**In vitro** activation of heat shock transcription factor DNA-binding by calcium and biochemical conditions that affect protein conformation

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**ABSTRACT** The transcription of heat shock genes in response to physiological stress requires activation of heat shock transcription factor (HSF). Although the transcriptional response is most commonly induced by temperature elevation, the biochemical events involved in HSF activation *in vivo* can also be triggered at normal physiological temperatures by chemicals that inhibit metabolic processes. We have used a HeLa cell-free system in which HSF DNA-binding is activated by conditions that affect protein conformation, including increasing concentrations of hydrogen ions, urea, or nonionic detergents. Treatment with calcium ions also results in a concentration- and time-dependent activation of HSF *in vitro*. Pretreatment with each of these biochemical conditions reduces the temperature dependence for HSF activation *in vitro*. These results suggest that HSF is activated either directly by undergoing a conformational change or indirectly through interactions with unfolded proteins.

A common feature of inducible gene expression is the activation of a preexisting transcription factor in response to a change in the environment (1). Since induction can occur in the absence of protein synthesis, activation of these transcription factors often requires posttranslational modifications including phosphorylation (2, 3), disruption of a factor-ligand complex (4–7), and protein conformational changes (8). These posttranslational events could alter the affinity or accessibility of the factor for its DNA-binding site, increase the ability of the factor to form a more stable transcription complex, or affect the multimeric state of the factor.

The transcriptional activation of heat shock genes represents a rapid response to environmental and physiological stress that is mediated by the conversion of a preexisting heat shock transcription factor (HSF) from an inactive to an active form (9–11). The signal that activates HSF can be generated both at elevated temperatures and by treating cells at nonstress temperatures with agents that affect the metabolic state of the cell, such as amino acid analogues and heavy metals (12). A central question is whether the pathway of HSF activation following incubation at extreme temperatures and the pathway following treatments that induce the stress response at 37°C occur through common mechanisms. In this study we demonstrate that DNA-binding ability of human HSF can be induced by biochemical conditions known to affect protein conformation and that these conditions act by reducing the temperature dependence for *in vitro* activation.

**MATERIALS AND METHODS**

Preparation of HeLa Extracts and Gel Mobility-Shift Assays. Cytoplasmic extract prepared from 4 liters of HeLa cells growing at 37°C was dialyzed into 20 mM Hepes, pH 7.9/20% (vol/vol) glycerol/0.1 M KCl/0.2 mM EDTA/0.5 mM phe- nylmethylsulfonyl fluoride/0.5 mM dithiothreitol (13). Binding reactions were performed by adding 10 μg of whole cell extract or 30 μg of cytoplasmic extract to a mixture containing 0.1 ng of a 32P-labeled, double-stranded heat shock element (HSE) oligonucleotide (nucleotides −107 to −83 of the promoter of a human gene encoding a 70-kDa heat shock protein (HSF70); ref. 14) in 25 μl of binding buffer (10 mM Tris, pH 7.8/50 mM NaCl/1 mM EDTA/0.5 mM dithiothreitol/5% glycerol containing 10 μg of bovine serum albumin and 0.5 μg of poly(dI-dC)poly(dI-dC)). Competition reaction mixtures contained either a 100-fold excess of nonradioactive HSE oligonucleotide, a 100-fold molar excess of a HSE mutant (HSE−) oligonucleotide, or no competitor. The HSE− oligonucleotide (upper strand, 5′-GAT-CTC-GGC-TTC-AAT-ATT-GTC-CAC-CTG-GCA-GCC-GA-3′) contained substitutions (indicated in bold type) in the guanine residues that are essential for HSF binding (12). The mixtures were incubated for 20 min at 25°C, and then free and bound DNAs were separated by electrophoresis in a nondenaturing 4% polyacrylamide gel in 6.7 mM Tris, pH 7.5/1 mM EDTA/3.3 mM sodium acetate (15, 16). Gels were run at 160 V for 2 hr at room temperature, dried, and exposed to film.

**Methylation Interference Assays.** For methylation experiments, binding mixtures contained 2 ng of a partially methylated HSE oligonucleotide radiolabeled on either the upper or the lower strand along with 400 ng of whole cell extract or 240 ng of cytoplasmic extract in 25 μl of binding buffer (17). The amount of the nonspecific carrier DNA, poly(dI- dC)poly(dI-dC), was increased to 15 μg in the binding mixture containing the whole cell extract and to 5 μg for the cytoplasmic extract. Complexes were separated in a nondenaturing gel and the free and bound DNAs were eluted and cleaved with piperidine as described (11). The cleaved products were then separated in a 16% polyacrylamide/7 M urea gel together with a guanine reaction "ladder."

**RESULTS**

In Vitro Activation of HSF. The *in vivo* levels of HSF detected in heat-shocked HeLa cells were compared with the levels of *in vitro* activated HSF in HeLa cell cytoplasmic extracts. Whole cell extracts were prepared from cells incubated for 1 hr at various temperatures from 37°C to 44°C and the levels of activated HSF were determined by the gel mobility-shift assay. Extracts from cells maintained at 37°C contained two binding activities (Fig. 1, lane 2). One of these, corresponding to the constitutive HSE-binding activity, was primarily found in control cells and its binding to the labeled HSE probe was specifically inhibited by the HSE oligonucleotide; the other activity (ns) corresponded to a nonspecific complex whose formation was not inhibited by either the

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**Abbreviations:** HSE, heat shock element; HSF, heat shock transcription factor; HSF70, 70-kDa heat shock protein; NP-40, Nonidet P-40.
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The presence of in vitro activated HSF, we performed competition binding experiments and identified the base-specific contacts in the HSE. The presence of excess nonradioactive HSE oligonucleotide in the binding reaction mixture with a whole cell extract from heated cells prevented the binding of HSF to the temperature. Among the many pleiotropic effects of temperature, we considered the possibility that a rise in temperature could affect the conformation of a component in HSF activation. For this reason we examined biochemical conditions that are known to affect protein conformation, such as changes in pH or treatment with detergent or urea, for their ability to activate HSF at 37°C in vitro.

Cytoplasmic extracts were mixed with phosphate buffers in which the final pH ranged from 5.0 to 7.8 and incubated at 37°C for 1 hr. Activation of HSF occurred in extracts adjusted to a pH from 5.8 to 6.4, with maximal activation occurring at pH 6.0 (Fig. 2A). Similar results were obtained using the same pH range for the buffers Hepes, Pipes, and sodium citrate. Incubation of cytoplasmic extract with the nonionic detergent NP-40 at 37°C also resulted in the activation of HSF (Fig. 2B).

The effect of NP-40 on HSF activation was concentration-dependent; the levels of activated HSF increased with detergent concentration from 0.02% to 2% (vol/vol). Identical results were obtained with two other detergents, Triton X-100 and 3-[3-(cholamidopropyl)dimethyammonio]-2-hydroxy-1-propanesulfonate (CHAPS). The third condition in which HSF activation in vitro was observed was following addition of urea to the cytoplasmic extract (Fig. 2C). Activated HSF appeared after urea was added at 0.5-1.0 M. Taken together, these results indicate that in vitro activation of HSF as directly measured by the formation of a HSF-HSE complex can be achieved not only by elevated temperatures but also by biochemical conditions that affect protein conformation.

Figs. 1 and 2 offer clear evidence that HSF can be activated by conditions that affect the physicochemical environment of this transcription factor. Our data can be explained by a number of possibilities. One model is that HSF itself undergoes conformational change and acquires DNA-binding ability. Alternatively, these conditions could indirectly activate HSF through interaction of HSF with unfolded or denatured proteins. Because the biochemical conditions in Fig. 2 have broad effects on proteins, we examined whether a specific modulator of protein conformation, Ca²⁺, would also activate HSF. Addition of Ca²⁺ to a cytoplasmic extract activated HSF in a concentration- and time-dependent manner. The effect was specific to Ca²⁺, as the addition of EGTA prior to Ca²⁺ prevented HSF activation (Fig. 3). Similar results of HSF activation were obtained with Mn²⁺ and La³⁺ but not with Mg²⁺ (data not shown).

**Fig. 1.** Activation of HSF by elevated temperature in vivo and in vitro. Gel mobility-shift analysis was used to assess HSE-binding activity in whole cell extracts from HeLa cells that were heated for 1 hr at the indicated temperatures in vivo (lanes 2-9) and in cytoplasmic extracts that were heated for 1 hr at the same temperatures in vitro (lanes 11-18) or left on ice (lane 10). The reaction mixture assayed in lane 1 did not contain extract (–). Solid arrowhead, induced HSF; open arrowhead, constitutive HSE-binding activity; ns, nonspecific DNA-binding complex; F, free HSE oligonucleotide.

HSE oligonucleotide or an oligonucleotide containing base substitutions in the HSE (12). The levels of the slowly migrating HSE-HSF complexes corresponding to the heat shock-induced form increased as the temperature was raised, with maximal levels detected at 41°C (Fig. 1, lanes 2-10). The rapid appearance of activated HSF in HeLa cells exposed to elevated temperatures parallels the kinetics of HSF70 gene transcription (12).

The appearance of in vitro activated HSF was examined by performing a gel mobility-shift assay using HeLa cell cytoplasmic extracts that were incubated for 1 hr at temperatures from 37°C to 44°C (18) (Fig. 1, lanes 11-18). Overall, the levels of HSF detected in vivo and in vitro were similar, although the levels of in vitro activated HSF continued to increase during incubation at elevated temperatures while the in vivo activated HSF attained maximum levels at slightly lower temperatures. The similarity between the temperatures that elicit activation in vitro in extracts and in vivo in intact cells suggested that some component of the heat shock pathway might directly sense and respond to changes in the

**Fig. 2.** Activation of HSF at 37°C in vitro by low pH, Nonidet P-40 (NP-40), or urea. Cytoplasmic extracts were mixed with 0.2 M phosphate buffers at pH 5.0-7.8 (A), with NP-40 to give a final concentration of 0.002-2.0% (vol/vol) (B), or with urea at 0-2.5 M (C) and incubated for 1 hr at 37°C. Symbols are as in Fig. 1.
radiolabeled HSE oligonucleotide probe (Fig. 4, lane 5). However, HSF–HSE binding was not inhibited by the HSE oligonucleotide, in which the guanines essential for specific complex formation had been replaced by other bases (Fig. 4, lane 6). The binding of HSF activated in vitro at 43°C (lanes 10–12) or by treatment with NP-40 (lanes 13–15) or low pH (lanes 16–18) at 37°C was also specifically inhibited by the HSE oligonucleotide but not by the HSE oligonucleotide.

A methylation interference assay was performed to identify the specific bases in contact with HSF activated either in vivo or in vitro at 43°C or by detergent treatment at 37°C in vitro. Methylation of the guanine residues within the conserved HSE (positions −105, −104, and −94 on the upper strand and positions −97 and −96 on the lower strand) interfered with the binding of activated HSF from cells heated in vivo (Fig. 5). HSF activated in vitro at 43°C or at 37°C by treatment with 2% NP-40 contacted the same guanine residues in the HSE (Fig. 5). These results demonstrate that HSF activated in vitro by elevated temperature or by detergent treatment has the same sequence specificity and base-specific contacts as HSF activated by heating cells in vivo.

**Thermal Denaturation Profile of HSF Activation.** We next examined whether incubation under these biochemical conditions reduced the temperature requirement for in vitro activation. Cytoplasmic extracts were treated with 2% NP-40 and incubated at various temperatures for 1 hr (Fig. 6). HSF activation did not occur when the extract was kept on ice or incubated at 25°C. Activated HSF was first detected at 30°C and reached half-maximal levels at 37°C. In contrast, in the untreated extracts HSF was not detected until the temper-
Fig. 6. Incubation with detergent reduces the temperature requirement for in vitro activation. Cytoplasmic extracts were mixed with NP-40 to give a final concentration of 2%, incubated at various temperatures for 60 min, and analyzed by the gel mobility-shift assay ( Insets). The relative levels of activated HSF were determined by scanning densitometry and graphed.

ture reached 39°C and required incubation at 43°C to reach maximal levels of HSF. Thus, for both the treated and the untreated extracts there was a sharp transition temperature at which activation occurred. However, in the presence of detergent, the transition temperature was shifted toward temperatures within the normal physiological range. Similar results were obtained with low pH, urea, and Ca²⁺.

The kinetics of HSF activation in the presence of detergent or low pH was examined in time-course experiments. Levels of activated HSF were measured in cytoplasmic extracts incubated at 25°C, 30°C, 37°C, and 43°C at pH 6.0 or with 2% NP-40 for periods of 15–90 min (data not shown). Even with prolonged incubation, HSF activation did not occur at 25°C and very little active HSF was present during incubation at 30°C. At 37°C, activated HSF appeared within 15 min and continued to increase for at least 90 min. This rate of activation is similar to that seen at elevated temperatures in vivo and in vitro. These results suggest that in vitro activation at 37°C by low pH or detergent treatment may occur via a mechanism similar to that which occurs at elevated temperatures either in vivo or in vitro.

Inhibition of in Vitro Activation by Conditions That Stabilize Proteins. Additional evidence to support the hypothesis that HSF activation involves protein conformational change was provided in experiments by others, which have demonstrated that pretreatment of cells with high concentrations of glycerol or H₂O, reagents widely used to stabilize protein structure, blocks the acquisition of thermotolerance (19–21) and interferes with activation of heat shock gene transcription (22). Based on these observations, we examined whether glycerol or H₂O could block the in vitro activation of HSF.

As shown in Fig. 7, activation of HSF in vitro was inhibited in a concentration-dependent manner by glycerol. Similar results were obtained with H₂O (data not shown). A concentration of 15% glycerol was sufficient to inhibit 40–60% of HSF activation in vitro by temperature elevation, pH 6.0, urea, or NP-40. Only 10% of the maximal levels of HSF were induced by these conditions when the glycerol concentrations were increased to 25%. We also examined whether Ca²⁺ activation of HSF could be inhibited by glycerol and found that higher levels of glycerol were required to prevent HSF activation in vitro. These results provide supporting evidence that HSF activation can occur through an effect on the biochemical environment and protein conformation.

DISCUSSION

A major goal in understanding the regulation of heat shock-inducible transcription is the identification of the intracellular signals that activate HSF. The data presented here reveal that a simple alteration in the biochemical environment is sufficient for human HSF to acquire DNA-binding activity. The biochemical events that occur when a cytoplasmic extract is heated in vitro can also occur at lower temperatures under conditions that affect protein conformation. A conformational change would provide a rapid mechanism for activating a transcription factor that must respond quickly to environmental changes.

The in vitro activation of HSF by low pH and detergent may be related to the events leading to HSF activation in vivo, since many of the conditions that we used for our in vitro studies were previously shown to activate the heat shock response. A transient reduction in intracellular pH in Drosophila salivary glands (23) and yeast (24) results in increased expression of stress genes. Furthermore, two inducers of the heat shock response, dinitrophenol and arsenite, are known to affect intracellular pH (24). The anion-transport inhibitor 4,4'-diisothiocyanatostilbene-2,2'-disulfonate (DIDS) can induce the heat shock response in Drosophila Kc cells (25). Detergent treatment has also been shown to activate heat shock gene transcription in Drosophila (26): treatment of Drosophila salivary glands with digitonin, Triton X-100, or NP-40 induced the formation of chromosomal puffs at the sites corresponding to heat shock genes.
Other examples in which conformational changes in transcription factors induce DNA-binding ability include the copper-induced factor for metallothionein gene transcription (7), steroid hormone receptors (4–6), and NF-kB (8). Although an important event in HSF activation is the acquisition of DNA-binding ability, a stress-induced conformational change in HSF could also expose a domain that is a target for posttranslational modifications. The activated forms of human and yeast HSF are phosphorylated in vivo (10, 11), and this modification may be required for HSF to become transcriptionally competent. Human HSF does not become phosphorylated when it is activated by heating in vitro (18); therefore, other biochemical events that cause a conformational change are likely to be responsible for DNA-binding activity. In this study and in a previous study (12) we have detected a constitutive HSE-binding activity that is predominant in extracts from control cells and that decreases upon stress. The constitutive HSE-binding activity and HSF exhibited similar DNA sequence specificity in competition and methylation interference experiments (12). In this study we observed that the constitutive HSE-binding activity decreased in many of the conditions in which HSF DNA binding activity was induced, suggesting a possible relationship.

Our results do not indicate whether HSF itself undergoes a direct conformational change or whether HSF responds indirectly to proteins that have undergone a conformational change as recently suggested for gr2 regulation by Parsell and Sauer (27). Although many of the biochemical conditions that cause in vitro HSF activation affect protein conformation, the ability of Ca2+ to activate HSF suggests that conformational change in a Ca2+-binding protein(s) (possibly HSF itself) may trigger the activation.

Another possibility is that HSF possesses DNA-binding ability prior to activation but is incapable of binding in vivo because it is held in a complex that does not bind to the HSE or is inaccessible to the HSE. For example, this could be accomplished by a complex in which HSF is maintained in an inert state through association with either the constitutive (P72/HSC70) or inducible (HSP70) members of the HSP70 family. Similar models have been proposed for complexes consisting of HSP90, steroid receptors, and HSP70 as a mechanism to maintain the inaccessibility of the DNA-binding domain (28). Disruption of such a HSF–HSP70 complex—during heat shock, for example—would release HSP70 to associate with unfolded domains of substrate proteins and HSF for binding to the HSE. The trigger for this response could be increased levels of denatured proteins that would compete with the HSF–HSP70 complex and result in the formation of a HSP70–unfolded protein complex. Such a model could also provide an explanation for the autoregulatory aspect of the heat shock response. As the level of stress-induced HSP70 increases in response to the need for HSP70 to interact with unfolded proteins, the heat shock promoter remains transcriptionally active. However, as the pool of free or unassociated HSP70 increases, as would occur during recovery from heat shock, the HSF–HSP70 complex would re-form in the cell nucleus, thus attenuating the heat shock response.

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