# Chapter 5: Hsp transcription and translation and trehalose mobilization during recovery from hyperthermia.

## 5.1 Introduction

It is now well established that cells upon exposure to heat shock temperature rapidly synthesize hsps (Lindquist and Craig, 1988; Watson, 1990; Piper, 1993). It is further recognised that hsps are highly conserved and some perform essential cellular functions in the normal unstressed cell (see Chapter 1). These functions include participation in protein folding and assembly and degradation of denatured proteins (Gething and Sambrook, 1992; Craig *et al.*, 1993). The term molecular chaperone has therefore been used to describe the function of some of the hsps (Ellis, 1987). The wealth of data supporting the role of hsps as molecular chaperones in the unstressed cell has lead to the proposal of a similar role in the stressed cell. Environmental insults such as elevated temperature, high ethanol concentrations, heavy metals and other inducing agents elicit protein denaturation (Casey and Ingeldew, 1986; Lepock, 1992), which would promote an immediate increase in the requirement for chaperone activity. There is considerable experimental support for this concept. For example, denatured or abnormal proteins trigger the heat shock response in mammalian cells (Ananthan *et al.*, 1986). In *Escherichia coli* the heat shock inducible *lon* gene product is a protease that functions to remove abnormal proteins (Neidhardt and vanBogelen, 1987). In the case of *Saccharomyces cerevisiae*, there is clear evidence that hsp 70 (Sanchez *et al.*, 1993; Nwaka *et al.*, 1996) and hsp 104 (Sanchez *et al.*, 1992; Lindquist and Kim, 1996) play roles in thermotolerance. In particular, hsp 104 has been implicated as a key protein for tolerance to various stresses including heat and ethanol (Sanchez *et al.*, 1992).

Despite the abundance of data supporting the concept of a prevalent role for hsps in the heat shock induction of thermotolerance in yeast, there exist significant contradictions in the literature (reviewed in Watson, 1990; Piper, 1993). These include acquisition of thermotolerance despite suppression of protein synthesis by cycloheximide (Hall, 1983; Watson *et al.*, 1984) and thermotolerance acquisition in mutants unable to synthesize hsps (DeVirgilio *et al.*, 1991; Smith and Yaffe, 1991). Also, acquired thermotolerance has been noted under conditions in which the full spectrum of hsps is not induced (Barnes *et al.*, 1990; Coote *et al.*, 1991). Similar contradictions with respect to the role of the

disaccharide trehalose in heat shock induced thermotolerance have been noted with evidence either supporting the greater importance of trehalose over hsps (De Virgilio *et al.*, 1991) or hsps over trehalose (Winkler *et al.*, 1991; Sanchez *et al.*, 1992). Although many of these contradictions may be attributable to strain specific differences or differences in experimental protocols, sufficient anomalies exist to raise significant contention with respect to the relative roles of hsps and trehalose in heat shock acquisition of thermotolerance. Moreover, while most reports have been concerned with one or other of these stress metabolites, results reported in Chapter 3 of this thesis, recently published (Gross and Watson, 1996a), demonstrated a lack of correlation between both hsp synthesis and trehalose accumulation with acquired thermotolerance in derepressed *S. cerevisiae*.

Although it appears well established that both hsps and trehalose are important to normal cellular metabolism, their relative contributions to thermotolerance as well as factors regulating their modes of action remain unresolved (Piper, 1993; Elliot *et al.*, 1996). An alternate approach to investigate these issues would be to compare levels of hsps and trehalose with loss of induced thermotolerance during recovery from sublethal heat treatment. In fact, while the induction of the heat shock response has been extensively investigated, there exists a dearth of information concerning its termination and the mechanism of recovery from the hyperthermic state. Few studies have addressed this question in detail. It has however, been previously demonstrated that suppression of hsp expression and resumption of normothermic protein synthesis in *E. coli* depends on the presence of intact dnaK (hsp 70 homolog) (Tilly *et al.*, 1983). Similarly, a critical level of hsp 70 was suggested to be required for restoration of normothermic protein synthesis in heat shocked *Drosophila* cells (DiDomenico *et al.*, 1982b). In mammalian cells, Pelham (1984) demonstrated that over-expression of hsp 70 promotes recovery of nucleolar morphology subsequent to heat shock. More recently, hsps 70 and 104 have been shown to be required for recovery of yeast cells after heat shock in exponential and stationary phase (Sanchez *et al.*, 1992) and to ameliorate trehalose mobilization upon temperature decrease from 40°C to 27°C (Hottiger *et al.*, 1992). Moreover, hsp 104 has also been shown to be functional in promoting recovery of splicing following return of yeast cells to 25°C (Vogel *et al.*, 1995) as well as facilitating folding and reactivation of damaged proteins subsequent to heat shock (Parsell *et al.*, 1994; Schirmer *et al.*, 1996).

In this chapter, the concept of recovery from hyperthermia was comprehensively examined in *S. cerevisiae* strains Yres and Ysen, grown on fermentable and non-

fermentable carbon sources. In Chapter 3 results showed a marked increase in intrinsic thermoresistance of both strains grown in acetate supplemented medium. This raised the question as to how the kinetics of induced thermotolerance decay may compare in derepressed and repressed cells. Transcriptional and translational regulation of the major hsps and patterns of trehalose mobilization during recovery of cells from thermal stress was investigated in parallel with cellular viability, an area in which previous investigations have fallen short. Results presented indicate that progressive loss of induced thermotolerance exhibited by repressed and derepressed cells was not closely correlated with either levels of hsp or trehalose. The implications for the protective mechanisms conferred by these stress metabolites are discussed.

## 5.2 Results

### 5.2.1 Intrinsic thermotolerance

Intrinsic levels of thermotolerance exhibited by Yres grown in glucose supplemented medium (YNBG) were 2-3 logs greater than those of Ysen after 10 min at 50°C (Fig. 5.1 A, C). This difference was maintained for the duration of the time course. These results were described in detail in Chapter 3. In addition, it was also shown that cells of these strains grown in acetate supplemented medium indicated a marked increase in thermoresistance, to a 50°C heat challenge. As a result, particularly for Yres, where levels of intrinsic tolerance were 90-100%, there was little difference between intrinsic and heat shock induced thermotolerance. Therefore, for the purposes of the present studies, in which the kinetics of loss of thermotolerance was of interest, stress temperatures were increased to 55°C and 52°C, for Yres and Ysen respectively, in YNBA medium. Under these conditions, similar levels of intrinsic tolerance were exhibited as in YNBG, although a significant retention of thermotolerance over the 60 min time course was noted (Fig. 5.2 A, C). Pre-incubation of cells with cycloheximide (20μg $ml^{-1}$) had no significant effect on intrinsic thermotolerance in YNBG or YNBA media for either strain (Fig. 5.1 B, D, Fig. 5.2 B, D).

### 5.2.2 Induced thermotolerance and recovery

As shown in Fig. 5.1 and Fig. 5.2, heat shock induced thermotolerance of cells grown in both YNBG (Fig. 5.1 A, C) and YNBA (Fig. 5.2 A, C) media was substantially (2-3

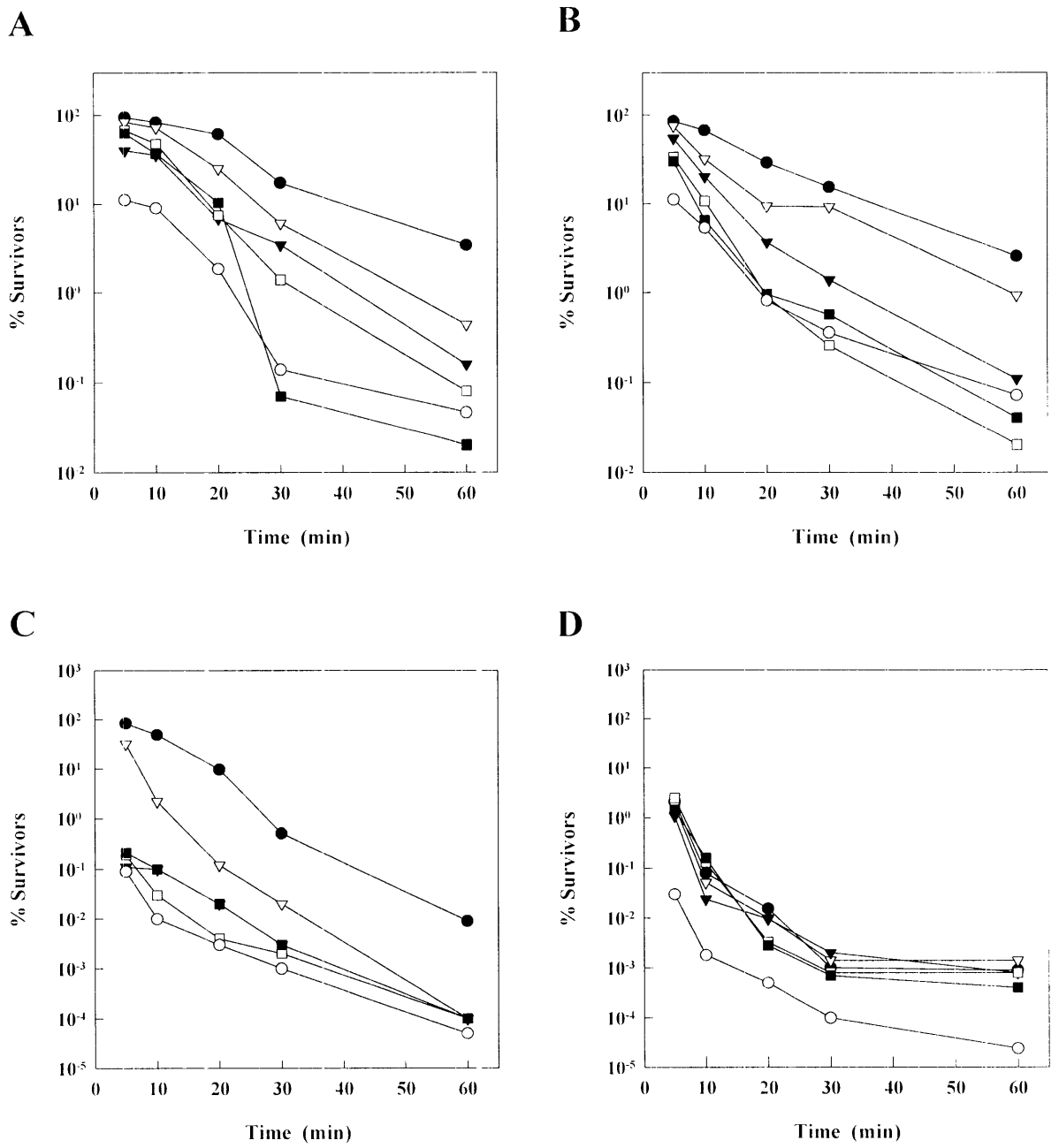


**Fig. 5.1.** Intrinsic ( ○ ) and induced ( ● ) thermotolerance and its decay during recovery in glucose grown (YNBG) cultures without (A, C) and with (B, D) cycloheximide (20μg $ml^{-1}$) treatment, for Yres (A, B) and Ysen (C, D). Intrinsic tolerance was assessed following incubation at 50°C for the times indicated. Induced tolerance was monitored at 50°C following a 30 min incubation at 37°C. Decay of induced thermotolerance was determined at 50°C following 30 ( ▽ ), 60 ( ▼ ), 90 ( □ ) and 240 ( ■ ) min recovery from 37°C at 25°C. Levels of thermotolerance are expressed as the percentage of viability with respect to a 25°C control sample in each case.

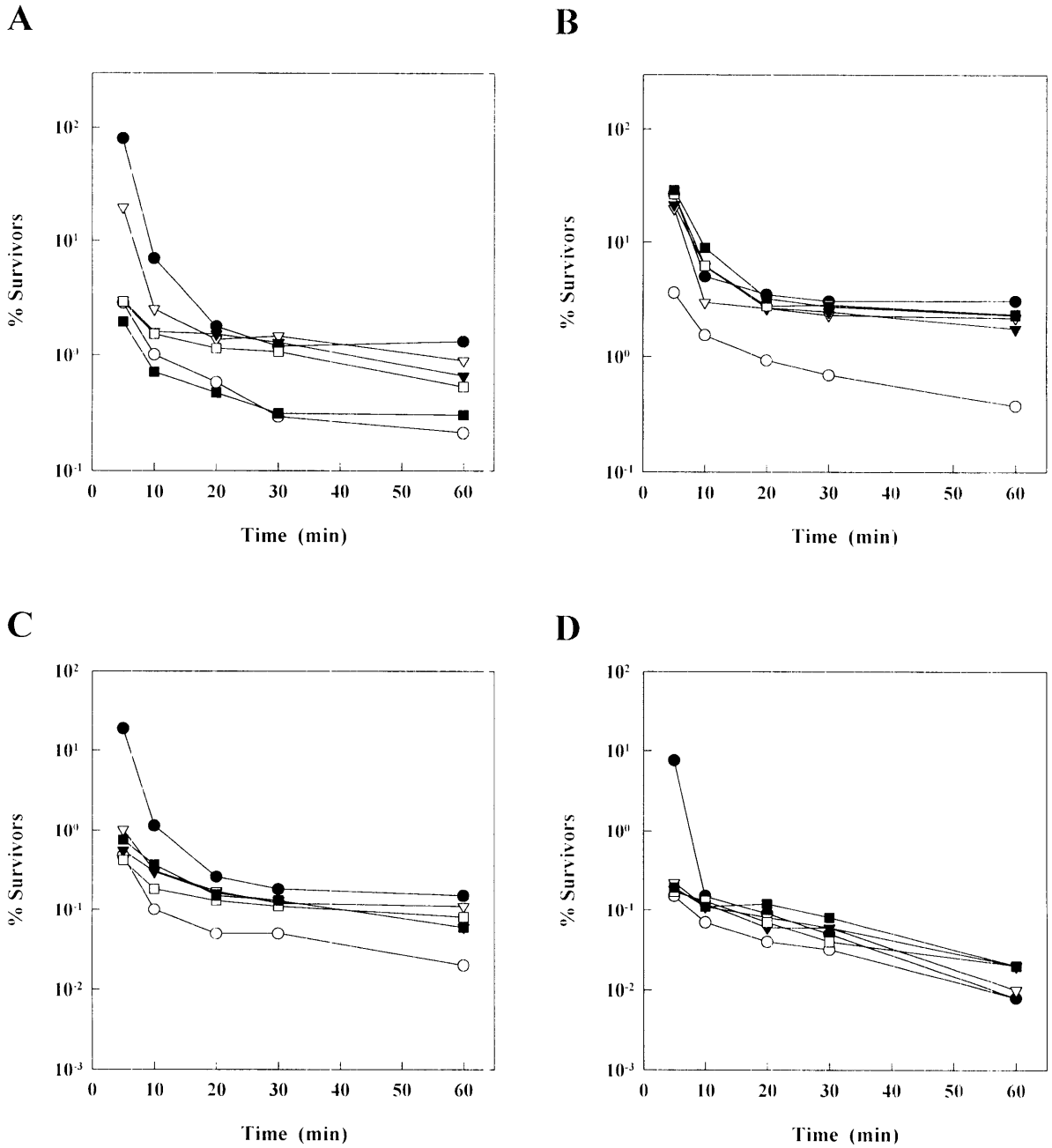


**Fig. 5.2.** Intrinsic ( ○ ) and induced ( ● ) thermotolerance and its decay during recovery in acetate grown (YNBA) cultures without (A, C) and with (B, D) cycloheximide (20μg $ml^{-1}$) treatment, for Yres (A, B) and Ysen (C, D). Intrinsic tolerance was assessed following incubation at 55°C (Yres) or 52°C (Yser) for the times indicated. Induced tolerance was monitored at 55°C (Yres) or 52°C (Ysen) following a 30 min incubation at 37°C. Decay of induced thermotolerance was determined at 55°C (Yres) or 52°C (Ysen) following 30 ( ▽ ), 60 ( ▼ ), 90 ( □ ) and 240 ( ■ ) min recovery from 37°C at 25°C. Levels of thermotolerance are expressed as the percentage of viabil ty with respect to a 25°C control sample in each case.

logs) greater than intrinsic thermotolerance. In the case of Yres grown in YNBG, this effect was less dramatic, attributable to the greater degree of intrinsic resistance exhibited by this strain. These results were previously described in Chapter 3. However, a further, notable difference for both strains in the two different media was the period of maintenance of acquired thermotolerance over the 60 min time courses. Although levels of induced thermotolerance decreased dramatically after 10 min at the stress temperature in YNBA cells, the level of cell survival after this point was relatively constant as compared with YNBG cells, despite the higher stress temperatures applied to the former. In general, survival of cells following 90 or 240 min recovery at 25°C reflected intrinsic values.

The affect of cycloheximide on heat shock induced thermotolerance and its maintenance was examined and results are shown in Fig. 5.1 (B, D) and Fig. 5.2 (B, D). Incubation with cycloheximide, prior to and during heat shock, was found to have a marginal effect on the acquisition and maintenance of thermotolerance in YNBG cultures of Yres with survival curves mirroring those of intrinsic levels following 90 min at the recovery temperature (Fig. 5.1 B). By contrast, glucose supplemented cultures of Ysen were deficient in thermotolerance acquisition, exhibiting an extremely transient response in comparison to their non-treated counterparts (Fig. 5.1 D). In YNBA medium however, while neither strain was significantly compromised in the magnitude of tolerance induction following 5 min at the stress temperature (Fig. 5.2 B, D), the response decayed rapidly in Ysen. Conversely, in the case of Yres, both magnitude and maintenance of induced tolerance persisted throughout the 240 min recovery period.

### 5.2.3 HSP transcription during heat shock and recovery

Patterns of HSP transcription during heat shock and recovery were monitored using total RNA slot blot hybridization. Fig. 5.3 shows heat shock induction of *HSP 104* (A) and *HSP 70* (C) mRNA in both strains grown in either YNBG or YNBA medium. In all cases, a general pattern of a marked decrease in transcription 30 min after heat shock was observed and this was followed by an increase back to constitutive levels after a 60-90 min recovery at 25°C. In addition, it was noted that Yres exhibited greater constitutive transcription of *HSP 104* and *HSP 70* than Ysen, in both media. This was in agreement with greater translational efficiency evident from immunoblot analyses (Fig. 5.8). An actin DNA probe (*ACT1*) was used to rehybridize the blots originally screened for *HSP 104* and *HSP 70* and was indicative of uniform loading of total RNA samples (Fig. 5.3 B, D).

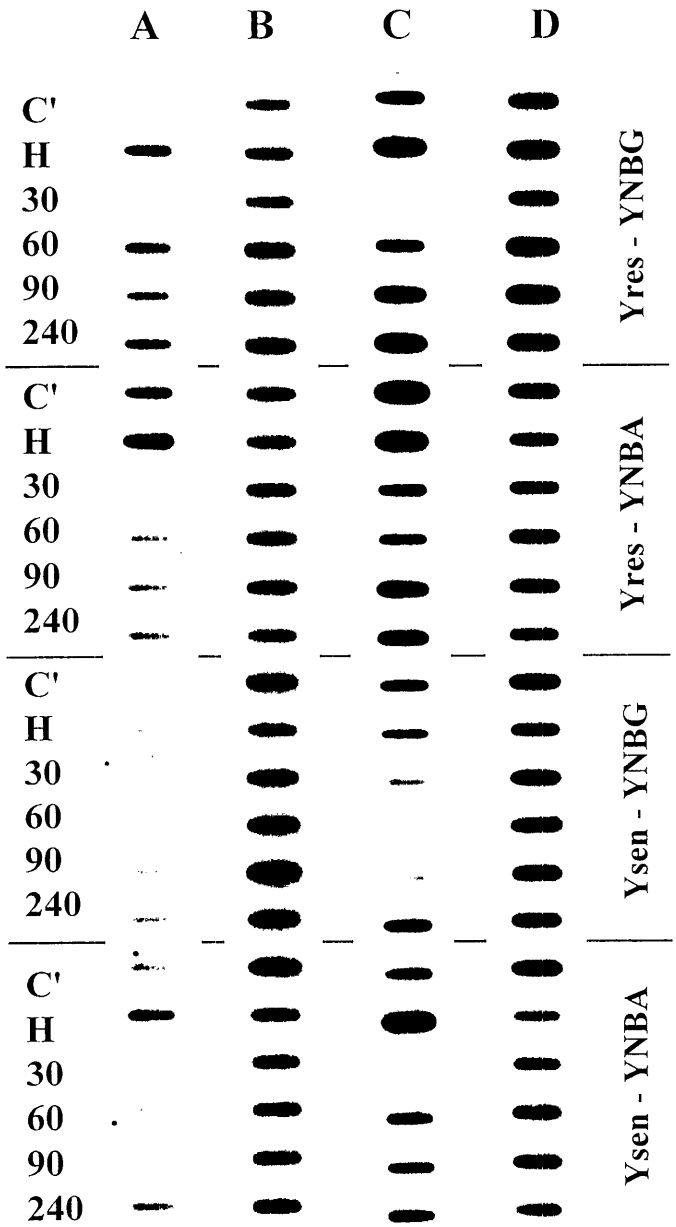


**Fig. 5.3.** Total RNA slot blot analysis of *HSP 104* (A) and *HSP 70* (C) expression from 25°C control (C'), 37°C (30 min) heat shocked (H) and 25°C recovering (30, 60, 90 and 240 min) cultures of Yres and Ysen. Cells were grown in either glucose (YNBG) or acetate (YNBA) supplemented medium. Membranes were rehybridized with *ACT1* (B and D) as a loading control.

### 5.2.4 *De novo* protein synthesis during heat shock and recovery

In vivo [$^{35}$S]-methionine labelling was employed to monitor the role of *de novo* protein synthesis in the heat shock acquisition of thermotolerance and its subsequent rate of decay during recovery. Protein profiles of control, heat shocked and recovering Yres and Ysen cells grown in either YNBG or YNBA are shown in Fig. 5.4-5.7, respectively. Both strains exhibited increased synthesis of typical heat shock inducible proteins at approximately 100, 90 and 70 kDa, in addition to a high molecular protein at approximately 130-150 kDa reported in Chapter 3, in both media types (indicated by arrows). However, after 30 min at the recovery temperature, all cultures exhibited a dramatic decrease in levels of *de novo* hsp synthesis. In fact, in all cases, intensities of *de novo* hsp synthesis following the 30 min recovery period at 25°C were less than those of constitutive bands, concurrent with transcriptional levels discussed above (Fig. 5.3). Resumption of translation activity to pre-heat shock levels was also in general agreement with transcriptional patterns for hsps 104 and 70. Cycloheximide treatment (20 μg ml$^{-1}$) resulted in negligible synthesis of constitutive and heat shock inducible protein (Fig. 5.4-5.7). However, gradual resumption of protein synthesis during the recovery period was observed. This attests to the efficacy of cycloheximide removal prior to return of cultures to 25°C. It was of interest to note that constitutive expression of members of the hsp 70 family and hsp 90 was amongst those proteins exhibiting earliest resumption of expression (Fig. 5.4-5-7). In this regard, it has been previously reported that certain members of the hsp 70 family and hsp 90 are essential for yeast viability (reviewed in Chapter 1).

### 5.3.5 Western blot analysis of proteins from heat shocked and recovering cells

Determination of levels of hsps following heat shock and recovery was conducted by immunological cross-reaction of total protein profiles with respective anti-hsp antibodies. Heat shock induction of hsps 104, 90, 70 and 60 was evident for both Yres and Ysen in glucose and acetate supplemented media (Fig. 5.8). However, following return to control temperature (25°C), high levels of the major hsps persisted in the cells for the duration of the recovery period. In the case of YNBG cultures of Ysen however, levels of hsps had decreased to those of control cells after 240 min recovery (Fig. 5.8 A). A further, notable trend was the presence of higher constitutive levels of hsps 104, 90 and 70 in both strains grown in YNBA medium, particularly for Yres (Fig. 5.8 B). It should be noted that the

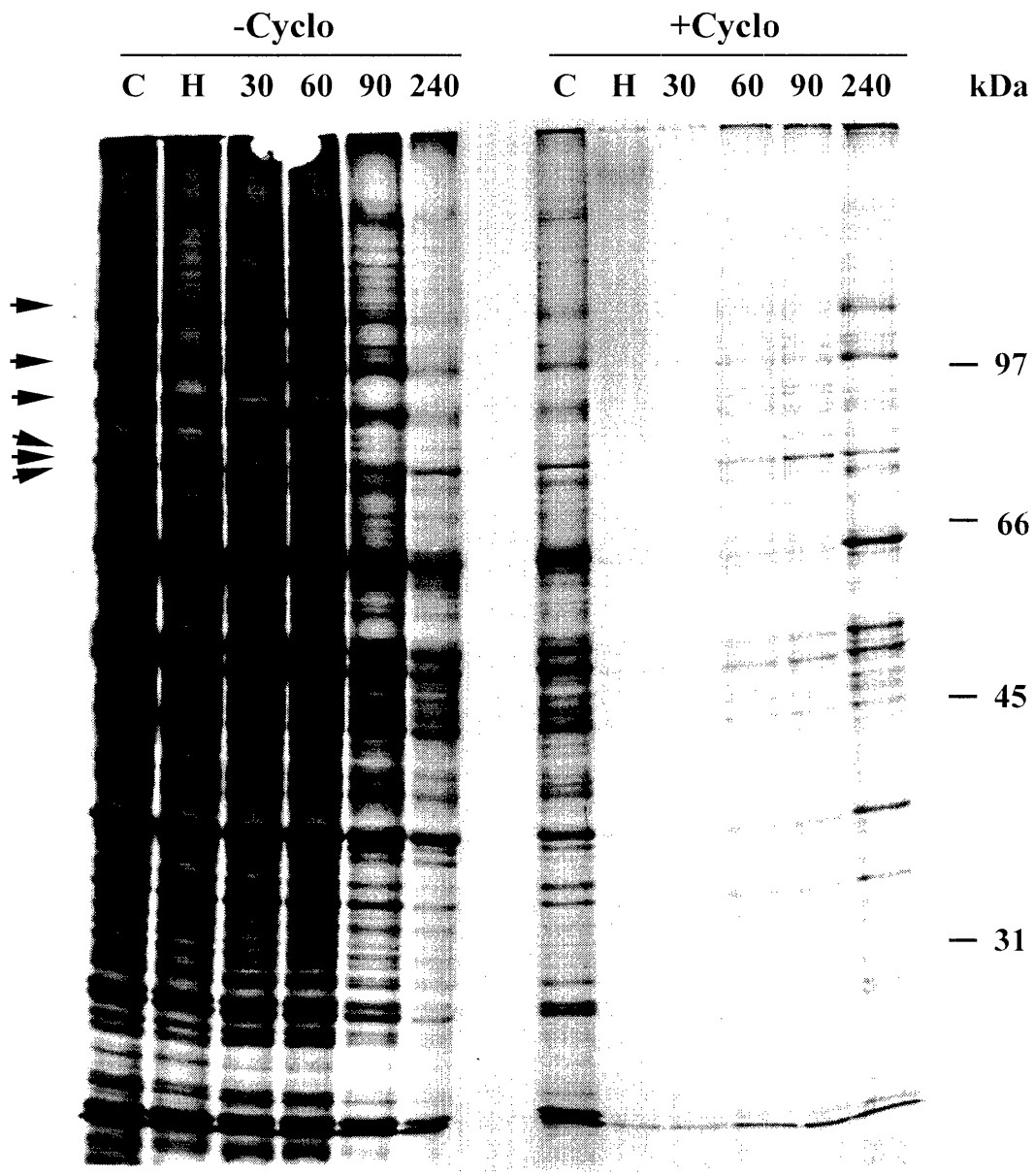


**Fig. 5.4.** SDS-polyacrylamide gel autoradiogram of [$^{35}$S]-methionine labelled protein extracts from control (C), heat shocked (H) and recovering (30, 60, 90 and 240 min) cultures of Yres grown in YNBG medium. Heat shock was carried out at 37°C without (-cyclo) or with (+cyclo) prior cycloheximide (20μg ml$^{-1}$) treatment. Cycloheximide was washed from cells prior to incubation at the recovery temperature (25°C). Arrows indicate new or increased protein bands in the heat shocked sample compared with control. Molecular mass standards (kDa) are as indicated.

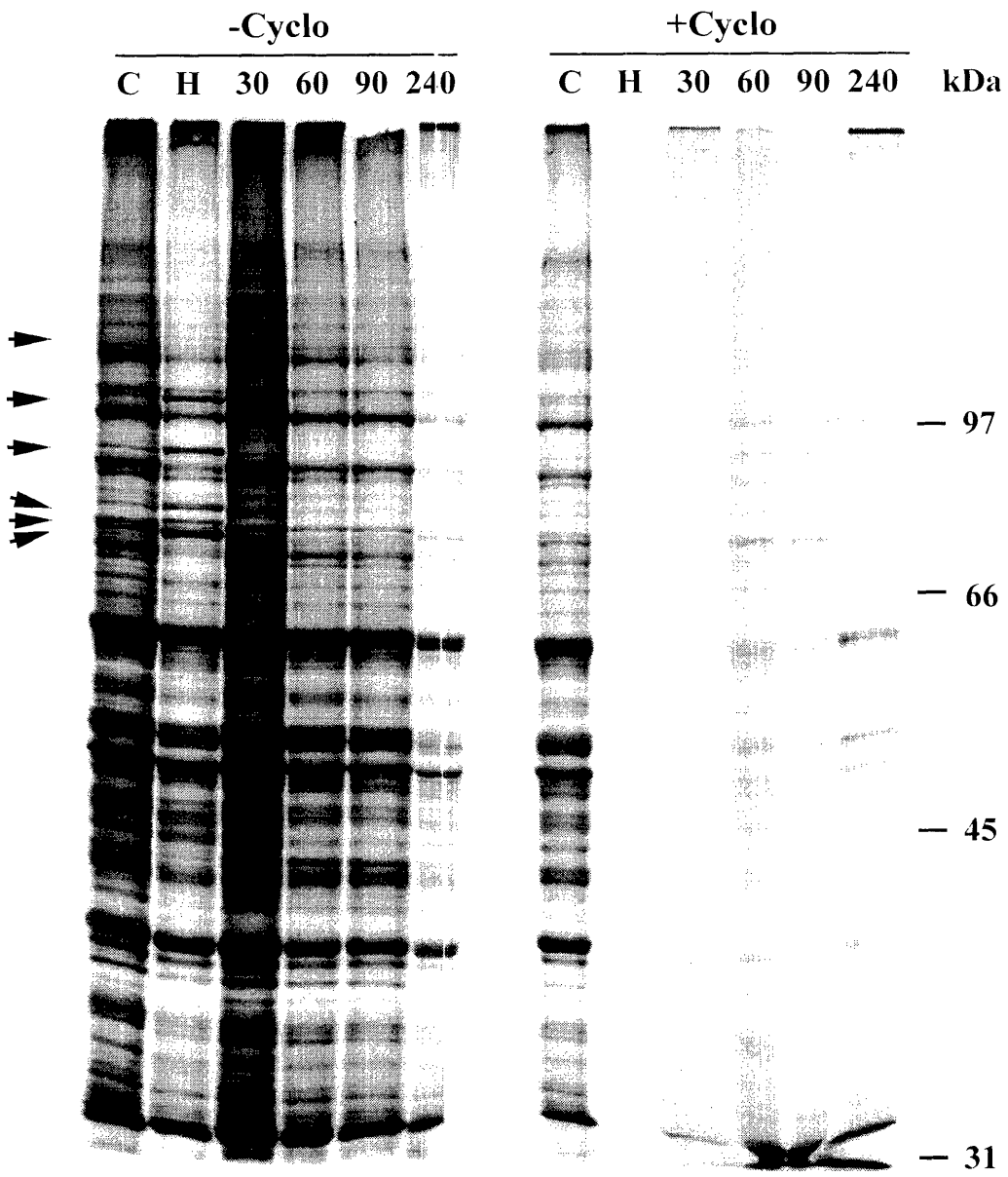


**Fig. 5.5.** SDS-polyacrylamide gel autoradiogram of [$^{35}$S]-methionine labelled protein extracts from control (C), heat shocked (H) and recovering (30, 60, 90 and 240 min) cultures of Ysen grown in YNBG medium. Heat shock was carried out at 37°C without (-cyclo) or with (+cyclo) prior cycloheximide (20μg ml$^{-1}$) treatment. Cycloheximide was washed from cells prior to incubation at the recovery temperature (25°C). Arrows indicate new or increased protein bands in the heat shocked sample compared with control. Molecular mass standards (kDa) are as indicated.

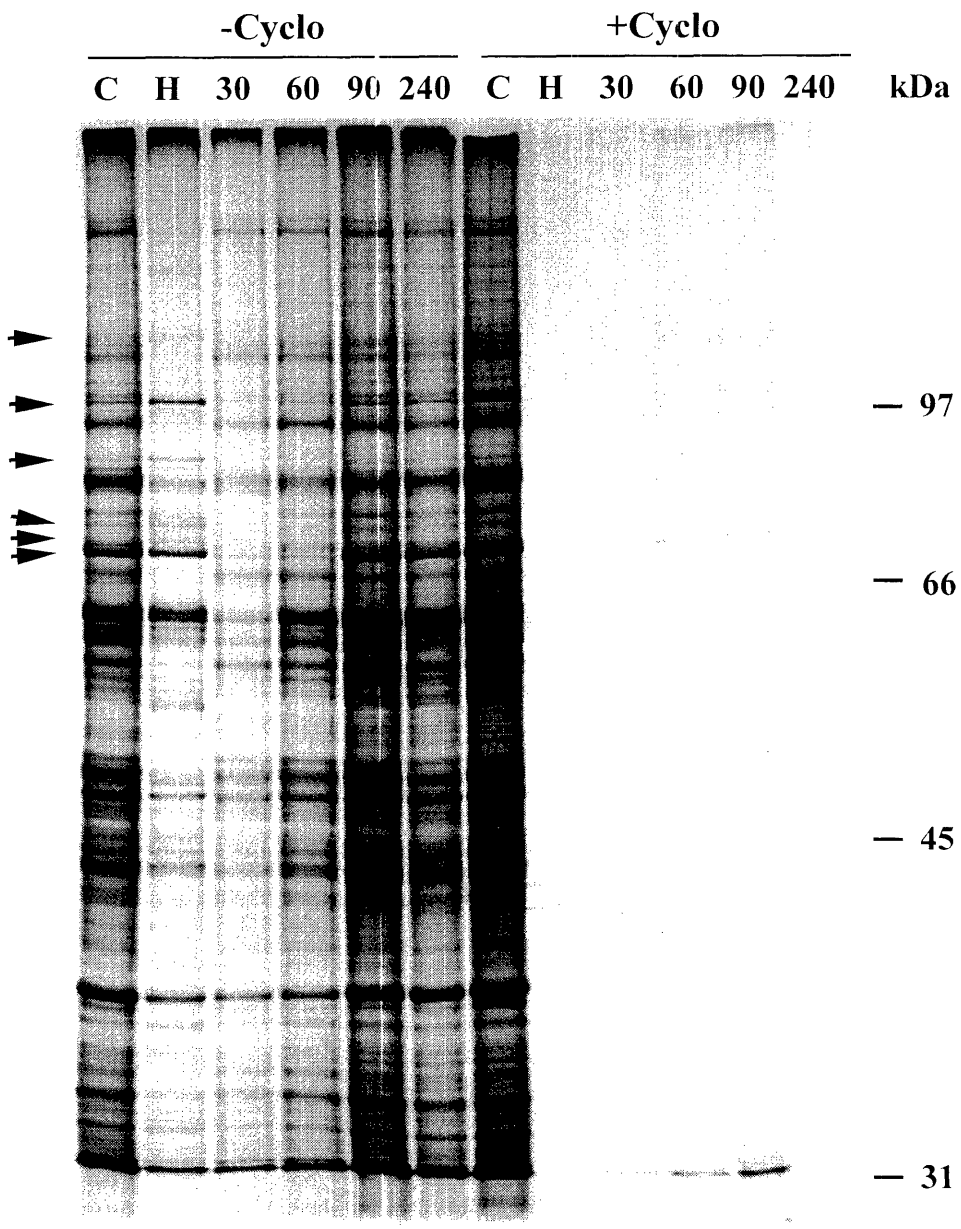


**Fig. 5.6.** SDS-polyacrylamide gel autoradiogram of [$^{35}$S]-methionine labelled protein extracts from control (C), heat shocked (H) and recovering (30, 60, 90 and 240 min) cultures of Yres grown in YNBA medium. Heat shock was carried out at 37°C without (-cyclo) or with (+cyclo) prior cycloheximide (20μg ml$^{-1}$) treatment. Cycloheximide was washed from cells prior to incubation at the recovery temperature (25°C). Arrows indicate new or increased protein bands in the heat shocked sample compared with control. Molecular mass standards (kDa) are as indicated.

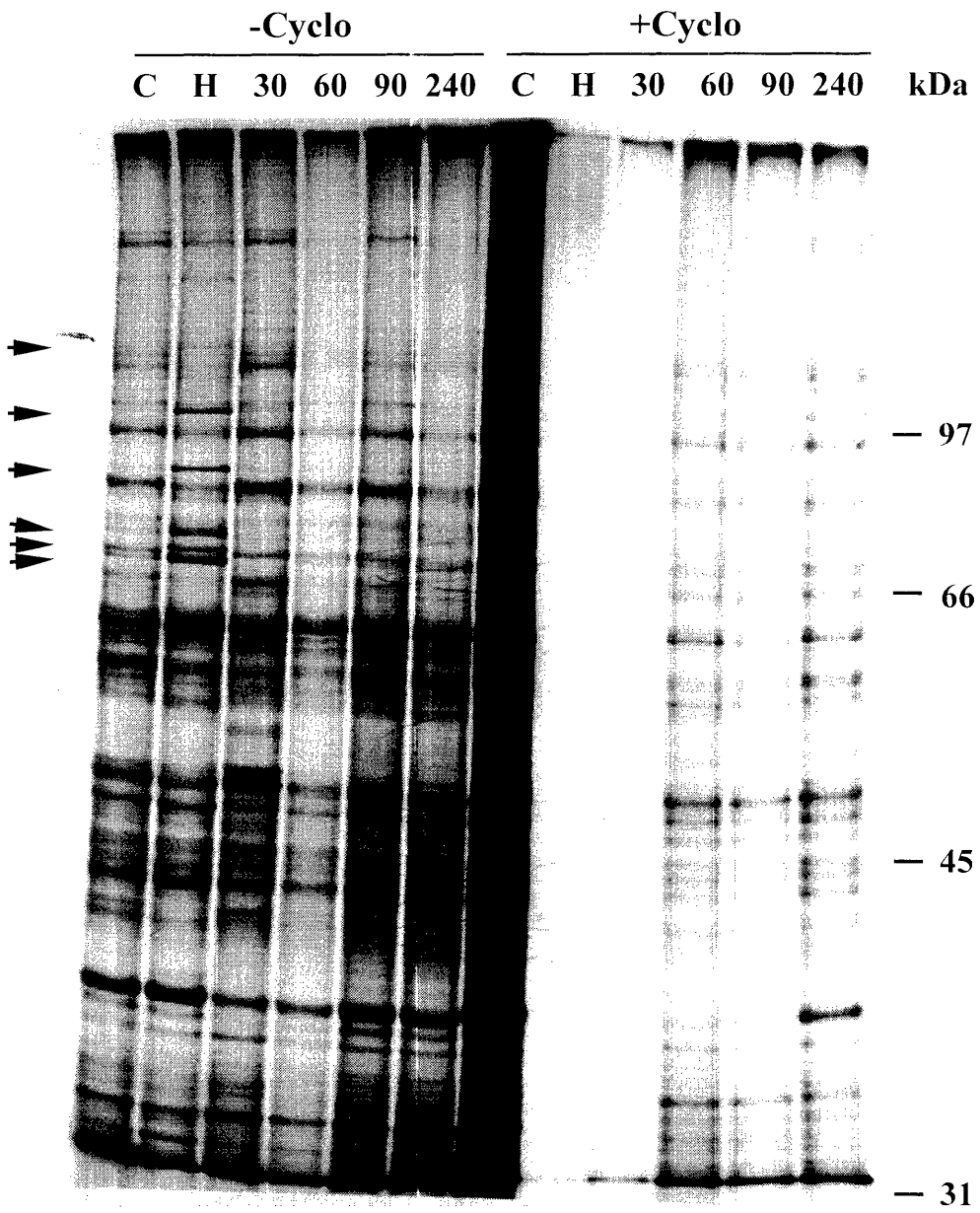


**Fig. 5.7.** SDS-polyacrylamide gel autoradiogram of [$^{35}$S]-methionine labelled protein extracts from control (C), heat shocked (H) and recovering (30, 60, 90 and 240 min) cultures of Ysen grown in YNBA medium. Heat shock was carried out at 37°C without (-cyclo) or with (+cyclo) prior cycloheximide (20μg ml$^{-1}$) treatment. Cycloheximide was washed from cells prior to incubation at the recovery temperature (25°C). Arrows indicate new or increased protein bands in the heat shocked sample compared with control. Molecular mass standards (kDa) are as indicated.

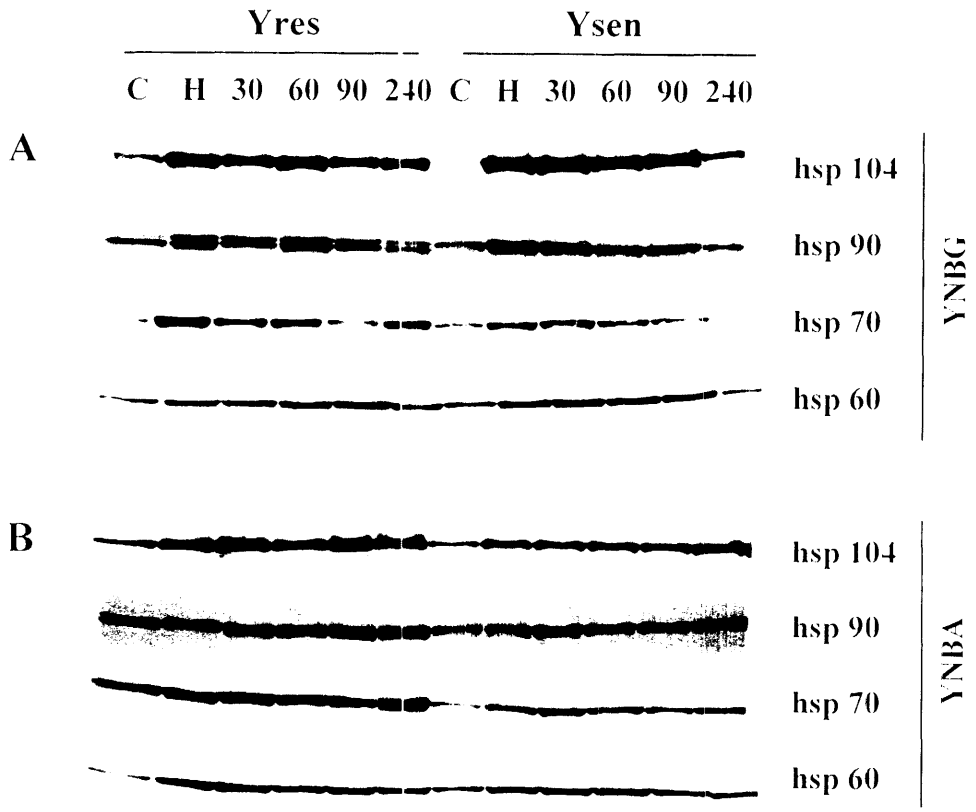


**Fig. 5.8.** Western immunoblot analysis of protein extracts from control (C), heat shocked (H) and recovering (30, 60, 90 and 240 min) cultures of Yres and Ysen grown on glucose (YNBG) (A) or acetate (YNBA) (B) medium. Immune complexes were generated for anti-hsp 104, 90, 70 and 60 antibodies.

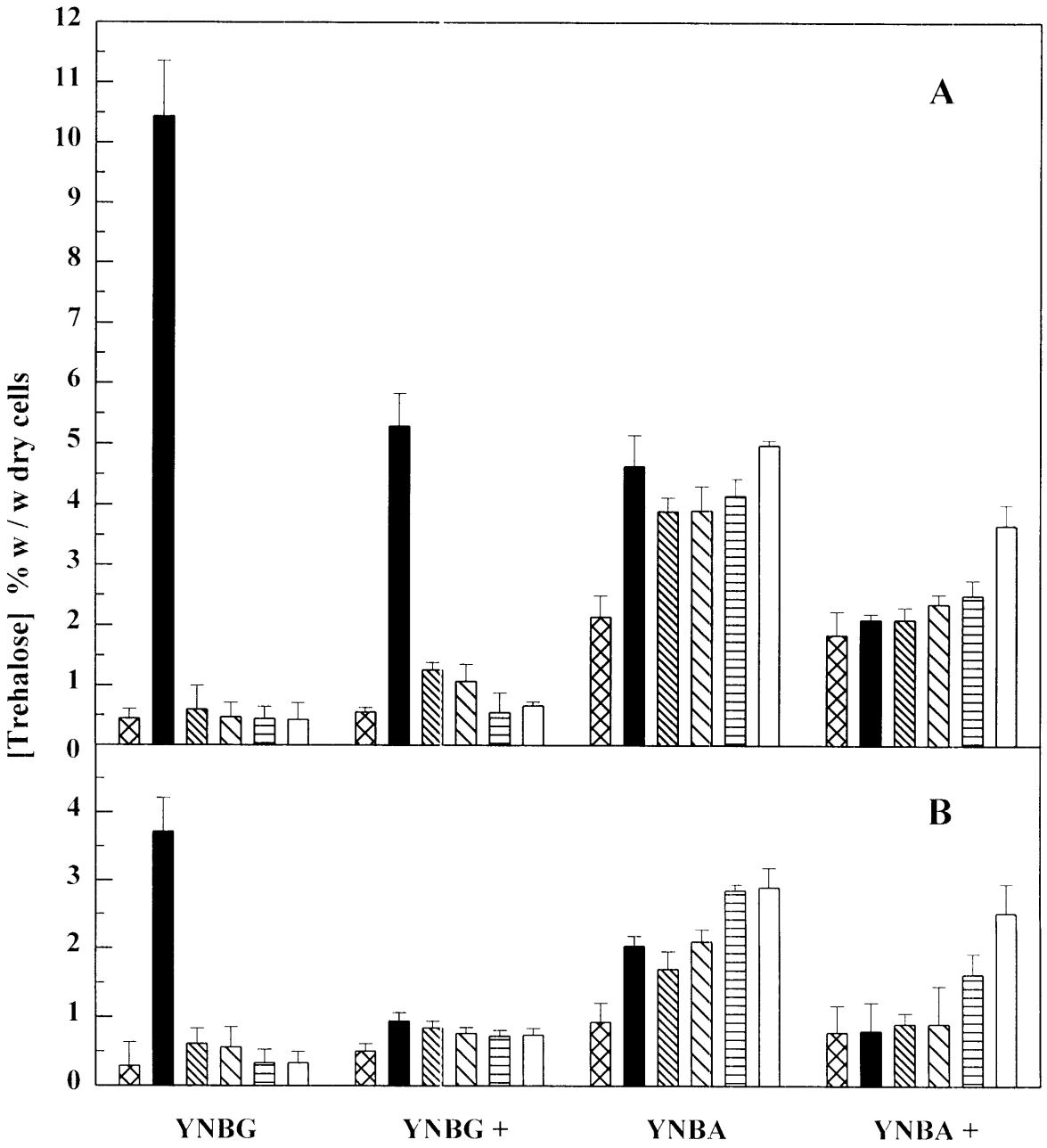


**Fig. 5.9.** Trehalose levels in control (▨) heat shocked (■) and recovering (30 ▨, 60 ▨, 90 ▭ and 240 □ min) cultures of Yres (A) and Ysen (B). Cells were grown to mid-log phase in either glucose (YNBG) or acetate (YNBA) medium (25°C) and heat shocked at 37°C (30 min) without or with (+) cycloheximide (20μg $ml^{-1}$) treatment. Cycloheximide was washed from cells prior to return of cultures to recovery temperature (25°C). Error bars represent the standard deviation of measurements from three experiments.

two bands observed in immunoblots with anti-hsp 90 antibody (Fig. 5.8) represent immune complexes of hsp 90 (upper band) and hsc 90 (lower band) (see 1.3.2).

### 5.3.6 Trehalose content in heat shocked and recovering cells

Levels of trehalose in control, heat shocked and recovering cells, in both YNBG and YNBA media, are presented in Fig. 5.9. In YNBG medium, trehalose content (0.5-0.7% w/w) was similar in Yres and Ysen. During heat shock treatment, the trehalose pool increased by a factor of 20-fold for Yres compared to a 6-fold increase noted for Ysen. Mobilization of trehalose was rapid following return to control temperature and had declined to pre-heat shock values following 60-90 min recovery. In the presence of cycloheximide however, heat shock induced accumulation of trehalose was reduced by at least 50%, to approximately 5.5% w/w and 1% w/w for Yres and Ysen respectively. Nevertheless, these levels were still significantly higher than in control cells, particularly for Yres. Rates of trehalose mobilization during recovery were marginally lower when heat shock was applied in the absence of protein synthesis.

Although similar trends were noted in acetate supplemented medium (Fig. 5.9 B), intrinsic trehalose content was higher than in YNBG, particularly for Yres. In addition, heat shock induced accumulation was notably lower, approximately 2-fold for both strains. Whilst cycloheximide had negligible effect on intrinsic trehalose content, YNBA cultures exhibited no significant accumulation upon heat shock. However, in marked contrast to glucose supplemented cultures, maintenance of the trehalose pool was evident during recovery in YNBA medium, with significant increases in content evident after 90 and 240 min at 25°C.

## 5.3 Discussion

A characteristic feature of the heat shock response is the transient nature of the acquired thermotolerance (Watson, 1990). In the present chapter, it was shown that biochemical events associated with the response are readily reversible subsequent to release from the heat shocked state and any possible correlation with loss of the thermotolerance phenotype was investigated. The current model system comprising *S. cerevisiae* strains Yres and Ysen, grown on a fermentable and a non-fermentable carbon source was employed.

Cells heat shocked at 37°C for 30 min initially acquired high thermotolerance which was progressively lost when cultures were allowed to recover at 25°C (Fig. 5.1, 5.2). Generally, by 240 min at 25°C, levels of tolerance were compatible to those of control or non-heat shocked cells. In Yres, inhibition of protein synthesis by cycloheximide during the heat shock had little effect on the kinetics of loss of thermotolerance. On the other hand, in Ysen, cycloheximide inhibited, but not entirely, the heat shock acquisition of thermotolerance and accelerated its subsequent loss. As reported in Chapter 3, these observations imply that heat shock protein synthesis was a prerequisite for acquired thermotolerance in Ysen but that in Yres some other, intrinsic factor(s) was operative. Similar results were observed for cells grown on fermentable or non-fermentable substrate. It was noteworthy however, that cells on YNBA were significantly more tolerant to heat that cells on YNBG, despite higher stress temperatures employed for the former.

It has been suggested that higher constitutive levels of hsp 104 are responsible for the higher thermotolerance of cells grown on a non-fermentable as compared to a fermentable carbon source (Sanchez *et al.*, 1992). This appears to be the case here, with Yres and Ysen cells grown on YNBA, as compared to YNBG, exhibiting higher constitutive levels of hsp104 (Fig. 5.8). Furthermore, it is also evident that higher constitutive levels of hsps 90, 70 and 60 are present in YNBA compared to YNBG. Support for these observations comes from results concerning the transcriptional levels of hsps, where higher constitutive levels of hsps 104 and 70 were exhibited by YNBA cells as compared with YNBG (Fig. 5.3). This was particularly evident in the case of Yres, although less obvious in Ysen. In a broad sense, constitutive levels of hsps thus appear to correlate with levels of intrinsic thermotolerance.

As previously demonstrated (Chapter 3), the now classic correlation of the rapid synthesis of hsps (Fig. 5.4-5.7) with acquisition of thermotolerance (Fig. 5.1, Fig. 5.2) was clearly shown in both Yres and Ysen, grown on both YNBG and YNBA. Most importantly however, cells allowed to recover at 25°C from a heat shock (37°C) were no longer able to synthesize hsps, after only 30 min at the recovery temperature (Fig. 5.4-5.7), as determined by [$^{35}$S]-methionine incorporation into protein. Furthermore, cells incubated at 25°C for up to 240 min following heat shock did not show any synthesis of hsps. Indeed, the protein patterns of cells from about 60 min onwards were similar to those of control cells. Nevertheless, to a large extent, these observations support the long adhered to

hypothesis that hsps are a significant, if not essential component of heat shock induced thermotolerance (Lindquist, 1986; Lindquist and Craig, 1988).

On the other hand, examination of the kinetics of loss of heat shock induced thermotolerance clearly showed that the loss was progressive rather than rapid (Fig. 5.1, Fig. 5.2). For example, cells incubated at 25°C for 30 min, although no longer engaged in hsp synthesis, still retained significant thermotolerance, relative to control non-heat shocked cells. Northern analysis (Fig. 5.3) revealed that hsp mRNAs increased immediately following a heat shock and then decreased markedly after 30 min incubation and recovery at 25°C. Interestingly, hsp mRNAs progressively increased thereafter over the 240 min recovery, approaching levels observed in non-heat shocked control cells. However, it was evident from [$^{35}$S]-methionine labelling experiments that cells recovering from heat shock were not synthesizing hsps in contrast to cells undergoing heat shock (Fig. 5.4-5.7). A two-fold picture emerges. Firstly, during heat shock at 37°C there was rapid transcriptional and translational increase in hsp expression and synthesis. Secondly, during recovery at 25°C there was a rapid transcriptional and translational decrease followed by a gradual increase in hsp mRNA transcription to levels observed prior to the heat shock. The key point being that translation of hsp mRNA was no longer observed during the recovery phase, as evident from [$^{35}$S]-methionine labelling. In this context, it is noteworthy that results of Fuge *et al.* (1994) indicated that synthesis of hsp 70 in stationary phase cells of *S. cerevisiae* did not correlate with accumulation of its mRNA. The authors concluded that synthesis was therefore regulated by mechanisms other than steady state mRNA accumulation.

The present results, recently accepted for publication (Gross and Watson, 1998b) allow the crucial and contentious issue as to the correlation of heat shock induced thermotolerance and hsp synthesis to be addressed. Western blot analyses unequivocally demonstrated the marked increase in hsp synthesis immediately following a heat shock (Fig. 5.8). Most pertinent however, was the observation that levels of hsps 104, 90, 70 and 60 remain high throughout the 240 min recovery period at 25°C. The only exception was for Ysen cells in YNBG, where levels of hsp 70 and hsp 60 markedly decreased but only after 240 min. The latter results however, do not inherently invalidate the observation that the products of hsp translation during heat shock persist in recovering cells at levels comparable to the those observed immediately following a heat shock. Indeed, in YNBA

cultures of Ysen, both hsps 104 and 90 have increased to levels above those of heat shocked cells (Fig. 5.8 B), despite the loss of acquired thermotolerance (Fig. 5.2 C). Overall, taking into consideration the results on transcription and translation and the kinetics of loss of heat shock induced thermotolerance, a direct correlation between *de novo* hsp synthesis and induced thermotolerance cannot be drawn. Previous studies on *S. cerevisiae* (McAlister and Finkelstien, 1980; Hall, 1983; Watson *et al.*, 1984; Cavicchioli and Watson, 1986) have shown the extremely transient nature of heat shock induced thermotolerance. The early studies of Cavicchioli and Watson (1986) also showed that loss of thermotolerance was not correlated with loss of hsps, particularly hsp 100 (hsp 104). More recently studies on *Salmonella typhimurium* (Mackey and Derrick, 1990) and *Listeria monocytogenes* (Jørgensen *et al.*, 1996) have indicated no correlation between levels of hsp and the loss of thermotolerance after heat shock. Both studies estimated levels of hsps, separated by one-dimensional SDS-PAGE gels, by following labelling of heat shocked cells with [$^{35}$S]-methionine and continued incubation at an appropriate recovery temperature. In both reports, there was a rapid loss of induced thermotolerance without concomitant decrease in levels of hsps. However, unlike the present work, none of these investigations clearly distinguished between repression of *de novo* synthesis of hsps upon return to control temperature and their subsequent persistence in the cell during the recovery period.

Cells respond to heat stress by activation and binding of a transcriptional activator protein, the heat shock factor, to the heat shock element, a specific DNA recognition sequence located in the 5'-region of heat shock genes (Sorger *et al.*, 1987; Sorger and Pelham, 1988; Larson *et al.*, 1988) (see 1.4.1). Moreover, under stress conditions, HSF undergoes reversible phosphorylation-dephosphorylation, which modifies its effect on transcriptional activity (Sorger, 1991). One of the ways that cells respond to rapid changes in environmental stress is thus by utilization of systems that are capable of reversible modification of cellular components, such as proteins. It is particularly relevant to the present discussion that heat shock in *E. coli* (Sherman and Goldberg, 1992; Sherman and Goldberg 1993) has been reported to induce phosphorylation of dnaK (hsp70 homolog) and groEL (hsp60 homolog). This modification of hsps was reversible on return of cells to control temperature. The authors also reported that acquired thermotolerance was lost upon return of heat shocked cells to a 25°C recovery period but that levels of hsps remained relatively unchanged. The authors proposed that the phosphorylated or activated

forms of dnaK and groEL may be critical in conferring acquired thermotolerance, perhaps as a result of an enhanced ability to bind damaged or misfolded proteins. Significantly, protein synthesis was not required for modification of dnaK and groEL. These observations thus offer a rational explanation for the present results in yeast. Heat shock at 37°C induces hsp synthesis and acquired thermotolerance. Incubation of cells at 25°C results in loss of thermotolerance but concurrent decrease in levels of hsp is not observed. By analogy to the observations in *E. coli* (Sherman and Goldberg, 1992, 1993) it seems feasible that in yeast, heat shock at 37°C induces activated, perhaps phosphorylated, hsps which confer thermotolerance. Subsequent return of cells to 25°C results in rapid deactivation or dephosphorylation of hsps and thus loss of thermotolerance. The fact that high levels of hsp remain without conferral of thermotolerance is thus explained by the latter proposal. It was noteworthy that Sherman and Goldberg (1993) suggested a common kinase and/or phosphatase, functioning in a temperature dependent fashion, is responsible for hsp modifications. In Chapter 7, further evidence of a temperature-regulated kinase, hexokinase A, is presented. Additional support for the concept of post-translational activation of hsps comes from observations that exposure of cells recovering at 25°C to a reheat shock at 37°C immediately confers thermotolerance (Cavicchioli and Watson, 1986; Chapter 6), presumably due to activation, perhaps phosphorylation of hsps. Whether this is due to activation of pre-existing hsps or newly synthesized hsps is explored in further detail in Chapter 6. However, the concept of heat shock activation of pre-existing hsps would serve to explain the current data concerning the compromise in tolerance induction in cycloheximide treated cells of Ysen (Fig. 5.1 D, Fig. 5.2 D). If hsps were to contribute to thermotolerance by alterations in their state rather than changes in absolute levels, then conceivably Yres may be sufficiently endowed with constitutive hsps, which following heat activation alleviate any such compromise (Fig. 5.1 B, Fig. 5.2 B). As an interesting corollary, Mackey and Derrick (1990) have proposed the existence of two mechanisms of thermotolerance, only one of which requires protein synthesis. Their evidence was derived from kinetics of thermotolerance acquisition in *Salmonella typhimurium*, constituting a rapid, initial increase that appeared to be unaffected by inhibition of protein synthesis with chloramphenicol and a slower second phase requiring protein synthesis. The present results, regarding cycloheximide treated heat shocked cells of Ysen (Fig. 5.1 D, Fig. 5.2 D) tend to support such a concept. Furthermore, it should be noted that an increase in *HSP 104* and *HSP 70* transcription following heat shock was not obvious in YNBG cells of

Ysen (Fig 5.3), despite a marked increase in translation (Fig. 5.5, Fig. 5.8A). These results suggest that the mechanism involved in tolerance conferral in this strain is post-transcriptional, resulting in preferential *de novo* translation. If this were the case, then it follows that inhibition of protein synthesis during heat shock would compromise Ysen in tolerance induction and subsequently, its maintenance. Inhibition of protein synthesis is known not to block transcriptional events associated with the heat shock response (McAlister and Finkelstein, 1980b; Craig, 1985; Hallberg *et al.*, 1985; Clarke *et al.*, 1996). Consequently, the question arises as to whether messages transcribed in heat shocked cells treated with cycloheximide could be translated during the recovery period subsequent to removal of cycloheximide from the culture medium. The data presented indicated that this was unlikely, as negligible hsp synthesis was evident in the latter cell type recovering from heat shock (Fig. 5.4-5.7). In addition, it has been previously reported that *S. cerevisiae* cannot sequester pre-existing messages from transcription (Lindquist, 1981) and furthermore, the typical half-life for yeast mRNAs is only 10-20 min (Tuite, 1996).

Related to the aforementioned observations in *E. coli* is a study by Seeger *et al.* (1996) using *Thiobacillus ferrioxidans*. Phosphorylation of dnaK and groEL was noted when cells were subjected to stresses other than heat shock, and more specifically in phosphate starved cells. The authors therefore proposed that phosphorylation of these two key proteins could be a general response of cells to stress conditions. Interestingly, the molecular chaperone Ydj1, a DnaJ homolog, has been shown to stimulate phosphorylation of the $G_1$ cyclin *Cln-3* of *S. cerevisiae*, in order that it may be degraded by the ubiquitin-proteosome proteolytic pathway (Yaglom *et al.*, 1996). Indeed, as outlined in the introduction to this chapter, it is now well accepted that several hsps function as molecular chaperones, preventing stress induced protein aggregation and assisting in refolding of damaged proteins during recovery from stress (Gething and Sambrook, 1992; Hartl, 1996) (see also Chapter 1). In addition, it has been revealed that hsp 104 disaggregates and reactivates stress damaged proteins (Parsell *et al.*, 1994; Vogel *et al.*, 1995). However, of significance to the present studies is the recent finding that a heat induced conformational change, resulting in increased hydrophobicity, activates hsp 90 whereupon it binds and protects proteins from irreversible aggregation (Yonehara *et al.*, 1996). This activity was shown to be latent under normal conditions and reversible following return to 25°C after heating. In this context, an alternate explanation concerning the current results of progressive decay of induced thermotolerance despite persisting hsp during recovery may

be proposed. Conceivably, a gradual saturation of the chaperone function of hsps may occur during the recovery phase. That is, during the recovery period, as heat shock induced damage is repaired, the supply of available chaperone molecules progressively becomes exhausted. This is coincident with loss of thermotolerance and resumption of further HSP transcription (Fig. 5.3) and translation (Fig. 5.4-5.7, Fig. 5.8) required for basal tolerance and cellular function. Also consistent with this model are observations of a more rapid decay of thermotolerance in cells treated with cycloheximide during heat shock (Fig 5.1 B, D). In this instance, it follows that the cells would rely on constitutively expressed hsps, a much smaller pool of chaperone molecules that would become depleted more rapidly following heat treatment. The importance of substrate binding by hsps to heat shock protection was recently demonstrated by Nwaka *et al.* (1996). It was shown that a conditional *SSC1* (mitochondrial *HSP 70*) mutant, deficient in ATP binding and hydrolysis, was unable to recover viability at 30°C following a 50°C treatment. The authors concluded that mitochondrial hsp 70 is involved in survival of cells after heat shock and suggested it may bind unfolded proteins in mitochondria to prevent their aggregation during hyperthermia.

In the present chapter work was also conducted to examine kinetics of trehalose mobilization with respect to acquired thermotolerance, on the basis that trehalose has been frequently implicated in yeast stress tolerance (Wiemken, 1990; Piper, 1993). The results demonstrated that the marked induction of trehalose subsequent to heat shock was followed by an equally marked decrease when cells were returned to 25°C (Fig. 5.9). Moreover, levels of trehalose remained essentially low throughout the recovery period. The salient feature of these observations was that the kinetics of trehalose accumulation and subsequent rapid mobilization did not match the kinetics of progressive loss of induced thermotolerance in YNBG cultures (Fig 5.1). These findings suggest that any protective role conferred by trehalose occurs during heat shock only. However, as previously discussed in Chapter 3, an obvious correlation between trehalose levels and intrinsic or induced thermotolerance was not evident. For example, the greater magnitude of tolerance induction in glucose grown Ysen compared to Yres was accompanied by only a 6-fold increase in trehalose compared to 20-fold in Yres. Furthermore, for cells grown on YNBA, although higher endogenous levels of trehalose were present and an increase in accumulation was observed upon heat shock, the levels were substantially less than in cells on YNBG, despite the fact that YNBA cells exhibited greater thermotolerance. Moreover,

trehalose content was not observed to decrease on return of cells to 25°C. Indeed the trend was an increase in the case of Ysen even though acquired tolerance was gradually lost during recovery, albeit less marked in YNBA cells compared to YNBG. Presumably, the latter case is attributable to higher constitutive hsp expression or some other intrinsic factor(s). In this regard, it was noteworthy that derepressed cells of a *tps1hsp104* mutant, deficient in trehalose and hsp 104 synthesis, were found to be still more resistant than wild type cells in logarithmic phase (Elliot *et al.*, 1996). The authors therefore suggested the existence of other, undefined mechanisms of resistance. Results generated in Chapter 7, currently in press (Gross and Watson, 1998a), using differential display of PCR amplified cDNA from Ysen and Yres show promise toward identification of other, unidentified mechanisms of stress resistance.

The current findings concerning trehalose both confirmed and extended those of Nwaka *et al.* (1994) who reported persistence of high levels of the disaccharide in a trehalase negative mutant, Δ*nth1*, following heat shock at 40°C and recovery at 30°C for 40 min, despite the loss of induced thermotolerance. In the present case, absence of mobilization of the trehalose pool during recovery in YNBA cells may be explained by low neutral trehalase activity observed in derepressed cells (De Virgilio, *et al.*, 1991; San Miguel and Argüelles, 1994). The presence of cycloheximide generally resulted in inhibition of heat shock induced trehalose accumulation in Yres by about 50% in YNBG and totally in YNBA and inhibited synthesis in Ysen in both media. However, all cell types exhibited at least some degree of heat shock induced thermotolerance.

Overall, coupled with conclusions arrived at in Chapter 4, the current results present a weak argument towards a role for trehalose in acquired thermotolerance, while hsps, in a heat shock activated state, appear to play a more significant role.

# Chapter 6: Heat shock, recovery and re-heat shock in repressed and derepressed cells

## 6.1 Introduction

As described in Chapters 3 and 5, there exists considerable controversy regarding the hypothesis that hsps are directly involved in the acquisition of thermotolerance. Such controversy may be attributed, in part, to observations that cells can acquire thermotolerance (as assayed by cell survival) following a prior heat shock in the absence of protein synthesis or induced hsps (Hall, 1983; Watson *et al.*, 1984; Smith and Yaffe, 1991). However, many such studies have ignored the role of constitutive stress proteins, several of which are present at significant levels in the cell at normal physiological temperatures (for eg, Fig. 5.8). Furthermore, some reports concentrate on the affects of trehalose on stress tolerance while dismissing a role for constitutive hsps (for eg, Eleutherio *et al.*, 1995). On the other hand, some investigations have attempted to make deductions concerning the role of hsps in thermotolerance based largely on their constitutive expression, disregarding heat shock induction (for eg, Hottiger *et al.*, 1992). To further compound these complexities, other studies still have been primarily concerned with evaluation of the function of hsps without relating cell survival data, making it difficult to assess whether the putative function may affect the thermotolerant state of the cell (for eg, Vogel *et al.*, 1995). In addition, there appears to be a general belief that innate and induced thermotolerance constitute the same mechanism, and few publications have dealt with or compared both. However, induced tolerance, being of a transient non-heritable nature (Watson, 1990; Watson *et al.*, 1996), may well comprise a related but distinct mechanism (a conclusion arrived at in Chapter 3). The question therefore remains whether constitutive hsps, while providing essential normothermic functions, may afford protective affects following an initial priming or activation event such as that provided by a heat shock pre-treatment.

In the previous chapter it was shown that cells recovering from a heat shock progressively lose induced thermotolerance to an extreme temperature challenge, despite the persistence in cells of elevated levels of hsps. Consequently, it was suggested that heat shock at 37°C induces activated, perhaps phosphorylated hsps that become deactivated (dephosphorylated) during the recovery period. Protein phosphorylation/dephosphorylation

is the major mechanism responsible for regulation of enzyme activity in response of cells to extracellular signals (for recent reviews see Hunter, 1995; Johnson and O'Reilly, 1996). Processes that are reversibly controlled by protein phosphorylation require a protein kinase and a protein phosphatase. These enzymes operate with different specificities so that reactions can be turned on or off in response to different stimuli (Cohen, 1992). Extracellular signals such as binding of hormones (Berridge, 1985) and growth factors (Cohen, 1986) to cell membrane receptors and agonists such as chemical stress and heat shock (Landry *et al.*, 1992; Rouse *et al.*, 1994), stimulate a series of intracellular reactions and recognition phenomena that result in the modification of proteins by a cascade of phosphorylation/dephosphorylation events. Phosphorylation may occur on single or multiple sites and leads to a variety of molecular responses including enzyme activation or inhibition, alteration of association/dissociation properties of protein-protein assemblies and alteration of protein surface-recognition properties (reviewed in Johnson and O'Reilly, 1996).

However, prior to consideration of investigations concerning hsp phosphorylation in *S. cerevisiae* strains, it was deemed necessary to further address the very concept of hsp activation, deactivation and subsequent reactivation. Consequently, work presented in the present chapter involves a comprehensive analysis of the kinetics of re-induction of thermotolerance subsequent to recovery of cells from an initial priming heat shock and describes any correlation between hsp transcription and translation, as well as trehalose mobilization. Results presented enabled the concept of reactivation of pre-existing hsps and their possible influence on thermotolerance to be addressed.

## 6.2 Results

In Chapter 3 the contrasting thermotolerance characteristics of *S. cerevisiae* strains Yres and Ysen were described. The progressive loss of induced thermotolerance during hyperthermic recovery at 25°C in repressed and derepressed cells of these strains was investigated in Chapter 5. In the present chapter, the kinetics of thermotolerance induced by two successive heat shock periods, subsequent to recovery of cells at 25°C from an initial 30 min heat shock at 37°C (termed re-heat shock) is demonstrated.

### 6.2.1 Induced thermotolerance

Results presented in Fig. 6.1 indicate that levels of thermotolerance induced by a second heat shock, following either 30 or 60 min at the recovery temperature, were compatible with those attained following the initial 37°C pre-treatment for both strains in YNBG and YNBA media. By contrast, recovering cultures not subjected to a second heat shock following either 30 or 60 min recovery, prior to exposure to heat stress, exhibited progressive loss of induced tolerance (as demonstrated in Chapter 5). While this was particularly evident in repressed cells of Ysen (Fig. 6.1 C), the trend was less obvious in derepressed cells (Fig. 6.1 B, D). The latter was observed despite higher stress temperatures employed (55°C and 52°C for Yres and Ysen respectively) due to the greater levels of intrinsic thermoresistance exhibited by these cells (see Chapter 5). A further, notable trend was that of increased maintenance of thermotolerance for the duration of the 60 min time course induced by a second heat shocked as compared with initial heat shock induction. This effect was not obvious in YNBA cultures, especially in the case of Ysen (Fig. 6.1 D).

### 6.2.2 *De novo* protein synthesis and trehalose accumulation

In Chapter 5 it was shown that a progressive loss of induced thermotolerance exhibited by recovering cells was not well correlated with levels of hsp or trehalose. This was also evident from the present data. A rapid suppression of synthesis of typical heat shock inducible proteins (Fig. 6.2, Fig 6.3) and substantial mobilization of heat shock induced trehalose (Fig. 6.4) was observed in recovering cells, despite significant retention of thermotolerance as compared with intrinsic values (Fig. 6.1). However, the present results have shown that cultures returned to control temperature for either 30 or 60 min following an initial inducing treatment, exhibited re-induction of hsp synthesis (Fig. 6.2, Fig. 6.3) and trehalose accumulation (Fig. 6.4) following a second heat shock treatment. This trend was apparent for cells grown in either YNBG or YNBA and was consistent with re-heat shock induced thermotolerance (Fig. 6.1). Levels of trehalose induced by a second heat shock exposure exceeded those accumulated following initial induction, with the exception of YNBG cultures of Yres. However, trehalose mobilization during recovery in YNBA cultures was less dramatic compared with cells grown in YNBG (Fig. 6.4). By contrast, trehalose content in recovering YNBG cultures was compatible with, if not less

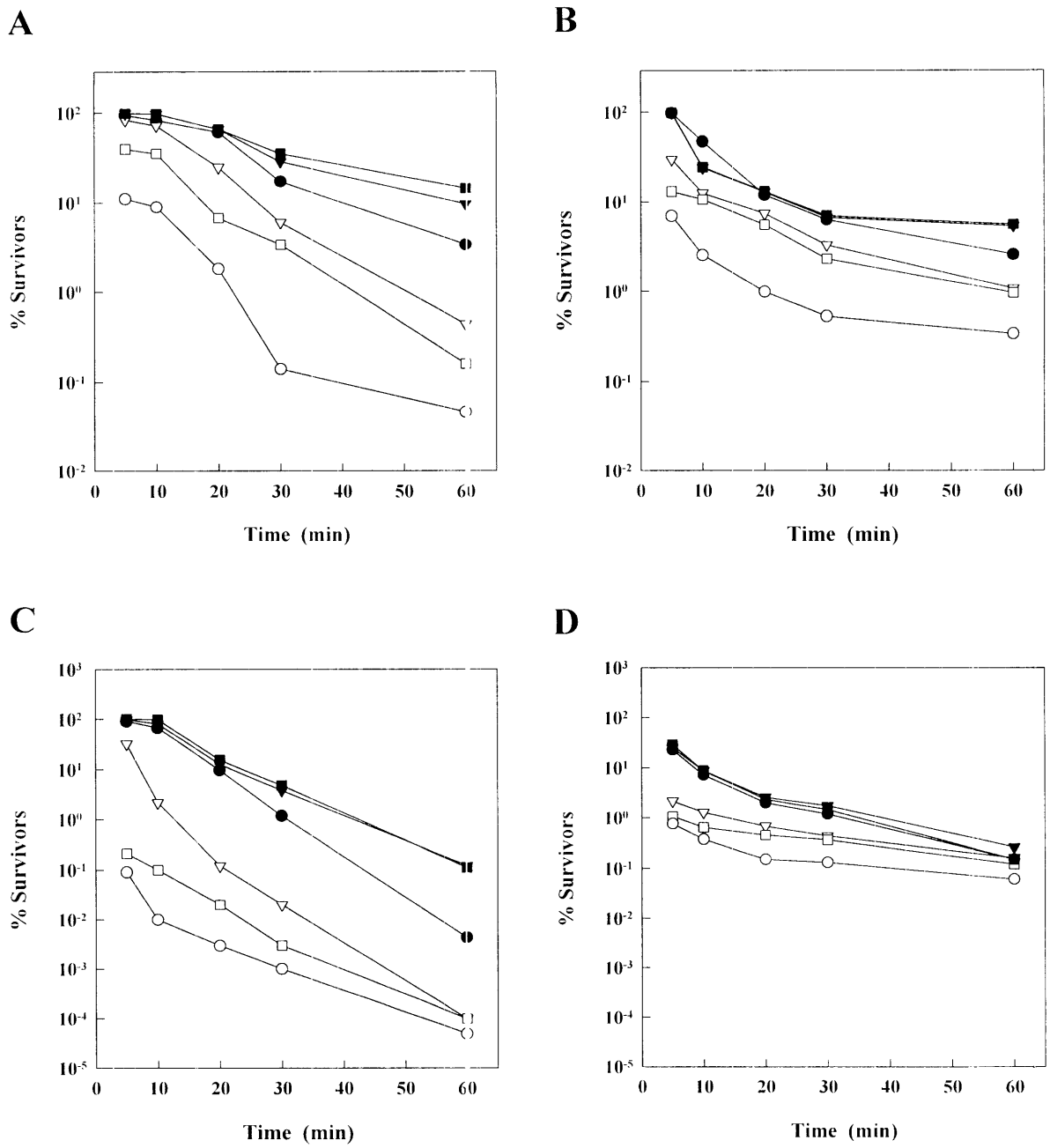


**Fig. 6.1.** Intrinsic (○) and induced (●) thermotolerance, its decay during recovery and subsequent re-induction in YNBG and YNBA media for Yres (A, B) and Ysen (C, D) respectively. Intrinsic tolerance was assessed following incubation at the following stress temperatures: 50°C (YNBG cultures), 55°C (Yres) or 52°C (Ysen) (YNBA cultures) for the times indicated. Similarly, induced tolerance was monitored at corresponding stress temperatures following a 30 min incubation at 37°C. Decay of induced thermotolerance was determined at stress temperatures following 30 (▽) and 60 (□) min recovery from heat shock at 25°C. Re-induced thermotolerance was determined at stress temperatures following 30 (▼) and 60 (■) min recovery at 25°C, subsequent to re-heat shock at 37°C for 30 min. Levels of thermotolerance are expressed as the percentage of viability with respect to a 25°C control sample in each case.

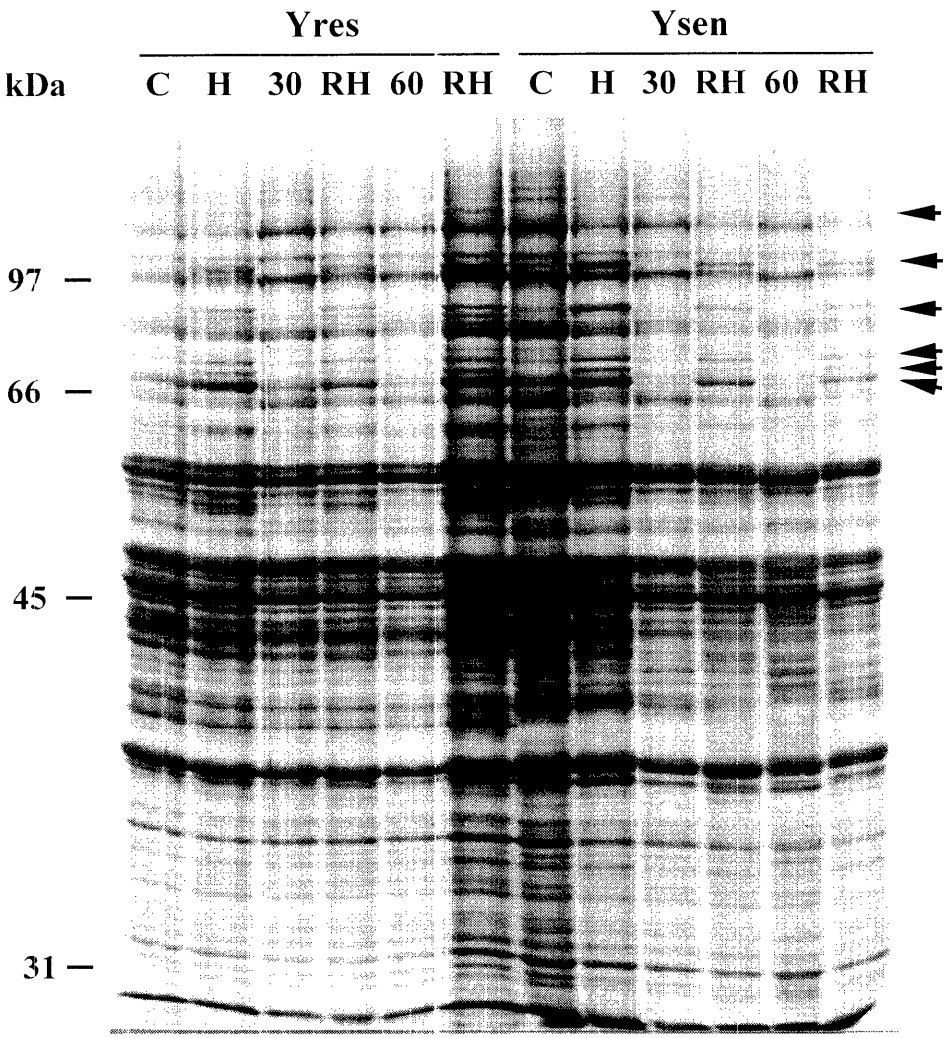


**Fig. 6.2.** SDS-polyacrylamide gel autoradiogram of [$^{35}$S]-methionine labelled protein extracts from control (C), heat shocked (H), recovering (30 and 60 min) and re-heat shocked (RH) cultures of Yres and Ysen grown in YNBG medium. Heat shock was carried out at 37°C (30 min). Cultures were returned to 25°C for either 30 or 60 min and subsequently re-heat shocked at 37°C (30 min). Arrows indicate new or increased protein bands in heat shocked samples compared with control. Molecular mass standards (kDa) are as indicated.

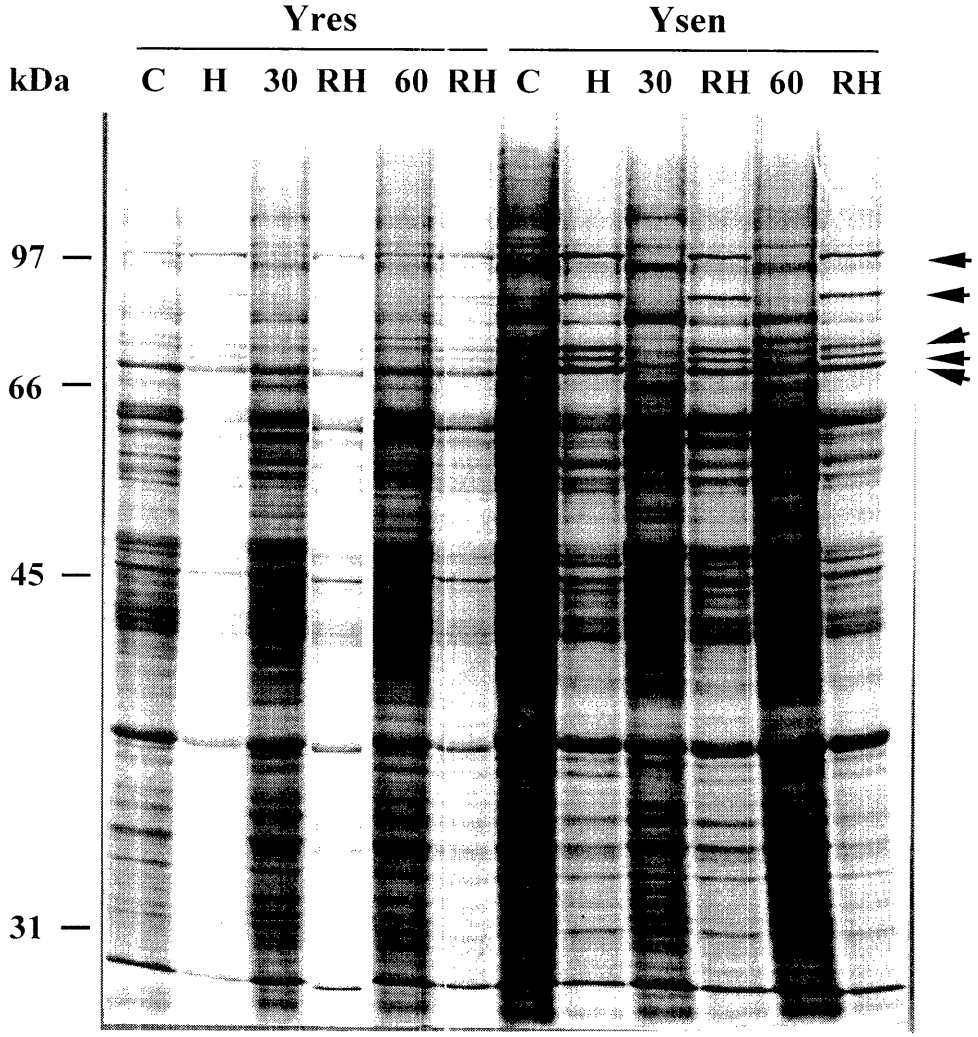


**Fig. 6.3.** SDS-polyacrylamide gel autoradiogram of [$^{35}$S]-methionine labelled protein extracts from control (C), heat shocked (H), recovering (30 and 60 min) and re-heat shocked (RH) cultures of Yres and Ysen grown in YNBA medium. Heat shock was carried out at 37°C (30 min). Cultures were returned to 25°C for either 30 or 60 min and subsequently re-heat shocked at 37°C (30 min). Arrows indicate new or increased protein bands in heat shocked samples compared with control. Molecular mass standards (kDa) are as indicated.

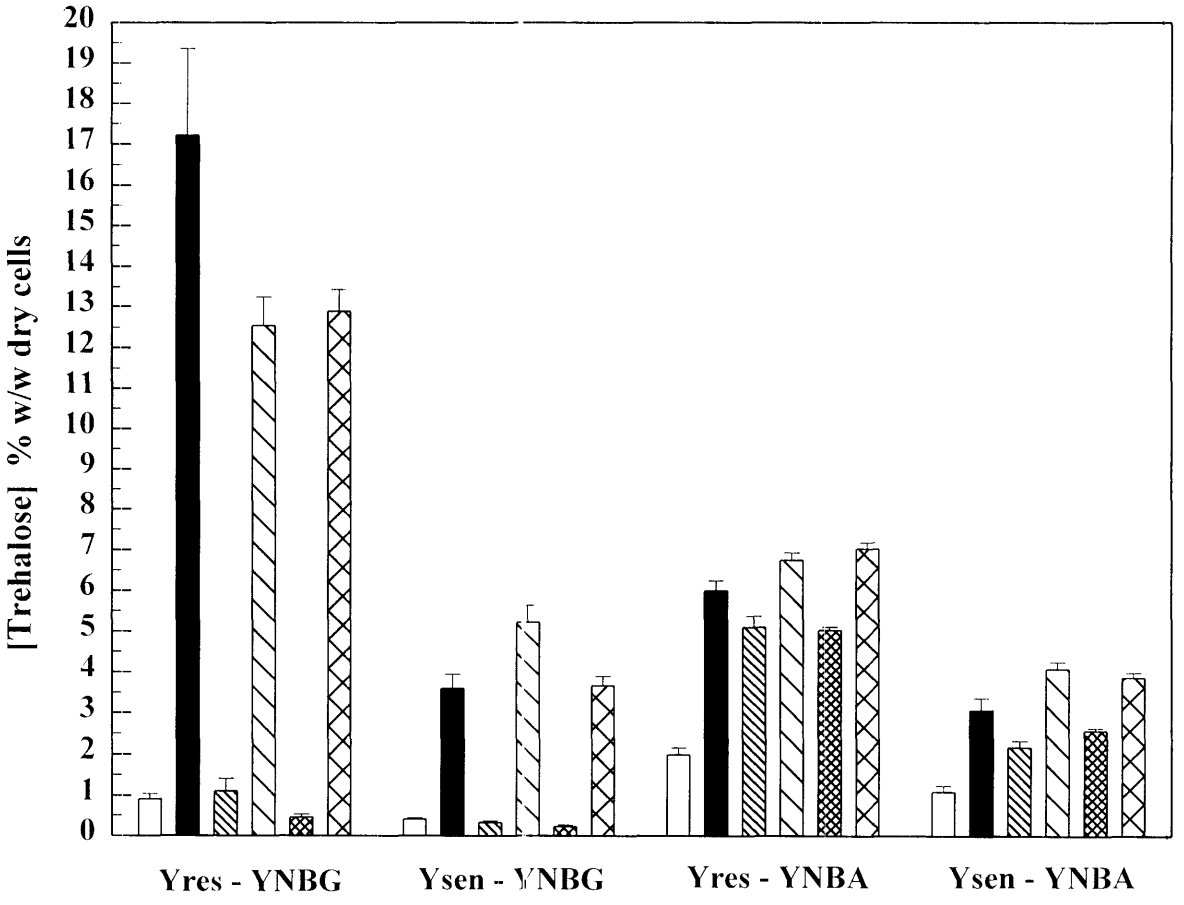


**Fig. 6.4.** Trehalose levels in control (□), heat shocked (■), recovering (30 ▨ and 60 ▩ min) and re-heat shocked cultures of Yres and Ysen. Cells were grown to mid-log phase in either glucose (YNBG) or acetate (YNBA) medium (25°C) and heat shocked at 37°C (30 min). Cultures were returned to 25°C and re-heat shocked following 30 ( ▨ ) or 60 ( ▩ ) min incubation. Error bars represent the standard deviation of measurements from three experiments.

than, that of control cells. This trend was also apparent in Fig. 6.2 and Fig. 6.3, where intensities of *de novo* hsp synthesis, following both 30 and 60 min recovery periods at 25°C appeared less than those of constitutive bands in control cells.

### 6.2.3 HSP transcription

Patterns of *de novo* protein synthesis during heat shock and recovery were in general agreement with transcriptional levels of *HSP 104* and *HSP 70* (Fig. 6.5). Heat shock induced transcriptional activity was evident for both genes in YNBA and YNBG cultures of Ysen and Yres. Following 30 and 60 minutes incubation at the recovery temperature, a general pattern of a marked decrease in transcription, to levels below those observed constitutively, particularly for *HSP 104*, was noted. However, re-induction of transcription, compatible with levels attained subsequent to initial induction, was evident for both *HSP 104* and *HSP 70* following a second heat shock treatment (Fig. 6.5). In addition, Yres exhibited greater constitutive transcription of *HSP 104* than Ysen, in both media, which was in agreement with greater translational efficiency observed from Western analysis (Fig. 6.6).

### 6.2.4 Western blot analysis of proteins

Levels of hsps 104 and 70 following heat shock, recovery and re-heat shock were determined by immunological cross-reaction of total protein profiles with respective anti-hsp antibodies. Contrary to the situation with either *de novo* protein synthesis, trehalose content or HSP transcription, high levels of the two proteins were shown to persist in cells following recovery at 25°C, for 30 and 60 minutes (Fig. 6.6). In most cases, these levels remained in excess of those exhibited by control cells and were only marginally lower than those produced subsequent to initial heat shock induction. Again, as observed in Chapter 5, this observation was not well correlated with the loss of thermotolerance exhibited by recovering cells, particularly after 60 minutes at 25°C (Fig. 6.1). However, consistent with results for *de novo* protein synthesis, trehalose content and HSP transcription, a second heat shock exposure was observed to re-induce hsps 104 and 70, although not to levels greater than those induced following the first heat shock treatment.

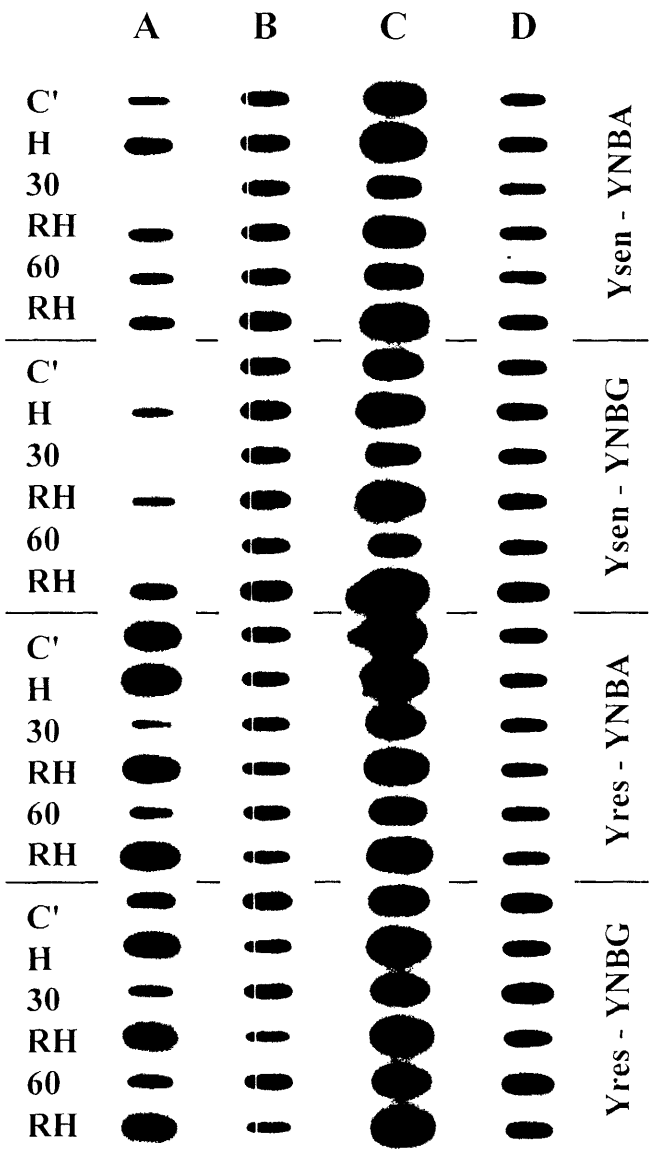


**Fig. 6.5.** Total RNA slot blot analysis of *HSP 104* (A) and *HSP 70* (C) expression from 25°C control (C'), heat shocked (H), recovering (30 and 60 min) and re-heat shocked (RH) cultures of Yres and Ysen. Heat shock was carried out at 37°C (30 min). Cultures were returned to 25°C for either 30 or 60 min and subsequently re-heat shocked at 37°C (30 min). Cells were grown in either glucose (YNBG) or acetate (YNBA) supplemented medium. Membranes were rehybridized with *ACT1* (B and D) as a loading control.

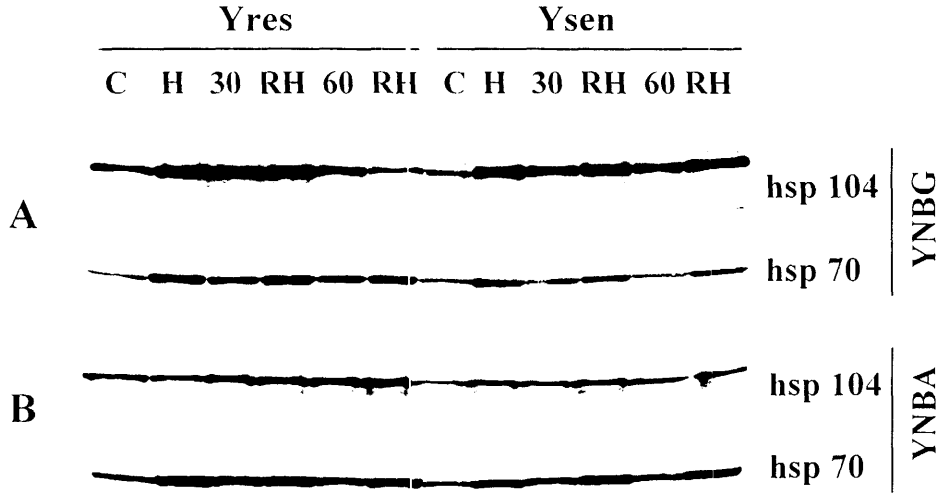


**Fig. 6.6.** Western immunoblot analysis of protein extracts from control (C), heat shocked (H), recovering (30 and 60 min) and re-heat shocked (RH) cultures of Yres and Ysen grown on glucose (YNBG) (A) or acetate (YNBA) (B) medium. Heat shock was carried out at 37°C (30 min). Cultures were returned to 25°C for either 30 or 60 min and subsequently re-heat shocked at 37°C (30 min). Immune complexes were generated for anti-hsp 104 and 70 antibodies.

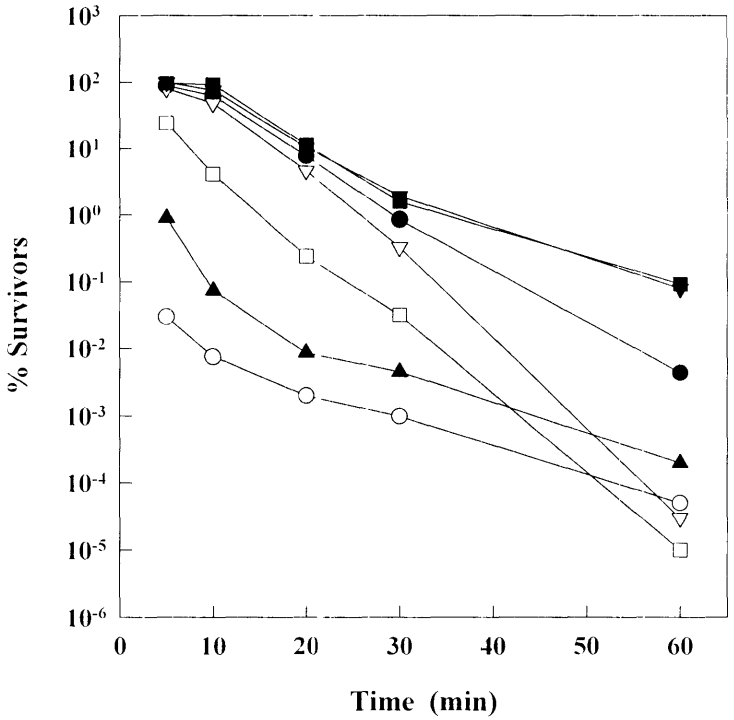


**Fig. 6.7.** Intrinsic (○), induced (●) and re-heat shock induced thermotolerance in the presence (▼,■) or absence (▽,□) of protein synthesis in YNBG cells of Ysen. Intrinsic tolerance was assessed following incubation at 50°C for the times indicated. Induced tolerance was determined at 50°C following a 30 min heat shock at 37°C. Re-induction of thermotolerance was determined at 50°C following a second heat shock treatment subsequent to either 30 (▼,▽) or 60 (■,□) min recovery from heat shock at 25°C. Protein synthesis was inhibited by incubation with cycloheximide (20μg $ml^{-1}$) for 20 min prior to and during a second heat shock.

### 6.2.5 Re-induced thermotolerance in the absence of protein synthesis

Results presented in Fig. 6.7 indicate that glucose grown cells of Ysen were compromised in thermotolerance acquisition to a 50°C heat challenge when protein synthesis was inhibited during the 37°C pre-treatment. This was in contrast to results obtained from cycloheximide treatment of Yres (see Fig. 3.7). Yet remarkably, recovering cells previously exposed to heat shock in the presence of protein synthesis, and thus able to accumulate hsps, exhibited substantial re-heat shock induced tolerance in the absence of protein synthesis, albeit of lower magn tude and maintenance than their counterparts not treated with cycloheximide (Fig. 6.7). However the intensity of this affect appeared to decrease over time. That is, cells allowed to recover at 25°C for 60 min prior to cycloheximide treatment and exposure to a second heat shock, acquired lower levels of tolerance to the subsequent 50°C challenge compared with cells recovered for only 30 min.

## 6.3 Discussion

In the previous chapter it was shown that molecular and physiological events associated with the heat shock response were readily reversible following removal of cells from the heat shock temperature. Cells allowed to recover exhibited rapid suppression of HSP transcription and *de novo* hsp synthesis, as well as mobilization of accumulated trehalose, accompanied by a distinctly more progressive loss of heat shock induced thermotolerance. The present experiments, reported in part by Watson *et al.* (1996), have expanded on these studies by demonstrating the kinetics of re-induction of thermotolerance, HSP transcriptional and translational activity and trehalose synthesis. These parameters were investigated in cells re-exposed to a second heat shock following recovery at 25°C, for either 30 or 60 min, from an initial heat shock at 37°C.

Fig. 6.1 indicates that re-heat shock induced thermotolerance was higher and maintained longer than levels attained after an initial heat shock treatment, particularly for cells grown in YNBG medium. These results therefore suggest a cumulative influence of the putative thermo-protective agents induced during the two successive heat treatments. However, further evidence of such an affect was not apparent from the data. Western blot analyses indicated that induced hsps persisted in cells during recovery, at levels compatible to those induced by the first heat shock, a subsequent re-heat shock did not produce accumulation of hsps to levels in excess of those observed following the initial pre-

conditioning treatment (Fig. 6.6). In addition, protein synthetic profiles showed that while the onset of thermotolerance was coincident with synthesis of the major hsps induced upon a second heat shock treatment, induction following the first heat shock appeared more intense (particularly evident for YNBA cultures) (Fig. 6.2, Fig. 6.3). Taken together, these results imply that cells exhibit re-heat shock induced supplementation of hsps to the pool induced by the first heat treatment. Such a concept was previously investigated in rodent and mammalian cells by Mizzen and Welch (1988) who described a negative 'titration' affect of hsp 70 as a function of pre-existing levels prior to a re-heat shock. However, the present results were consistent with more recent findings by investigators who observed hsp re-induction, albeit at reduced values, following sequential application of stress treatment to rat hepatoma cells (Weigant *et al.*, 1995), an effect precluded by the former titration hypothesis. Further evidence that favours a supplementation affect rather than hsp accumulation may be derived from results concerning re-heat shock induced transcriptional activity of HSPs 104 and 70 (Fig. 6.5). Following a marked decrease in transcription during recovery, which is consistent with suppression of *de novo* protein synthesis (Fig. 6.2, Fig. 6.3), mRNAs induced by a second heat shock increased to the same level as induced by the initial heat treatment. Given the observation that induced hsps persisted at high levels in cells during recovery, one would therefore expect to see re-heat shock augmentation of hsp levels, that is, to levels in excess of those induced by the initial heat shock, upon western analysis. However, this was not the case. Consequently the notion of increased re-heat shock induced thermoprotection afforded by successively accumulated quantities of hsps may be eliminated.

These observations raised the issue of whether all re-heat shock induced HSP transcripts are actually translated. The absence of hsp hyper-accumulation following two successive heat shock treatments discussed above suggests that this was not the case. It is possible that not all re-heat shock induced hsp mRNAs are required to be translated due to the persistence in cells of hsps induced by the first heat treatment, which conceivably, may be reactivated as a consequence of the second heat shock (proposed in Chapter 5, expanded upon below). The data therefore implies that inducibility of hsps occurs by a mechanism involving feedback by previously produced or pre-existing quantities. Unlike previous findings however, which demonstrated that yeast hsp 70s act as transcriptional repressors of their own genes (Stone and Craig, 1990), the present results tended towards negative regulation at the post-transcriptional level. Consistent with this concept was the

observation that in most cases, post-heat shock transcription of HSPs 104 and 70 had increased to greater levels subsequent to 60 min at the recovery temperature as compared with 30 min (Fig. 6.5). As previously described in Chapter 5, this increase continued throughout a 240 min recovery period. However, as evident from *in vivo* [$^{35}$S]-methionine labelling of proteins, these transcripts were not being translated (Fig. 6.2, Fig. 6.3). Also in Chapter 5, the concept of heat shock induced hsps becoming either de-activated or progressively substrate bound during recovery was proposed. Following this line of thought, it is possible that as the pool of heat shock induced non-associated or free hsps becomes depleted, or heat shock activated hsps become deactivated, any negative regulation on basal transcription is alleviated and it gradually increases. However, it is unlikely that these messages be sequestered for translation during an ensuing re-heat shock as earlier studies have shown that yeast cells, in contrast to *Drosophila*, do not induce a mechanism for this purpose (Lindquist, 1981).

It was observed that transcription of HSPs 104 and 70 was consistently more intense in Yres as compared with Ysen (Fig. 6.5, see also Fig. 5.3), whereas heat shock induced translation of hsps appeared more intense in Ysen (Fig. 6.2, Fig 6.3, see also Fig. 3.6). Although this apparent anomaly could be related to the greater magnitude of thermotolerance induction observed in Ysen (Fig. 6.1), it provided further evidence in support of a post-transcriptional influence on auto-regulation of hsps. That is, the greater constitutive transcription of HSPs in Yres (Fig. 6.5) and consequent constitutive translation (Fig. 6.6) may constitute a pool of pre-existing hsps that exert a negative regulatory affect on the extent of induced translational activity during heat shock. Whereas in Ysen, basal expression is comparatively limited and upon heat shock, the majority of induced transcripts would be translated.

During the course of experimentation for various aspects of this thesis it was observed that hsp induction in YNBA medium was consistently more intense than in YNBG (Fig. 3.6, Figs. 5.4-5.7, Fig. 6.2 and Fig. 6.3) particularly for Ysen. As previously described (Chapters 1 and 5), involvement of hsps in repair of damage to denatured, aggregated and misfolded proteins during recovery has been well documented (Ananthan *et al.*, 1986; Gething and Sambrook, 1992; Parsell *et al.*, 1994; Hartl, 1996). In this context, it is conceivable that following hyperthermia a pre-requisite level of chaperone activity, proportional to the extent of damage incurred would be required. Intuitively, YNBG cells are more obvious candidates for susceptibility to heat induced damage than

YNBA cells, the latter exhibiting markedly greater intrinsic resistance (Fig. 6.1, see also Fig. 3.1). However, an explanation exists for this apparent paradox. Nutritionally compromised cells such as those grown with a limited carbon or nitrogen source, may already be in a stressed state (Werner-Washburne *et al.*, 1993; Nwaka *et al.*, 1995). Indeed, the present data demonstrates that they exhibit greater constitutive levels of hsps 104 and 70 (Fig. 6.5, Fig. 6.6, see also Fig. 5.8), confirming previous observations (Sanchez *et al.*, 1992; Werner-Washburne *et al.*, 1993; Nwaka *et al.*, 1995). Subjection of the latter cell type to a heat shock may be perceived as further stress and consequently registered as a synergism of deleterious influences. It follows that sensory mechanisms in these cells may be stimulated to a greater extent, effectively priming them for repair of a greater amount of stress induced damage. The emphasis for hsp function in this instance would be on restoration of cellular homeostasis, consistent with results that indicated, in the case of Yres, *de novo* hsp synthesis was not required for induced thermotolerance *per se* (see Chapter 3). Therefore, it is possible that absolute levels of hsps do not influence the extent of tolerance at all (see discussion below). However, this would be in contrast to the situation in mammalian cells. Levels of hsp synthesis and degradation have been shown to be well correlated with the kinetics of thermotolerance induction and decay respectively (Landry *et al.*, 1982; Li and Werb, 1982, Subjeck *et al.*, 1982), particularly for hsp 70 (Li and Mak, 1989).

In contrast to the situation with Yres, results presented in Chapter 3 of this thesis demonstrated a necessity for *de novo* protein synthesis, if not specifically hsp synthesis, during heat shock to facilitate maximal induction of thermotolerance in Ysen to a subsequent heat stress. This situation provided a model system with which to attempt evaluation of some of the theories and speculations proposed in the current and preceding chapters. That is, to pre-load cells of Ysen with putative thermoprotective hsps during an initial heat shock treatment, followed by recovery periods at 25°C and subsequent exposure to a second heat shock, but this time in the absence of protein synthesis. Indeed, it was observed that cells of Ysen, pre-loaded with excess levels of hsps, were able to acquire thermotolerance in the absence of protein synthesis (Fig. 6.7), albeit less marked than their synthesising counterparts. Results presented in Chapter 5 indicated that thermotolerance waned during recovery despite persisting hsps induced as a consequence of a prior heat shock. It seems unlikely therefore that Ysen should develop thermotolerance during re-heat shock in the absence of further protein synthesis unless this

second heat treatment mediates re-activation of pre-existing hsps or other thermoprotective agents. The results therefore support the hypothesis concerning hsp re-activation. In addition, it was also observed that the extent of tolerance induction following a 60 min recovery period was less than that attained following 30 min recovery and had decreased substantially in comparison with tolerance levels exhibited by cells not treated with cycloheximide prior to and during a second heat shock. The most salient point in this respect was that levels of thermotolerance acquired in the absence of protein synthesis by cells pre-loaded with hsps, were still distinctly higher than tolerance levels remaining in cells recovered for equal periods but not subjected to a pre-conditioning heat shock (Chapter 5, Fig. 5.1). It follows, and supports the previous proposal, that supplementation of hsps is required to confer maximal thermotolerance upon successive heat treatments, with only a proportion of those hsps remaining in the cell from the previous heat shock being available for re-activation. As the duration of the recovery period increases, the pool of pre-existing hsps would become saturated with chaperone functions or substrate associations, depleting the proportion of molecules available for re-activation upon a subsequent heat shock. The major aspects of this proposed model are summarised in Fig. 6.8.

As a corollary from the preceding arguments, cells constitutively over-expressing hsps would have an increased inherent capacity to acquire thermotolerance. However, there exists considerable evidence in the literature to the contrary. In a recent study it was shown that over expression of either *SSA1*, *SSA2* or *SSA4* (members of the hsp 70 family) in *S. cerevisiae*, neither protected cells from thermal stress at 50°C, nor had a significant affect on the development of thermotolerance following a 40°C heat shock (Weitzel and Li, 1993). Presumably, under these conditions, activation of pre-existing hsp 70 was insufficient to confer thermotolerance. Similar results, also in *S. cerevisiae*, have indicated that over expression of neither hsp 26 (Tuite *et al.*, 1990; Lindquist, 1991) nor hsp 90 (Finkelstein and Stausberg, 1983) enhanced heat shock associated thermotolerance. On the other hand, it has been shown that over expression of hsps 27 (Landry *et al.*, 1989) and 70 (Li *et al.*, 1992) in the mammalian system does increase thermal resistance. Perhaps the situation in yeast is such that high concentrations of any one hsp alone, with the possible exception of hsp 104 (Lindquist and Kim, 1996) are not sufficient to produce the thermotolerance phenotype. Indeed recent investigations have shown that hsp 70, hsp 90

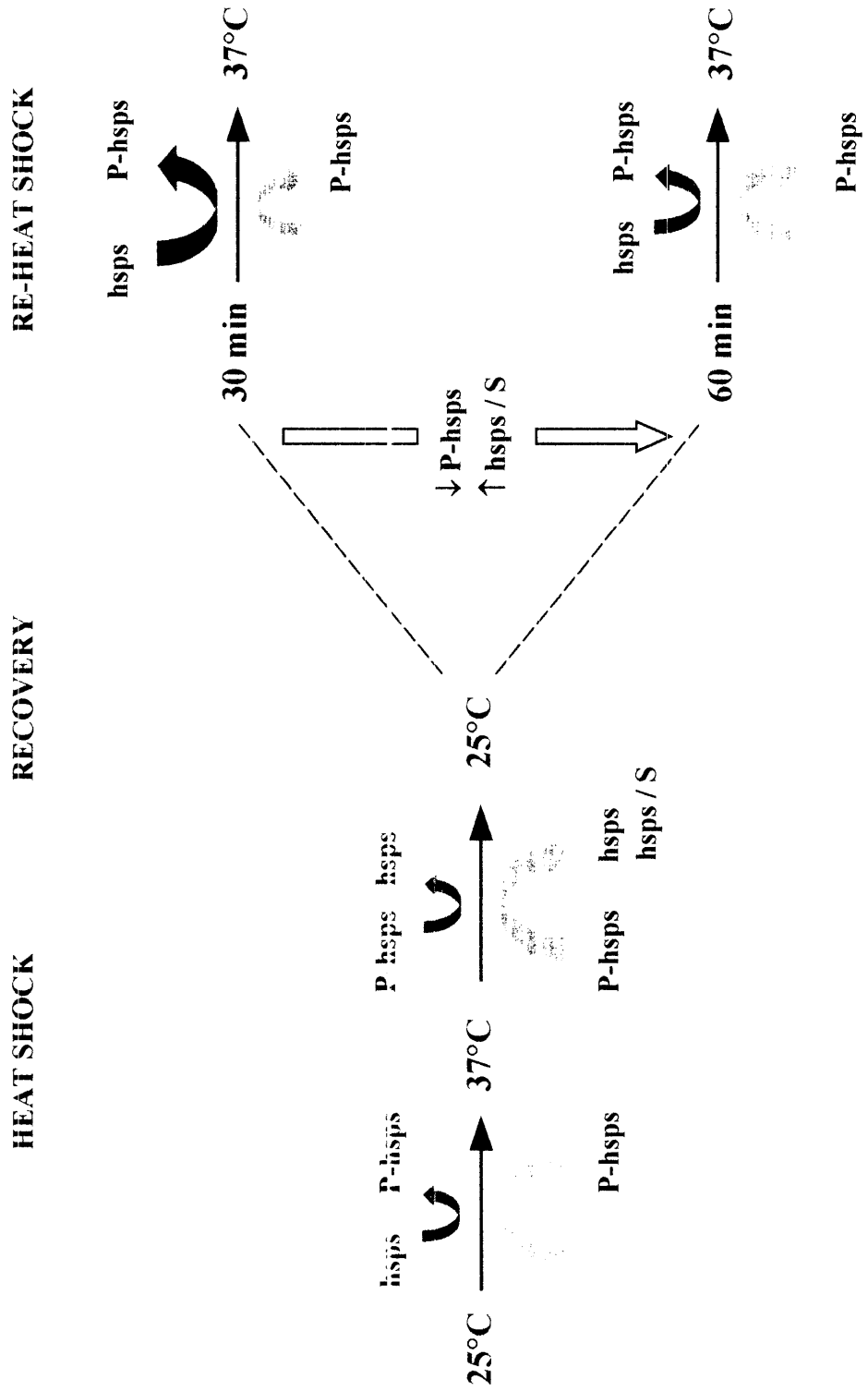


**Fig. 6.8.** Diagram representing proposed model of hsp activation, deactivation and reactivation following heat shock, recovery and re-heat shock, respectively. Red arrows denote activated/phosphorylated hsps synthesised *de novo* during heat shock at 37°C (P-hsps), subsequent deactivation (hsps) and/or hsp substrate association (hsps / S) during recovery at 25°C and reactivation following a second heat shock after 30 or 60 min recovery. Blue arrows denote activation, deactivation and reactivation of constitutive or pre-existing deactivated hsps, persisting in cells from a previous heat shock. Sizes of arrows indicate the relative quantities of activated, *de novo* synthesized or deactivated hsps. Open arrow represents progressive deactivation/dephosphorylation of hsps and/or increase in hsp substrate association as duration of the recovery period increases.

and DnaJ (or the yeast homolog YDJ-1) function cooperatively to renature damaged proteins in the eukaryotic cytoplasm (Schumacher *et al.*, 1996). Furthermore, Vogel *et al.* (1995) have shown that the combined function of hsps 104 and 70 was able to restore RNA splicing to a greater degree than optimal concentrations of either protein alone. In support of this concept, the present studies indicated that all major hsps exhibited heat shock induction, followed by cessation of *de novo* synthesis during recovery and subsequent re-heat shock induction, in concert (Fig. 6.2, Fig. 6.3). The sensitivity of major hsps to induction by heat thereby appears to occur via a common mechanism that ensures cooperative action in the protection from or restoration of the presumable multitude of ensuing lesions. Over- expression studies therefore do not preclude the possibility of re-activation of pre-existing hsps.

The latter concept has, to date, remained largely unaddressed. An earlier study conducted on *E. coli* demonstrated that cells containing elevated levels of hsps at 28°C did not acquire thermotolerance at 42°C if further protein synthesis was prevented with chloramphenicol (Van Bogelen *et al* 1987). The authors therefore concluded that activation of pre-existing hsp did not occur and *de novo* protein synthesis was required for tolerance acquisition during heat shock. While these findings were inconsistent with the present results, closer examination of the experimental protocols leads to an alternative interpretation. Pre-existing hsps in the *E. coli* cells, containing multiple copies of the heat shock regulatory gene *htpR* (*rpoH*) under the control of an isopropylthiogalactoside (IPTG) inducible promoter, were induced in response to IPTG treatment rather than heat. It is possible that the pattern, structure or even intracellular localization of hsps (see below) induced in this manner is different to that induced by heat. Consequently, pre-existing hsps in this case may not have had the potential to confer thermotolerance. This notion was consistent with more recent findings in mammalian cells. It was shown that the pattern of hsp re-induction following a second stress treatment is independent of any pre-treatment and influenced mainly by the second stress applied (Weigant *et al.*, 1995).

Recent investigations in yeast, which support the current work, comprised unexpected results concerning the HSF mutant, *hsf1-m3*. It had been previously shown that mutant cells were deficient in hsp induction but not thermotolerance acquisition (Smith and Yaffe, 1991). This had provided one of the most compelling contradictions concerning the involvement of hsps in thermotolerance. However, Lindquist and Kim (1996) demonstrated that *hsf1-m3* constitutively expresses hsp 104 at higher levels than wild type

cells and that the mutation reduces but does not completely block heat induction of hsp 104. Using a heterologous promoter to express hsp 104 at different levels in strains carrying hormone regulated expression plasmids, it was found that thermotolerance provided by even low levels of the protein was enhanced by moderate heat pre-treatments, as compared with control strains not expressing hsp 104. It was consequently suggested that constitutively expressed hsp 104 may be less active and that mild heat pre-treatments potentiate its capacity to confer thermotolerance. It follows therefore, that the higher degree of constitutive expression of hsp 104 in YNBG cultures of Yres and YNBA cultures of Yres and Ysen (Fig. 5.8, Fig. 6.6) rendered these cell types less sensitive to the deleterious effect of cycloheximide on tolerance acquisition observed in YNBG cells of Ysen (Fig. 3.7, Fig. 5.1 D).

In the preceding chapter the possibility that the mechanism responsible for re-activation of pre-existing hsps may involve heat induced phosphorylation was discussed. While the existence of such a mechanism for hsps has been sought in bacteria (Sherman and Goldberg, 1992; Seeger *et al.*, 1996), there is a dearth of evidence concerning its presence in yeast. However, detailed structural data concerning the phospho and dephospho forms of yeast glycogen phosphorylase (ygp) have recently become available (Lin *et* al., 1996). These results highlighted the importance of phosphoamino acids in the organization of local regions of protein structure and in the promotion of long-range conformational responses (Johnson and O'Reilly, 1996). Ygp is mobilized during the approach to stationary phase, under transcriptional control, to enable cells to utilize glycogen under conditions of substrate depletion. Of pertinence to the present discussion was the observation that phosphorylation of ygp resulted in over a 1000-fold increase in its activity (Newgard *et al.*, 1989). It was also noteworthy that a recent study has demonstrated a rapid increase in the protein kinase activity of yeast *SNF1* as a result of phosphorylation of Snf1p following removal of glucose from the culture medium (Wilson *et al.*, 1996). Genetic studies of *S. cerevisiae* have indicated that Snf1p and Snf4p, which together form the SNF1 complex, are essential for derepression of glucose repressed genes (Zimmermann *et al.*, 1977; Carlson *et al.*, 1981). Furthermore, SNF1 was shown to be inactivated by dephosphorylation and reactivated by phosphorylation (Wilson *et al.*, 1996), an ability it has in common with its mammalian homologue, the AMP-activated protein kinase (AMPK) (Carling *et al.*, 1994; Mitchelhill, 1994). The mammalian AMPK is activated in response to environmental stress (Hardie *et al.*, 1994) where it is postulated to

function in inactivating non-essential pathways that deplete ATP (Corton *et al.*, 1994). The significance of these findings to the present work constitutes the possibility that derepressed cultures of Yres and Ysen, exhibiting markedly higher levels of intrinsic thermotolerance (Fig. 3.1), may not only be endowed with higher constitutive hsp expression but activated, phosphorylated hsps. The further increase in tolerance observed in such cells following heat shock would be explained by the observed increase in *de novo* hsp synthesis, supplementing the pre-existing pool (see Fig. 6.8).

Other evidence exists in the literature for heat induced phosphorylation of hsps in the mammalian system. Of particular note, a dramatic increase in the phosphorylation state of hsp 27 within minutes of exposure to heat or chemical stress has been previously documented (Arrigo and Welch, 1987; Chrétien and Landry, 1988; Landry *et al.*, 1991; Landry *et al.*, 1992). While studies of mouse or Chinese hamster cell lines transformed with the human *HSP 27* gene have provided direct evidence that elevated levels of hsp 27 play a major role in establishing thermotolerance (Landry *et al.*, 1989), changes in hsp 27 phosphorylation state in the absence of its accumulation have also been associated with acquisition of thermotolerance (Landry *et al.*, 1991). More recently, a novel protein kinase cascade responsible for the phosphorylation of hsp 27 following cellular stress or heat shock was identified (Rouse *et al.*, 1994). However, in some cases the situation is less clear. For example, a dramatic increase in the rate of both phosphate uptake and dephosphorylation of hsp 90 has been demonstrated in HeLa cells following acute stress (Legagneux *et al.*, 1991). In plant cells, a soluble, short lived hsp 95 has been shown to be a phospho-protein not detectable in control cells (Key *et al.*, 1982; Nover and Scharf, 1984), whereas hsps 80 and 70 have been shown to be modified by both phosphorylation and methylation (Nover and Scharf, 1984). Significantly, Duncan and Hershey (1989) demonstrated that the phosphorylation states of eukaryotic protein synthesis initiation factors eIF-2$\alpha$ and eIF-4B acquired during heat stress in HeLa cells, are reversed within 30 min following return to control temperature. Whether such a mechanism is responsible for re-activation of pre-existing hsps in yeast remains to be resolved. However the present data suggested that any such process was reversible and in addition, was independent of active protein synthesis. Additional recovery experiments similar to those described in Chapter 5 were performed on glucose grown Yres and Ysen. However, cycloheximide was added to the culture medium towards the end of the heat shock period, prior to restoration of control temperature (results not shown). It was observed that inhibition of

protein synthesis prior to and during recovery in this manner, did not prevent the progressive loss of thermotolerance previously observed (Fig. 5.1), in either Yres or Ysen. Similar results have been previously obtained following chloramphenicol treatment of heat shocked *Salmonella typhimurium*, prior to temperature shift down (Mackey and Derrick, 1989).

Significantly, Sherman and Goldberg (1992, 1993) demonstrated heat shock associated phosphorylation of hsps in the absence of protein synthesis in *E. coli*. Similarly, Legagneux *et al.* (1991) reported that increased [$^{32}$P]-labelling of hsp 90 in heat stressed HeLa cells was not affected by the presence of cycloheximide in the culture medium. Conversely, dephosphorylation of *S. cerevisiae* sip1p upon addition of glucose to derepressed glycerol/lactate grown cells has been shown to occur in the absence of protein synthesis; inhibited by addition of 100μg ml$^{-1}$ cycloheximide (Long and Hopper, 1995). Consequently, the currently proposed function of phosphorylation dependent activation of pre-existing hsps during heat shock in yeast (summarised in Fig. 6.8) may extend to cells heat shocked in the absence of *de novo* protein synthesis. In this regard, data presented in Chapter 5 indicated that the rate of loss of thermotolerance was greater in cells heat shocked in the absence of protein synthesis (Fig. 5.1). In the context of the present discussion, the inference from the latter would be that the smaller constitutive pool of pre-existing hsps was deactivated more rapidly during recovery from hyperthermia. Interestingly, Jämsä *et al.* (1995) reported that a fusion protein, designated hsp150Δ-β-lactamase, accumulated in the yeast endoplasmic reticulum following a 37°C heat shock and became inactivated following a 48-50°C heat stress. Return of cells to 24°C resulted in an ATP-dependent reactivation of β-lactamase activity, even in the absence of *de novo* protein synthesis. The 321 aa segment of hsp 150 incorporated into the fusion protein facilitates folding of the β-lactamase portion to a secretion-competent and bioactive conformation. Consequently, the authors concluded that in addition to cytoplasmic and nuclear events, refolding of heat damaged proteins in the endoplasmic reticulum forms part of the heat stress survival mechanism in yeast (Jämsä *et al.* 1995). As noted in Chapter 3, a 130-150 kDa heat inducible protein was evident in both yeast strains under investigation in the present work. Taken together, results of Jämsä *et al* (1995) and the present findings appear to suggest that heat shock associated activation of hsps is necessary for enhanced chaperone function resulting in reactivation of normothermic proteins following hyperthermia.

There are other mechanisms that may be responsible for the re-activation of pre-existing hsps. However of relevance to the current work, were findings concerning the glycosylation of phosphoglucomutase (pgm) in *S. cerevisiae* (Dey, *et al.*, 1994). These investigators demonstrated that pgm, the synthesis of which is subject to glucose repression and increased following a 37°C heat shock, is under-glycosylated *in vivo* as a consequence of both hyperthermia and growth on derepressive carbon sources. In addition, over expression of pgm from the *GAL5* gene on a multicopy plasmid was assessed to substantially increase its capacity to act as an acceptor in *in vitro* glucose phosphotransferase reactions following a heat shock or during stationary phase growth. Pgm catalyzes the interconversion of g-6-p and g-1-p (the latter being required for UDPG synthesis, a substrate for t-6-p synthase) and its activity is essential for the synthesis of trehalose during nutrient deprivation or stress (Lillie and Pringle, 1980). The current investigations concerning trehalose were therefore consistent with these findings, as evident from heat shock induced accumulation of the disaccharide and a higher constitutive content in derepressed cells (Fig. 6.4). Dey *et al.*, (1994) also demonstrated complete inhibition of the increase in net glucose phosphotransferase activity following heat shock in the presence of cycloheximide, indicative of a requirement for *de novo* synthesis of at least one of the two pgm isoforms. However this observation fails to provide an explanation for the marked decrease in heat shock induced trehalose accumulation exhibited by cells in the absence of protein synthesis (Chapter 5, Fig. 5.9). Other findings have indicated that trehalose synthesis was only partially decreased even in the absence of both *PGM1* and *PGM2* (Boles *et al.*, 1994). Apparently other hexose mutases substitute for the response under these conditions.

Re-heat shock induced trehalose accumulation was demonstrated in repressed and derepressed cells of both Yres and Ysen (Fig. 6.4). While this trend was concordant with re-heat shock induced thermotolerance (Fig. 6.1), an apparent incongruity was evident. The magnitude of trehalose accumulated following a second heat shock in YNBG cells of Yres was decreased, as compared with the level observed following an initial heat shock. It is conceivable that greater re-induced HSP transcription (Fig. 6.5) and hsp content, particularly hsp 70 (Fig. 6.6) supplements for decreased trehalose accumulation in these cells. Whereas in YNBG cells of Ysen, which exhibited levels of re-induced trehalose accumulation compatible with those following the initial heat shock, re-induced HSP transcription is less dramatic. In this context it follows that Ysen would be compromised

in tolerance induction in the absence of protein synthesis, described in Chapters 3 and 5 (Fig. 3.7, Fig. 5.1), as cycloheximide was shown to severely inhibit heat shock induced trehalose accumulation in this strain (Fig. 3.8, Fig. 5.9). Conversely, Yres, even in the absence of protein synthesis exhibited a 10-12-fold increase in trehalose content, presumably heat shock stimulates the activity of pre-existing t-6-p synthase in this strain. In addition, derepressed cells of both strains, particularly Yres, exhibited lower levels of heat shock induced and re-induced trehalose accumulation (Fig. 6.4), conditions under which HSP transcription (Fig. 6.5), and to a lesser extent translation (Fig. 6.2, Fig. 6.3), were more intense. Therefore it seems that trehalose and hsps may be able to compensate for each other as constituents of a thermoprotective mechanism, should levels of either parameter alone be insufficient. Previous evidence has led to proposal of such a cooperative function between some hsps and trehalose (Hottiger *et al.*, 1992; Piper, 1993). Recently it was demonstrated that mutants of *S. cerevisiae* lacking both trehalose and hsp 104 (*tps2hsp104*) were extremely heat shock sensitive, even at moderate temperatures, where neither of the single mutations caused a thermotolerance defect (Elliot *et al.*, 1996). It was interesting to note that in the same study, investigators isolated a suppressor (*PMU1*) of the temperature sensitivity for growth exhibited by the *tps2* mutant. The protein encoded by *PMU1* was found to have a region homologous to the active site of phospho-mutases.

In previous chapters a common theme that emerged was a lack of convincing evidence in favour of trehalose acting as a thermoprotectant. While a theory involving synergistic cooperation between hsps and trehalose discussed above may seem appealing, comparison of absolute levels of trehalose induction between Yres and Ysen indicated a lack of correlation with the magnitudes of tolerance induction (discussed in Chapter 3). In addition, if synergy were to hold true, one might expect coregulation of the genes encoding hsps and trehalose under stress conditions. Transcriptional regulation of many genes, whose expression is elevated by conditions that also enhance trehalose content, is mediated by the stress activated HSF, which recognises the cis-acting DNA element HSE (Jakobsen and Pelham, 1988; Sorger and Pelham, 1988). More recently, a novel DNA element, the stress responsive element (STRE), which also mediates stress induced transcriptional activity, was identified (Kobayashi and McEntee, 1993). This element has since been shown to be present in promoter regions of several stress inducible genes including *CTT1* (Marchler *et al.*, 1993) and *UB14, HSP12, HSP26, HSP104* (Schüller *et al.*, 1994). In

addition, STRE motifs have also been identified in the promoter of *TPS2* (Gounalaki and Thireos, 1994) and in promoters of other genes encoding the trehalose synthase complex, *TPS1*, *TPS3* and *TSL1* (Mager and De Kruijff, 1995; Varela *et al.*, 1995; reviewed in Ruis and Schüller, 1995). However, in spite of this, it has been shown that expression of the trehalose synthase genes differs from the expression of typical STRE regulated genes (Winderickx *et al.*, 1996). Undoubtedly there exist many mechanisms and levels of control of the stress response. However, results presented in Fig. 6.7 cast further uncertainty on the importance of trehalose. It was shown that heat shock accumulated trehalose is rapidly mobilized during recovery (Fig. 6.4) and in the case of Ysen, accumulation was inhibited in the absence of protein synthesis (Fig. 3.8, Fig. 5.9). It follows therefore that the induced tolerance observed subsequent to a second heat shock in the absence of protein synthesis was independent of trehalose accumulation but dependent on pre-existing hsps.

One further point should be considered in light of the present discussion. It has been shown that heat shock affects the intracellular location of hsps. In stressed *Drosophila* cells, hsp 70 migrates from the cytoplasm to the nucleus. Following recovery, it returns to the cytoplasm but moves back to the nucleus upon a subsequent re-heat shock (Velazquez and Lindquist, 1984). A similar relocalization response has been reported for a member of the plant low molecular mass hsp family in *Chlamydomonas reinhardtii* (Eisenberg-Domovich *et al.*, 1994). Hsp 22 was demonstrated to associate with a membrane-enriched fraction during heat shock treatment. Of significance to the present discussion was the observation that hsp 22 dissociated from membranes during recovery from heat shock, in a protein synthesis independent manner, persisted in cells throughout recovery and rebound to membranes during a subsequent heat shock *in vivo*. These findings were in agreement with the putative role previously ascribed the low molecular mass hsps of plants of acting during recovery or in short term induced thermotolerance processes (De Rocher *et al.*, 1991; Vierling, 1991). In plant and mammalian systems, heat shock associated relocalization of hsp 70 has been well documented. The highly induced protein is observed to rapidly relocate from the cytoplasm to the nucleus and nucleolus after heat shock (not stress) treatment (Welch and Feramisco, 1984; Neumann *et al.*, 1987). It has been suggested that hsp 70 facilitates recovery of nucleolar morphology and function during recovery from heat shock (Lewis *et al.*, 1985; Pelham, 1984; Welch and Feramisco, 1984; Welch and Suhan, 1985). Specifically, hsp 70 has been shown to localize predominantly within that region of the nucleoli involved in ribosomal assembly early

during the recovery period (Welch and Suhan, 1986), corroborated more recently by Beck and De Maio (1994). As nucleoli regain normal morphology, during latter periods of recovery, hsp 70 relocalizes back to the cytoplasm. In yeast, the intracellular localization of hsp 26 is thought to be dependent on the physiological state of the cell (Rossi and Lindquist, 1989). Therefore, in addition to re-activation of pre-existing hsps, their re-location may be necessary to facilitate execution of thermoprotective functions. Post-translational modifications such as phosphorylation may mediate this process via appropriate conformational changes.

In summary, the present results suggest that induction of the heat shock response may be modified by the previous thermal history of the cell, to the extent that exposure of cells to a second heat shock results in hsp supplementation to the pre-existing pool of hsps induced by a prior heat treatment. Protein synthesis inhibition prior to and during re-heat shock in pre-heat shocked, recovered cells that contained elevated levels of hsps did not suppress induction of thermotolerance in strain Ysen. It was therefore concluded that pre-existing hsps might be re-activated following a subsequent re-heat shock treatment. However, the extent of thermotolerance induced upon successive treatments decreased with increased recovery periods. Consequently, it was proposed that hsps are either progressively deactivated and/or saturated in chaperone function, resulting in increased requirement for newly synthesised hsps or *de novo* proteins (Fig. 6.8). An interesting corollary exists to the latter point. The longer cells are recovered, the more constitutive protein synthesis would have resumed and the more damage that would ensue during a subsequent heat shock resulting in an even greater requirement for chaperone activity. Clearly, further investigations concerning the phosphorylation state of hsps in heat shocked, recovering and re-heat shocked cells are warranted. Specifically, immunoblot analyses of immuno-precipitated [$^{32}$P] orthophosphate-labelled cell extracts such as those described by Long and Hopper (1995), or SDS-PAGE analysis of [$\gamma^{32}$P] ATP labelled extracts as outlined by Tourinho dos Santos *et al.* (1997), would be suitable approaches. Finally, evidence presented in the current and preceding chapters collectively suggests that trehalose was not a vital factor in tolerance under the present conditions.