the physiological effects of inactivating the p66 gene; also site-directed mutagenesis of the site(s) on which p66 is phosphorylated *in vitro* in extracts from heat shocked cells, followed by study of the effects of abolishing this N-linked phosphorylation *in vivo.*

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A SMALL INFLUENCE OF HSP90 LEVELS ON THE TREHALOSE AND HEAT SHOCK ELEMENT INDUCTIONS OF THE YEAST HEAT SHOCK RESPONSE

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SUMMARY: Heat shock protein (HSP) induction is only one change elicited in yeast by heat shock. Trehalose is also accumulated, while declining intracellular pH stimulates plasma membrane ATPase activity. Recently the trehalose induction was shown to be regulated by levels of HSP70 and, to a lesser extent, HSP104. Another HSP which might contribute to regulation is HSP90, especially as HSP90 forms complexes with heat shock transcription factor and several of the regulatory proteins of eukaryotic cells. This possibility was investigated using isogenic yeast strains with normal, decreased or elevated HSP90. The results show HSP90 levels having a small negative influence over the heat inductions of trehalose and the heat shock element, a minor effect compared with the major regulation exerted by HSP70.

A sublethal heat shock leads to simultaneous induction of both heat shock proteins (HSPs) and thermotolerance in the cells of practically all species (1-4). This "heat shock response" is thought to result from the sensing of intracellular protein damage and, in eukaryotic cells, to be regulated primarily by the levels of HSP70 (1). Its trigger is possibly an intracellular shortage of free, uncomplexed HSP70, as all the available HSP70 binds to the damaged protein accumulating within the stressed cell (1). Heat induction of HSP70 genes can then restore free HSP70, leading to a switch off of the response (see Fig.1). Most heat shock studies have concentrated on the induction of heat shock genes and the functions of individual HSPs (1-4). A broader perspective must also consider the physiological changes triggered by heat stress, events which may be important in thermotolerance determination (4). In yeast heat shock induces a rapid cytoplasmic accumulation of trehalose and a reduction intracellular pH (pHi), the latter due partly to proton influx into the cell as a consequence of stress-induced increases in membrane permeability (for literature see refs. 4,7,8). This reduction in pHi stimulates both cyclic AMP synthesis and plasma membrane ATPase activity (4,5), the ATPase activation causing an increased *catalysed* proton extrusion from the cell that helps to restore normal pHi values and homeostasis (Fig.1).

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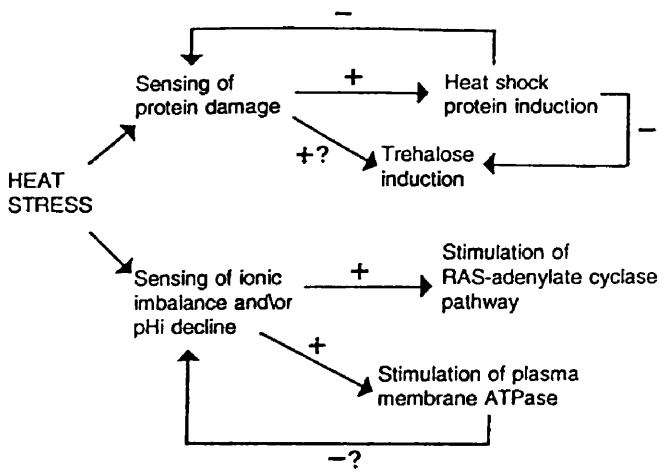

Fig.1. *Regulatory circuits known, or suspected of operating, in the yeast response to heat stress (for explanation, see Introduction).* pHi; intracellular pH.

Trehalose is thought to act primarily as a stress protectant of yeast rather than as a storage carbohydrate (4,6,8). It accumulates both during heat shock and when cells undergo growth arrest due to nutrient limitation (4-8). The inactivation of certain yeast HSP genes was recently shown to have dramatic effects on the trehalose accumulated with heat shock. A strain lacking three members of the *SSA*-subfamily of HSP70 genes overproduced trehalose during heat shock to 37°C or 40°C and showed abnormally slow degradation of this trehalose upon shift-down from 40°C to 27°C (9). Similar, although smaller, effects on trehalose were also seen in a strain lacking HSP104, but there was no altered trehalose accumulation in a strain disrupted in the heat-inducible polyubiquitin gene *UBI4* (9). The mechanisms for modulating the trehalose and HSP inductions of the heat shock response might therefore be the same, namely critical levels of certain specific HSPs (Fig.1).

Another protein which might be suspected of participating in control of the eukaryotic heat shock response is HSP90. HSP90 has recently been found to bind to the transcriptional transactivator of heat shock genes (heat shock transcription factor, HSTF)(10). HSTF binds at a specific heat shock gene promoter element (heat shock element: HSE). Also HSP90, as part of its natural functioning, complexes several of the regulatory proteins of eukaryotic cells (see refs. 3,11 for literature). In addition changes to HSP90 level can alter both high temperature growth and thermotolerance (11,12). We therefore investigated if HSP90 levels affect the HSE element and trehalose inductions of the yeast heat shock response, by measuring trehalose and HSE induction in a series of isogenic yeast strains shown in earlier studies to possess normal, reduced or elevated amounts of HSP90 protein (11-14).

MATERIALS AND METHODS

Yeast strains, media and growth conditions. The yeast strains used for this study (Table 1)were all derived from W303-1A. W303Leu, PLD82 and CLD82 were grown on YPD medium (1% yeast extract, 2% bactopeptone, 2% glucose, all w\v). Transformants W303-3a

and W303-82 (Table 1) were cultivated on standard defined minimal medium (SD) plus supplements, as in ref.11. Shake-flask 25°C cultures were used for heat shock experiments when in exponential growth at 5-7x10^6 cells ml^{-1} (YPD) or 1-2x10^6 cells ml^{-1} (SD).

Trehalose determinations. At intervals following a 25°C-39°C heat shock portions of each culture were harvested, washed and extracted with ice-cold 5%(w\v) trichloroacetic acid (9,15). Trehalose in these acid extracts was determined by the anthrone procedure (15) and glucose using the Sigma HK(20) kit. Since the anthrone procedure detects both trehalose and glucose, trehalose was obtained by subtracting the low glucose assay values from the anthrone assay values. Protein of the cells in culture aliquots was determined as in ref. 11.

Measurement of HSE expression. Strains W303Leu, PLD82 and CLD82 were transformed with the HSE-*lacZ* expression plasmid pHSE2 by selection for uracil prototrophy (16). The resulting transformants (denoted by a -pHSE2 suffix in conjunction with strain designations; e.g. PLD82-pHSE2), were grown on SD medium plus adenine, histidine and tryptophan and their β-galactosidase activity measured as in ref. 16. Plasmid pHSE2 carries a tandem HSE sequence within a *CYC1* promoter-*lacZ* gene fusion (HSE-*lacZ*), this HSE replacing the normal *CYC1* upstream activating sequences (16).

RESULTS

HSP90 levels exert a small negative effect on heat-induced trehalose accumulation. *S. cerevisiae* has two HSP90 genes, these encoding products 97% identical in amino acid sequence (12). The *HSC82* gene displays a high constitutive expression that increases only slightly with heat shock. *HSP82*, the other HSP90 gene, displays 10-fold lower constitutive expression yet is activated strongly by heat shock. *HSC82* expression therefore contributes approximately 90% of the HSP90 protein present during normal vegetative growth, while *HSP82* is primarily responsible for the approximate two-fold increase in HSP90 level with a 25°C-39°C heat shock (12-14). For this study the strains in Table 1 were heat shocked using the same rapid 25°C-39°C temperature upshift that was employed for the earlier characterisations of their HSP90 changes (11-14). Fortunately the HSP90 changes of these strains have no effect on 25°C growth (11,12), thus enabling any differences in the immediate responses of cultures grown to the same physiological state at this temperature to be attributed to the altered HSP90 levels.

We initially investigated the effects of 25°C-39°C heat shock on trehalose in the three isogenic diploid strains, W303Leu, CLD82 and PLD82. W303Leu is wild-type with respect to HSP90 genes. CLD82, due to insertional inactivation of both *HSC82* alleles (Table 1), has lower HSP90 levels during 25°C growth but exhibits strong HSP90 induction with heat shock (12). PLD82, homozygous for an insertional inactivation of *HSP82* (Table 1), has almost normal HSP90 at 25°C but displays little HSP90 increase with heat shock (12). Trehalose was very low at 25°C in all three strains, but showed the expected large increase with heat shock (Fig.2A). There was a small, yet reproducible trehalose overproduction in PLD82 as compared to either W303Leu or CLD82 (Fig.2A). Since these experiments were on completely isogenic strains and used cultures which were in the same physiological state prior to the stress, this trehalose overproduction can be attributed to the lack of a normal increase in HSP90 with heat shock in PLD82. In all three strains a shift-down to 25°C after 1h at 39°C resulted in rapid mobilisation of the accumulated trehalose (Fig.2A), in agreement with previous reports of trehalose in other *S. cerevisiae* strains (4-9).

Table 1. **Yeast strains used for this study**

Strain	Relevant genotype[a]	Reference
W303leu	**a** *LEU2,ura3-1,HSP82,HSC82* α *LEU2,ura3-1,HSP82,HSC82*	(12)
CLD82	**a** *leu2-3,112,ura3-1,HSP82,hsc82::LEU2* α *leu2-3,112,ura3-1,HSP82,hsc82::LEU2*	(12)
PLD82	**a** *leu2-3,112,ura3-1,hsp82::LEU2,HSC82* α *leu2-3,112,ura3-1,hsp82::LEU2,HSC82*	(12)
W303-1A[b]	**a** *leu2-3,112,ura3-1,HSP82,HSC82*	(11)

[a] All strains also carry the mutations *ade2-1, can1-100, his3-12,16* and *trp1.1.*
[b] Two transformants of W303-1A were employed. One (W303-3a) carries the 2μORI-*STB* yeast-*E.coli* shuttle vector pMA3a, a plasmid which has sequences from pBR322, the ORI-*STB* region of the yeast 2μ plasmid, and *LEU2d* for plasmid selection and maintenance at 50-150 copies per cell in *leu2* yeast strains. The other transformant (W303-82) carries a plasmid that comprises pMA3a with a 2.4kb restriction fragment insert that contains the *HSP82* gene for HSP90 protein (11). W303-82 is therefore isogenic with W303-3a except in its possession of 50-150 extra episomal copies of *HSP82*. The low basal expression of these multiple *HSP82* gene copies in W303-82 causes a 3 to 7-fold HSP90 overproduction in this transformant at 25°C as compared to W303-3a (11). The HSP90 level of W303-82 increases 10-fold, to 30-40% of total cell protein, following temperature upshift to 39°C, while the HSP90 levels in W303-3a are as in wild-type strains (11).

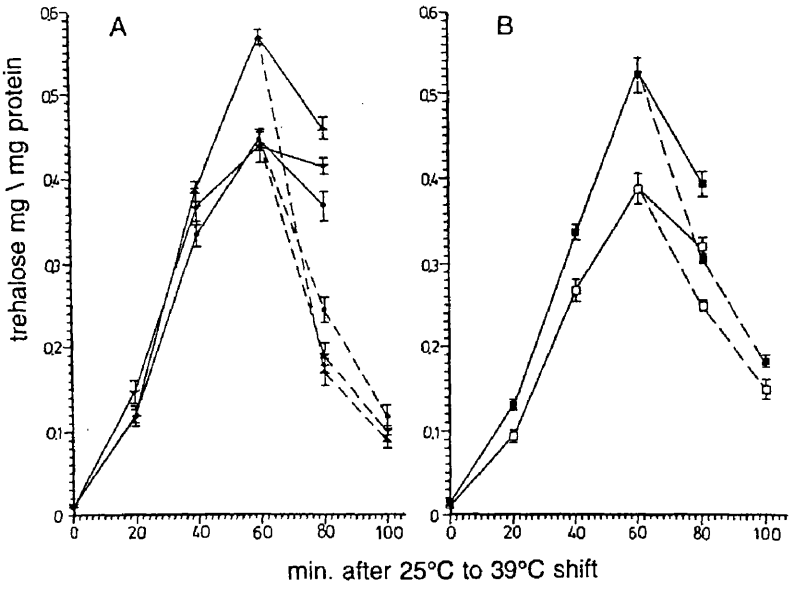

Fig.2. *HSP90 exerts a small negative influence on the trehalose accumulated with heat shock.* A: YPD medium cultures of W303Leu (●), CLD82 (▼) and PLD82 (▲). B: SD medium cultures of W303-3a (■) and W303-82 (□). All cultures were grown to early exponential phase at 25°C and heat shocked from 25°C to 39°C at time zero. Samples were taken for trehalose determination at 20min intervals at 39°C (continuous lines). Part of each culture was heat shocked to 39°C for 60min and then allowed to recover at 25°C (broken lines). Error bars represent SEM for triplicate determinations.

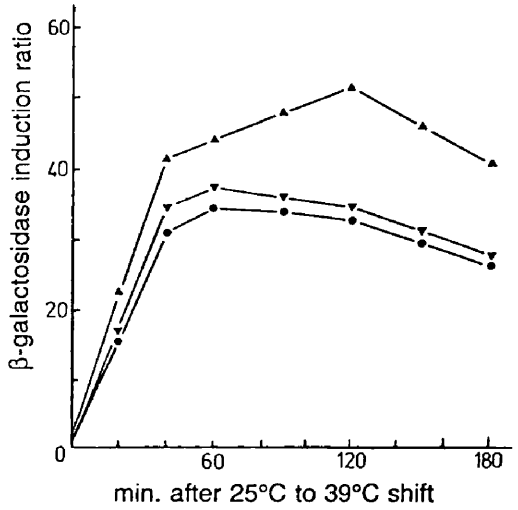

Fig.3. *Prevention of the normal HSP90 induction during heat shock leads to more sustained HSE-lacZ expression.* 25°C SD medium cultures of transformants W303Leu-pHSE2, CLD82-pHSE2 and PLD82-pHSE2 were heat shocked to 39°C and β-galactosidase activity, due to induction of the HSE-*lacZ* gene, measured at 20min intervals. β-galactosidase activity is presented as fold-induction relative to the low constant level of β-galactosidase in uninduced 25°C cultures (for this activity level see ref. 24) and were the mean of two experimental determinations. HSE-*lacZ* expression is normally switched off 40-60min after a 25°C-39°C heat shock with the modulation of the heat shock response [16]. This was apparent with W303Leu-pHSE2 (●) and CLD82-pHSE2 (▼), but PLD82-pHSE2 (▲) displayed a more sustained HSE-*lacZ* expression.

Since substantially preventing the HSP90 increase with heat shock causes trehalose hyperaccumulation (Fig.2A), it might be expected that HSP90 overexpression would reduce trehalose induction. The measurements in Fig.2B showed this to be the case. They employed two transformants of strain W303-1A (W303-3a and W303-82) which are isogenic but for the presence of 50-150 extra episomal copies of the *HSP82* gene in W303-82. As a consequence W303-82 overexpresses HSP90 (Table 1 and ref. 11). At 39°C the trehalose accumulation of W303-82 was less than of that of W303-3a (Fig.2B), providing further evidence of a small negative influence of HSP90 on heat-induced trehalose accumulation.

HSP90 levels influence *HSE-LacZ* expression.

If the trehalose and heat shock protein inductions of the heat shock response are both under similar control (Fig.1), the activity of the HSE promoter element might resemble trehalose in displaying a small dependence on HSP90 level. HSE expression was studied by measuring the activity of a HSE-*lacZ* gene fusion inserted into strains W303Leu, CLD82 and PLD82 (Table 1)(see Materials and Methods). Transformants of these strains (W303Leu-pHSE2, CLD82-pHSE2 and PLD82-pHSE2) were then grown and their β-galactosidase levels determined at intervals after a 25°C-39°C heat shock (Fig.3). PLD82-pHSE2 showed a greater and more prolonged HSE-*lacZ* expression as compared to either W303Leu-pHSE2 or CLD82-pHSE2 (Fig.3). This effect, though small, was reproducible by reporter enzyme assay and is the first demonstration of HSP90 levels influencing HSE activity in any eukaryotic system. HSE-*lacZ* expression was not investigated in W303-3a or W303-82 (Table 1). This was because the several additional heat-inducible (*HSP82*) genes

in W303-82 might cause any differences in HSE-directed expression in this transformant to be a reflection of titration of the available HSTF of the cell, rather than any influence of HSP90 levels on the regulation of the heat shock response. Any such titration effect should however be small, since a multicopy heat shock gene introduced into *S. cerevisiae* shows almost normal heat induction [11].

DISCUSSION

The heat shock response has an inbuilt autoregulation, HSP synthesis becoming repressed a short while after it has been induced by moderate heat shock (1-4). This autoregulation is best-understood in *E. coli*, where it reflects the heat-activated expression of more than one gene; the products of genes *DnaK*, *DnaJ*, *GrpE*, *rpoD* and various proteolytic activities having been implicated (1,3,17,18). Downregulation of the eukaryotic heat shock response is probably also influenced by the expression of more than one heat shock gene. Inhibitor studies indicate that levels of the eukaryotic homologue of *E. coli dnaK* protein, HSP70, are important (19). Also both HSP70 (20) and HSP90 (10) have been shown to bind forms of HSTF *in vitro*. Eukaryotes have multiple HSP70-related proteins, some of which (HSP70s) are synthesised in response to heat shock while others (HSP70-related or HSC70 proteins) are made constitutively. Deletion of genes for the cytoplasmic HSC70s of yeast (*SSA1* and *SSA2*) leads to a constitutive heat shock response (1,21,22). Conversely overproduction of either HSC70 (*SSA1* overexpression) or HSP70 (*SSA4* overexpression) suppresses the heat inducibility of the *SSA1* promoter (23). HSP70 therefore autoregulates itself at the level of transcription in yeast, although the *SSA1* product does not apparently directly repress many other yeast heat shock promoters (23). Also substantially preventing the normal heat shock induction of HSP70 (as in the *ssa3,ssa4* mutant) does not markedly alter the heat shock response (23). It appears therefore that constitutive levels of *SSA* products are more important than the heat-induction of HSP70 in regulating heat shock gene expression in yeast (1-3,21-23).

In this paper we show that HSP90 levels have relatively small effects on heat-induced trehalose (Fig.2) and HSE activity (Fig.3), despite the recently-demonstrated association of HSP90 with HSTF (10). Effects of HSP90 on trehalose, like those of HSP104 (9), are less marked than those of HSP70 (9). It is probable, therefore, that HSP70 levels are a major controller of both HSP and trehalose synthesis in the yeast heat shock response (Fig.1), while HSP90, HSP104 and possibly other heat shock proteins play more minor roles. The differences in HSP90 level between strains W303Leu, CLD82 and PLD82 correspond to the changes in HSP90 that occur during the normal physiology of the yeast heat shock response (12-14). Prevention of the normal heat induction of HSP90 in PLD82 causes small enhancements to heat inductions of both trehalose (Fig.2A) and HSE activity (Fig.3). HSP90 changes during the heat shock response must therefore be considered to influence this response even though changes in HSP70 level are probably more important in its control.

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Authentic temperature-regulation of a heat shock gene inserted into yeast on a high copy number vector. Influences of overexpression of HSP90 protein on high temperature growth and thermotolerance

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Key words: Heat shock; HSP90 overexpression; Thermotolerance; (*S. cerevisiae*)

Heat shock protein HSP90 is relatively abundant in eukaryotic cells even in the absence of heat shock. Its precise function is still unclear, although it is apparently required in higher levels for growth at high temperatures. In this study *Saccharomyces cerevisiae* transformants were constructed with 50–150 copies of the homologous heat-inducible gene for HSP90 (*HSP82*) present on a high copy number episomal vector. These transformants were then used to demonstrate: (i) that this heat shock gene displays essentially normal regulation when present in yeast at high copy numbers; (ii) that yeast is an expression host suitable for the high level synthesis of HSP90; and (iii) that increasing normal cellular levels of HSP90 affects a number of physiological properties. The *HSP82* gene is normally single-copy in the haploid yeast genome, yet even at 50 to 150 copies per cell it displayed almost normal basal and heat shock-induced levels of expression. Proper regulation of the heat shock element sequence controlling *HSP82* is therefore not lost at high gene copy levels. In unstressed cultures in exponential growth at 25°C the low basal expression of the multiple *HSP82* gene copies caused a 3 to 7-fold HSP90 overproduction, but HSP90 levels increased 10-fold to 30–40% of total cell protein following temperature upshift to 39°C for 75 min. Heat induction of the chromosomal genes for other heat shock proteins in the same cells was not suppressed relative to cells which were isogenic but for the possession of just a single *HSP82* gene, this constituting further evidence that yeast can authentically regulate a large number of heat shock genes. HSP90 overproduction was not protective against heat killing, causing strain-dependent reductions in growth at 37.5°C and in thermotolerance.

Introduction

A heat shock response is normally induced when cells are suddenly exposed to temperatures close to, or slightly above, the maximum permitting growth. At the molecular level the response causes strong induction of heat shock proteins, while at the physiological level it acts to increase thermotolerance (the tolerance of still higher, potentially lethal temperatures). The heat shock proteins are generally thought to help cells withstand heat-induced damage, even if they may act to confer increased thermotolerance by indirect mechanisms [1–8]. Studies on organisms that are readily amenable to molecular genetic analysis, notably *Esherichia coli* and *Saccharomyces cerevisiae*, are proving especially informative for uncovering the roles of the individual heat shock proteins. The high evolutionary conservation of many of these proteins means that such studies also provide insight into the roles of the stress proteins of higher organisms.

Proteins that are identical with, or closely related to, heat shock proteins are synthesised during normal growth and development and serve to provide essential functions in the absence of stress [5,9–11]. Thus, all cells constitutively synthesise homologues of the highly-conserved, major heat shock protein of eukaryotic cells (HSP70). These HSP70-related proteins constitute some of the major 'chaperonins' in unstressed cells, and as such assist in the unfolding and refolding of polypeptide chains during the events whereby such proteins are transported across membranes. Gene disruption studies in yeast have revealed that individual members of this family of HSP70-related proteins func-

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tion in the cytosol, in the mitochondrion or within the lumen of the endoplasmic reticulum [2,9].

HSP90 is another highly-conserved heat shock protein. There is more than 50% amino acid sequence identity between the HSP90s from different eukaryotes and 40% identity between these eukaryotic HSP90s and *htpG* protein of *E. coli* [3,5]. Although usually considered a true heat shock protein since its levels increase with heat shock [1–5], HSP90 is nevertheless abundant in the absence of stress. Mammalian HSP90 exists either as a homodimer or in heterodimeric association with a number of important regulatory proteins [2]. These HSP90-associating proteins include steroid hormone receptors, protein kinases, actin and tubulin [12–17]. A major tumour-specific transplantation antigen of carcinogen-treated mice is identical with HSP90 [18], while a proportion of patients with the autoimmune disease systemic lupus erythematosus exhibit a grossly elevated lymphocyte HSP90 which fluctuates with disease activity [19]. However, despite so many pointers to the importance of HSP90 and suspicions that HSP90 associations might be crucial for the intracellular transport or regulation of steroid hormone receptors and protein kinases, the true function of HSP90 is still far from clear. That it is an essential protein in normal growth has been shown by gene disruption of the two *S. cerevisiae* genes for HSP90 (*HSC82* and *HSP82*). Strains with just one of these genes inactivated (*HSC82,hsp82* or *hsc82,HSP82* strains) are viable, whereas a double disruption mutant (*hsc82,hsp82*) is inviable [5].

It is known that higher HSP90 levels are required for growth of both yeast [5] and mouse L cells [20] at high growth temperatures. These higher HSP90 levels probably serve to re-establish equilibria of heterodimer formation, compensating for the weakening of the non-covalent interaction of HSP90 with other proteins as temperature increases. There have been no reports of the effects of increasing normal cellular HSP90 levels. It might be anticipated that HSP90 overexpression would affect stress tolerances, since this protein is induced as thermotolerance increases with the triggering of the heat shock response. Also a heat-resistant mutant Chinese hamster cell line has been shown to overexpress HSP90 [21]. This report describes a study in which the homologous yeast HSP90 was overexpressed in three *S. cerevisiae* strains, an investigation conducted with three aims: (i) to determine if a heat shock gene can display essentially normal regulation when present in yeast at high gene copy number; (ii) to gain evidence for the physiological consequences of elevating HSP90 levels; and (iii) to demonstrate that this expression host is capable of high level synthesis of HSP90, a capability which can be exploited to purify HSP90 in quantity for structural investigations on this protein.

Materials and Methods

Construction of plasmid p82-2B. Plasmid pU84, the original source of *HSP82* gene sequences for this study, comprises a 5.6 kb *Hin*dIII restriction fragment of the DNA of *S. cerevisiae* X2180-1B inserted at the single *Hin*dIII site of vector pUC8. A 2.6 kb *Hin*dIII + *Mlu*I restriction fragment from pU84 (*HSP82* sequences from the *Hin*dIII site at −331 relative to the initiation codon to the *Mlu*I site at +2168, 27 nucleotides 3′ to the major site of polyadenylation of *HSP82* mRNA [22]) was made blunt-ended, and tailored with *Bam*HI termini using *Bam*HI linkers, as in Ref. 23. It was then inserted at the unique *Bam*HI site of the 2μORI-*STB* yeast-*E. coli* shuttle vector pMA3a [24], thereby generating the plasmid p82-2B shown in Fig. 1. pMA3a, the parent plasmid, contains sequences from pBR322, the ORI-*STB* region of the yeast 2μ plasmid, and *LEU2d* for plasmid selection and maintenance at high copy levels in *leu2* yeast strains [24,25].

Yeast transformations. Three haploid *cir*$^+$ *S. cerevisiae* strains were employed for this study. These were: W3031A (*a ade2-1, can1-100,his3-12,16,leu2-3,112,trp1-1,ura3-1*; gift of R. Rothstein); PMY1 (*α leu2-3,112,his4*; gift of P. Meacock); and MD40-4c (*α leu2-3,112, ura2,his3–15,trp-1*; gift of A. and S.M. Kingsman). Each of these strains was transformed to leucine prototropy using plasmid pMA3a or p82-2B, as in Ref. 26. Transformants carrying the parent plasmid pMA3a (designated W303-3a, PMY1-3a and MD40-3a, respectively) were used as control cultures, cells that were maintaining the sequences of the high copy number vector yet which possessed normal *HSP82* gene and HSP90 protein levels. Transformants maintaining p82-2B (designated W303-82, PMY1-82 and MD40-82, respectively) were each shown to be overexpressing

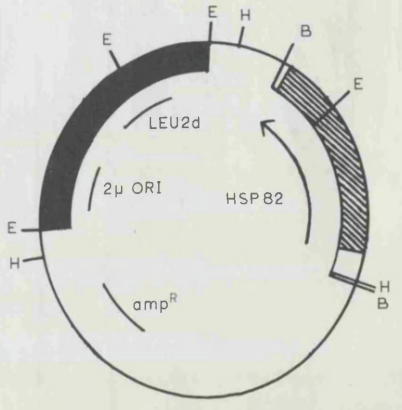

Fig. 1. The HSP90 expression vector p82-2B. Thin line, pBR322 sequences; solid line, 3.25 kb double *Eco*RI fragment of yeast DNA containing 2 μORI-*STB* and *LEU2d*; open box, 2.6 kb fragment from plasmid pU84 (see Materials and Methods) containing the *HSP82* gene (coding region hatched) and part of the pUC8 polylinker. E, *Eco*RI; H, *Hin*dIII; B, *Bam*HI.

HSP90 (see Results). Diploid strains were created by mating haploids derived from independent transformations.

Yeast culture and heat shock. Transformants were grown in shake-flask cultures at either 25°C or 37.5°C in minimal dextrose (SD) medium [27] with necessary aminoacid supplements (omitting leucine to ensure plasmid maintenance). For analysis of mid-log phase cultures, experiments were conducted at $5 \cdot 10^6$ cells per ml. Stationary phase cultures were analysed after 24 h of further growth at 25°C. For sublethal heat shock treatment, cultures in log phase at 25°C were placed at 39°C for 40 or 75 min, as indicated. Plasmid copy levels were determined as in Ref. 25.

Pulse-labelling of S. cerevisiae before and during heat shock. Pulse-labelling of proteins in vivo with [^{3}H]leucine was as in Ref. 28.

Protein isolation and immunological detection of HSP90. Samples of total cell protein were isolated as in Ref. 28. Protein determinations were performed using the Bio-Rad Protein Assay Kit and bovine serum albumin as standard. Preparation of protein samples for gel electrophoresis, analysis of proteins on one-dimensional 12.5% or 10% sodium dodecyl sulfate (SDS) polyacrylamide gels, gel staining with Coomassie brilliant blue, and detection of labelled proteins in gels by fluorography were according to standard procedures [28,29]. A polyclonal antiserum which cross-reacts with both isoforms of yeast HSP90 was raised in rabbits by subcutaneous injection of polyacrylamide gel fragments, these being derived from the HSP90 band from multiple one-dimensional SDS gel fractionations of the total protein from heat shocked yeast. Relative levels of HSP90 production were measured by loading known quantities of total cell protein, also sequential dilutions of these same samples, into adjacent wells on polyacrylamide gels. Then, after these gels had been electrophoresed and the separated proteins semidry electroblotted onto nitrocellulose, the blots were probed with the anti-HSP90 antiserum according to Ref. 29 (a typical estimation of the relative levels of HSP90 in two samples of total cell protein is shown in Fig. 3b).

Thermotolerance measurements. Thermotolerance was measured as in Ref. 30.

Results

Plasmid p82-2B caused moderate overexpression of HSP90 in the absence of heat shock stress, an overexpression markedly increased by growth at high temperature

The two *S. cerevisiae* genes for HSP90 encode products which are 97% identical at the amino acid sequence level and which appear from genetic analysis to be functionally equivalent [5]. One gene (*HSC82*) dis-

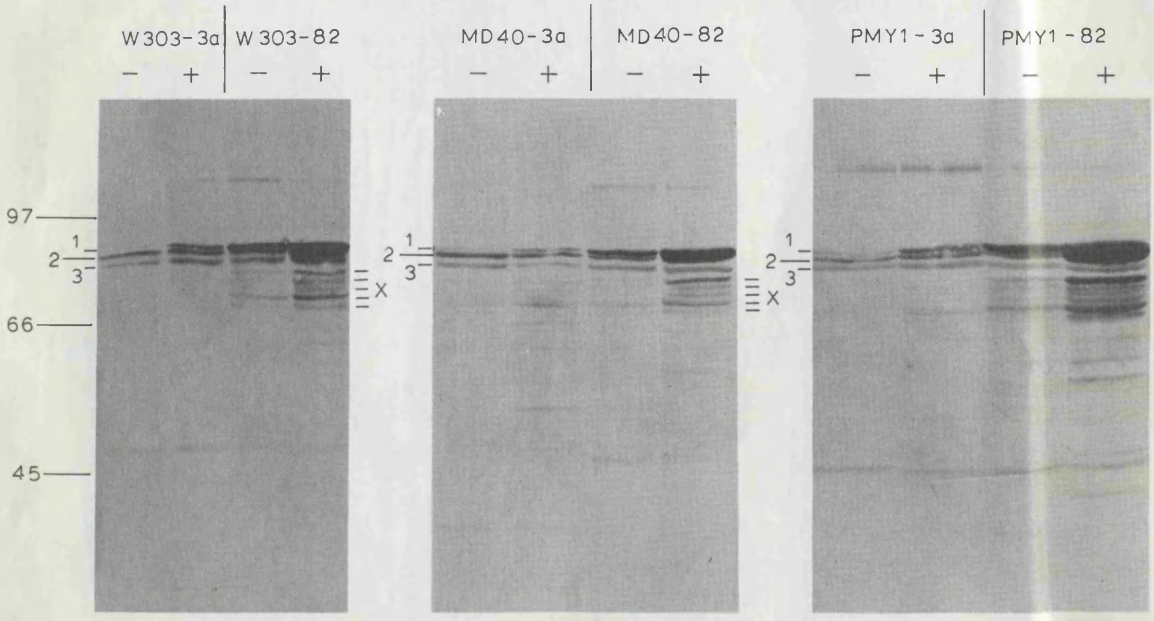

Fig. 2. Western blot analysis of the levels of HSP90 protein in individual transformants. Cultures were all initially in the exponential phase of growth at 25°C, samples of total cell protein being prepared before (−) or 75 min after (+) a 25–39°C heat shock. The polyclonal antiserum used in HSP90 detection crossreacts with both the *HSC82* and *HSP82* gene products, the former migrating slightly faster than the latter on SDS polyacrylamide gels [5]. The *HSP82* and *HSC82* products are bands 1 and 2, respectively. Certain lower molecular mass bands cross-reacting with the antiserum (×) are minor, degraded forms of HSP90 that were consistently observed in cells overexpressing HSP90. They were observed even though protease inhibitors were used during the preparation of samples for gel analysis, although as total protein their amount was less than 10% of the amount of intact HSP90. The band (3) detected by this antiserum between the degraded HSP90 (×) and *HSC82* protein (2) is not related to HSP90. The positions of molecular weight markers (phosphorylase *a*, 97 kDa; bovine serum albumin, 67 kDa and ovalbumin, 43 kDa) are indicated to the left of the figure. 10 μg of cell protein was loaded in each gel track.

plays a high constitutive expression that increases only slightly with heat shock. The other (*HSP82*) displays a 10-fold lower constitutive expression yet is subject to strong activation by heat shock. *HSC82* expression therefore contributes approx. 90% of the HSP90 present in normal vegetative growth, while *HSP82* expression is primarily responsible for the increase in HSP90 with heat shock [5,31,32].

Overexpression of HSP90 due to high *HSP82* gene dosage was investigated in three *leu2-3,112 S. cerevisiae* strains (W303A, PMY1 and MD40-4c; see Materials and Methods). This was because it was uncertain at the commencement of this work which genetic background might be most suitable for HSP90 production. Many molecular genetic studies of the yeast heat shock response have been on strains of W303 genetic background [5–8,31,32]. However, there is considerable previous experience of high level expression of genes carried on *LEU2d*-based vectors in the strain MD40-4c [24]. Also many laboratory strains of yeast grow adequately in shake-flask culture but do not display particularly good growth in fermenter culture. Strain PMY1 has been shown not to suffer from this restriction (D. Pioli, personal communication). While these considerations led us to investigate HSP90 overproduction in all three strains, it was only with the investigation of the effects of high *HSP82* gene dosage on high temperature growth and thermotolerance that significant strain differences were observed (see below).

Cultures of these different strains possessing normal or high *HSP82* gene dosage (transformants maintaining either plasmid pMA3a or plasmid p82-2B, respectively) were harvested in the exponential phase of growth at 25°C and 37.5°C, and their HSP90 levels analysed. Coomassie blue-staining of the proteins of these transformants separated on 12.5% gels showed that the maintenance of p82-2B caused HSP90 to be only slightly overexpressed at 25°C in the absence of heat shock (2–5% of total cell protein; data not shown). Western blots of identical gel fractionations probed with an anti-HSP90 antiserum (Fig. 2) showed that the HSP90 levels of 25°C log phase cultures of W303-82, PMY1-82 and MD40-82 were elevated 3 to 7-fold relative to the normal HSP90 levels in the corresponding control cultures containing plasmid pMA3a (W303-3a, PMY1-3a and MD40-3a; Fig.2). In contrast log phase cultures growing at 37.5°C showed considerably greater overexpression of HSP90 (approx. 30-fold; Fig.

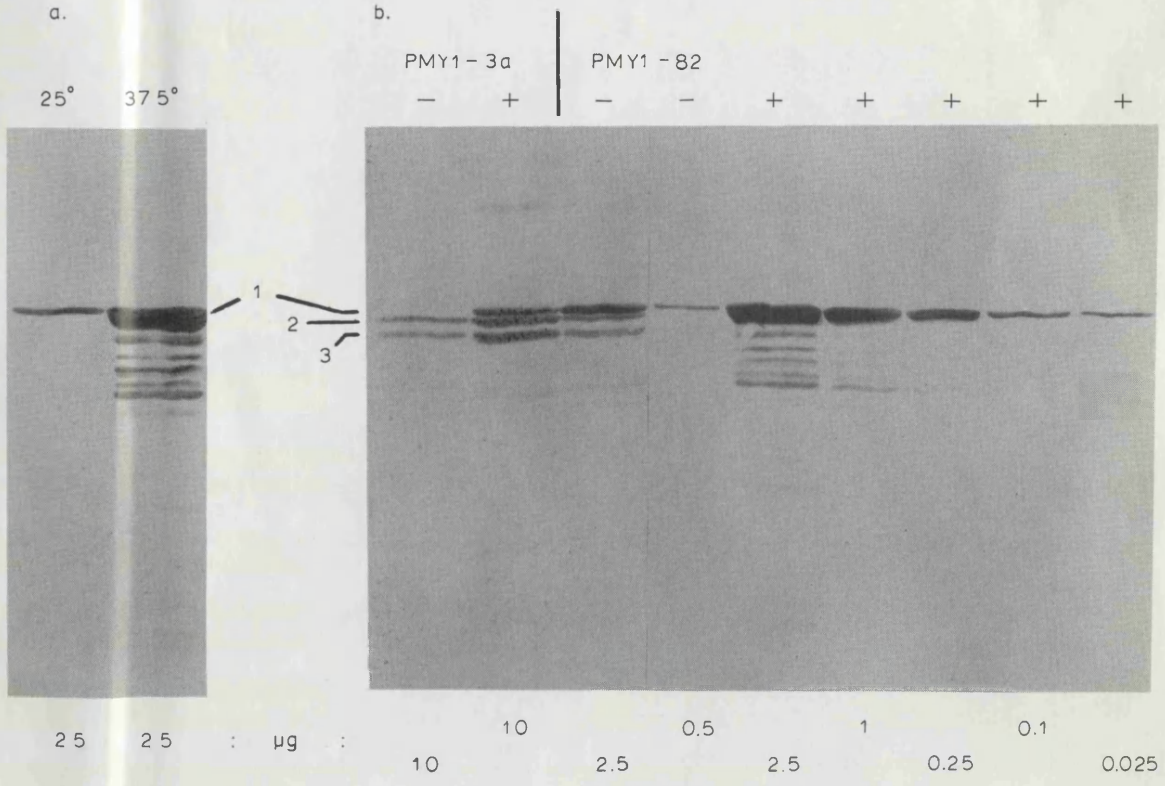

Fig. 3. Western blot analysis of HSP90 protein levels. (a) Samples from PMY1-82 maintained in log growth for several generations either at 25°C or at 37.5°C. (b) Comparison of relative HSP90 expression levels through serial dilution of total cell protein samples. The analysis of PMY1-3a and PMY1-82 protein before (–) or 75 min after (+) a 25–39°C heat shock is shown, the amount of protein loaded into each gel track being given at the base of the figure. Bands 1–3 are as indicated in the legend to Fig. 2.

3a). This increased expression at higher temperature can be attributed to the appreciable basal expression of sequences under heat shock element control at 37.5°C [33,34],

LEU2d-containing yeast episomal vectors such as pMA3a and p82-2B are normally maintained at between 50 and 150 copies per cell in *cir*$^+$ yeast strains when selection is applied for the *LEU2d* gene on the plasmid [24,25]. Southern blot analysis of the DNA of W303-82, PMY1-82 and MD40-82 showed that p82-2B was being maintained at around 100 copies per cell in the same log phase cultures of these transformants that were employed for HSP90 protein analysis; also that this plasmid was monomeric and substantially unconcatenated in these cultures (data not shown). The low basal expression of *HSP82* at 25°C in the absence of heat shock (10% of *HSP82* expression [5,31,32]) multiplied by this gene dosage factor of 50–150 would produce a basal HSP90 overproduction of 5 to 15-fold in the absence of heat shock. The Western blot analysis (Figs. 2 and 3b) indicated HSP90 overproduction at 25°C was approx. 2-fold less than this in the absence of heat shock in each of transformants W303-82, PMY-82 and MD40-82.

HSP90 overexpression showed an approximate 10-fold increase in all three expression strains in response to a 25–39°C heat shock

Following a 25–39°C heat shock the HSP90 of transformants W303-82, PMY1-82 and MD40-82 increased approx. 10-fold, becoming 30–40% of total cell protein 75 min after temperature upshift (Figs. 2 and 3b; also data not shown). To quantitate the extent of HSP90 overexpression samples of total cell protein from unstressed and heat shocked cultures of W303-82, PMY1-82 and MD40-82 were sequentially diluted, then compared by Western blotting. A typical dilution analysis is shown in Fig. 3b.

In yeast cells with normal HSP90 gene levels (not overexpressing HSP90) *HSP82* mRNA increases 14-fold with a 25–39°C heat shock [31,32] and total HSP90 protein approximately doubles as the *HSP82* product accumulates to a level approximating to that of the *HSC82* product (see samples from W303-3a, PMY1-3a and MD40-3a in Figs. 2 and 3b; also data in Ref. 5). HSP90 measurements for transformants W303-82, PMY1-82 and MD40-82 revealed that the induction ratio of the *HSP82* product with heat shock was relatively unaffected by an approximate 100-fold increase

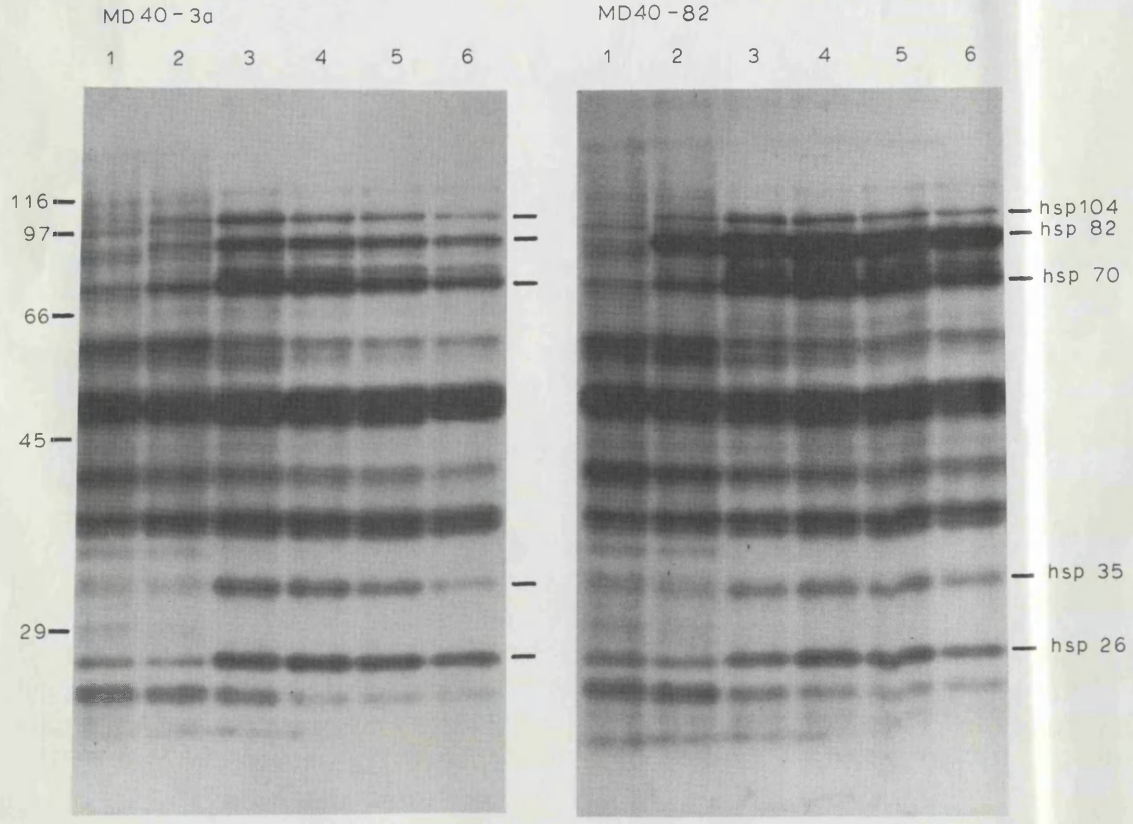

Fig. 4. [^{3}H]Leucine pulse-labelling of the proteins of transformants MD40-3a and MD40–2B before and after heat shock. Cultures initially in log growth at 25°C were pulse-labelled for 15 min at this temperature (1); and at 0–15 min (2), 15–30 min (3), 30–45 min (4), 45–60 min (5) or 60–75 min (6) following a 25–39°C heat shock. Positions of unlabelled molecular mass markers are indicated on the left in kilodaltons, while strongly-labelled protein bands corresponding to major yeast heat shock proteins [1,2,5–8] are indicated on the right.

in *HSP82* gene copy levels (Figs. 2 and 3b). That this induction ratio has not undergone major change shows that yeast can correctly regulate a large number of genes subject to heat shock element regulation (see Discussion).

HSP90 overexpression does not strongly suppress the induction of other heat shock proteins in log phase MD40-82 heat shocked to 39°C

If heat shock gene regulation is substantially unaltered in cells with multiple episomal *HSP82* genes, such cells should still display an essentially normal heat shock activation of chromosomal heat shock protein genes. In vivo protein pulse-labelling showed this to be the case. Fig. 4 shows pulse-labelling of MD40-3a and MD40-82 before and during heat shock, the heat induction of HSP104, HSP70, HSP35 and HSP26 in MD40-82 being similar to the induction of these same heat shock proteins in cells isogenic to MD40-82 but for the presence of just a single *HSP82* gene (MD40-3a; Fig. 4). Similar results (not shown) were obtained by comparison of the proteins pulse-labelled during heat shock in W303-82 as compared to W303-3a; also PMY1-82 as compared to PMY1-3a.

Effects of HSP90 overexpression on the high temperature growth and thermotolerance of log phase cultures

Reducing the HSP90 of yeast has little effect on growth at 25°C, but causes major reductions in growth at 37.5°C (Ref. 5; a study conducted in strains of the same genetic background as W303-3a). Reductions in the HSP90 of mouse L cells also prevent growth at high temperatures [20]. In this study the 3 to 7-fold overexpression of HSP90 in W303-82, PMY1-82 and MD40-82 at 25°C (Figs. 2 and 3b) also had minimal effect on growth at this temperature (compare these transformants with W303-3a, PMY1-3a and MD40-3a; Fig. 5a). However, the much higher overexpression of HSP90 in W303-82, PMY1-82 and MD40-82 cultures growing at 37.5°C (Fig. 3a) was associated with an appreciable reduction in growth rate at this temperature (Fig. 5b). This reduction in 37.5°C growth was least marked in cells of W303-3a genetic background, although W303-3a transformants grew less well than transformants of the two other expression strains (MD40-4c and PMY1) at this temperature (Fig. 5b). Since therefore both underproduction and high overproduction of HSP90 reduce growth at 37.5°C, a temperature close to the maximum for growth of *S. cerevisiae* [28], there is probably an optimal HSP90 level for cell proliferation at higher growth temperatures.

Thermotolerance can be measured as cell survival during short exposures to high, potentially-lethal temperatures, well above the maximum temperature of growth. It is usually increased by a prior nonacute heat shock, conditions that allow the induction of the heat shock response [1–8,30]. High *HSP82* gene dosage reduced thermotolerance at 52°C in PMY1-82 and MD40-82 initially in log growth at 25°C (compare wih PMY1-3a and MD40-3a; Fig. 6a,b). 52°C is approx. 10°C above the maximum temperature permitting protein synthesis in *S. cerevisiae* [28]. Even after prior shift from 25°C to 39°C for 40 min, a sublethal heat shock which will increase thermotolerance and cause appreciable increases in HSP90 (Figs. 2 and 3b), the presence of multiple *HSP82* genes still caused a reduction in thermotolerance (Fig. 6a–c). These effects were however strain-dependent, since in W303-82 the high *HSP82*gene dosage caused only a small thermotolerance reduction (Fig. 6c). This was apparently due to recessive mutations in the W303 genetic background, since the diploid strain formed by mating haploid transformants PMY1-82 and W303-82 displayed thermotolerance levels that were practically identical to those observed with its PMY1-82 parent (data not shown).

Effects of HSP90 overexpression on the thermotolerance of stationary phase cultures

High *HSP82* gene dosage was also found to cause strain-dependent reductions in the thermotolerance of

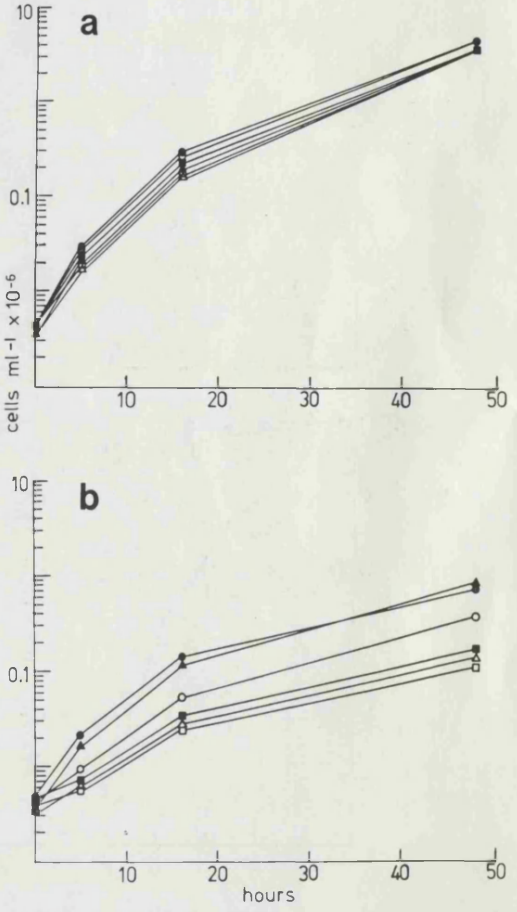

Fig. 5. Growth at 25°C (a) and 37.5°C (b) of transformants MD40-3a (●), MD40-82 (○), PMY1-3a (▲), PMY1-82 (△), W303-3a (■) and W303-82 (□).

stationary phase yeast cultures (Fig. 6d–f). As with the log phase cultures, the smallest reductions in thermotolerance were those shown by W303-82 as compared to W303-3a (Fig. 6f).

Discussion

When regulatable promoters are used to express genes on high copy number yeast expression vectors it is frequently found that regulation of these promoters has become defective. This is due to an insufficiency of key transcription factor molecules now that multiple DNA binding sites for these factors have been introduced on the plasmid. An example of such loss of tight regulation is that shown by the galactose-inducible *GAL1* promoter at moderate copy levels, this resulting from the low cellular abundance of the GAL4 regulator protein [35,36]. In contrast, this study shows that the *HSP82* heat shock gene is still subject to almost normal heat-inducible regulation when present at 50–150 copies per cell on a high copy episomal vector. Cells maintaining plasmid p82-2B showed an approximate 10-fold induction of the *HSP82* product following a 25–39°C heat shock (Figs. 2 and 3b), an induction level comparable to that seen in cells with the same gene as a single chromosomal copy [5,31,32].

HSP82 is probably a good model sequence with which to study the effects of gene dosage on heat shock gene expression since its transcription is apparently under the control of a single heat shock element sequence [31,32]. The heat shock element is the binding site for the well-characterised heat shock transcription factor (HSF) of yeast [33,37–39]. Of all the temperature-regulated systems for controlling gene expression in *S. cerevisiae*, heat shock element-directed expression is probably the most practicable and versatile [34]. That heat shock element-directed expression is still authentically regulated at high gene copy levels further in-

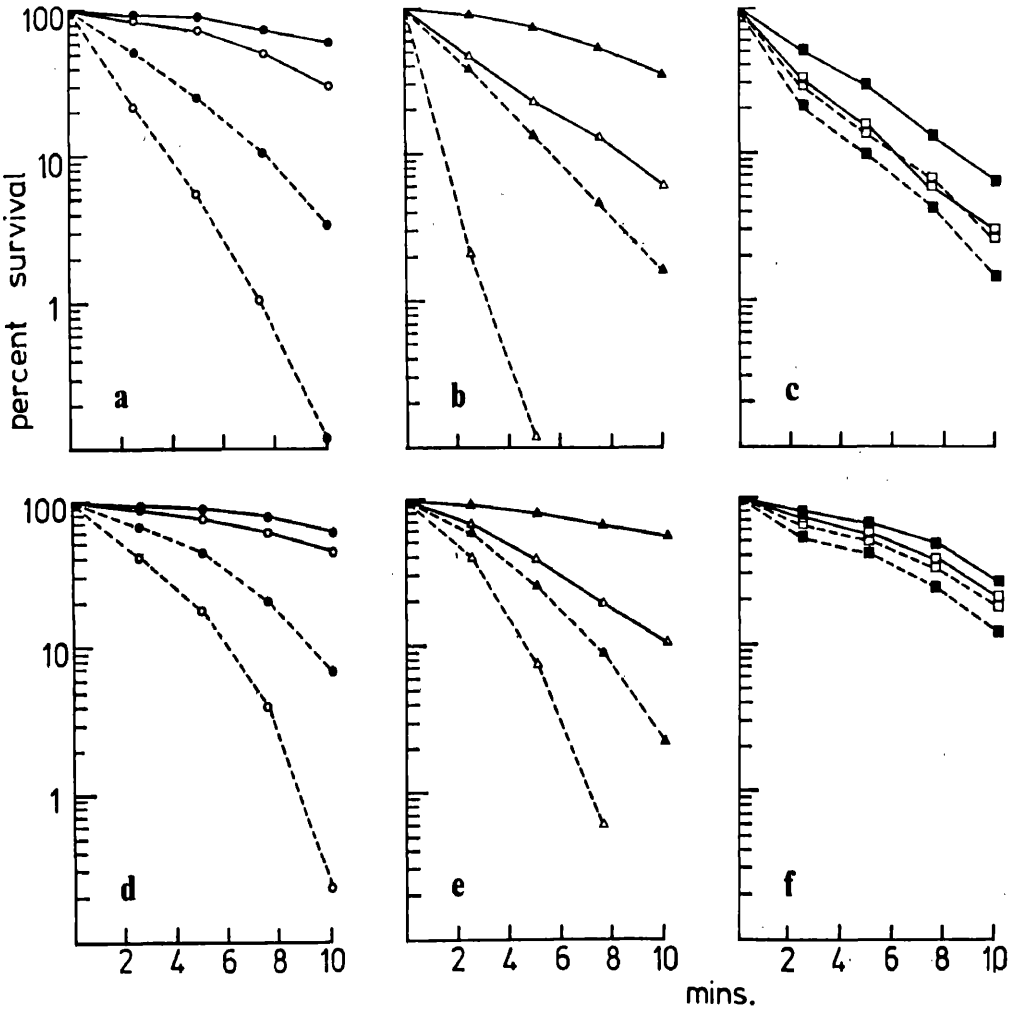

Fig. 6. Thermotolerance of exponential (a–c) or stationary phase (d–f) 25°C cultures of transformants of strains MD40-4c (a,d), PMY1 (b,e) and W303-3a (c,f) containing either plasmid pMA3a (solid symbols) or p82-2B (open symbols). Viability was measured at intervals after a direct shift to 52°C from 25°C (dashed lines), or a shift to 52°C following a sublethal heat shock (25°C to 39°C for 40 min; continuous lines).

creases the versatility of this expression system. If HSF, or another factor needed for the induction of heat shock genes, was to become limiting in cells with multiple episomal *HSP82* copies this would result in a pronounced perturbation of the heat shock response. This was not observed, there being no appreciable loss of the inducibility of chromosomal heat shock protein genes in cells with 50–150 episomal *HSP82* gene copies (Fig. 4).

Log phase cultures of W303-82, PMY1-82 and MD40-82 that have been heat shocked to 39°C for a short period are a suitable starting point for large-scale isolation of substantially-undegraded yeast HSP90. HSP90 isolated from these cultures was greater than 90% intact, as judged by Western blotting (Figs. 2 and 3b). It should also be possible to isolate HSP90 in substantial quantity from stationary cultures of W303-82, PMY1-82 and MD40-82 since *HSP82*, in common with many other heat shock genes of *S. cerevisiae* [40–43], is induced both by heat shock and as cells undergo the transition from exponential growth to stationary phase [2]. However, all our attempts to isolate HSP90 from stationary MD40-82, PMY1-82 and W303-82 cultures were unsuccessful, this protein being extensively degraded when the crude cell extracts were analysed by Western blotting (not shown). In an attempt to avoid this problem the p82-2B expression plasmid was transformed into the multiply proteinase-deficient strain BJ2168 [33]. However, the resultant transformants grew poorly and they were therefore not subjected to detailed analysis.

This study did not find that high *HSP82* gene dosage and the resultant increases in HSP90 level elevated thermotolerance. Instead moderate thermotolerance decreases were consistently observed with HSP90 overexpression (Fig. 6). This protein in high level is therefore not protective against heat damage, which is perhaps surprising in view of the evidence for protective effects of HSP70 overproduction [2]. Also apparent with HSP90 overexpression were reductions in growth at 37.5°C, a temperature close to the maximum for *S. cerevisiae* (Fig. 5b). Taken together with a previous study showing that HSP90 underexpression reduces high temperature growth [5], this indicates that there is an optimum HSP90 level for the growth of yeast at 37.5°C.

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