

Activation of heat shock transcription factor in yeast is not influenced by the levels of expression of heat shock proteins

Bjørn Hjorth-Sørensen, Eva R. Hoffmann,[†]
Nikolai M. Lissin, Andrew K. Sewell and
Bent K. Jakobsen*

University of Oxford, Institute of Molecular Medicine,
John Radcliffe Hospital, Headington, Oxford,
OX3 9DS, UK.

Summary

Heat shock transcription factor (HSF) transiently induces the expression of a universally conserved set of proteins, the heat shock proteins (Hsps), when cells are exposed to elevated temperatures as well as to a wide range of other environmental stresses. The tight control of heat shock gene expression has prompted a model, according to which HSF activity and 'free' heat shock protein levels are tied up in a regulatory loop. Other data have indicated that HSF senses stress directly. Here, we report that yeast cells in which the basal expression levels of Hsps have been significantly increased exhibit improved thermotolerance but display no detectable difference in the temperature required for transient activation of HSF. In a separate experiment, overexpression of SSA2, a member of the Hsp70 family and a prominent candidate for the feedback regulation of HSF, did not inhibit the heat shock response. Our findings challenge the dogma that relief of the suppression of HSF activity by Hsps can account for the acute heat shock response.

Introduction

When exposed to various environmental stresses, cells dramatically increase the expression of a characteristic set of proteins, the heat shock proteins (Hsps). Although the precise functions of most Hsps are not yet fully understood, it is clear that they play essential roles in protecting the cell against the damaging effects of stressful conditions. It has been widely documented that

many Hsps act as molecular chaperones (Pelham, 1986; Ellis, 1987) that facilitate the refolding of denatured proteins (Parsell *et al.*, 1994; Nathan *et al.*, 1997; Glover and Lindquist, 1998) and can, for example, clear amyloid-like misfolded protein formations (Chernoff *et al.*, 1995). Hsps can be upregulated by cellular exposure to a broad range of conditions, including hyperthermia, heavy metal ions, oxidants and amino acid analogues, all of which are known to contribute to protein denaturation and misfolding. Injection of heat-denatured but not native proteins into *Xenopus* oocytes results in sharp upregulation of Hsps (Ananthan *et al.*, 1986). It has become the predominant notion that the heat shock induction is, at least in part, a response to the accumulation of denatured and misfolded proteins.

The heat shock response in eukaryotic cells is controlled at the transcriptional level by the ubiquitous heat shock transcription factors (HSFs) that bind to arrays of the 5 bp DNA consensus element nGAAn (heat shock elements, HSEs) in the Hsp gene promoter regions. HSF is encoded by a single gene (*HSF1*) in yeasts (Sorgner and Pelham, 1988; Jakobsen and Pelham, 1991), whereas three to four different HSFs can co-exist in plant, avian and mammalian cells (Scharf *et al.*, 1990; Rabindran *et al.*, 1991; Nakai and Morimoto, 1993; Hubel *et al.*, 1995). All HSFs share conserved DNA-binding and trimerization domains but are otherwise quite variable between species. However, examples of functional interchangeability of HSFs from organisms as diverse as yeast, fruit fly and man have been demonstrated (Clos *et al.*, 1993; Liu *et al.*, 1997). Interestingly, the activation temperature of human HSF was lowered by about 10°C in *Drosophila* cells, whereas the *Drosophila* HSF was constitutively active in human cells (Clos *et al.*, 1993). In the fission yeast *Schizosaccharomyces pombe* and all higher eukaryotes, activation of HSF is a two-stage process. Monomeric HSF with low affinity for DNA is converted into a high-affinity trimer when cells are exposed to heat shock. Conditions sufficient to induce trimerization normally lead to transcriptional competency, but trimerization and transcriptional activation can be separated (Jurivich *et al.*, 1992). In the budding yeasts *Saccharomyces cerevisiae* and *Kluyveromyces lactis*, HSF is constitutively present as DNA-bound trimers and undergoes only the second regulatory step, transcriptional activation, upon

Accepted 8 November, 2000. *For correspondence. E-mail jakobsen@pinnacle.jr2.ox.ac.uk; Tel. (+44) 1865 222 332; Fax (+44) 1865 222 502. [†]Present address: Genome Stability Group, Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU, UK.

heat shock (Sorger *et al.*, 1987; Jakobsen and Pelham, 1988). The acute heat shock response is transient and attenuates upon prolonged stress or recovery. Genetic observations show that some mutations of Hsp genes in *S. cerevisiae* can result in increased expression from Hsp gene promoters (Duina *et al.*, 1998). Biochemical evidence also suggests that some Hsps can physically interact with HSFs (Nadeau *et al.*, 1993; Shi *et al.*, 1998; Zou *et al.*, 1998; Bonner *et al.*, 2000). This has led to a model according to which the cellular level of Hsps, directly or indirectly, feedback regulates HSF activity. Heat shock, or the exposure to certain other stresses, leads to a depletion of free Hsps in the cell as they become engaged with denatured or misfolded protein substrates. The competitive depletion of Hsps in turn leads to a relieve of the control over HSF suppression, triggering the heat shock response. Once the denatured substrates are saturated through the dramatic induction of Hsp genes, and the free Hsp levels are replenished, suppression of HSF activity is re-established. This 'feedback' model is attractive, as it agrees with the highly diverse set of conditions capable of inducing the heat shock response.

In *S. cerevisiae*, proteins of the Hsp70 family have been shown to feedback regulate their own gene promoters (Park and Craig, 1989). However, this regulation is exercised through the promoter elements that are overlapping with, but distinct from, those bound by HSF (Park and Craig, 1989; 1991). It is therefore unclear to what extent the feedback regulation of Hsp genes could account for the control of the heat shock response. Here, we examine the above feedback model using yeast strains expressing Hsps at increased levels. Our findings clearly indicate that the transient activation of HSF upon heat shock is independent of expression levels of Hsp genes. The mechanism responsible for setting the activation temperature for HSF in yeast is capable of 'recalibration' to match the growth conditions, but it is not influenced by Hsps.

Results

In *S. cerevisiae*, HSF trimers constitutively bound to HSEs in the heat shock gene promoters support a basal level of transcription that is slightly regulated with growth temperature. When cells are exposed to acute hyperthermia, HSF transiently becomes highly active, resulting in a dramatic upregulation of expression of Hsps. These two modes of transcriptional regulation by HSF are referred to as the sustained and the transient activity respectively (Sorger, 1990). The transcriptional activity of HSF is thought to result from an interplay between separate activation and deactivation mechanisms (Hoj and Jakobsen, 1994). This study concentrates solely on transient activation.

Heat shock activation temperature in yeast adjusts with the growth temperature in the same way in wild-type cells and in the KIHSF/S.cerΔhsf1 construct

It has been demonstrated previously that the heat shock responses in *Drosophila* and in mammalian cells are the reaction to a change in temperature rather than to an absolute temperature (Abravaya *et al.*, 1991; Sarge *et al.*, 1995). We sought to establish whether the heat shock activation in yeast follows similar rules. *S. cerevisiae* Y700 cells bearing an HSE-*lacZ* reporter plasmid were grown at 18°C or 30°C, after which the cultures were shifted to different temperatures at 3°C intervals. Indeed, although the threshold of the transient activation in cells grown at 18°C was between 27°C and 30°C, it was found to be between 33°C and 36°C in the yeast cultivated at 30°C (data not shown). Virtually indistinguishable profiles were obtained with a model strain (KIHSF/*S.cerΔhsf1*) in which the endogenous *S. cerevisiae* HSF (ScHSF) had been substituted for the functionally interchangeable *K. lactis* HSF (KIHSF) expressed from an ARS/CEN plasmid (Fig. 1). The use of the KIHSF/*S.cerΔhsf1* construct in this study was prompted by the fact that mutations that influence the sustained activity have been extensively characterized in KIHSF. We conclude that the transient heat shock activation temperature in yeast is adjusted in accordance with the state of physiological adaptation of cells to the environmental changes. It can also be observed that the KIHSF-bearing strain reacts to heat shock in a manner that is indistinguishable from the reaction of the wild-type *S. cerevisiae* strain.

Increased basal expression of Hsps as a result of a mutation in HSF does not affect the heat shock activation temperature

Conflicting results from studies in mammalian cells suggest that the levels of one or other Hsp either directly control the trimerization/DNA binding of HSF (Zou *et al.*, 1998) or act as transcriptional repressors (Abravaya *et al.*, 1991). To establish whether increased expression of Hsps could influence the transient activation of HSF, we first compared the heat shock responses in two strains in which the levels of sustained Hsp gene expression are different. At growth temperatures over 15°C, the strain KIHSF-R₄₅₁→A/*S.cerΔhsf1* displays a higher sustained HSF activity from a synthetic indicator construct than does the control strain with the wild-type KIHSF (KIHSF/*S.cerΔhsf1*). This is caused by a single amino acid substitution in a short sequence element, the CE2, that is conserved between the HSFs of *S. cerevisiae* and *K. lactis* (Hoj and Jakobsen, 1994). It is important to note that, although this mutation increases the sustained activity of HSF, it does not affect the maximal levels of

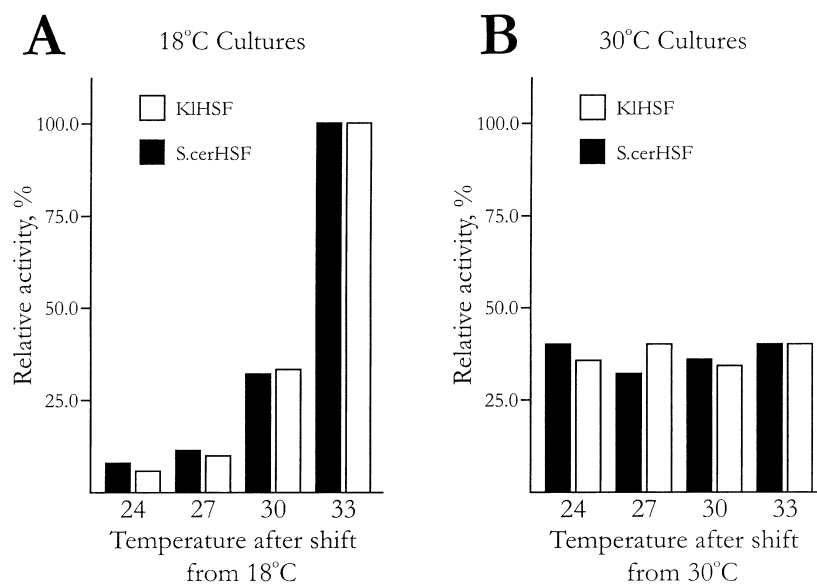


Fig. 1. Heat shock activation temperature in both the wild-type strain Y700 and KIHSF/*S.cerΔ hsf1* is affected by the growth temperature in the same way. A. Relative β -galactosidase activity (from HSE2-lacZ reporter) in the wild-type Y700 versus KIHSF/*S.cerΔ hsf1* cells grown at 18°C, after shifting to different temperatures as indicated. B. As above, but the cells were cultivated at 30°C before the shift. The measured activities were normalized against the maximal activity (at 33°C) for each strain (taken as 100%).

the transient activity (Hoj and Jakobsen, 1994). In Fig. 2A, we demonstrate, using a reverse transcriptase-polymerase chain reaction (RT-PCR) assay, the increased levels of sustained expression from the cognate *SSA2*, *HSC82*, *HSP82* and *HSP104* promoters caused by the $R_{451} \rightarrow A$ mutation compared with those observed in the control. The direct quantification of Hsp levels using conventional Western blotting proved difficult as a result, at least in part, of the existence of highly homologous protein species within several Hsp subfamilies that exhibit different levels of constitutive expression and inducibility. To overcome this difficulty, we used an *in vivo* tagging approach based on the insertion by homologous recombination of a coding sequence for the haemagglutinin (HA) epitope at the end of the open reading frame (ORF) of a target protein (Jungmann *et al.*, 1999). This allows the levels of individual proteins to be monitored using a single anti-HA monoclonal antibody. We tested the effect of the HSF mutation on two members of Hsc70/Hsp70 family, *SSA2* and *SSA4*, versus actin as a control (Fig. 2B). As expected, a moderate increase was observed for *SSA2* on the background of its strong constitutive expression, whereas the level of the highly inducible *SSA4* increased dramatically in cells bearing $R_{451} \rightarrow A$ HSF. The level of actin was found to decline slightly, which may be explained by the increased proportion of Hsps, bearing in mind that the total protein concentration in the extracts was maintained at the same level. To confirm that the higher Hsp expression has phenotypic consequences, the survival rates of the two strains were measured after severe heat shocks. The cells were grown at 23°C, then incubated for either 1 min at 52°C (Fig. 2C) or 1, 5 and 15 min at 50°C (Fig. 2D), by diluting 100-fold into preheated medium. On average, the survival rates of the strain KIHSF- $R_{451} \rightarrow A$ /*S.cerΔ hsf1* were about one order

of magnitude higher than those of the control strain with the wild-type KIHSF. Clearly, the mutation supports a significantly higher degree of thermotolerance – a phenotype that is normally acquired through exposure to a milder heat shock before the shift to 50–52°C. Thus, the $R_{451} \rightarrow A$ mutation alters a physiological response that is related to the Hsps levels (Sanchez and Lindquist, 1990; Parsell *et al.*, 1993; Lindquist and Kim, 1996).

If the transient activation of HSF indeed results from the depletion of the pool of free Hsps, the temperature of the heat shock response must be expected to be higher in cells with the elevated sustained expression of Hsps. However, the transient heat shock response in the two strains showed no difference. At 18°C, the sustained level of HSF activity caused by the $R_{451} \rightarrow A$ substitution is approximately three times higher than that supported by the wild-type KIHSF (compare the induction temperatures of two strains in Fig. 2E). At 30°C, that activity is about five times higher relative to the control (Fig. 2F). Despite the KIHSF- $R_{451} \rightarrow A$ /*S.cerΔ hsf1* strain exhibiting, at 18°C, a sustained transcriptional activity equal to that observed in the control strain after a heat shock at around 34°C, this has no influence on its transient activation temperature, which is approximately 27°C in both strains (Fig. 2E). Similarly, when cultured at 30°C, the KIHSF- $R_{451} \rightarrow A$ /*S.cerΔ hsf1* strain supports a sustained activity level that is comparable with the activity in the control strain after the induction at $\approx 37^\circ\text{C}$. However, in both strains, the transient activation temperatures ($\approx 33^\circ\text{C}$) are identical (Fig. 2F).

Overexpression of SSA2 under the TPI promoter does not suppress the heat shock response

An increase in overall expression levels of Hsps showed

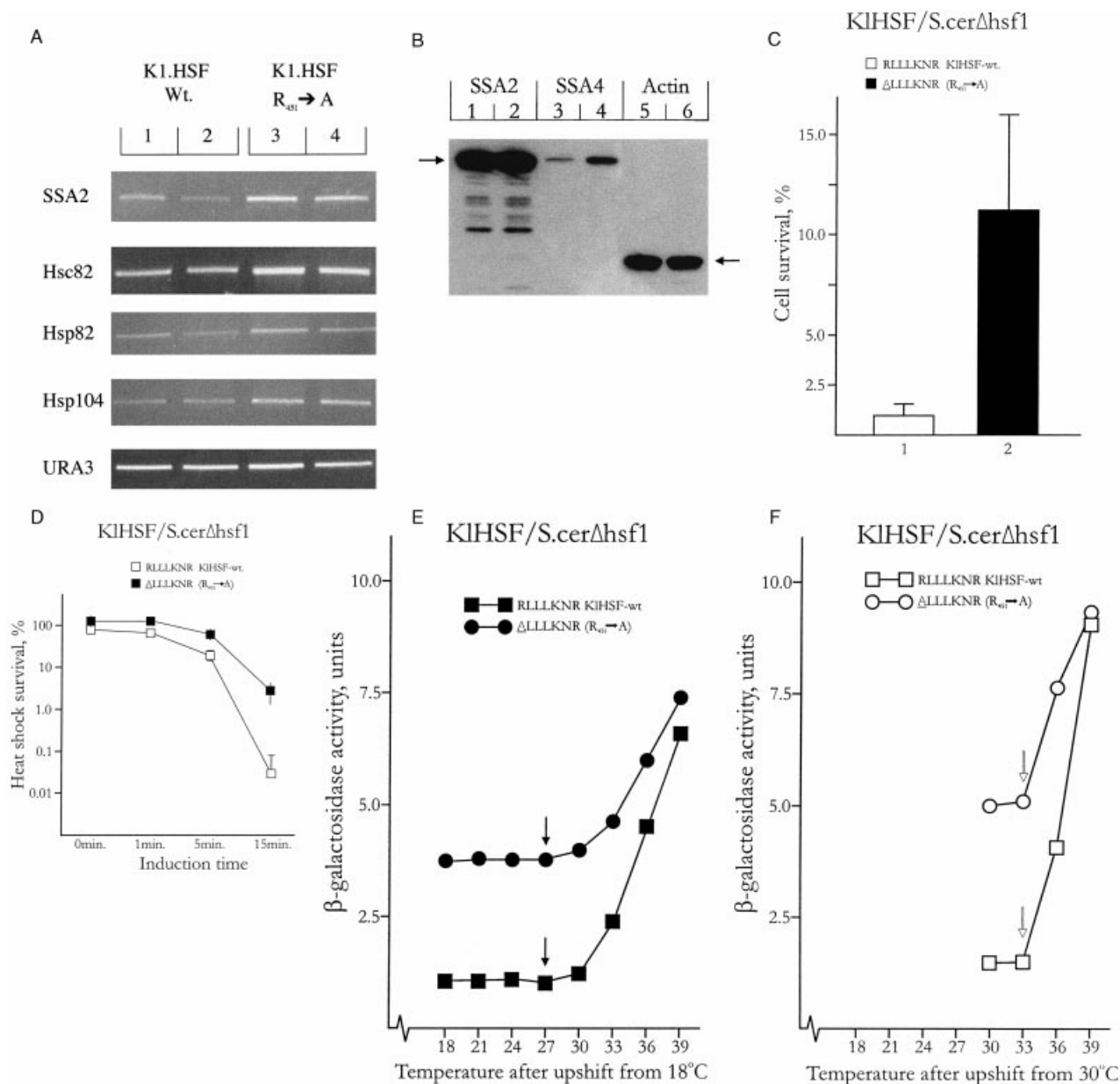


Fig. 2. Level of basal expression of HSF-regulated genes does not affect heat shock response in yeast.

A. Gel analysis (in duplicate) of quantitative RT-PCR products obtained with Hsp gene-specific (*SSA2*, *HSC82*, *HSP82*, *HSP104*) or control (*URA3*) gene-specific primers on cDNA templates derived from K1HSF/*S.cerΔhsf1* cells (lanes 1 and 2) or K1HSF-R₄₅₁→A/*S.cerΔhsf1* cells (lanes 3 and 4).

B. Western blotting analysis of HA-tagged proteins, SSA2 (lanes 1 and 2), SSA4 (lanes 3 and 4) and actin (lanes 5 and 6) in the extracts prepared from K1HSF/*S.cerΔhsf1* (lanes 1, 3 and 5) or K1HSF-R₄₅₁→A/*S.cerΔhsf1* cells (lanes 2, 4 and 6). The total protein content in all samples was equalized based on a Coomassie protein assay.

C. Relative cell survival from cultures grown at 23°C after 1 min exposure to heat shock at 52°C.

D. Relative cell survival from cultures grown at 23°C after exposure to heat shock at 50°C as indicated.

E and F. β-Galactosidase activity from HSE2-lacZ reporter in K1HSF/*S.cerΔhsf1* (square symbols) and K1HSF-R₄₅₁→A/*S.cerΔhsf1* (circular symbols). Activities were determined after growth at 18°C (**E**) or 30°C (**F**) and exposure to heat shock (30 min heat shock, 30 min recovery) at the indicated temperatures. Arrows show the highest temperature at which transient heat shock activation is not observed.

no effect on the regulation of transient heat shock activation. We then sought to examine whether a dramatic overexpression of an Hsp gene could still be capable of causing such an effect. *SSA* genes, which encode

members of the Hsp70 family of molecular chaperones, provide an example of interdependence in their regulation. Expression of the *SSA4* gene is almost undetectable during normal growth but undergoes a dramatic induction

with acute heat shock (Boorstein and Craig (1990). In *ssa1ssa2* cells, however, high constitutive *SSA4* expression is observed (Werner Washburne *et al.*, 1987; Boorstein and Craig, 1990; Unno *et al.*, 1997). As *SSA4* induction seems to depend on the levels of *SSA1/SSA2* gene products, the latter proteins, which share 97% of their amino acid sequences (Craig and Jacobsen, 1984), appear to be likely candidates to be involved in the suppression of HSF activity. To examine this possibility, the *SSA2* gene was expressed under the control of the strong constitutive promoter from the triose phosphate isomerase (TPI) gene in the *S. cerevisiae* strain expressing the wild-type *K. lactis* HSF (KIHSF/*S.cerΔhsf1*). Levels of *SSA2* transcription were confirmed to be greatly increased in the transformed cells (Fig. 3A). The heat shock response curves of cells overexpressing *SSA2* versus the control strain, grown at either 18°C or 30°C, are shown in Fig. 3B and C respectively. The high level of expression of *SSA2* appears to cause a slight increase in the sustained activity of HSF (compare the activities in the two strains at the respective growth temperatures in Fig. 3B and C). However, no differences in the heat shock activation temperatures were detected between the two strains in the cultures grown at either 18°C (Fig. 3B) or 30°C (Fig. 3C). The high level of *SSA2* expression is therefore not sufficient to offset the triggering effect on HSF of the heat shock activation temperature.

Discussion

Similar to the situation in mammalian cells, the temperature required for the transient activation of HSF in yeast adjusts in accordance with the conditions. Cells grown at 18°C induce a heat shock response at a significantly lower temperature compared with those maintained at 30°C. The mechanism that sets the activation threshold appears to work identically for both *S. cerevisiae* and *K. lactis* HSFs. The activation temperature would appear to be, at least in part, a function of some adaptations inside the cell, rather than being determined by HSF itself. Perhaps the most obvious candidates to be involved in such regulation are Hsps themselves, and the negative feedback loop invoked has become the prevalent theory for the control of HSF activation. Our experiments have tested this notion and rule out the possibility that Hsps, or genes under the direct control of HSF, are solely or mainly responsible for such feedback. A mutation in HSF that alters its sustained activity leading to a significant increase in the basal expression of Hsps, but does not influence the ability of the molecule to react transiently to an acute heat shock, has no effect on the transient activation temperature (Fig. 2E and F). This mutation should upregulate all genes under the control of HSF, which was exemplified by three different classes of Hsps: *SSA2/SSA4* (Hsp70), *Hsc82/Hsp82* and *Hsp104* (Fig. 2A

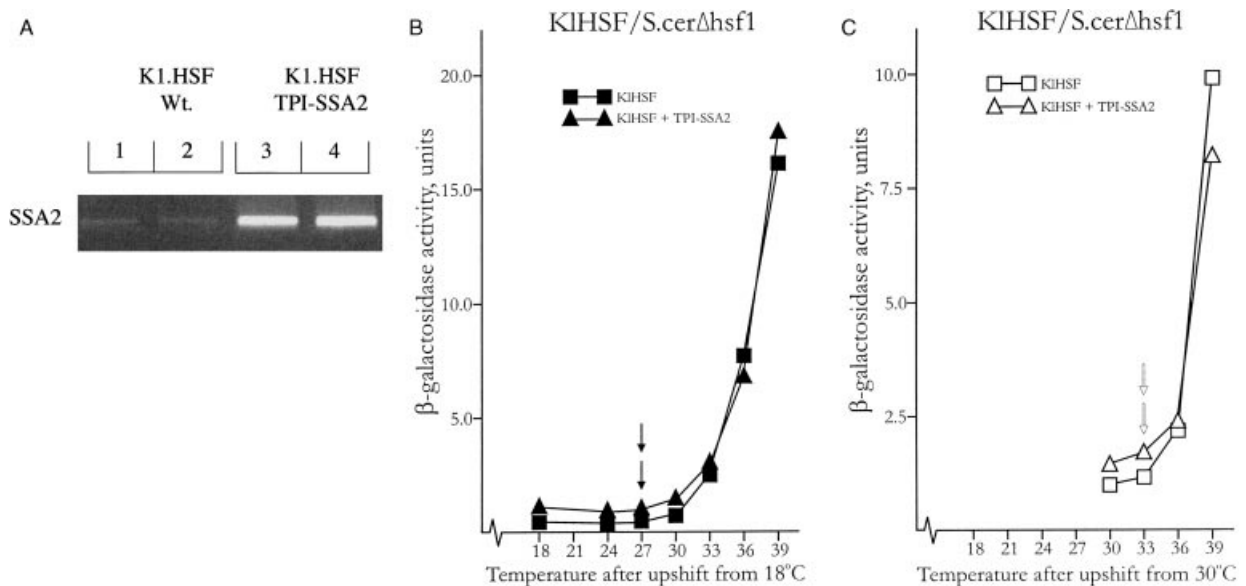


Fig. 3. Overexpression of *SSA2* does not suppress HSF activation.

A. Gel analysis (in duplicate) of quantitative RT-PCR products obtained with *SSA2*-specific primers on cDNA templates derived from KIHSF/*S.cerΔhsf1* cells bearing control plasmid without insert (lanes 1 and 2) or the plasmid with the *SSA2* gene under the control of a strong constitutive TPI promoter (lanes 3 and 4).

B and C. β -Galactosidase activity in KIHSF/*S.cerΔhsf1* cells, co-transformed with HSE2-lacZ reporter and the TPI plasmid either without insert (square symbols) or with the *SSA2* gene insert (triangular symbols). Activities were determined after growth at 18°C (B) or 30°C (C) followed by exposure (30 min) at the indicated temperatures and recovery (30 min). Arrows indicate the highest temperatures at which transient heat shock activation is not observed.

and B). Moreover, in a different set of experiments, we tested whether the heat shock induction in yeast is suppressed by a massive overexpression of one of the above proteins, the SSA2. The transient activation temperature remained unchanged (Fig. 3).

In the experiments presented here, we only tested the effect of increased sustained levels of Hsps and not that of decreased levels. It is possible to hypothesize that the elevated expression of Hsps does not influence the transient response, because the normal cellular levels of Hsps are already sufficient for the stress-sensing mechanism. Lowering the levels of all or some Hsps must potentially stress the cell, making such experiments unfeasible. Indeed, underexpression of Hsp90, for example, induces a sustained heat shock response (Duina *et al.*, 1998). However, as it is expected according to the tested model that the levels of free Hsps are depleted gradually depending on the severity of the stress stimuli, measuring the activity of HSF in cells exposed to different induction temperatures allows the overcoming of this difficulty. It is logical to expect that a higher 'starting' level would lead to retarded depletion of Hsps, which, if the feedback model was right, would result in a higher temperature for the acute heat shock response.

Our findings provide an explanation for earlier observations on yeast cells expressing truncated HSF proteins. A potent transactivator domain has been located to the C-terminal portion of *S. cerevisiae* and *K. lactis* HSFs (Chen *et al.*, 1993). The C-terminal activator domain (CTA) is involved in supporting the sustained activity level (Sorger, 1990; Jakobsen and Pelham, 1991). Cells expressing HSF protein that lack the C-terminal activator domain (HSF Δ CTA) have lower sustained HSF activity and are unable to grow at high temperatures ($\approx > 35^\circ\text{C}$). However, the truncated HSF retains the ability to support a substantial response to heat shock. If transient activation was triggered by depletion of free Hsps, it would therefore have been expected that cells expressing HSF Δ CTA would have induced the transient response when growing at higher temperatures, rescuing cell viability.

One can imagine that the significantly increased sustained expression levels could result in the accumulation of misfolded, aggregated or otherwise inactive proteins. In other words, an increase in expression of some proteins might not, at least in theory, lead to the proportional increase in their activity. This is extremely unlikely in the context of our experiments, as Hsps in general are known to be exceptionally stable, being designed by nature to deal specifically with most difficult conditions. A dramatically enhanced thermotolerance of KHSF-R₄₅₁→A/*S.cer* Δ *hsf1* cells versus the control strain (Fig. 2C and D) provides indirect evidence in support of this notion. Dilution of a cofactor or the disruption of normal cellular mechanisms in some other ways can

potentially be a problem during the overexpression of some proteins. However, in most of our experiments (except those describing overexpression of SSA2 under the TPI promoter), the levels of expression of Hsps were well within the range of physiological response (roughly one-third of the levels seen during the acute heat shock). One can hypothesize further that an increased expression of some Hsp(s) could still fail to result in the proportional increase in its activity, as the latter can be modulated through a separate mechanism. Although such a possibility cannot be totally excluded, this will mean that the simple negative feedback loop does not hold for the regulatory mechanism we were examining.

The conclusion that there is no link between the levels of Hsps and transient activation of HSF conflicts with the conclusions of another recent study (Duina *et al.*, 1998). *S. cerevisiae* has two Hsp90 genes, *HSC82* and *HSP82*. *HSC82* is constitutively expressed, whereas *HSP82* exhibits tight heat shock regulation. The simultaneous deletion of both genes is lethal to the cell. In a Δ *hsc82* Δ *hsp82* background, cells carrying *hsp82*^{G170D}, a temperature-sensitive allele of *HSP82*, exhibit growth arrest at 34°C and die after a few hours' incubation (Nathan and Lindquist, 1995; Nathan *et al.*, 1997). The observation that an HSE2-*LacZ* reporter was activated in a Δ *hsc82* Δ *hsp82hsp82*^{G170D} strain at 34°C was taken as evidence that Hsp82 negatively regulates HSF (Duina *et al.*, 1998). However, it is easy to assume that Hsp82_{G170D} mutant protein denatures under the heat shock conditions, causing cell death. Clearly, transfer to 34°C must constitute a severe stress to Δ *hsc82* Δ *hsp82hsp82*^{G170D} cells, and it is perhaps not surprising that the reporter construct activates at this temperature. If Hsp82 was responsible for the negative feedback regulation of HSF, increased expression of Hsp82 would be expected to suppress HSF activation. However, this does not happen (Fig. 2). Our results are in agreement with the findings of another group showing that overexpression of Hsp82 from a high-copy-number vector did not suppress heat shock induction of Hsp104, Hsp70, Hsp35 or Hsp26 and had little effect on thermotolerance (Cheng *et al.*, 1992). Specific repression of HSF1 by Hsp90 and the formation of reversible stress-sensitive complexes between the two proteins have been observed *in vitro* using human HeLa cell extracts (Zou *et al.*, 1998). However, the authors were uncertain whether the inhibitory effect was caused by Hsp90 directly or was a result of the formation of more complex multiprotein associations. The use of trimerization/DNA-binding assay rather than measuring the transcriptional potency of HSF makes these results inapplicable to the yeast system in which HSF is constitutively DNA bound.

Whether the heat shock activation temperature is controlled by the HSF molecule itself (e.g. through some gradual temperature-inducible changes in its

conformation) or is dependent on some unknown pathway(s) that signals the physiological state of the cell to HSF remains an open question. Such signals may comprise covalent modifications of HSF, as well as various interactions with some repressors and/or activators. Phosphorylation was found to play a role in the control of HSF on the stage of either activation (Chu *et al.*, 1996) or deactivation (Hoj and Jakobsen, 1994). Despite several proteins having been implicated in interactions with HSF (Reindl and Schoeffl, 1998; Satyal *et al.*, 1998; Scharf *et al.*, 1998; Stephanou *et al.*, 1999), very little is known about the mechanism and primary source of the cellular signals controlling the induction of heat shock response. Recently (Bonner *et al.*, 2000), an ATP-dependent interaction was reported between the yeast HSF and SSB1/2p protein, members of the Hsp70/Hsc70 family, which was found to have some moderate effect on HSF activity. However, SSB1/2p itself is not regulated by heat shock. One possibility is that, as is generally believed, the signals regulating HSF might arise from the accumulation of misfolded and denatured proteins. Alternatively, some totally different mechanisms have been proposed, ranging from temperature-dependent changes in the physical state of cellular membranes (Horvath *et al.*, 1998) to the stress-induced accumulation of superoxide (Bonner *et al.*, 2000). In any case, our results presented here exclude the possibility that a principal role in the control of heat shock activation is played directly by Hsps. This, however, does not contradict the notion that Hsps, e.g. Hsp90 and the components of the 'multichaperone complexes', could participate in suppressing HSF activity after the heat shock response (i.e. through the deactivation mechanism) (Rabindran *et al.*, 1994; Bharadwaj *et al.*, 1999). Whether this is the case or not, the regulatory loop model of Hsps controlling HSF activity cannot account for the transient activation of HSF upon acute heat shock.

Experimental procedures

Yeast strains and plasmids

S. cerevisiae wild-type strain Y700 (*MAT α* , *ade2-1*, *his3-11*, *-15*, *leu2-3*, *-112*, *trp1*, *ura3*, *can1-100*), transformed with a *URA3* plasmid containing a synthetic heat shock element-*lacZ* reporter gene, pH2G0 (Jakobsen and Pelham, 1988), was used to determine the effect of growth temperature on the transient activation temperature of the endogenous *S. cerevisiae* HSF (ScHSF) (Fig. 1). In all other experiments (Figs 1–3), the coding sequences of *K. lactis* HSF (KIHSF) or its R₄₅₁→A mutant (KIHSF-R₄₅₁→A) were expressed downstream of a 782 bp fragment from the *S. cerevisiae* HSF gene promoter as described previously (Jakobsen and Pelham, 1991; Hoj and Jakobsen, 1994). The KIHSF-expressing genes were maintained on *ARS/CEN* plasmids with *HIS3* as the selectable marker (derived from pRS313;

Sikorski and Hieter, 1989) in a haploid *S. cerevisiae* strain BJ100 (*MAT α* , *ade2-1*, *his3-11*, *-15*, *leu2-3*, *-112*, *trp1*, *ura3*, *can1-100*, *HSF Δ ::TRP1*), a derivative of W303 (Shore and Nasmyth, 1987) carrying a chromosomal *HSF Δ ::TRP1* disruption and expressing ScHSF from a vector maintained with *URA3* as the selectable marker (Jakobsen and Pelham, 1991). ScHSF expression was then eliminated from the strains transformed with either of the KIHSF plasmids by two rounds of counterselection on plates containing 1 mg ml⁻¹ 5-fluoro-orotic acid (Boeke *et al.*, 1987). The resulting *ura3* cells (KIHSF/*S.cer Δ hsf1* or KIHSF-R₄₅₁→A/*S.cer Δ hsf1* respectively) were transformed with the *URA3* plasmid, pH2G0, containing the synthetic heat shock element-*lacZ* reporter gene (Jakobsen and Pelham, 1988). The *SSA2* structural gene was obtained by PCR using *S. cerevisiae* genomic DNA as template and the following primers: 5'-GGGGGGGGGGG ATCCATGTCTAAAGCTGTCGGTATTGATTTAGG-3' (coding) and 5'-GGGGGGGGGGCTCGAGAAGCTTAATCAACTTCTTCGACAGTTGG-3' (non-coding). The PCR product corresponding to the *SSA2* gene was inserted using the unique *Bam*HI and *Xho*I sites into a derivative, pBP13, of the pYX242 plasmid (2 μ , *LEU2*; R and D Systems) downstream of the triose phosphate isomerase (TPI) promoter, generating plasmid pBP13SSA2 (the *SSA2* insert and junctions were verified by DNA sequencing). Cells were transformed using a lithium acetate procedure (Agatep *et al.*, 1988).

Heat shock induction

Cells were grown in the appropriate selective media to an OD₆₀₀ of \approx 0.4. Aliquots of the culture were then incubated at the indicated temperatures for 30 min (15, 45 or 90 min, respectively, for experiments in Fig. 3B and C) followed by 30 min recovery at the initial growth temperature. A control aliquot from the same original culture was maintained at the growth temperature for 60 min for the determination of reference activity.

Permeabilized cell ONPG assay for β -galactosidase activity

Cells were pelleted at 3500 *g* for 3 min in a microcentrifuge and resuspended in 1 ml of assay buffer (82 mM Na₂HPO₄, 18 mM Na₂HPO₄, 1 mM MgCl₂). Aliquots of 20 μ l of 0.1% SDS and 20 μ l of chloroform were added, and the samples were vortexed briefly, then incubated at the growth temperature for 5 min to allow permeabilization of the cells. ONPG solution (200 μ l, 4 mg ml⁻¹) was added, and incubation was allowed to proceed until a pale yellow colour developed (OD₄₂₀ of 0.1–0.4 corresponding to the linear assay range) before the reaction was stopped by adding 450 μ l of 1 M Na₂CO₃. OD₄₂₀ in the supernatant was measured after cells were spun at 10 000 *g* for 5 min in a microcentrifuge. β -Galactosidase activity (units) was calculated as: OD₄₂₀/OD₆₀₀ \times volume \times time.

Overexpression of SSA2

The strains used for examining the effect of overexpression of *SSA2* were constructed by co-transformation of the BJ100/

KIHSF expressing strain (KIHSF/*S.cerΔhsf1*) with either pBP13SSA2 or pBP13 without insert (control) together with the reporter plasmid pH2G0.

Preparation of cDNA for PCR assay

Total RNA from yeast was isolated using a Qiagen RNeasy mini kit. cDNA synthesis was performed with 1 µg of total RNA in 10 µl of sterile H₂O as template and 2.5 µl of 100 pmol µl⁻¹ random hexamer (N6) primer. The mixture was incubated at 70°C for 10 min in an Eppendorf tube and quickly transferred to ice. An aliquot of 4 µl of 5× first strand buffer [250 mM Tris-HCl, pH 8.3, 375 mM KCl, 15 mM MgCl₂, 2 µl 0.1 M dithiothreitol (DTT) and 1 µl 10 mM dNTPs] was added. The mixture was initially incubated at 25°C for 10 min, then 1 µl (200 units) of SuperScript II RNase H⁻ reverse transcriptase (Gibco BRL) was added, and the reaction was incubated for 50 min at 42°C. The reverse transcriptase was inactivated by heating to 70°C for 10 min.

Quantitative PCR

All PCRs were performed in 50 µl reaction volumes containing 2.5 U of cloned *Pfu* DNA polymerase (Stratagene) and 5 µl of 10× reaction buffer [200 mM Tris-HCl, pH 8.8, 100 mM KCl, 100 mM (NH₄)₂SO₄, 20 mM MgSO₄, 1% Triton X-100, 1 mg ml⁻¹ nuclease-free BSA and 0.2 mM dNTPs]. The PCR reactions used 25 pmol of the primer pairs: 5'-GGGGG GGGGGGATCCATGTCTAAAGCTGTCGGTATTGATTTAG G-3' (SSA2 coding) and 5'-GGGGGGGGCTCGAGAAGCT TAATCAACTTCTTCGACAGTTGG-3' (SSA2 non-coding); 5'-ATGGCTGGTAAAACCTTTTGAATTTCAAGC-3' (Hsc82 coding) and 5'-TTAATCAACTTCTTCATCTCGGTGTC-3' (Hsc82 non-coding); 5'-GGGGGGCATATGGCTAGTAAAACCTTTTGAATTT CAAGC-3' (Hsp82 coding) and 5'-GGGGGGCTCGAGCTAATC TACCTCTTCCATTTTCGGTGTGACG-3' (Hsp82 non-coding); 5'-GACGTTGGCTCAAAAATTGGCTTCGG-3' (Hsp104 coding) and 5'-CGTCATCACCTAACGTGTCAGCCCC-3' (Hsp104 non-coding); 5'-ATGTCGAAAGCTACATATAAGGAACGTG C-3' (URA3 coding) and 5'-GGGGGGTTCGACGGGTAA-TAACTGATATAATTAATTGAAGC-3' (URA3 non-coding). A sample of 2 µg of cDNA, at standardized concentration and prepared from total yeast RNA, was used as template. The PCR amplifications were carried out for 32–38 cycles (depending on the primer pair) with denaturation at 95°C (1 min), annealing at 57°C (1 min) and extension at 72°C (10 min). An aliquot of 15 µl from each reaction was separated by electrophoresis on a standard 1% agarose gel, the bands being visualized in the presence of ethidium bromide using a UVP gel documentation system.

In vivo protein tagging

The HSF mutant strain (KIHSF-R₄₅₁→A/*S.cerΔhsf1*) and the control strain (KIHSF/*S.cerΔhsf1*) for use in haemagglutinin (HA) epitope tagging experiments were constructed as described above except that *LEU2* was used as the selective marker. The plasmid p3XHA-HIS5 (a generous gift from Dr S. Munro, LMB Cambridge, UK; described by Jungmann *et al.*, 1999), which encodes a linker of the amino acid sequence

GAGAGA, followed by three copies of the epitope sequence YPYDVPDYA (the first and third repeats followed by a G-residue) was used as a template in PCR with the following primer pairs: 5'-GGTGCTCCTCCAGCTCCAGAAGCTGAAG GTCCAACGTGCGAAGAAGTTGATGGAGCAGGGGCGGGTG C-3' (SSA2-forward) and 5'-CAAGTTTTTATTATTAAGAGCT CTTTTATTTTTATTTATAGGGGAGGTCGACGGTATCGA TAAG-3' (SSA2-reverse); 5'-AGGCCCCACTGGAGCACCAG ACAACGGCCCAACGGTTGAAGAGTTGATGGAGCAGGGG CGGGTGC-3' (SSA4-forward) and 5'-CTTTCTTATTGTCAT TTCGGCTTTGTATATGAACGCGAAATCGCATCCCCGGGAGG TCGACGGTATCGATAAG-3' (SSA4-reverse); 5'-CAAGAATA CGACGAAAGTGGTCCATCTATCGTTCACCAACAAGTGTTC GGAGCAGGGGCGGGTGC-3' (actin-forward) and 5'-ATTAT ATCAATGAAAACGTACAAAAAGTAGATAAAAGTCAGTGCTTA AACACGGAGGTCGACGGTATCGATAAG-3' (actin-reverse).

The reactions were run for 35 cycles, with denaturation at 95°C for 1 min, annealing at 54°C for 1 min and extension at 72°C for 4 min. The DNA products (≈1.5 kb) containing the *HIS5* gene of *S. pombe* and the flanking sequences of the sites of integration (the 5'-flanking sequence being in frame with the 3 × HA repeats) were transformed into the corresponding (KIHSF-R₄₅₁→A/*S.cerΔhsf1*) and (KIHSF/*S.cerΔhsf1*) strains.

Protein extract preparation and Western blotting

Yeast cultures (20 ml of YEPD medium inoculated with 2 ml of overnight cultures) were grown for 4 h at 30°C on a shaker, cooled on ice and centrifuged at 3000 r.p.m. for 5 min. Cells were resuspended in 1 ml of H₂O containing Complete protease inhibitor (Roche Molecular Biochemicals) and centrifuged in Eppendorf tubes at 7500 r.p.m. for 3 min. The cell pellets were resuspended in 200 µl of buffer (82 mM Na₂HPO₄, 18 mM Na₂HPO₄, 1 mM MgCl₂) supplemented with the protease inhibitor. Acid-washed glass beads (Sigma, G-8772) were added, and the samples were vortexed vigorously for 5 min followed by centrifugation. The protein concentration in the extracts was determined using a Coomassie Plus protein assay reagent (Pierce). The samples were diluted to an equal total protein concentration and run on a 10% denaturing polyacrylamide gel according to standard procedures. Proteins were transferred to nitrocellulose using a Mini Trans-Blot Cell (Bio-Rad). A rat anti-HA monoclonal antibody (clone 3F10; Roche Molecular Biochemicals) followed by anti-rat IgG peroxidase conjugate and a chemiluminescence blotting substrate (Roche Molecular Biochemicals) were used for the visualization of HA-tagged proteins.

Thermotolerance

Aliquots (100 µl) of cultures grown at 23°C to OD₆₀₀ of ≈ 0.6 were added to 10 ml of fresh medium either at 23°C (control) or preheated at 52°C (Fig. 1B) or 50°C (Fig. 1C). Cell survival was measured as the ability to form colonies after dilution and plating on yeast extract–peptone–dextrose plates that were incubated at 30°C.

Acknowledgements

We would like to thank our colleagues at the Institute of

Molecular Medicine, especially Jonathan Boulter, John Bell and Ian Hickson. We are also grateful to Ed Louis and Rhona Borts for help and advice, and to Sean Munro (MRC Laboratory of Molecular Biology, Cambridge, UK) for his advice on protein tagging and the generous gift of the plasmid p3XHA-HIS5. This work was supported by a project grant from the Leverhulme Trust.

References

- Abravaya, K., Phillips, B., and Morimoto, R.I. (1991) Attenuation of the heat shock response in HeLa cells is mediated by the release of bound heat shock transcription factor and is modulated by changes in growth and in heat shock temperatures. *Genes Dev* **5**: 2117–2127.
- Agatep, R., Kirkpatrick, R.D., Parchaliuk, D.L., Woods, R.A., and Geitz, R.D. (1988) Transformation of *Saccharomyces cerevisiae* by the lithium acetate/single stranded carrier DNA/polyethylene glycol (LiAc/ss-DNA/PEG) protocol. Technical Tips Online. [WWW document] URL <http://www.tto.trends.com>.
- Ananthan, J., Goldberg, A.L., and Voellmy, R. (1986) Abnormal proteins serve as eukaryotic stress signals and trigger the activation of heat shock genes. *Science* **232**: 522–524.
- Bharadwaj, S., Ali, A., and Ovsenek, N. (1999) Multiple components of the Hsp90 chaperone complex function in regulation of heat shock factor 1 *in vivo*. *Mol Cell Biol* **19**: 8033–8041.
- Boeke, J.D., Trueheart, J., Natsoulis, G., and Fink, G.R. (1987) 5-Fluoroorotic acid as a selective agent in yeast molecular genetics. *Methods Enzymol* **154**: 164–175.
- Bonner, J.J., Carlson, T., Fackenthal, D.L., Paddock, D., Storey, K., and Lea, K. (2000) Complex regulation of the yeast heat shock transcription factor. *Mol Biol Cell* **11**: 1739–1751.
- Boorstein, W.R., and Craig, E.A. (1990) Structure and regulation of the *SSA4* Hsp70 gene of *Saccharomyces cerevisiae*. *J Biol Chem* **265**: 18912–18921.
- Chen, Y., Barlev, N.A., Westergaard, O., and Jakobsen, B.K. (1993) Identification of the C-terminal activator domain in yeast heat shock factor: independent control of transient and sustained transcriptional activity. *EMBO J* **12**: 5007–5018.
- Cheng, L., Hirst, K., and Piper, P.W. (1992) Authentic temperature-regulation of a heat shock gene inserted into yeast on a high copy number vector. Influences of overexpression of Hsp90 protein on high temperature growth and thermotolerance. *Biochim Biophys Acta* **1132**: 26–34.
- Chernoff, Y.O., Lindquist, S.L., Ono, B., Inge-Vechtomov, S.G., and Liebman, S.W. (1995) Role of the chaperone protein Hsp104 in propagation of the yeast prion-like factor [psi⁺]. *Science* **268**: 880–884.
- Chu, B., Soncin, F., Price, B.D., Stevenson, M.A., and Calderwood, S.K. (1996) Sequential phosphorylation by mitogen-activated protein kinase and glycogen synthase kinase 3 represses transcriptional activation by heat shock factor-1. *J Biol Chem* **271**: 30847–30857.
- Clos, J., Rabindran, S., Wisniewski, J., and Wu, C. (1993) Induction temperature of human heat shock factor is reprogrammed in a *Drosophila* cell environment. *Nature* **364**: 252–255.
- Craig, E.A., and Jacobsen, K. (1984) Mutations of the heat inducible 70 kilodalton genes of yeast confer temperature sensitive growth. *Cell* **38**: 841–849.
- Duina, A.A., Kalton, H.M., and Gaber, R.F. (1998) Requirement for Hsp90 and a CyP-40-type cyclophilin in negative regulation of the heat shock response. *J Biol Chem* **273**: 18974–18978.
- Ellis, J. (1987) Proteins as molecular chaperones. *Nature* **328**: 378–379.
- Glover, J.R., and Lindquist, S. (1998) Hsp104, Hsp70, and Hsp40: a novel chaperone system that rescues previously aggregated proteins. *Cell* **94**: 73–82.
- Hoj, A., and Jakobsen, B.K. (1994) A short element required for turning off heat shock transcription factor: evidence that phosphorylation enhances deactivation. *EMBO J* **13**: 2617–2624.
- Horvath, I., Glatz, A., Varvasovszki, V., Torok, Z., Pali, T., Balogh, G., *et al.* (1998) Membrane physical state controls the signaling mechanism of the heat shock response in *Synechocystis* PCC 6803: identification of Hsp17 as a 'fluidity gene'. *Proc Natl Acad Sci USA* **95**: 3513–3518.
- Hubel, A., Lee, J.H., Wu, C., and Schoffl, F. (1995) *Arabidopsis* heat shock factor is constitutively active in *Drosophila* and human cells. *Mol Gen Genet* **248**: 136–141.
- Jakobsen, B.K., and Pelham, H.R. (1988) Constitutive binding of yeast heat shock factor to DNA *in vivo*. *Mol Cell Biol* **8**: 5040–5042.
- Jakobsen, B.K., and Pelham, H.R. (1991) A conserved heptapeptide restrains the activity of the yeast heat shock transcription factor. *EMBO J* **10**: 369–375.
- Jungmann, J., Rayner, J.C., and Munro, S. (1999) The *Saccharomyces cerevisiae* protein Mnn10p/Bed1p is a subunit of a Golgi mannosyltransferase complex. *J Biol Chem* **274**: 6579–6585.
- Jurivich, D.A., Sistonen, L., Kroes, R.A., and Morimoto, R.I. (1992) Effect of sodium salicylate on the human heat shock response. *Science* **255**: 1243–1245.
- Lindquist, S., and Kim, G. (1996) Heat-shock protein 104 expression is sufficient for thermotolerance in yeast. *Proc Natl Acad Sci USA* **93**: 5301–5306.
- Liu, X.D., Liu-Phillip, C.C., Santoro, N., and Thiele, D.J. (1997) Conservation of a stress response: human heat shock transcription factors functionally substitute for yeast HSF. *EMBO J* **16**: 6466–6477.
- Nadeau, K., Das, A., and Walsh, C.T. (1993) Hsp90 chaperonins possess ATPase activity and bind heat shock transcription factors and peptidyl prolyl isomerases. *J Biol Chem* **268**: 1479–1487.
- Nakai, A., and Morimoto, R.I. (1993) Characterization of a novel chicken heat shock transcription factor, heat shock factor 3, suggests a new regulatory pathway. *Mol Cell Biol* **13**: 1983–1997.
- Nathan, D.F., and Lindquist, S. (1995) Mutational analysis of Hsp90 function: interactions with a steroid receptor and a protein kinase. *Mol Cell Biol* **15**: 3917–3925.
- Nathan, D.F., Vos, M.H., and Lindquist, S. (1997) *In vivo* functions of the *Saccharomyces cerevisiae* Hsp90 chaperone. *Proc Natl Acad Sci USA* **94**: 12949–12956.

- Park, H.O., and Craig, E.A. (1989) Positive and negative regulation of basal expression of a yeast Hsp70 gene. *Mol Cell Biol* **9**: 2025–2033.
- Park, H.O., and Craig, E.A. (1991) Transcriptional regulation of a yeast Hsp70 gene by heat shock factor and an upstream repression site-binding factor. *Genes Dev* **5**: 1299–1308.
- Parsell, D.A., Taulien, J., and Lindquist, S. (1993) The role of heat-shock proteins in thermotolerance. *Phil Trans R Soc Lond B Biol Sci* **339**: 279–285.
- Parsell, D.A., Kowal, A.S., Singer, M.A., and Lindquist, S. (1994) Protein disaggregation mediated by heat-shock protein Hsp104. *Nature* **372**: 475–478.
- Pelham, H.R.B. (1986) Speculations on the functions of the major heat shock and glucose-regulated proteins. *Cell* **46**: 959–961.
- Rabindran, S.K., Giorgi, G., Clos, J., and Wu, C. (1991) Molecular cloning and expression of a human heat shock factor, HSF1. *Proc Natl Acad Sci USA* **88**: 6906–6910.
- Rabindran, S.K., Wisniewski, L., Li, L., Li, G.C., and Wu, C. (1994) Interaction between heat shock factor and Hsp70 is insufficient to suppress induction of DNA-binding activity *in vivo*. *Mol Cell Biol* **14**: 6552–6560.
- Reindl, A., and Schoeffl, F. (1998) Interaction between the *Arabidopsis thaliana* heat shock transcription factor HSF1 and the TATA binding protein TBP. *FEBS Lett* **436**: 318–322.
- Sanchez, Y., and Lindquist, S.L. (1990) Hsp104 required for induced thermotolerance. *Science* **248**: 1112–1115.
- Sarge, K.D., Bray, A.E., and Goodson, M.L. (1995) Altered stress response in testis (letter). *Nature* **374**: 126.
- Satyal, S.H., Chen, D., Fox, S.G., Kramer, J.M., and Morimoto, R. (1998) Negative regulation of the heat shock transcriptional response by HSBP1. *Genes Dev* **12**: 1962–1974.
- Scharf, K.D., Rose, S., Zott, W., Schoffl, F., Nover, L., and Schoff, F. (1990) Three tomato genes code for heat stress transcription factors with a region of remarkable homology to the DNA-binding domain of the yeast HSF. *EMBO J* **9**: 4495–4501.
- Scharf, K.D., Hoehfeld, I., and Nover, L. (1998) Heat stress response and heat stress transcription factors. *J Biosci Bangalore* **23**: 313–329.
- Shi, Y., Mosser, D.D., and Morimoto, R.I. (1998) Molecular chaperones as HSF1-specific transcriptional repressors. *Genes Dev* **12**: 654–666.
- Shore, D., and Nasmyth, K. (1987) Purification and cloning of a DNA binding protein from yeast that binds to both silencer and activator elements. *Cell* **51**: 721–732.
- Sikorski, R.S., and Hieter, P. (1989) A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. *Genetics* **122**: 19–27.
- Sorger, P.K. (1990) Yeast heat shock factor contains separable transient and sustained response transcriptional activators. *Cell* **62**: 793–805.
- Sorger, P.K., and Pelham, H.R. (1988) Yeast heat shock factor is an essential DNA-binding protein that exhibits temperature-dependent phosphorylation. *Cell* **54**: 855–864.
- Sorger, P.K., Lewis, M.J., and Pelham, H.R. (1987) Heat shock factor is regulated differently in yeast and HeLa cells. *Nature* **329**: 81–84.
- Stephanou, A., Isenberg, D.A., Nakajima, K., and Latchman, D.S. (1999) Signal transducer and activator of transcription-1 and heat shock factor-1 interact and activate the transcription of the Hsp70 and Hsp90beta gene promoters. *J Biol Chem* **274**: 1723–1728.
- Unno, K., Kishido, T., Hosaka, M., and Okada, S. (1997) Role of Hsp70 subfamily, SSA, in protein folding in yeast cells, seen in luciferase-transformed *ssa* mutants. *Biol Pharm Bull* **20**: 1240–1244.
- Werner Washburne, M., Stone, D.E., and Craig, E.A. (1987) Complex interactions among members of an essential subfamily of Hsp70 genes in *Saccharomyces cerevisiae*. *Mol Cell Biol* **7**: 2568–2577.
- Zou, J., Guo, Y., Guettouche, T., Smith, D.F., and Voellmy, R. (1998) Repression of heat shock transcription factor HSF1 activation by Hsp90 (Hsp90 complex) that forms a stress-sensitive complex with HSF1. *Cell* **94**: 471–480.