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Complex Regulation of the Yeast Heat Shock Transcription Factor

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The yeast heat shock transcription factor (HSF) is regulated by posttranslational modification. Heat and superoxide can induce the conformational change associated with the heat shock response. Interaction between HSF and the chaperone hsp70 is also thought to play a role in HSF regulation. Here, we show that the Ssb1/2p member of the hsp70 family can form a stable, ATP-sensitive complex with HSF—a surprising finding because Ssb1/2p is not induced by heat shock. Phosphorylation and the assembly of HSF into larger, ATP-sensitive complexes both occur when HSF activity decreases, whether during adaptation to a raised temperature or during growth at low glucose concentrations. These larger HSF complexes also form during recovery from heat shock. However, if HSF is assembled into ATP-sensitive complexes (during growth at a low glucose concentration), heat shock does not stimulate the dissociation of the complexes. Nor does induction of the conformational change induce their dissociation. Modulation of the in vivo concentrations of the SSA and SSB proteins by deletion or overexpression affects HSF activity in a manner that is consistent with these findings and suggests the model that the SSA and SSB proteins perform distinct roles in the regulation of HSF activity.

INTRODUCTION

It has long been known that in cells of many species, including *Escherichia coli* and *Saccharomyces cerevisiae*, cell division rates are tightly coupled with the steady-state levels and rates of synthesis of ribosomal proteins and rRNA. For example, cells in rich media, with a short generation time, display higher abundance of rRNA and higher rates of synthesis of ribosomal proteins than do cells in poor media, with a long generation time. In *E. coli*, some of this regulation is achieved by transcriptional attenuation and translational regulation *via* binding of ribosomal proteins to the RNAs of target operons (Freedman *et al.*, 1985; Cole and Nomura, 1986). In yeast, regulation is partly transcriptional, via RAP1 and its binding site, the upstream activating sequence $(UAS)_{rpg}$ (Herruer *et al.*, 1987; Moehle and Hinnebusch, 1991; Kraakman *et al.*, 1993), although much of the regulation of ribosomal protein abundance is achieved by a competition between the assembly into ribosomes and the rapid degradation of unassembled ribosomal proteins (Warner *et al.*, 1985; Maicas *et al.*, 1988).

Growth rate control clearly modulates the level of the translational machinery, which in turn influences the overall rate of protein synthesis. Changes in the rates of protein synthesis must, in turn, have profound implications for the protein chaperone system. Newly synthesized polypeptides typically do not adopt their mature conformation immediately but instead follow a complex folding pathway. For many proteins, the cytoplasmic chaperone system plays an integral role in helping these polypeptides adopt their mature conformations (for review, see Hendrick and Hartl, 1995). The hsp70 proteins are generally believed to bind efficiently to nascent or newly synthesized polypeptides, recognizing exposed hydrophobic sequences. Binding of hsp70 thus plays a role in protein folding as well as serving the general function of decreasing the likelihood of nonspecific aggregation. Later steps in the catalysis of protein folding are thought to use the groE (bacterial) or TRiC (eukaryotic) complex.

As the rate of protein synthesis increases, and the cell cycle time decreases, cells are faced with the need to produce the same quantity of protein (enough to supply the daughter cell) in a shorter time. Not only will these proteins require generalized, chaperone-mediated protein folding, but many will be targeted to the various membrane systems. Transport into mitochondria and posttranslational transport into the ER both depend on hsp70 chaperone activity (Deshaies *et al.*, 1988). Thus, the overall rate of protein synthesis should have a direct influence on the demand for chaperone activity.

The regulation of chaperone synthesis is complex. The promoter of the hsp70 gene of humans, for example, contains binding sites for multiple transcriptional regulators

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Table 1. Plasmids used in this study

Plasmid	Description	Source
pJB254	YEpHSF$^{1-390}$ LEU2	Kopczynski *et al.*, 1992
pJB398	YEpHSF LEU2	*Eco*RI–*Xho*I fragment from YEpHSF LEu (Torres and Bonner, 1995) transferred to YEplac181 (Gietz and Sugino, 1988), with *Bgl*II and *Kpn*I engineered into codons 168 and 440, respectively
pJB330	YEpHSF$^{1-583}$ LEU2	Bonner *et al.*, 1994
pJB490	YCpHSF LEU2	*Eco*RI–*Pst*I fragment from pJB398 transferred into YCplac111 (Gietz and Sugino, 1988)
pJB491	YEpSSB1 LEU2	*SSB1* gene from pYW13 (E. A. Craig) cloned into YEplac181 (Gietz and Sugino, 1988)
pZ4G	YEpHSE-*lacZ* URA3	Synthetic HSE inserted into the *Xho*I site of pLGΔ178 (Guarente and Mason, 1983; Bonner *et al.*, 1994)
pLGΔ312	YEp-CYC1-*lacZ* URA3	CYC1 promoter driving *lacZ* (Guarente and Mason, 1983)

(Morgan *et al.*, 1987; Morgan, 1989). This gene can thus be expressed in response to stress as well as in response to various developmental or other cues. Regulatory complexity is magnified by the fact that the hsp70 gene is only one of a multigene family, each gene of which may have multiple regulators. The regulators themselves are often not unique, including (in vertebrates) the heat shock transcription factor (HSF) that elevates hsp70 expression during stress. It is therefore challenging to tease apart the individual controls that conspire to generate the final level of chaperone activity in multicellular organisms.

In the yeast *S. cerevisiae*, there is but a single heat shock transcription factor gene, *HSF1*, encoding HSF. All of the hsp70 genes are known and have been sorted into functional families (Craig *et al.*, 1993). Of these families, the *SSA* and *SSB* families produce proteins that are cytoplasmically localized and are thus most directly involved in cytoplasmic chaperone activity. The *SSA* family, comprised of genes *SSA1, SSA2, SSA3,* and *SSA4*, includes the traditional hsp70 (heat shock–inducible) and hsc70 (constitutive) proteins that have been implicated in protein chaperone activities. The *SSB* family, comprised of genes *SSB1* and *SSB2*, encodes proteins that bear significant similarity to the Ssa proteins but that are nonetheless functionally distinct (Craig and Jacobsen, 1985; James *et al.*, 1997). The Ssb proteins are regulated quite differently from the Ssa proteins, in that their expression is elevated by growth at low temperature and is repressed during heat shock (Werner-Washburne *et al.*, 1989; Iwahashi *et al.*, 1995; Lopez *et al.*, 1999). The Ssb proteins are ribosome-associated chaperones that associate with nascent peptides and probably participate in early steps in folding (Nelson *et al.*, 1992; Pfund *et al.*, 1998).

HSF (i.e., HSF1) is generally believed to be primarily responsible for modulating the expression of chaperones such as hsp70 during stress and, in yeast, for stress-dependent expression of *SSA1* and *SSA4* (for review, see Voellmy, 1994). It has been assumed that the main role of HSF is to elevate the expression of chaperones during periods of acute stress, in response to dramatic elevation of the level of partially unfolded proteins. Consistent with this idea are the observations that unfolded or aberrant proteins can induce the heat shock response when injected into cells in the absence of a temperature shift (Ananthan *et al.*, 1986; Mifflin and Cohen, 1994) and that recovery from heat shock depends critically on the amount of hsp70 that has been produced (DiDomenico *et al.*, 1982).

The activation of HSF by unfolded protein, at least at normal growth temperatures, most likely involves feedback regulation from chaperones like hsp70 and hsp90 (Craig and Gross, 1991; Zou *et al.*, 1998). It has been shown recently that HSF from both *Drosophila* and yeast is directly responsive to temperature (Zhong *et al.*, 1998; Lee *et al.*, 2000). It is thus possible that both HSF itself and hsp70 can serve as "cellular thermometers" in the activation of the heat shock response.

It is less clear what roles HSF may play outside the context of the traditional heat shock response. In yeast, deletion of *HSF1* is lethal, indicating a necessary function of HSF even during normal growth. Although it is possible that this function is to modulate the expression of chaperones such as hsp70, it is likely that HSF is involved in the regulation of other genes as well. It is the goal of this study to examine the roles of different aspects of HSF posttranslational modification. In particular, we sought to distinguish between HSF regulation caused by heat shock per se and regulatory modifications that are *not* specifically a part of the traditional heat shock response. We reasoned that a better understanding of the variety of HSF modifications would help disentangle our views of the roles of each and help identify those aspects of the heat shock system that are fundamental to the stress response, as compared with those aspects of HSF regulation that are involved in its other functions.

MATERIALS AND METHODS

Strains and Plasmids

Table 1 lists the plasmids that were used in this study. Table 2 lists the yeast strains that were used. Reagents were from Sigma (St. Louis, MO).

β-Galactosidase Assays

Cultures were grown at 25°C, unless otherwise indicated, in minimal medium supplemented with appropriate amino acids and sugars (glucose at various concentrations, as indicated in the text, or galactose at 2%) until the cell density reached A_{600} = 0.7–1.0. Cells were heat shocked at 41–42°C in a shaking water bath. After the appropriate time, cells were harvested and assayed as described previously (Bonner *et al.*, 1992). For the heat shock time course (see Figure 3A), both shocked and nonshocked cultures were diluted periodically to keep the cell density below A_{600} = 1 to maintain logarithmic growth.

Table 2. Strains used in this study

Strain	Genotype	Source
RE1006 Z46	**a** *can1.100 his3-11,15 leu2-3,112 trp1-1 ura3-52* [pZ4G]	RE1006 (R. Strich) bearing pZ4G
YJJ159 Z4G	**a** *trp1-289 ura3-52 met2 his3Δ1 leu2-3, 112* [pZ4G]	YJJ159 (J. Jaehning) bearing pZ4G
RE1006 Z4G *snf1*	**a** *can1.100 his3-11,15 leu2-3,112 trp1-1 ura3-52 snf1Δ* [pZ4G]	Derived from RE1006 Z4G
YJB270	**a** *prc1-407 prb1-1122 pep4-3 leu2 trp1 ura3-52 hsf1Δ* [YEpHSF$^{1-390}$ URA]	Kopczynski *et al.*, 1992
YJB341	**a**/α *trp1-289::TRP1-HSE-lacZ/trp1-289::TRP1-HSE-lacZ ura3-52/ura3-52 leu2-3,112;leu2-3,112 his3Δ1/his3ΔHindIII met2/MET2 his4-519/HIS4 ade1-100/ADE1 LYS2/LYS2::HSE-HIS3 hsf1Δ/hsf1Δ* [YEpHSF$^{1-583}$ URA]	Torres and Bonner, 1995
YJB371	**a** *prc1-407 prb1-1122 pep4-3 leu2 trp1 ura3-52 hsf1Δ* [pJB398]	Derived from YJB270 by plasmid shuffle
YJB378	**a**/α *trp1-289::TRP1-HSE-lacZ/trp1-289::TRP1-HSE-lacZ ura3-52/ura3-52 leu2-3,112;leu2-3,112 his3Δ1/his3ΔHindIII met2/MET2 his4-519/HIS4 ade1-100/ADE1 LYS2/LYS2::HSE-HIS3 hsf1Δ/hsf1Δ* [pJB398]	Derived from YJB341 by plasmid shuffle
YJB399	**a**/α *trp1-289::TRP1-HSE-lacZ/trp1-289::TRP1-HSE-lacZ ura3-52/ura3-52 leu2-3,112;leu2-3,112 his3Δ1/his3ΔHindIII met2/MET2 his4-519/HIS4 ade1-100/ADE1 LYS2/LYS2::HSE-HIS3 hsfΔ/hsf1Δ* [pJB490]	Derived from YJB341 by plasmid shuffle
ECY406	**a** *his3-11,15 leu2-3,112 ura3-52 trp1-Δ1 lys2?*	E. A. Craig
ECY419	**a** *his3-11,15 leu2-3,112 ura3-52 trp1-Δ1 lys2? ssb1-1::LEU2 ssb2-1::HIS3*	Derived from ECY406 E. A. Craig
T127	**a** *leu2-3 leu2-112 ade2-101 trp1Δ ura3-52 lys2*	E. A. Craig
T128	**a** *leu2-3 leu2-112 ade2-101 trp1Δ ura3-52 lys2 ssa1::LEU2 ssa2::LEU2*	Derived from T127 E. A. Craig

Gel Mobility Shift DNA-Binding Assays

Extracts were prepared from YJB371 as described previously (Bonner *et al.*, 1994). For each binding assay, extract (35 μg of protein) was suspended in 15 μl of binding reaction (20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES], pH 7.9, 1 mM EDTA, 60 mM KCl, 12% glycerol, 1 mM dithiothreitol, 1 μg/l bovine serum albumin [BSA], 0.1 μg/ml sonicated *E. coli* DNA). The binding reaction was initiated by the addition of 1.3 ng of oligonucleotide probe BS4T (5′-ACAGGGATCCTGAAGCTTCTAGAAGCTTCCTAGAGTCGACCTGCAG-3′) labeled with [α-^{32}P]dCTP by fill-in synthesis of a second strand after priming with oligo Bsb (5′-CTGCAGGTCGACTCTAG-3′). In one experiment (see Figure 6), the probe was BS6T, which differs from BS4T only in containing an additional AGAAGCTTCT repeat. ATP (1 mM) was included in the reactions as noted in the text (results were identical whether or not the reactions were supplemented with 3 mM $MgCl_2$.) Reactions were incubated at room temperature for 30 min and then chilled and loaded at 4°C onto a 4–10% polyacrylamide gel (prepared and run in 22.5 mM Tris-base, 22.5 mM boric acid, 0.63 mM EDTA). Gels were run at 4°C for 4800 V-h. In the experiment shown in Figure 1, monoclonal antibody MA3-007 (Affinity Bioreagents, Golden, CO) was added at the beginning of the incubations, as indicated in the text.

Westerns and Coimmunoprecipitation Assays

For coimmunoprecipitation, extract (600 μg of protein) prepared as described previously (Bonner *et al.*, 1994) was diluted into 1 ml of XLB buffer (175 mM NaCl, 15 mM Tris, pH 8.0, 1 mM EDTA, 1.5 mM $MgCl_2$). Cross-linking was initiated by the addition of 15 μl of 10 mM dithiobis[succinimidylproprionate] (DSSP; Pierce Chemical, Rockford, IL). Cross-linking was terminated after 10 min by the addition of 10 μl of 1 M lysine, and affinity-purified anti-HSF antibody was added. After binding, IgG was bound to protein A Sepharose, and samples were washed 10 times in XLB and eluted into SDS sample buffer. Bound protein was divided into two samples, either with or without ∃-mercaptoethanol to dissociate the cross-links, and run on a 4–10% SDS polyacrylamide gel. For the experiment shown in Figure 2B, samples were prepared as described by Sadler *et al.* (1989). For Western analysis, gels were blotted onto nitrocellulose in 50 mM Tris, 380 mM glycine, 0.1% SDS, and 20% methanol, blocked in 3% Carnation nonfat dry milk in 8 g/l NaCl, 0.2 g/l KCl, 3 g/l Tris, pH 8, and 0.1% Tween 20), and processed for immunodetection with antibody directed against HSF or against sp70 (the latter was the generous gift of E. A. Craig, University of Wisconsin, Madison, WI). Westerns were visualized by enhanced chemiluminescence, using reagents purchased from Pierce Chemical (Rockford, IL).

RESULTS

The Ssb1/2p Members of the hsp70 Family Bind to Yeast HSF

Mammalian HSF1 has been shown to form a complex with hsp70 (Abravaya *et al.*, 1992; Baler *et al.*, 1992), leading to the compelling idea that HSF activity is regulated by interaction with this protein. To determine whether yeast HSF is also capable of direct association with any of the hsp70 proteins of yeast, we performed two kinds of experiments: coimmu-

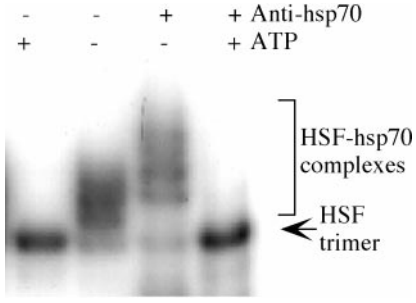

Figure 1. Demonstration of HSF–hsp70 complexes. An extract that displays the multiple HSF bands characteristic of cells grown in low glucose was used for a gel mobility shift DNA-binding assay. ATP was added to the two outer lanes, and antibody to hsp70 was added to the two right lanes. The ladder of bands above the HSF trimer was specifically supershifted by the antibody and was specifically eliminated by ATP.

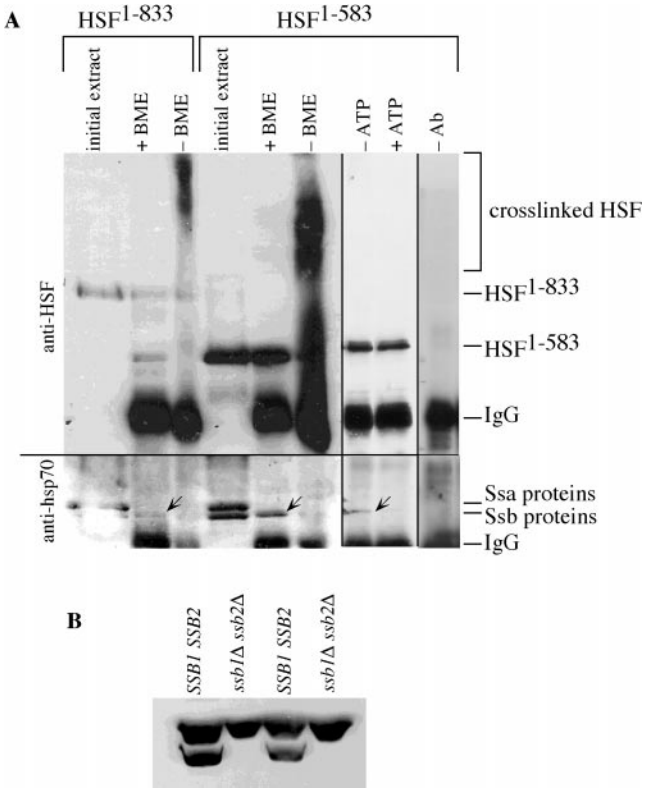

Figure 2. Identification of the hsp70 species that binds HSF. (A) Coimmunoprecipitation of hsp70 and HSF is shown. Extracts of cells containing either full-length HSF (HSF^{1-833}) or the C-terminally truncated HSF^{1-583} were diluted and incubated with the reversible cross-linker DSSP. After cross-linking was terminated by addition of lysine, samples were immunoprecipitated with antibody to HSF. Immunoprecipitates were run on a 4–10% SDS acrylamide gel either with or without the addition of β-mercaptoethanol (BME) to reverse the cross-links. The gel was blotted to nitrocellulose and analyzed immunochemically with antibody to HSF (top) or antibody to hsp70 (bottom). Arrows (bottom) indicate the hsp70 band that is specifically coimmunoprecipitated. Using HSF^{1-583}, two controls (right) were also run: immunoprecipitations with and without the addition of 1 mM ATP to the immunoprecipitation mixture and a mock immunoprecipitation without the anti-HSF antibody. (B) The lower band is Ssb1/2p. Extracts were run in duplicate from cells carrying the wild-type alleles of *SSB1* and *SSB2* (ECY406) or the *ssb1*Δ, *ssb2*Δ double disruption (ECY419). Large quantities of extract were used in an attempt to reveal cross-reacting material at the position of the Ssb1/2p band in the double-disruption strain. The blot was probed with the same antibody to hsp70 that was used in A.

noprecipitation and a gel mobility shift assay. We will first consider the gel mobility shift. Yeast HSF, when provided with ^{32}P-labeled DNA containing the sequence GAAnnTTCnnGAAnnTTC, forms a DNA–HSF complex containing a single HSF trimer (Bonner *et al.*, 1994). As shown in Figure 1, lane 1 (on left), this is the sole complex that is visible when an HSF-containing cell extract is incubated with the probe in the presence of 1 mM ATP. In the absence of ATP, however (Figure 1, lane 2 from left), additional bands are visible that migrate more slowly than does the trimer. These slow-migrating bands, but not the HSF trimer band, are retarded by the addition of a monoclonal antibody directed against hsp70 (Figure 1, lane 3 from left). This result identifies the upper bands as complexes that contain, in addition to HSF, one or more molecules of hsp70. That hsp70 is induced to dissociate by ATP is consistent with the findings of Palleros *et al.* (1993) that ATP induces hsp70 to adopt a conformation that has low affinity for its peptide substrates. The multiple bands may represent HSF trimers that contain different numbers of hsp70 molecules or that contain hsp70 in addition to other, as yet unidentified, proteins. Whatever additional proteins may be present in the HSF–hsp70 complexes, they are apparently all released by the addition of ATP.

The results presented in Figure 1 demonstrate that HSF can be found in a complex that contains one or more species of hsp70, but they do not identify the hsp70 species that binds to HSF. To ascertain which of the numerous hsp70 family members binds to yeast HSF, we performed a coimmunoprecipitation experiment. We used two strains, one of which expresses full-length HSF and the other of which expresses a truncated version of HSF (HSF^{1-583}), which is more abundant in cell extracts. The latter was used to increase the signal strength in the experiment. Extracts were diluted, treated with the reversible cross-linker DSSP, and then immunoprecipitated with antibody to HSF. Samples were separated by SDS gel electrophoresis either with or without reversal of the cross-links with β-mercaptoethanol. Proteins were detected by immunoblot, using antibody to HSF (Figure 2A, top) or antibody to hsp70 (Figure 2A, bottom).

In yeast cells, the cytoplasmic hsp70 proteins resolve into two bands, both of which are detectable with our antibody (the generous gift of E. A. Craig). The upper band has been identified by Gao *et al.* (1991) as the product of the *SSA* gene family, and the lower product has been shown to be the product of the *SSB* gene family (Gao *et al.*, 1991). The lower band (Ssb1/2p) was specifically coimmunoprecipitated with HSF (Figure 2A). Coimmunoprecipitation was more readily detected with the truncated HSF but was nonetheless evident with full-length HSF. Precipitation of the lower band was eliminated by the addition of ATP to the incubation with anti-HSF antibody and was eliminated by a mock immunoprecipitation without the anti-HSF antibody (Figure 2A). To confirm that our antibody recognized no other proteins with the mobility of Ssb1/2p and thus to reconfirm the identity of the lower band, we used the same antibody to probe a blot of wild-type cells compared with an *ssb1*Δ, *ssb2*Δ double mutant (Figure 2B). The deletion of the *SSB1* and *SSB2* genes specifically eliminated the lower band, thereby identifying this band as the Ssb1/2p proteins and confirming the specificity of the antibody.

Taken together, the results presented in Figures 1 and 2 demonstrate that HSF can exist in a complex that contains the SSB family of hsp70 proteins. The results do not exclude the possibility that other proteins, including the SSA family of hsp70, might also associate with HSF under these or other conditions. No other proteins were detected using antibodies specific to the SSA family, or to hsp90, but this negative result must be interpreted only to mean that we were unsuccessful in detecting such associations. Nonetheless, the finding that Ssb1/2p *does* associate with HSF is surprising. The Ssb1/2p proteins are not heat shock inducible (Werner-

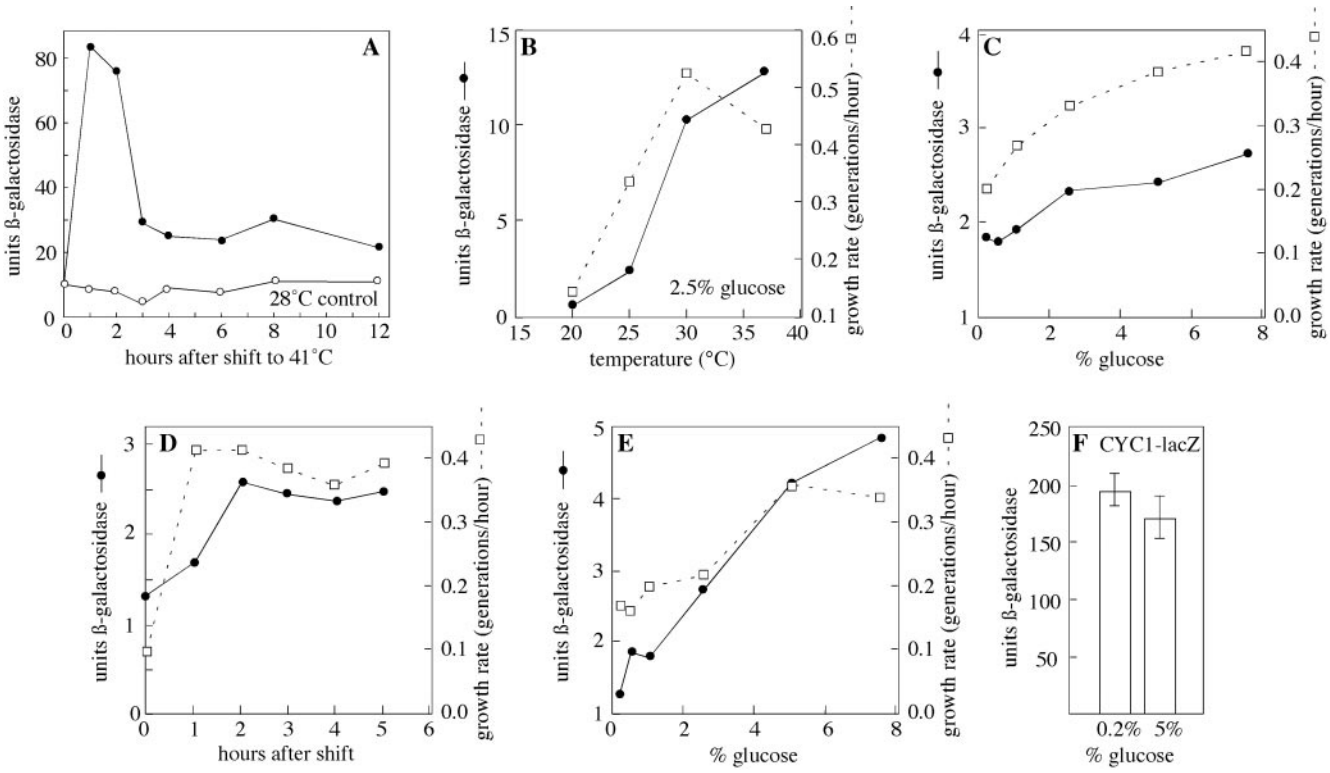

Figure 3. β-Galactosidase activity varies with the physiological state of the cells. Cells carrying the HSE–*lacZ* reporter on an episomal plasmid were grown under various conditions and then assayed for β-galactosidase activity. (A) Cells (strain YJJ159 Z4G) were grown at 28°C, and the culture was split. One-half was transferred to 41°C; the other remained at 28°C. At hourly intervals, samples were removed for analysis. The cultures were diluted periodically to maintain logarithmic growth. The data present a representative experiment. (B) Cells (strain RE1006 Z4G) were grown at various temperatures in medium containing 2.5% glucose, and the steady-state level of β-galactosidase was measured. Data are averages of two independent experiments. (C) Cells (strain RE1006 Z4G) were grown at 25°C in media containing various concentrations of glucose, and the steady-state level of β-galactosidase was measured. Data are averages of two independent experiments. (D) Cells (strain RE1006 Z4G) were grown at 0.2% glucose and then shifted to 2% glucose. β-Galactosidase and growth rate were measured at hourly intervals. Data present a representative experiment. (E) This experiment is similar to that in C, except that the strain RE1006 *snf1*Δ Z4G was used. (F) β-Galactosidase activities in cells (strain RE1006 carrying pLGΔ312) grown in 0.2 or 5% glucose were measured.

Washburne *et al.*, 1989; Iwahashi *et al.*, 1995). This suggests that this particular HSF–hsp70 interaction may reflect a function of HSF in a regulatory system distinct from the heat shock response per se. We therefore sought to determine whether other physiological states *besides* heat shock might influence the activity and modification of HSF and what roles (if any) the SSB proteins might play.

HSF Activity Can Be Regulated Independent of Stress

To examine the activity of HSF in the absence of additional, potentially interacting transcription factors, we prepared a β-galactosidase reporter gene that has, as its sole regulatory element, a synthetic heat shock element (HSE–*lacZ* [Bonner *et al.*, 1992, 1994]). The *lacZ* vector pLGΔ178, originally built by Guarente and Mason (1983), has been used by many laboratories and shown to have essentially no activity without the insertion of an appropriate UAS; to the best of our knowledge, this reporter gene responds only to HSF. Thus, to a first approximation, the level of β-galactosidase activity in cells carrying this reporter gene reflects the activity of HSF (although the *kinetics* of changes in activity are not accurately reported, because of the inherent stability of β-galactosidase). In those instances in which they have been tested, other UASs do not give similar results to those described here, suggesting that fluctuations in β-galactosidase mRNA or protein stability are unlikely to account for our observations.

In a typical heat shock response (Figure 3A), β-galactosidase activity increases rapidly from a distinct basal level to an induced level; with time, activity decreases again to a new steady-state level. The general outlines of the response are seen for nearly all temperature upshifts within the growth range; only the absolute values of β-galactosidase activity differ. This suggests that the regulation of HSF has at least three components: an "acute phase" response that occurs in the first minutes of the temperature shift to elevate the transcriptional activity of HSF, an "adaptation" phase in which HSF activity is downregulated as cells adapt to the new temperature, and a "steady-state" level of activity that

is seen in cells that are adapted to a particular temperature. We prefer to refer to this as "steady-state" activity, rather than "basal," because the steady-state level of activity that cells display after adaptation to a mild temperature upshift becomes the basal level of activity from which they mount their heat shock response upon a more severe temperature upshift.

The steady-state level of activity depends critically on the temperature to which the cells are adapted. As shown in Figure 3B, the steady-state level of β-galactosidase activity varies with growth temperature, indicating that steady-state activity is under some form of regulation.

Figure 3B also shows that *growth rate* increases with temperature, at least until stressful temperatures are met. It is therefore unclear whether the effect of temperature on the steady-state level of HSF activity, as measured by HSE–*lacZ*, reflects the temperature itself (and thus the degree of stress to which cells are exposed) or the cell division time per se. We therefore sought to vary growth rates without altering the temperature. To do so, we examined the activity of the HSE–*lacZ* reporter in cells grown in differing concentrations of glucose. The results, shown in Figure 3C, indicate that HSF activity increases in relationship to growth rate.

The traditional view of the heat shock system is that HSF activity should increase in response to stress. Yet, in the experiment shown in Figure 3C, HSF activity is highest in cells growing the fastest, which seems unlikely to represent the most stressful conditions. A possible explanation might lie in the fact that yeast fermenting glucose produce ethanol, which is a known inducer of the heat shock response. To determine whether ethanol stress might underlie the results of Figure 3C, we performed a nutritional upshift experiment, shifting cells from low to high glucose. We reasoned that growth rate would increase rapidly, although it would take a significant time period for the cells to produce sufficient ethanol to render their growth medium stressful. The result of a typical experiment is shown in Figure 3D. The β-galactosidase activity shifted from a low steady-state level to a higher steady-state level within the first 2 h; thereafter it remained constant. This pattern of expression is remarkably similar to the pattern of expression of ribosomal proteins after nutritional upshift (Griffioen *et al*., 1994). This result argues against the idea that the higher β-galactosidase level in rapidly growing cells is a response to ethanol stress.

The interpretation of the experiment shown in Figure 3C is clouded by the fact that yeast preferentially degrade glucose by fermentation and switch to oxidative phosphorylation when glucose is deprived. Thus, the experiment shown in Figure 3C actually varies both growth rate and physiological state. To prevent cells from activating the genes that are subject to glucose repression and thus to restrict cells to the fermentative pathway, we repeated the experiment in cells deleted for *SNF1* kinase, which is required to relieve glucose repression (Celenza and Carlson, 1984). The result, shown in Figure 3E, was striking; the effect of lowered glucose on both growth rate and β-galactosidase activity was much more pronounced in *snf1* cells than in the *SNF1* cells shown in Figure 3C.

Although our HSE–*lacZ* reporter gene indicates that HSF activity increases with increased glucose concentration, it is conceivable that such changes might reflect properties of β-galactosidase expression in general. That these results are *not* reproduced by all *lacZ* reporter genes is shown by the behavior of the *CYC1–lacZ* reporter gene (from which our HSE–*lacZ* reporter gene was derived; Figure 3F). Here, β-galactosidase activity was somewhat lower in cells grown at high glucose concentration, the opposite pattern to that seen with the HSE–*lacZ* reporter gene.

Taken together, these several experiments support the view that steady-state HSF activity is tied to growth rate. That is, there appear to be other controls over HSF activity besides the traditional stress response. To investigate possible mechanisms for the nonstress modulation of HSF activity, we investigated HSF phosphorylation and the formation of ATP-sensitive complexes, as described below.

HSF Phosphorylation and Downregulation of HSF Activity

In gel mobility shift experiments such as that shown in Figure 1, the mobility of HSF is influenced not only by the binding of other proteins but also by phosphorylation. Because the conditions that stimulate phosphorylation are not mutually exclusive with those that stimulate the formation of larger HSF complexes, the interpretation of experiments to be presented later will be more straightforward if we present a discussion of phosphorylation first. To a large extent, our observations corroborate the findings of others (Sorger *et al*., 1987; Høj and Jakobsen, 1994) and are to some extent confirmatory. However, the new findings extend our understanding in an unexpected way.

It was shown first by Sorger *et al.* (1987) that HSF phosphorylated at specific, but as yet undefined, sites exhibits a slow electrophoretic mobility on native gels. It has been shown numerous times (Sorger *et al*., 1987; Sorger and Pelham, 1988; Bonner, unpublished observations) that this mobility shift can be reversed by treatment with phosphatase. This mobility shift is, at best, an imperfect surrogate for the direct measurement of phosphorylation but serves as a very good assay for phosphorylation of those sites that cause the mobility change. By the use of this assay, it is possible to examine this aspect of phosphorylation of HSF from cells grown or shocked under different conditions.

Figure 4A (left half, +ATP) illustrates the effect of a heat shock on the mobility of HSF. In confirmation of the findings of others (Sorger *et al*., 1987; Sorger and Pelham, 1988; Høj and Jakobsen, 1994), the heat shock induces a detectable change in mobility. As has been shown previously (Sorger *et al*., 1987; Sorger and Pelham, 1988), the mobility shift is completely reversible by treatment with shrimp alkaline phosphatase (our unpublished observations). Surprisingly, however, the extent of the mobility shift is dependent on the length of the heat shock. Upon longer heat shock, the mobility of HSF becomes even slower and more heterodisperse (Figure 4A, lanes 3 and 4 from left), suggesting that additional phosphorylation occurs during adaptation.

The gel mobility effect caused by HSF phosphorylation is not confined to heat shock. It can also be seen in nonshocked cells at the lowest glucose concentrations, with the lowest steady-state level of activity. This is evident in the experiment shown in Figure 4B, which examines HSF from cells grown in various concentrations of glucose. In the presence of ATP, the mobility of HSF from cells grown in 0.2% glucose is somewhat heterodisperse, whereas the mobility of HSF from cells grown in the absence of glucose (2% glycerol)

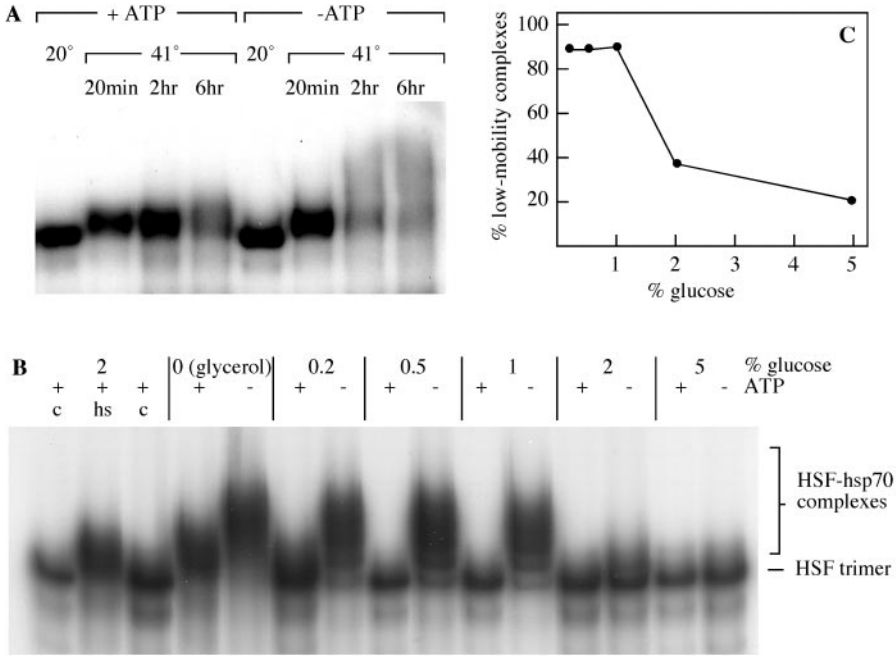

Figure 4. HSF mobility and association with hsp70 vary with the physiological state of the cells. (A) Duration of heat shock. Cells were grown at 20°C and then shifted to 41°C. At the indicated times, samples were harvested, and extracts were prepared. Samples were analyzed by gel mobility shift DNA-binding assay, either with (left 4 lanes) or without (right 4 lanes) the addition of 1 mM ATP. (B) Steady-state growth conditions. Cells were grown at 25°C in media containing the indicated concentrations of glucose, and extracts were prepared. For comparison, cells grown in standard 2% glucose medium were either heat shocked (hs) or left as unshocked controls (c). Gel mobility shift DNA-binding assays were performed, either with (+) or without (−) the addition of ATP. (C) Quantitation (by phosphorimager) of the gel shown in B.

is markedly slower than normal, similar to the mobility of HSF from cells that have been heat shocked for an hour. These mobilities suggest that HSF is phosphorylated during steady-state growth at low glucose. This suggestion was confirmed by a Western blot of HSF run on SDS gels (our unpublished observations), which is also sensitive to phosphorylation-induced mobility changes.

Taken together, these results are most consistent with the suggestion of Høj and Jakobsen (1994) that the aspect of HSF phosphorylation that is revealed in these assays is likely to be involved in the downregulation of HSF activity. These authors concluded that phosphorylation participates in the downregulation of HSF activity as cells adapt to heat shock, a conclusion entirely consistent with the data of Figure 4A. The data of Figure 4B extend this conclusion to suggest that phosphorylation may downregulate HSF activity in low growth rate conditions as well. However, phosphorylation alone is clearly insufficient to account for all of the HSF regulation. First, cells show adaptation to heat shock at high, medium, and low glucose concentrations, despite significant differences in the extent of HSF phosphorylation. Second, steady-state HSF activity is regulated at intermediate glucose concentrations at which HSF phosphorylation is not readily detectable. The following experiments help address this issue by revealing that HSF phosphorylation can occur simultaneously with the assembly of larger HSF complexes, thus allowing combinatorial regulatory mechanisms.

Sequestration of HSF in Larger Complexes Varies with the Physiological Conditions

The data presented above suggest that HSF phosphorylation may play a role in the downregulation of HSF activity but also emphasize that phosphorylation is insufficient to account for all of the regulation of HSF activity that can be measured. It is therefore likely that there are additional controls over HSF activity besides phosphorylation, both during steady-state growth and during adaptation to elevated temperature. As has been suggested by others, hsp70 binding is likely to downregulate HSF activity. The results shown above (Figures 1 and 2) indicate that the ATP sensitivity of slowly migrating complexes represents an assay for the assembly of HSF–hsp70 complexes; we therefore used this assay to examine the assembly of such HSF complexes under different physiological conditions.

We will first consider the formation of ATP-sensitive HSF complexes in extracts from cells grown at different glucose concentrations (i.e., steady-state conditions), shown in Figure 4B. At the highest glucose concentration (5%), all of the HSF runs at the position of the HSF trimer. There are no ATP-sensitive bands. This result indicates that HSF is not sequestered into larger complexes in cells grown in 5% glucose. Similarly, for cells grown in 2% glucose, very little of the HSF is found in ATP-sensitive complexes. At lower glucose concentrations, however, the majority of the HSF is in a ladder of bands of slower mobility than that of the trimer. From these results, we infer that the assembly of larger HSF complexes is regulated and varies with growth conditions.

Phosphorimager analysis of the gel shown in Figure 4B (Figure 4C) indicates that association of other proteins with HSF does not exactly follow the HSF activity curve shown in Figure 3C. β-Galactosidase activity declines more smoothly with decreasing glucose concentrations than does the assembly of larger HSF complexes. This suggests that, although sequestration of HSF into larger complexes could participate in downregulating steady-state HSF activity, it is unlikely to be wholly responsible. However, HSF is also phosphorylated at the lowest glucose concentrations, as noted previously. Thus, phosphorylation can occur simultaneously with

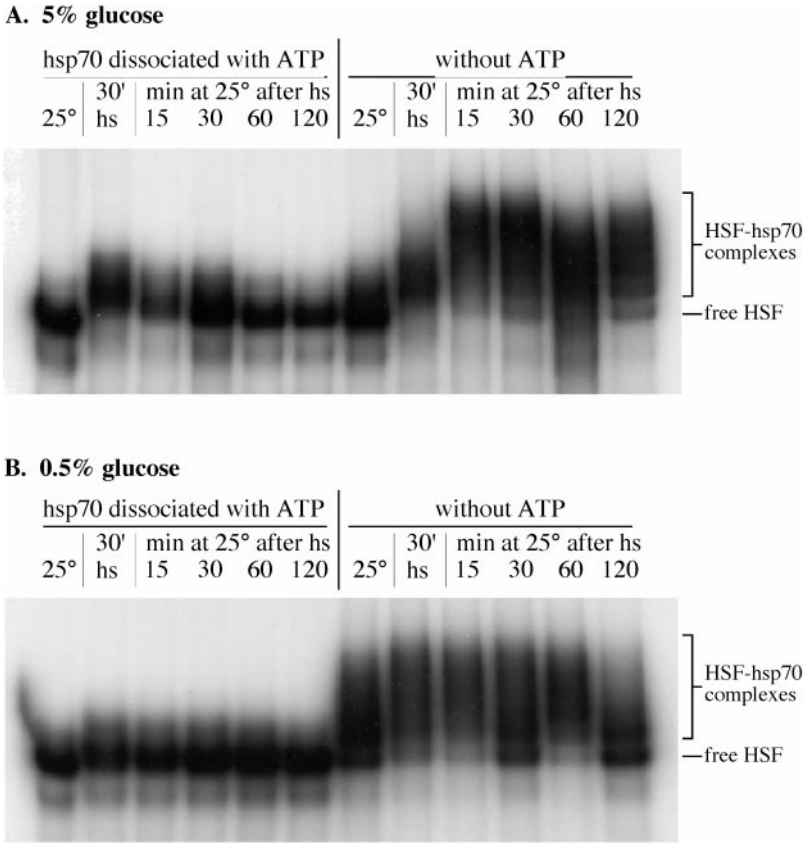

Figure 5. Behavior of HSF–hsp70 complexes during and after heat shock. Cells were grown at 25°C and heat shocked for 30 min at 41°C before being returned to 25°C. Extracts were prepared, and gel mobility shift DNA-binding assays were performed. Samples were run with (left lanes) or without (right lanes) the addition of ATP to dissociate hsp70. (A) Cells were grown in 5% glucose to prevent the binding of Ssb1/2p to HSF under normal growth conditions. ATP-sensitive complexes form only during recovery from heat shock. (B) Cells were grown in 0.5% glucose to induce the binding of Ssb1/2p to HSF. The heat shock did not dissociate Ssb1/2p from the HSF.

the binding of additional proteins to HSF. If both act to downregulate HSF activity, it might well be possible to create the pattern of HSF activity shown in Figure 3C.

We also examined the effect of heat shock on the formation of ATP-sensitive complexes. Figure 4A shows an analysis of such complexes in cells that have been heat shocked for various time periods. During steady-state growth (in 2% glucose), essentially none of the HSF is in ATP-sensitive, slow-mobility complexes, consistent with the observations reported above. HSF from cells heat shocked briefly also shows very little effect of ATP (although the effect of heat shock–induced phosphorylation is evident in the change in mobility). Apparently, under these conditions, little of the HSF is sequestered into larger complexes. HSF from cells subjected to a long heat shock, however, shows a dramatic difference. In the absence of ATP, much of the HSF is retarded to much slower mobility. The HSF changes its mobility when ATP is added, suggesting that much of the HSF is bound to other proteins. This observation indicates that, under these growth conditions, the assembly of HSF into larger complexes occurs during adaptation to heat shock. This finding parallels the findings described above with HSF phosphorylation, suggesting that HSF sequestration could cooperate with phosphorylation to downregulate HSF activity during adaptation to elevated temperature.

We last sought to determine how HSF responds to a brief heat shock, followed by recovery. We present data for cells grown in high glucose (Figure 5A) and in low glucose (Figure 5B). Cells grown under normal conditions (2% glucose) behaved identically to cells grown at higher glucose (our unpublished observations). There are several significant observations. First, if HSF is not assembled into larger complexes during steady-state growth (Figure 5A), complex formation does not occur during the short heat shock (consistent with the data of Figure 4A). Second, larger HSF complexes do form upon recovery from the shock, when HSF activity is decreasing. This is reminiscent of HSF behavior during adaptation to elevated temperature. Interestingly, the cells did not return to the identical physiological state in which they were before the shock; presumably, a longer recovery period is required. Third, if larger HSF complexes do form during steady-state growth (Figure 5B), the heat shock and recovery period have little effect on the ATP-sensitive complexes. This is somewhat surprising, in that one might have expected these complexes to dissociate during heat shock. However, this was not observed.

Together, the data of Figures 4 and 5 suggest that the function of HSF assembly into larger complexes is not part of the heat shock response per se. Figures 4 and 5A suggest that the assembly of ATP-sensitive complexes could play a role in the downregulation of HSF activity, consistent with previous suggestions that hsp70 helps to depress HSF activity in other organisms, but the findings of Figure 5B indicate that such complexes can exist before, during, and after a heat shock.

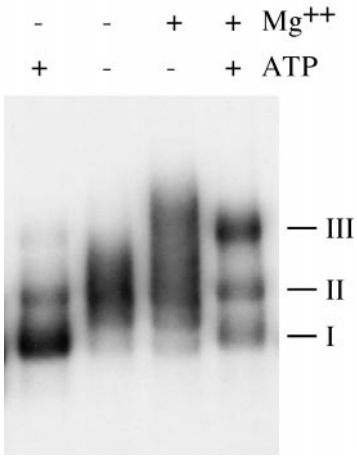

Figure 6. Ssb1/2p binds to HSF in both high- and low-activity conformations. A DNA-binding assay was performed using probe 6T, to which two trimers can bind. ATP was added to the incubations run in the outer two lanes to dissociate Ssb1/2p. $MgCl_2$ (10 mM) was added to the incubations run in the two right lanes to induce the formation of the high-activity conformation (complex III).

HSF Complexes Do Not Dissociate upon Superoxide-stimulated Conformational Change

We have shown recently (Lee *et al.*, 2000) that HSF undergoes a conformational change upon heat shock. This conformational change is detectable only in the context of two HSF trimers bound cooperatively to DNA. It is induced by temperature or by superoxide-dependent HSF modification, which can be induced in vitro by addition of high concentrations of Mg^{+2}. The HSF assays described above used single, DNA-bound trimers and thus did not assess the possibility that the active conformation of HSF might be unable to assemble into slowly migrating, ATP-sensitive complexes. To examine the behavior of this conformational state of HSF, we performed a gel mobility shift using a DNA probe to which two trimers can bind (Figure 6). In the absence of Mg^{+2} (to stabilize the low-activity conformation) and with added ATP (to release bound proteins), HSF binds to this DNA probe to give two bands, representing the binding of one trimer (band I) and two trimers (band II). Addition of 10 mM $MgCl_2$ induces the formation of the active conformation (Figure 6, band III). The same experiments, performed in the absence of ATP, demonstrate that all three forms of HSF participate in the ladder of ATP-sensitive bands. We interpret this result to mean that, even in the active conformation, HSF can bind to other proteins readily and, conversely, that the HSF conformational change does not cause these other proteins to dissociate.

Altering the Dose of SSB1 Modulates HSF Activity In Vivo, But Not Very Much

We have shown above (Figure 2) that HSF can bind the *SSB* family of hsp70 proteins, suggesting that the Ssb proteins could play a role in regulating HSF activity. It is also possible that the *SSA* family of hsp70 proteins may interact with HSF, either transiently during the interconversion of inactive and active conformations of HSF or stably under conditions we have not examined. It is therefore essential to examine the role of the hsp70 proteins in vivo. It has been shown previously that deletion of *SSA1* and *SSA2* results in temperature-sensitive growth and elevation of expression of other heat shock proteins (Craig and Jacobsen, 1984), whereas deletion of the entire SSA gene family is lethal (Werner-Washburne *et al.*, 1987). As shown in Figure 7A, deletion of *SSA1* and *SSA2* dramatically elevates HSF activity, consistent with these previous observations. The effect of the *ssa1*Δ, *ssa2*Δ double disruption on HSF could reflect feedback regulation of the Ssa proteins on HSF directly or could result from a generalized growth defect that stimulates a stress response. If it were the former and if it were attributable to stoichiometric binding of the Ssa proteins to HSF in a stable, low-activity complex, then one would expect that overproduction of the Ssa proteins should have the converse effect.

Stone and Craig (1990) have shown previously that overproduction of Ssa1p has little effect on the expression of an hsp70–*lacZ* reporter gene unless additional regulatory sequences are included besides the HSE. We observed a similar lack of effect on our HSE–*lacZ* reporter gene (Figure 7B). Indeed, in nonshocked cells, overexpression of Ssa1p *elevated* β-galactosidase activity slightly. This result argues against the idea that the Ssa proteins bind stably to HSF to lower its activity. If they act in a feedback system, they are more likely to function by refolding HSF after it has been activated, in which case the deletion of *SSA* genes might affect steady-state HSF activity, but overexpression would not produce an observable effect.

To complement these studies, we have examined the effects of deletion and overexpression of the *SSB* family of hsp70 proteins. The *ssb*Δ*,1 ssb2*Δ double disruption is viable (but slow growing and cold sensitive [Craig and Jacobsen, 1985]), thus allowing a stringent test of the maximum effect of the Ssb proteins on HSF. Figure 7C shows that the *ssb1*Δ, *ssb2*Δ double disruption causes the elevation of HSF activity both in unstressed and in heat-shocked cells, but only a fewfold. Because the *ssb1*Δ, *ssb2*Δ double deletion neither rendered β-galactosidase constitutive nor prevented the heat shock response, it appears that Ssb1/2p cannot be required to maintain HSF in a low-activity state before heat shock or to activate HSF in response to heat shock. This result is consistent with the behavior of the ATP-sensitive complexes described above. They formed when HSF activity was being downregulated by a fewfold and were able to assemble whether cells were, or were not, subjected to heat shock. Thus, these findings show that Ssb1/2p is unlikely to regulate the heat shock response per se; at best, it may be able to fine-tune HSF activity both during steady-state growth and during heat shock.

Cells deleted for *SSB1* and *SSB2* are cold sensitive and grow poorly. [Indeed, this growth defect precludes disrupting the *SSB1* and *SSB2* genes in the protease-deficient strain from which we prepare HSF for DNA-binding assays.] Thus, the results shown in Figure 7C, like the results shown in Figure 7A, are subject to the caveat that the physiological problems associated with the growth defect could result in a form of stress, which induces a

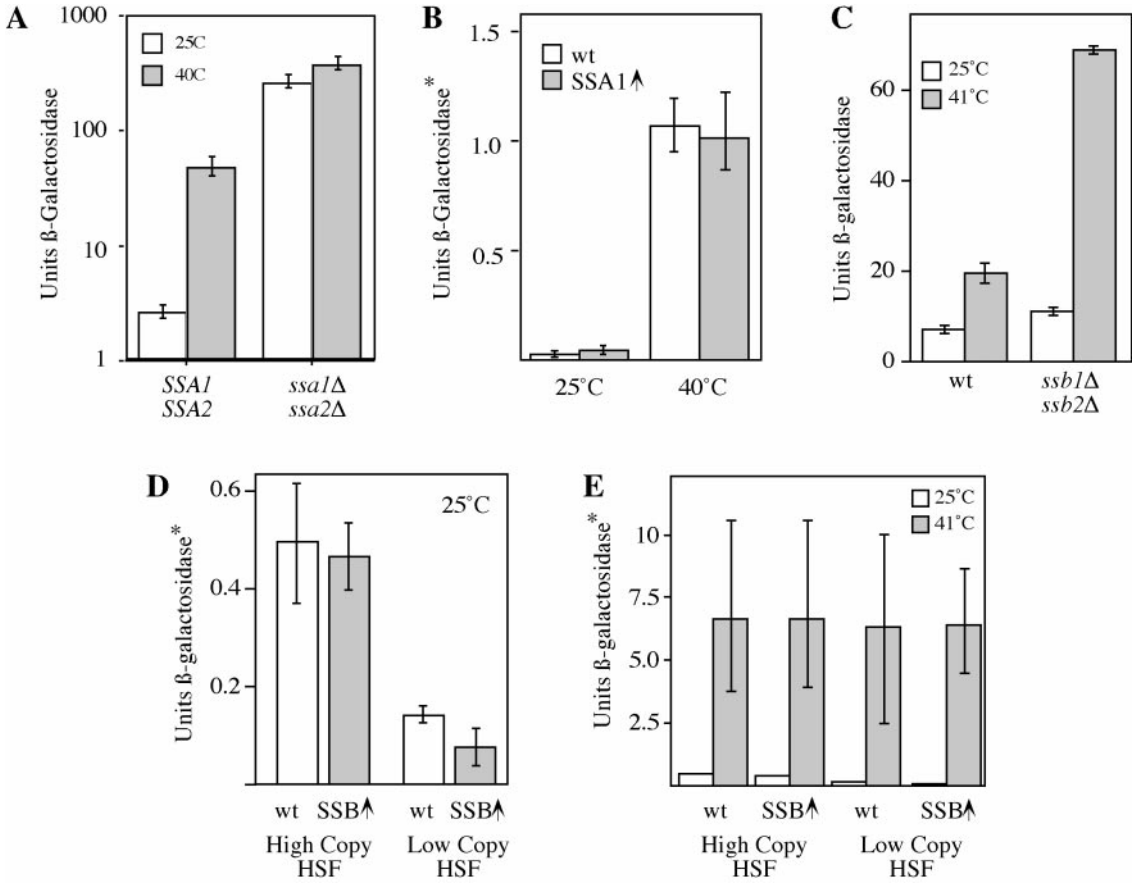

Figure 7. Variation of HSF activity as a function of the dose of SSA and SSB proteins. (A) Deletion of *SSA1* and *SSA2*. Strains T127 and T128 were transformed with an (episomal) HSE–*lacZ* reporter plasmid and assayed for β-galactosidase activity either before (25°C) or after a 1-h heat shock at 40°C. (B) Overproduction of Ssa1p. Isogenic strains carrying the HSE–*lacZ* reporter gene integrated into the *TRP1* locus were transformed with a plasmid carrying the *SSA1* gene under control of the *GAL1* promoter (SSA1 ↑) or with the vector alone (wt). Cells were grown in galactose to induce overexpression of Ssa1p; β-galactosidase activity was assayed either before (25°C) or after a 1-h heat shock at 40°C. (C) Deletion of *SSB1* and *SSB2*. The strains used in Figure 2B were transformed with an (episomal) HSE–*lacZ* reporter plasmid and assayed for β-galactosidase activity either before (25°C) or after a 1-h heat shock at 41°C. (D and E) Overproduction of Ssb1p. Isogenic strains carrying the HSE–*lacZ* reporter gene integrated into the *TRP1* locus and carrying HSF on an episomal plasmid (high-copy HSF) or a centromere-containing plasmid (low-copy HSF) were transformed with an episomal plasmid carrying the *SSB1* gene (SSB ↑) or with the vector alone (wt). β-Galactosidase activity was assayed either before (25°C) or after a 1-h heat shock at 41°C. D shows the 25°C activities only; E shows 25°C activity and heat-shocked activity. Data represent the averages of three or four measurements. *, the lacZ reporter gene was integrated into the chromosome in the strains used for B, D, and E. Hence, the overall units of β-galactosidase are lower than that when the reporter gene is on an episomal plasmid.

heat shock response. To address this caveat, we again predicted that overexpression of the Ssb proteins should have an effect opposite to that of the deletion. That is, if Ssb1/2p does downregulate HSF activity, then overexpression of Ssb1p should depress HSF activity; furthermore, any depression of activity should be dependent on the relative concentrations of HSF and Ssb1p. The effect of Ssb1p overproduction should be reversed by overproducing HSF as well.

To test these predictions, we prepared strains bearing a high-copy plasmid carrying *SSB1* (or vector alone, as a control) as well as either a high-copy or low-copy plasmid carrying *HSF1*. To avoid problems associated with plasmid maintenance, we integrated the HSE–*lacZ* reporter gene into the *TRP1* gene. We then examined the heat shock response in these strains. As shown in Figure 7D, the effect of overproduction of HSF was revealed in the elevation of steady-state β-galactosidase activity, as has been reported previously (Wiedderecht *et al.*, 1988). In heat-shocked cells (Figure 7E), no significant effect of Ssb1p overproduction could be detected. In unstressed cells (Figure 7, D and E), Ssb1p overproduction decreased β-galactosidase activity slightly relative to the isogenic controls, provided HSF was present on a low-copy plasmid. The effect was very modest, as would be predicted from the observation (Figure 7C) that complete removal of Ssb1/2p elevated HSF activity only a fewfold. Overproduction of HSF eliminated this effect of overproduction of Ssb1p, suggesting that the effect of Ssb1p could be stoichiometric, rather than enzymatic.

DISCUSSION

In initiating these experiments, we had expected, on the basis of current models of HSF regulation, that the *SSA* family of hsp70 proteins binds to HSF and that binding would be eliminated by heat shock. We were therefore surprised to obtain results that were quite different. In particular, we found that hsp70-containing, ATP-sensitive HSF complexes did not dissociate in response to heat shock, suggesting that these complexes perform a different role than we had imagined. In coimmunoprecipitation experiments, we showed that it is possible for the nonheat shock–inducible Ssb proteins to bind HSF, thereby suggesting that these HSF–hsp70 complexes might be involved in some other aspect of HSF regulation besides the stress response per se. How can we integrate our findings with the current body of information about HSF and its regulation? The simplest view is that HSF is subject to several distinct modes of modification, each of which is somewhat independent of the others.

With respect to the heat shock response per se, we adopt the concept that HSF responds to heat shock by conformational change, as suggested previously (Carr and Kim, 1993; Westwood and Wu, 1993; Zhong and Wu, 1996; Lee *et al.*, 2000). We have shown that the conformational change can be induced by heat or superoxide acting directly on HSF (Lee *et al.*, 2000), which suggests that a direct role of hsp70 may not be required for the activation of the heat shock response. We therefore seek a model for the role of the hsp70 proteins that accommodates both our biochemical data and the genetic data.

We consider two possible roles for the hsp70 proteins, both based on the preponderance of data that suggest they function to restrain HSF activity. One role, which is consistent with the biochemical activity of the hsp70s, is the modulation of HSF conformation. That is, if HSF is activated by conformational change, it is eventually necessary for HSF to revert to its low-activity conformation. The protein chaperone activity of hsp70 proteins may help catalyze HSF refolding. Our data are most consistent with the idea that the Ssa proteins perform this role. Deletion of *SSA1* and *SSA2* renders HSF constitutive, as would be expected if HSF could not refold effectively after activation by even a low level of stress. We did not detect Ssa1p in our coimmunoprecipitation experiments, consistent with the idea that it might interact with HSF transiently.

The second possible role for the hsp70 proteins is that of forming stable complexes with HSF and downregulating activity (perhaps by steric interference with the transcriptional activation domains). On the basis of our ability to coimmunoprecipitate the Ssb proteins with HSF, coupled with the finding that neither heat shock nor the HSF conformational change disrupted the ATP-sensitive HSF complexes, we suggest that this role may be performed by the Ssb proteins. The in vivo data suggest that any role of the Ssb proteins in the downregulation of HSF activity is relatively minor; the deletion of the *SSB1* and *SSB2* genes elevated HSF activity only a fewfold. However, the effect was apparent for both steady-state and heat-shocked conditions, consistent with the finding that the ATP-sensitive complexes bind HSF independently of heat shock, whether HSF is in its higher-activity (stressed) or lower-activity (unstressed) conformation.

Together, these considerations suggest a model in which HSF is activated by the direct sensing of heat or superoxide, which triggers a conformational change, and is then refolded with the possible assistance of the Ssa proteins. In either conformation, HSF is subject to the phosphorylation and assembly of larger complexes that may contain the Ssb proteins. These latter modifications serve to fine-tune HSF activity modestly and may act independently or simultaneously.

This model is supported by our observations on steady-state HSF activity in cells grown in different concentrations of glucose, which suggest that HSF can be sequestered into larger complexes that may downregulate its activity when the growth rate falls slightly but that this kind of sequestration *and* phosphorylation are both used when the growth rate falls more significantly. Similarly, sequestration of HSF into larger complexes may be sufficient to downregulate the lower-activity conformation of HSF during recovery from heat shock, but both sequestration and phosphorylation may be needed to downregulate the higher-activity conformation during adaptation to growth at higher temperature.

It is clear that in yeast, at least, HSF is not simply an on–off switch. Rather, it is tightly regulated over a wide range of activities. Certainly, the dramatic elevation of HSF activity depends on stress, but there appear to be signals besides stress that influence HSF activity. This conclusion is strongly supported by the observation that rapid growth at an optimal glucose concentration results in higher HSF activity than does slow growth in limiting glucose. Although the idea is not new that HSF activity might be tied to growth rate, the mechanism has been uncertain. The finding that the Ssb members of the hsp70 family can interact with HSF offers a possible mechanism.

The Ssb proteins have been shown to function both in binding nascent peptides (Nelson *et al.*, 1992; Pfund *et al.*, 1998) and in the degradation of short-lived proteins (Ohba, 1997). Thus, the availability of Ssb1/2p should be sensitive to changes in the abundance of these substrates. As noted in the INTRODUCTION, cells growing at different rates exhibit different overall rates of protein synthesis. This has an obvious and direct effect on the concentration of nascent peptides and thus on one major class of substrates for Ssb1/2p. The growth rate dependence of protein synthesis is also reflected in changes in the concentration of rRNA and ribosomal proteins (r-proteins). Because r-proteins are synthesized in excess of what can be assembled into ribosomes (Warner *et al.*, 1985; Maicas *et al.*, 1988), r-proteins represent a major class of short-lived proteins, with half-lives of 1–5 min (Warner, 1989). Therefore, r-proteins represent a second major class of Ssb1/2p substrates.

These considerations suggest that the Ssb proteins might function in a feedback loop with yeast HSF, essentially analogous to the feedback loop that has been proposed for metazoan HSF and hsp70 (Morimoto, 1993; Shi *et al.*, 1998), but not dependent on stress per se. When the rate of protein synthesis is high and the concentration of Ssb1/2p substrates is therefore also high, the Ssb proteins would be occupied and would be unavailable to bind HSF. When the concentration of substrates falls, the pool of free Ssb1/2p proteins increases, allowing Ssb1/2p to bind to HSF and decrease its activity. This model offers a reasonable link between HSF, growth rate, and the overall rate of protein

synthesis. It also explains why the binding of Ssb1/2p to HSF might be independent of heat shock.

The concept that the Ssb proteins may interact with HSF independent of heat shock does not eliminate the potential for regulation via the Ssa proteins by a distinct mechanism. If, as we think likely, the Ssa proteins help catalyze the reversal of the HSF conformational change, then the SSA proteins could also influence HSF activity via a similar feedback mechanism, such that titration of the Ssa proteins would result in the accumulation of HSF in its active conformation. There may be additional controls on HSF activity as well. Lin and Lis (1999) have reported the binding of Gac1p to yeast HSF, and Zou *et al*. (1998) have demonstrated that metazoan HSF is readily bound by hsp90. Our gel mobility shift assays and coimmunoprecipitations did not detect hsp90, but this could well be caused by inadequate methodology. We therefore cannot assess the roles of these other proteins in the regulation of yeast HSF but suspect that the scheme we have described here represents only part of the story.

The finding that HSF can respond to normal physiological conditions that differ from traditional "stress" suggests that HSF may be responsive to other signals as well. This possibility is especially significant when viewed in combination with the findings of Liu and Thiele (1996) and Cahill *et al*. (1996) that there are genes that are regulated by HSF in distinctly different ways than the traditional heat shock genes. Human IL1β is *repressed* by heat shock, and yeast *CUP1* is induced by oxidative stress under conditions that have little effect on hsp70. *CUP1* expression appears to correlate with phosphorylation at sites different from those that are phosphorylated during heat shock (Liu and Thiele, 1996). These differences in HSF function appear to be determined in part by the nature of the HSF-binding site (Santoro *et al*., 1998). *CUP1* and *IL1*β have noncanonical arrangements of the GAA-binding motifs, which may allow HSF to interact differently with other transcription factors. Taken together, these observations suggest that HSF is subject to several kinds of posttranslational modifications, only some of which are directly tied to the heat shock response. It will be interesting to learn what these different modes of regulation may be.

ACKNOWLEDGMENTS

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