# In Vitro Activation of Purified Human Heat Shock Factor by Heat†

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ABSTRACT: A major regulatory step in the heat-induced transcription of heat shock protein (hsp) genes in eukaryotes is the activation of heat shock factor (HSF). In metazoans and *Schizosaccharomyces pombe*, HSF is present in unstressed cells but is unable to bind to its target DNA sequence element, the heat shock element (HSE). Heat induction of the DNA binding activity of HSF is a critical component required for activation of heat shock genes. Inactive HSF in extracts of non-heat shocked human cells can be heated in vitro to activate HSF, suggesting the factors required to sense temperature and activate HSF are soluble factors [Larson, J. S., Schuetz, T. J., & Kingston, R. E. (1988) *Nature 335*, 372−375]. We utilized the ability to purify human HSF in the active form to characterize further the in vitro activation of HSF. Here we have developed a procedure to deactivate the DNA binding ability of HSF. When purified and deactivated HSF is heated, the DNA binding ability of HSF is activated. This activation occurs most efficiently at 43 °C (heat shock temperature), but, in contrast to activation in the crude system, some activation of HSF is observed at 37 °C (non-heat shock temperature). We show that purified and deactivated HSF is similar to natural inactive HSF in both size and shape. Thus, the monomer to trimer transition that activates HSF can occur in a temperature-dependent fashion in the absence of other proteins. It is possible that these biochemical properties of HSF contribute to the ability of HSF to respond to heat in vivo.

When cells are heat shocked above their normal growth temperatures, they synthesize a set of proteins called the heat shock proteins (hsps;[1] reviewed in Lindquist & Craig, 1988; Hightower, 1991). Accumulating evidence indicates these proteins may work to combat the deleterious effects of heat shock, for example, by preventing extensive protein aggregation. The conservation of the heat shock response and the hsp amino acid sequences in all organisms testifies to its importance. The induction of hsp synthesis can be caused by a variety of effectors in addition to heat, including heavy metals, ethanol, developmental programs, and many diseases. The promoters of the hsp genes in eukaryotes contain a heat shock element (HSE), which has been shown to be both necessary and sufficient to confer heat inducible transcription (Pelham & Bienz, 1982). The HSE contains tandem repeats of the sequence _GAA_ arranged in inverted orientation (Amin et al., 1988; Xiao & Lis, 1988).

A protein that binds to the HSE and stimulates transcription in response to a heat shock, termed heat shock factor (HSF), has been analyzed in several eukaryotes (reviewed in Sorger, 1991; Lis & Wu, 1993; Morimoto, 1993). In yeast (Sorger & Nelson, 1989; Peteranderl & Nelson, 1992) and *Drosophila* (Perisic et al., 1989; Clos et al., 1990) evidence suggests HSF binds to the HSE as a trimer. In the yeasts *Saccharomyces cerevisiae* and *Kluyveromyces lactis*, HSF is constitutively bound to the HSE (Sorger et al., 1987; Jakobsen et al., 1991). Heat shock causes an increase in phosphorylation of HSF that correlates with an increase in hsp gene transcription (Sorger et al., 1987; Sorger & Pelham, 1988). However, in the yeast *S. pombe* (Gallo et al., 1991), *Drosophila* (Wu, 1984; Zimarino et al., 1990a), and humans (Kingston et al., 1987; Sorger et al., 1987; Larson et al., 1988; Zimarino et al., 1990a), HSE binding by HSF is induced by heat. Under normal growth conditions in these latter organisms, HSF is present but is unable to bind DNA (Kingston et al., 1987; Zimarino & Wu, 1987; Larson et al., 1988; Zimarino et al., 1990a). Induction of the DNA binding of HSF in metazoans is a major regulatory step in the activation of the stress response.

HSF genes from several species have been isolated. Only one HSF gene has been found in yeast (Sorger & Pelham, 1988; Wiederrecht et al., 1988; Jakobsen & Pelham, 1991; Gallo et al., 1993) and *Drosophila* (Clos et al., 1990), while two HSF genes have been identified in mice (Sarge et al., 1991) and humans (Rabindran et al., 1991; Schuetz et al., 1991) and three in tomatoes (Scharf et al., 1990) and chickens (Nakai & Morimoto, 1993). Although the heat shock response and hsps are extensively conserved across these species, the complete amino acid sequence of HSF is not (Clos et al., 1990). There are, however, two regions of conservation in the amino terminus of the protein, one believed to be involved in DNA binding (Wiederrecht et al., 1988; Harrison et al., 1994) and the other in oligomerization (Sorger & Nelson, 1989; Peteranderl & Nelson, 1992). A comparison of the HSFs that possess an inducible DNA binding activity, however, reveals a third conserved region (Rabindran et al., 1991; Gallo et al., 1993) that contains a hydrophobic repeat in the carboxy terminus of the protein.

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[1] Abbreviations: HSF, heat shock factor; HSE, heat shock DNA sequence element; hsp, heat shock protein; SDS−PAGE, SDS−polyacrylamide gel electrophoresis; S100, cytoplasmic extracts from non-heat shocked cells; HSNE, nuclear extracts from heat shocked cells; ELISA, enzyme linked immunosorbent assay; NP-40, Nonidet P40 [(ethylphenyl)poly(ethylene glycol]; BSA, bovine serum albumin; EMSA, electrophoretic mobility shift assay.

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Since the heat inducibility of DNA binding by HSF is an important regulatory event in the stress response in metazoans, we and others have sought to determine the mechanism of activation of HSF. Human HSF becomes phosphorylated upon heat shock; however, this is not required for activation of DNA binding and does not appear to affect DNA binding (Larson et al., 1988, and unpublished observations). The observation that HSF binds to the HSE as a trimer (Perisic et al., 1989; Sorger & Nelson, 1989) led these researchers to postulate that the regulation of DNA binding in metazoans might be caused by the regulation of trimerization. A change in oligomeric status in HSF has been observed upon heat shock, and it appears this activates DNA binding (Westwood et al., 1991; Baler et al., 1993; Sarge et al., 1993). Current data are consistent with the hypothesis that heat shock causes the conversion of HSF monomers to trimers and the trimerization of HSF allows high affinity DNA binding (Westwood & Wu, 1993; Sistonen et al., 1994).

Metazoan cells must then contain a factor or factors that can sense a heat shock and cause trimerization of HSF. These factors have been called the "biological thermometers". To examine this phenomenon, we initially established an in vitro system that recreates the DNA binding activation of human HSF (Larson et al., 1988). Cytoplasmic extracts (S100) from HeLa cells grown at normal temperatures contained little HSF binding activity. Incubating these extracts at heat shock temperatures in vitro induced HSF DNA binding activity, implying the biological thermometer consists of a soluble factor(s). The changes to HSF that allow trimerization and activation of DNA binding appear to be conformational changes. Antibodies to *Drosophila* HSF can activate the DNA binding of HSF in vitro (Zimarino et al., 1990b), suggesting a heat shock-induced enzymatic activity is not necessary for the activation of DNA binding of HSF.

Our goal was to determine whether HSF has biochemical properties consistent with a role as part of the biological thermometer. It appears that factors other than HSF must be involved in sensing temperature stress. The absolute temperature that induces a heat shock response in a cell population changes as the basal growth temperature of the cell changes, implying that the "biological thermometers" monitor change in temperature, not just the absolute temperature (Abravaya et al., 1991). It is difficult to envision how a single factor could play this role. In addition, human HSF1 that is expressed in *Drosophila* cells is activated at the heat shock temperature for *Drosophila* (37 °C; the normal growth temperature for humans; Clos et al., 1993). Thus factors exist that can induce binding of HSF at normal growth temperatures (see also Mezger et al., 1989), as is also evidenced by the observation that denatured protein alone can induce the heat shock response at normal growth temperatures (Ananthan et al., 1986). Thus, induction of HSF binding is likely to be a complicated process, involving several proteins. We were interested in examining the possibility that HSF itself is one of the factors that is capable of sensing heat.

HSF can be purified from heat shocked human cells (Schuetz et al., 1991); however, it is in the active form. Here we report the development of a procedure that deactivates the DNA binding of HSF and demonstrate that this purified protein can be induced to bind DNA in a temperature-dependent manner. We show that purified and deactivated HSF is similar to natural HSF in both size and shape. We confirm that activation of HSF in this purified system is caused by the conversion of HSF monomers to trimers. This can occur without other factors or DNA. Our data imply that heating homogeneous, deactivated HSF in vitro causes a direct structural transition in HSF that allows trimerization and subsequent high affinity DNA binding. Activation of purified, deactivated HSF occurred maximally at heat shock temperatures; however, some activation was seen at non-heat shock temperatures.

## MATERIALS AND METHODS

*HeLa Cell Extracts and Purification.* HeLa cells were grown and heat shocked where indicated, and nuclear and cytoplasmic extracts were made as described (Larson et al., 1988). HSF was purified from nuclear extracts of heat shocked HeLa cells precisely as described (Schuetz et al., 1991; estimated purification of 2000-fold). To purify HSF from non-heat shocked HeLa cells, S100 extracts (0.8 g) were fractionated on a single strand DNA cellulose column in buffer D (Dignam et al., 1983; 20 mM Hepes, pH 7.9, 20% (v/v) glycerol, 100 mM KCl, 0.2 mM EDTA, 0.5 mM phenylmethanesulfonyl fluoride, 0.5 mM dithiothreitol) supplemented with protease inhibitors (0.5 mM benzamidine, 1.0 mg/L leupeptin, 1.0 mg/L aprotinin, and 1.0 mg/L pepstatin A). The flow through was heat activated in vitro by incubation at 43 °C for 1 h and then purified as described (Schuetz et al., 1991), except only a single HSE affinity column run was used. Total recovery of HSF DNA binding activity was approximately 6%, and estimated purification (as monitored by measuring DNA binding activity by electrophoretic mobility shift assay (EMSA)) was 5300-fold.

*Deactivation of HSF.* Samples containing HSF were denatured by the addition of guanidine hydrochloride (Gdn-HCl) powder to a 6 M concentration. Samples were then typically diluted to 2 mL with buffer D containing 6 M Gdn-HCl and 0.1 mg/mL BSA. Renaturation was performed by dialysis against a 500−1000-fold volume excess of buffer D at 4 °C for 16−20 h. Samples were then concentrated by centrifugation in centricon-30 concentrators (Amicon) in a JA 20.1 rotor at 4 °C to a final volume of 43 $\mu$L. Extensive loss of HSF protein occurred during this procedure. Recovery of total HSF DNA binding activity after heat activation was approximately 5−10%. Estimations of HSF protein by western analysis revealed most of the loss of binding activity occurred through loss of HSF protein.

In some instances, samples were first isolated from SDS−PAGE gel slices prior to deactivation. Samples were electrophoresed on 10% acrylamide SDS−PAGE. Slices containing approximately 5% of gel length were made, electroeluted, precipitated with acetone, and washed twice with 80% acetone. Samples were then resuspended in buffer D containing 6 M Gdn-HCl and 0.1 mg/mL BSA, renatured, and concentrated as described above. Slices containing HSF activity were determined by in vitro heat activation and electrophoretic mobility shift assay. Extensive loss of HSF protein occurred during this procedure. Recovery of total HSF DNA binding activity after heat activation was 2−5%. Estimations of HSF protein by western analysis revealed most of the loss of binding activity occurred through loss of HSF protein.

*In Vitro Activation and DNA Binding Assay.* In vitro heat incubation and gel electrophoretic mobility shift assays were

performed as described (Larson et al., 1988) using a $^{32}$P-labeled synthetic oligonucleotide containing the HSE (Kingston et al., 1987; bases −115 to −80 of the human hsp70 gene) as a probe. Where indicated, binding reactions contained a 100-fold molar excess of a 50 bp double strand synthetic oligonucleotide containing hsp70 HSE sequences −128 to −81 ("WT") within *Hin*dIII and *Sac*I linkers or the same oligonucleotide substituted at 4 bp in the HSE ("MUT": relevant changes are from tgGAAtaTTCccGAC to tgTACtaCTGccGAC, where capital letters represent consensus HSE bases). Gels were dried and exposed using film except Figure 2D, where a PhosphorImager was used.

*Pore Gradient Gel Electrophoresis Assay.* Samples were electrophoresed at 20 V/cm on acrylamide gradient gels as described (Weber & Osborn, 1969; Clos et al., 1990) with a 4−25% acrylamide gradient and a 1.5−7.5% glycerol gradient for 24−48 h at 4 °C in 0.5× TBE. Protein markers were visualized by Coomassie staining. HSF bound to DNA was visualized by performing an electrophoretic mobility shift assay on a gradient gel. Western staining analysis to visualize unbound HSF utilized mouse α-HSF polyclonal ascites fluid (Schuetz et al., 1991) as primary antibodies and visualized with ECL staining (Amersham).

*Physical Analysis of Native HSF Structure. (i) Analytical Gel Filtration.* Approximately 20 ng of active purified HSF was applied to a 1 cm × 85 cm Sephacryl S-300 HR column equilibrated with buffer D supplemented with protease inhibitors (1.0 mg/L aprotinin, 1.0 mg/L leupeptin, 0.5 mM benzamidine, 1.0 mg/L pepstatin A), 1.0 mg/mL hemoglobin, 0.1% (v/v) NP-40, and 5 mM *n*-octyl glucoside (Smith, 1991). Fractions (1% of column volume) were analyzed by electrophoretic mobility shift assay, and fractions containing HSF activity were further analyzed by pore gradient gel electrophoresis followed by western staining. The Stokes radius ($r$) was determined from the average distribution coefficient, $k_{av}$, which was calculated from the equation $k_{av} = (V_e - V_o)/(V_t - V_o)$, where $V_e$ represents elution volume, $V_o$ represents void volume, and $V_t$ represents total column volume. The column was calibrated with protein markers to determine the relationship between the Stokes radius and $k_{av}$ for the column. Inactive HSF (5 ng) was analyzed as described above except equilibration buffer did not contain hemoglobin, NP-40, or *n*-octyl glucoside, and fractions were analyzed by ELISA using mouse α-HSF polyclonal antibodies (Hornbeck, 1991; Schuetz et al., 1991).

*(ii) Sedimentation Velocity Analysis.* Approximately 6 ng of active purified HSF was layered on a 5 mL isokinetic gradient of 10−30% sucrose in glycerol free gel filtration buffer. Samples were centrifuged in a SW55Ti rotor at 4 °C for 18 h at 42 000 rpm. Fractions (1−2% of gradient) were analyzed by electrophoretic mobility assay, and fractions containing HSF activity were further analyzed by the pore gradient assay visualized with western staining. The sedimentation coefficients ($S_{20,w}$) were determined by using the $\omega^2 t$ integration function (Beckman Instruments) and by cosedimentation of standards with known $S_{20,w}$ values. Inactive HSF (5 ng) was analyzed as described above except equilibration buffer did not contain hemoglobin, NP-40, or *n*-octyl glucoside, and fractions were analyzed by ELISA using mouse α-HSF polyclonal antibodies (Hornbeck, 1991; Schuetz et al., 1991).

*(iii) Native Molecular Mass and Frictional Ratio Determination.* Native molecular mass was calculated from the Svedberg equation: $m = S_{20,w}RT/[D_{20,w}(1 - \upsilon\varrho)]$, where $S_{20,w}$ was determined by sucrose gradient analysis, $R$ is the gas constant, $T$ is temperature in degrees kelvin, $\upsilon$ is partial specific volume of the sample (0.74 cm$^3$/g, the average for proteins, was used (Smith, 1966)), and $\varrho$ is the solvent density at 20 °C. $D_{20,w}$, the diffusion coefficient, was calculated from the Stokes−Einstein equation ($D_{20,w} = RT/6\pi nrN_a$), where $n$ is the solvent viscosity at 20 °C, $r$ is the Stokes radius from gel filtration, and $N_a$ is Avogadro's number. The Perrin factor ($f/f_{sph}$) was determined from the equation $f/f_{sph} = r/[3m(\upsilon + \delta_1 V_1)/4\pi N_a]^{1/3}$, where $\delta_1$ is the grams of water bound per gram of protein (0.34) and $V_1$ is the partial specific volume of water (1 cm$^3$/g).

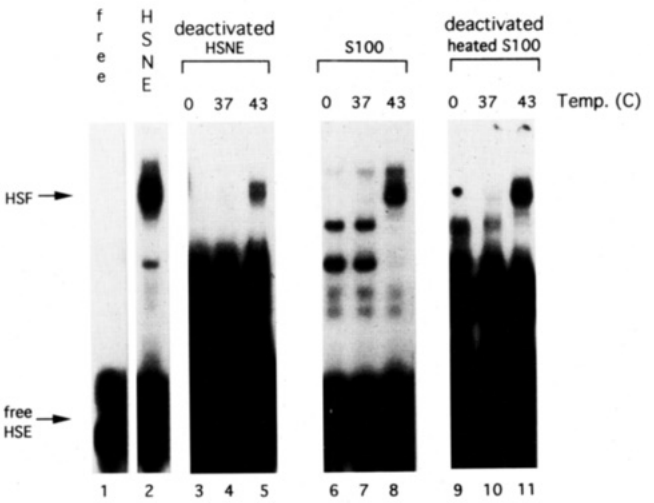


FIGURE 1: Deactivation assay of HSF in extracts and reactivation by heat in vitro. Nuclear extracts from heat shocked HeLa cells (0.68 mg) and S100 extracts from control HeLa cells (1.5 mg) heat activated in vitro were subjected to the deactivation protocol (see Materials and Methods). Briefly, extracts were denatured and then diluted to 1.0 mL with 6 M Gdn-HCl, renatured by dialysis, and concentrated by centrifugation. Untreated extracts (4.5 μg, lane 2; 13 μg, lanes 6−8) or deactivated extracts (3.0 μL, lanes 3−5: 2.0 μL, lanes 9−11) were then incubated for 60 min at the indicated temperatures. Samples were then analyzed by the gel shift assay by incubation for 30 min at 30 °C with a $^{32}$P-labeled synthetic oligonucleotide containing the HSE (bases −115 to −80 of the human hsp70 promoter; Kingston et al., 1987) followed by electrophoresis on a nondenaturing 4% polyacrylamide gel. Different panels represent different exposures of the same gel. HSNE, heat shocked nuclear extracts; S100, cytoplasmic extracts from non-heat shocked cells; HSF, heat shock factor; HSE, radioactive heat shock element DNA probe; C, degrees centigrade.

## RESULTS

*Activation of Purified HSF by Heat in Vitro.* To determine potential mechanisms that allow HSF to respond to heat, we took advantage of our previous observation that the DNA binding ability of human HSF can be activated in a cell-free extract in a temperature specific manner (Larson et al., 1988). Cytoplasmic extracts (S100) from HeLa cells grown at the normal growth temperature (37 °C) contain low levels of active HSF when analyzed by the electrophoretic mobility shift assay. Heating these extracts at heat shock temperatures in vitro caused a 5−20-fold increase in HSE binding activity (Larson et al., 1988; see lanes 6−8 of Figure 1). We were unable to separate activities required for this induction from inactive HSF after numerous different chromatographic procedures. This raised the possibility that HSF itself could sense temperature, so we sought to determine whether

homogeneous HSF was capable of responding to heat. It is possible to purify active HSF to apparent homogeneity by DNA affinity chromatography, so we developed protocols that allow us to inactivate the DNA binding activity of human HSF and study its reactivation by heat in vitro.

We first demonstrated that crude preparations of HSF can be cycled between active and inactive forms by denaturation, dilution of the denatured factor, renaturation, and concentration (see Materials and Methods). This process, referred to as deactivation, inactivated HSF from nuclear extracts of heat shocked cells (Figure 1, compare lanes 2 and 3). This HSF could be reactivated by heating the sample at 43 °C but not by heating at 37 °C (lanes 4 and 5). Similar results were obtained when the starting material was a heat activated S100 extract (lanes 9−11) or inactive HSF from non-heat shocked S100 (data not shown). These data demonstrate that the factor(s) that are required for temperature specific activation of HSF in vitro are present and functional following denaturation and renaturation of both nuclear and cytoplasmic extracts. If the dilution and concentration steps are omitted from this protocol, then a substantial amount of HSF is renatured in an active form (data not shown). Therefore, dilution prior to renaturation is required to produce inactive HSF. If the inactive HSF is not concentrated prior to heating, its binding activity will not be activated by heat (data not shown). These observations are in agreement with our earlier observation concerning the dramatic inhibitory effects of dilution on activation of the HSF in S100 extracts (Larson et al., 1988) and suggest that the ability to form active HSF is affected by the absolute concentration of HSF (see below).

We next applied this deactivation procedure to extensively purified HSF to determine if other factors are required for heat activation. Active HSF was purified from heat shocked HeLa cells to apparent homogeneity by DNA affinity chromatography (Schuetz et al., 1991; Figure 2, panels A and B, lane 1). The purity of this preparation of HSF was confirmed by microsequencing the protein (Schuetz et al., 1991). To ensure further the purity of HSF, 350 ng of the 87 kDa HSF peptide was excised from an acrylamide gel following SDS−PAGE, and the deactivation protocol was then used to produce inactive HSF (Figure 2B, lane 2). We found that this deactivated homogeneous HSF could be reactivated by heat without addition of other factors (lanes 3−6). The overall yield of DNA binding activity for this procedure was 2−5%. Estimations of HSF protein revealed most of this loss occurred through the loss of HSF protein. Thus, a significant percentage of purified, deactivated HSF could be reactivated by incubation at heat shock temperatures. This activation was dependent on time and temperature in a manner similar to the S100 extract (compare lanes 2−6 with 7−11). However, more activity was observed after incubation at 37 °C with the purified factor than was observed with the crude extract (compare lanes 3, 5, 8 and 10). Isolation of HSF in S100 extracts by excision from SDS−PAGE followed by deactivation caused a similar decrease in the temperature specificity of activation (lanes 12−16). This demonstrates that the decrease in temperature specificity was not unique to HSF from heat shocked cells. These data indicate that homogeneous, deactivated HSF can be activated for DNA binding by heat.

The homogeneous HSF used in these experiments was purified from heat shocked HeLa cells. Changes in HSF during heat activation in intact cells (e.g., phosphorylation; Larson et al., 1988) might have affected the observations. Therefore, we developed a procedure to purify HSF from S100 extracts of non-heat shocked HeLa cells (Figure 2C, see Materials and Methods). Briefly, S100 extracts were chromatographed on single stranded DNA cellulose, heat activated in vitro, and purified by DNA affinity chromatography. HSF is not phosphorylated when activated in vitro (Larson et al., 1988); in fact, no alterations of HSF migration in SDS−PAGE are observed when S100 extracts containing inactive HSF are heat activated in vitro (data not shown). The two purified HSF fractions in Figure 2C were pooled, and 230 ng of HSF was excised from an acrylamide gel following SDS−PAGE. HSF was renatured under deactivating conditions to produce inactive HSF (Figure 2D, lane 6). Aliquots of this deactivated HSF were then heated at different temperatures in vitro and assayed for DNA binding ability (Figure 2D, lanes 1−6). Maximal activation was seen at heat shock temperatures (43 °C, lane 2; 6-fold induction), and induction decreased at higher temperatures (lane 1). Activation also occurred upon incubation at non-heat shock temperatures (37 and 34 °C, lanes 4 and5), although to a lesser extent than incubation at heat shock temperatures (lane 2). Activation of HSF at temperatures that do not elicit the heat shock response was not observed in crude S100 extracts (Larson et al., 1988; Figure 2B and data not shown), but was observed with HSF purified from either heat shocked or non-heat shocked cells (Figures 2B,D). However, maximal induction with either purified fraction required heat shock temperatures. These results demonstrate that, in vitro, purified and deactivated HSF is maximally induced to bind DNA by incubation at heat shock temperatures. Other factors are not required to maintain HSF in an inactive form or to convert inactive HSF to active HSF upon heat shock. These results also suggest non-HSF factors may regulate HSF DNA binding, and these may also sense temperature changes.

*Induction of Binding Alters the Oligomeric State of HSF.* The observation that deactivated HSF purified from HeLa cells can respond to temperature prompted us to ask what changes occur to HSF upon induction of binding activity. Standard western analyses of human HSF before or after heat activation of S100 extracts in vitro revealed no detectable changes in HSF size (data not shown). HSF in *Drosophila* (Perisic et al., 1989; Clos et al., 1990) and *S. cerevisiae* (Sorger & Nelson, 1989; Peteranderl & Nelson, 1992) has been shown to bind to DNA as a trimer, and circumstantial evidence has accumulated that the conformation of HSF is important to its ability to bind DNA (Mosser et al., 1990; Zimarino et al., 1990b). We reasoned that heat stress could cause a conformational change in homogeneous HSF that enables it to form homo-oligomers that are then capable of binding DNA (Lis & Wu, 1993; Morimoto, 1993; Rabindran et al., 1993; Sheldon & Kingston, 1993; Westwood & Wu, 1993). Pore-exclusion limit gradient gel analysis allows a crude estimation of size in native acrylamide gels. Protein samples are loaded on a native gel consisting of a gradient that increases in acrylamide concentration as the sample migrates; proteins move through the gel until they reach their pore-exclusion limit. This assay has been used to demonstrate an apparent change in size upon activation of Drosophila (Westwood et al., 1991), mouse (Sarge et al., 1993), and human (Baler et al., 1993) HSF when extracts containing HSF were analyzed, and it is believed this is the change that activates DNA binding. We used this assay to verify that

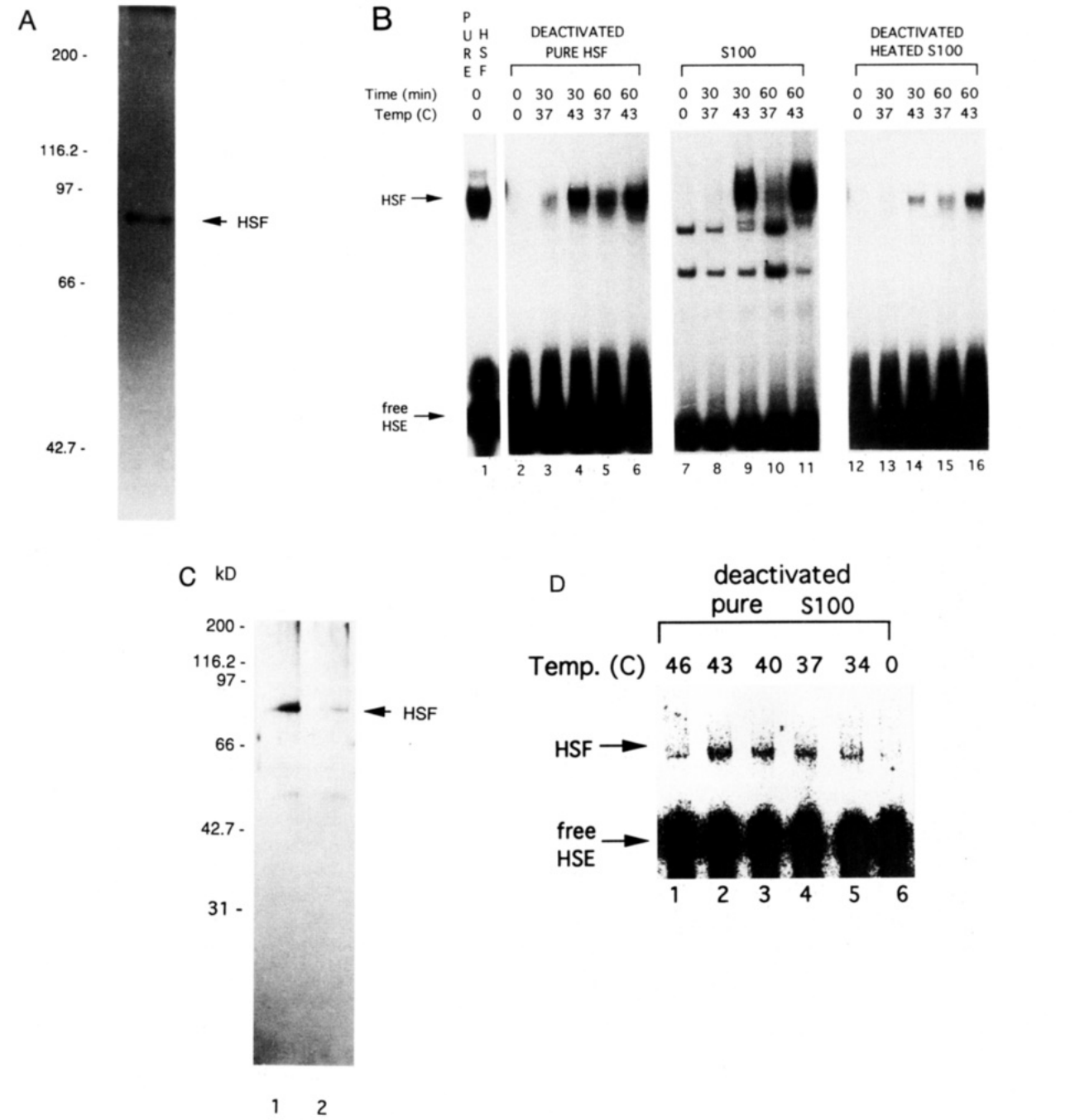


FIGURE 2: Pure deactivated HSF can respond to heat by altering its DNA binding capabilities. (A) Purification of HSF from nuclear extracts of heat shocked cells. HSF was purified as described (Schuetz et al., 1991), and 120 ng of HSF was subject to SDS−PAGE and visualized by reducing silver stain. The position of markers in kDa are indicated. (B) Heat activation of deactivated pure HSF. Pure active HSF (350 ng) or control cell (37 °C) S100 that had been heat-activated in vitro (650 $\mu$g) was eluted from SDS−PAGE slices and subjected to the deactivation protocol. Deactivated samples (4 $\mu$L, lanes 2−6, 12−16) or untreated samples (3 ng, lane 1; 13 $\mu$g, lanes 7−11) were incubated at the indicated temperatures for the indicated time and then analyzed by the electrophoretic mobility shift assay. Different panels represent different exposures of the same gel except that lane 1 is from a different gel. (C) Purification of HSF from S100 extracts of non-heat shocked cells. HSF was purified from non-heat shocked cells as described in Materials and Methods, and 50−100 ng was analyzed as described as described in A. (D) Heat activation of pure HSF from S100. Pure HSF (230 ng) was eluted from SDS−PAGE slices, renatured under deactivating conditions, incubated at the indicated temperature for 1 h, and analyzed by the electrophoretic mobility shift assay, except DNA binding reactions did not contain poly(dI·dC).

the purified deactivated HSF used in Figure 2 had characteristics that were similar to naturally occurring inactive HSF. Using purified HSF, we could also determine if the oligomeric change was a homo-oligomeric change.

A gel shift analysis of human HSF in extracts from HeLa cells on a pore gradient gel revealed that HSF bound DNA as a complex with an exclusion limit greater than the 669 kDa marker (Figure 3A). This complex was HSF, as its formation was dependent on heat-activating the S100 extract (compare lanes 1 and 2) and it could be competed by the inclusion of excess unlabeled HSE fragments (lane 3) but not by unlabeled mutant HSE fragments (lane 4). The complexes observed in unheated S100 (lane 1) were nonspecific (data not shown). The apparent size of HSF without DNA in untreated and heat activated S100 was assessed by using pore gradient native gel electrophoresis visualized by

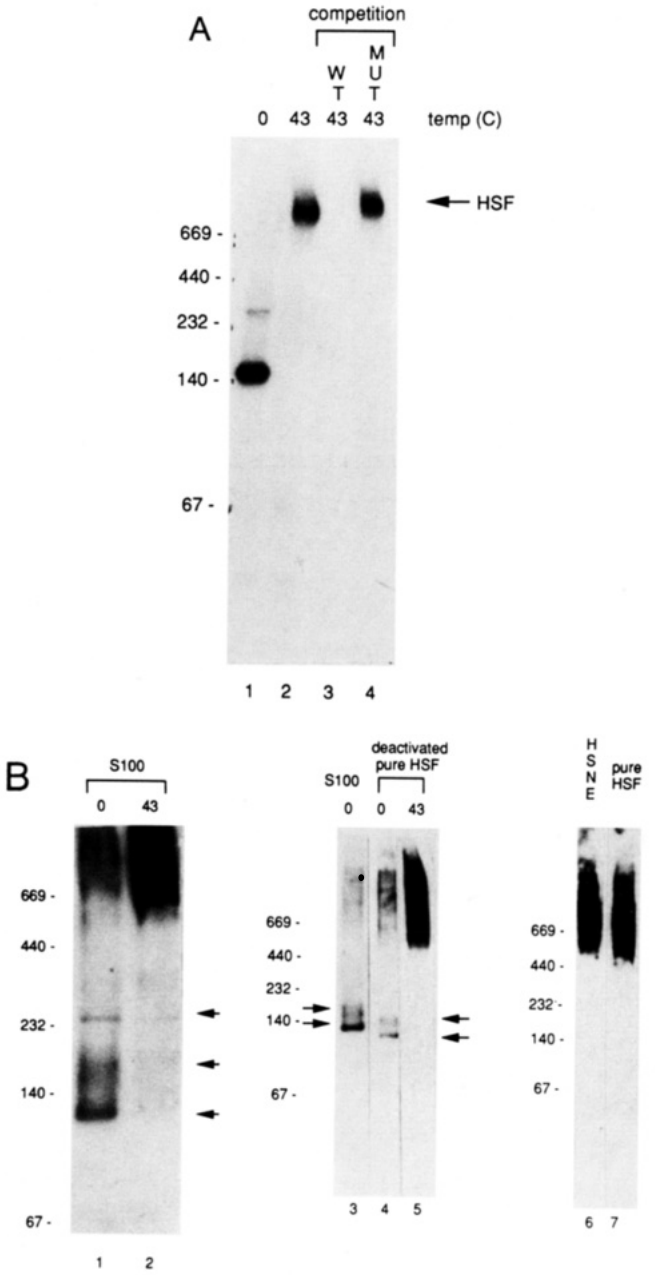


FIGURE 3: Pore gradient analysis of HeLa cell HSF. (A) HSF when bound to DNA is present in a high molecular weight complex. S100 extracts (32 $\mu$g) from control cells were incubated for 60 min at the indicated temperature, and a gel shift analysis was performed with (lanes 3, 4) or without (lanes 1, 2) the indicated unlabeled competitors. The WT competitor contains a wild-type HSE, while MUT contains a 4 bp substitution in the HSE (see Materials and Methods for relevant sequences). After the DNA binding reaction was performed, the samples were electrophoresed on a 4−30% native acrylamide gel. Positions of protein markers in kDa are indicated. (B) Pore gradient analysis of HSF without DNA. HSNE extracts (14 $\mu$g, lane 6), control cell S100 extracts (240 $\mu$g, lanes 1 and 2), or pure active HSF (2 ng, lane 7) were (lanes 1, 2) or were not (lanes 6, 7) incubated for 60 min at the indicated temperatures. For lanes 3−5, pure active HSF (45 ng) or control cell S100 (1.87 mg) was subjected to the deactivation protocol. Deactivated pure HSF (10 $\mu$L), incubated for 60 min at 43 °C (lane 5) or 0 °C (lane 4), and deactivated S100 (10 $\mu$L, lane 3) were subject to pore gradient analysis followed by western staining with anti-HSF antibodies (Schuetz et al., 1991). Blots were visualized with ECL staining (Amersham). Each panel represents a different experiment.

western staining with anti-HSF antibodies (Schuetz et al., 1991). When unheated and heat activated S100 extracts were compared in this protocol, we observed a size change in HSF similar to that seen by other researchers (Westwood et al., 1991; Baler et al., 1993; Sarge et al., 1993). HSF in non-heat shocked S100 extracts contained low molecular weight species (Figure 3B, lane 1) that were apparently converted to a high molecular weight species upon heat activation in vitro (lane 2). The species migrating directly above the 232 kDa marker is not always observed and is not as dramatically reduced following heat activation. Activation therefore correlates with an apparent increase in size of human HSF, as has previously been demonstrated with *Drosophila* HSF (Westwood et al., 1991), mouse HSF (Sarge et al., 1993), and human HSF (Baler et al., 1993) when HSF was activated in cells by heat shock. The total amount of HSF protein appeared to increase upon heat activation in this assay (lanes 1 and 2). An increase has also been observed by some researchers (Baler et al., 1993), while a decrease in activity upon heat activation has been observed by others (Sarge et al., 1993). Thus, the apparent change in HSF protein amount probably reflects the limitations of the assay and not an actual change in the amount of HSF.

We next used this assay to compare the artificially deactivated HSF purified from heat shocked HeLa cells (see Figure 2A,B) with naturally inactive HSF in S100 extracts. Homogeneous deactivated HSF contained two lower molecular weight species similar to those seen in unheated S100 (Figure 3B, lanes 3 and 4). This preparation of HSF was not fully deactivated and, therefore, retained a small amount of DNA binding activity (data not shown) and also retained some of the high molecular weight species (lane 4). The lower molecular weight species were not seen in homogeneous HSF prior to deactivation (lane 7), implying that they formed as a result of the deactivation procedure (lanes 4 and 7). The slight difference in migration between the two lower molecular weight species in deactivated homogeneous HSF (lane 4) and unheated S100 (lane 3) probably reflects differences in post-translational modification (Larson et al., 1988; Schuetz et al., 1991; and data not shown). Reactivation of this preparation of deactivated homogeneous HSF by heat increased binding activity approximately 3- to 5-fold (data not shown) and caused a disappearance of the lower molecular weight species and an increase in the high molecular weight complex (lanes 4 and 5). Thus, the deactivated purified HSF and naturally inactive HSF behaved similarly in response to heat as analyzed by native gel electrophoresis in both the size and number of complexes. Further, our results using pure substrates demonstrate that HSF homo-oligomerizes in response to heat shock temperatures. This can be accomplished without other factors or DNA.

To characterize the oligomeric composition of active and inactive HSF, analytical gel filtration and sedimentation velocity analysis was performed. These experiments have been performed previously with *Drosophila* HSF (Westwood & Wu, 1993) and human HSF (Sistonen et al., 1994) in extracts. The use of purified forms of HSF here allows an unambiguous determination of homo-oligomeric states. We used active HSF purified from nuclear extracts of heat shocked HeLa cells and analyzed columns and gradients by the electrophoretic mobility shift assay. The active fractions were then analyzed by the pore gradient gel analysis, and this demonstrated they consisted of the high molecular weight active complex shown in Figure 3B (data not shown).

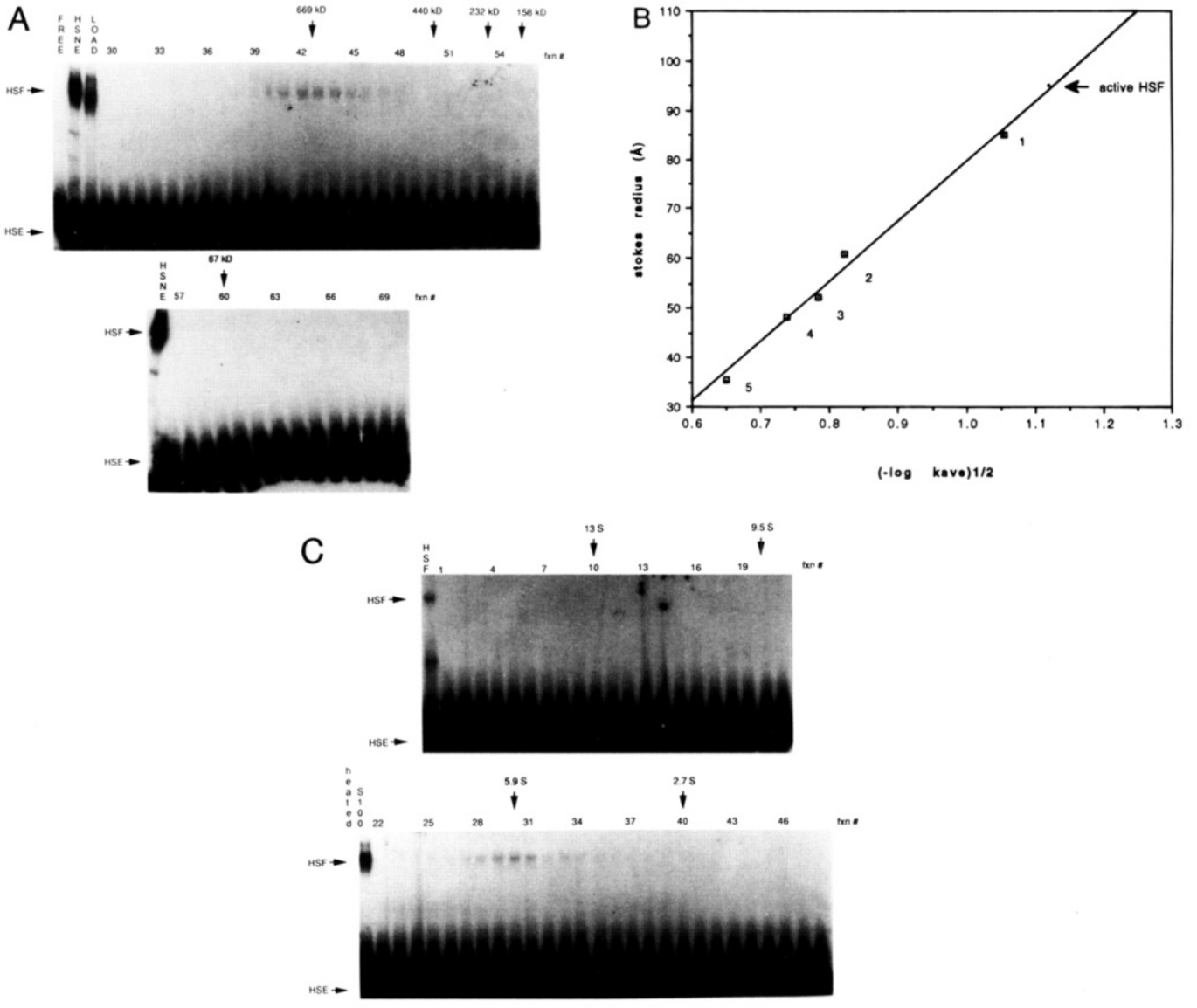


FIGURE 4: Gel filtration and sedimentation velocity analysis of active, purified HSF. (A) Gel filtration analysis of active HSF. Approximately 20 ng of active, purified HSF (Schuetz et al., 1991) was loaded on a Sephacryl S300-SF column. Fractions were collected, and 10$\mu$L aliquots were analyzed by electrophoretic mobility shift assay. HSNE and load material lanes were included as a reference point for the HSF−HSE complex. The elution profile of protein standards used to calibrate the column is depicted above the corresponding lanes in kDa. Protein standards were visualized by SDS−PAGE followed by silver or Coomassie staining. The protein standards were as follows: 1, thyroglobulin, 669 kDa, 85.0 Å; 2, ferritin, 440 kDa, 61.0 Å; 3, catalase, 232 kDa, 52.2 Å; 4, aldolase, 158 kDa, 48.1 Å; and 5, albumin, 67 kDa, 35.5 Å. fxn #, fraction number. (B) Determination of the Stokes radius of active HSF. To determine the relationship between Stokes radius ($r$) and $(-\log k_{av})^{1/2}$, protein standards were plotted. $k_{av}$ was calculated from the equation: $k_{av} = (V_e - V_o)/(V_t - V_e)$, where $V_e$ (elution volume), $V_o$ (void volume), and $V_t$ (total column volume) were all measured. By calculating the $k_{av}$ of active HSF from the mobility shift assay, one can determine the Stokes radius (Table 1). (A) and (B) are from separate trials. (C) Sedimentation velocity analysis of active HSF. Approximately 6 ng of purified HSF was analyzed by sedimentation in a 10−30% sucrose gradient. Fractions were collected starting with the bottom of the gradient (30% sucrose), and 10 $\mu$L aliquots were analyzed by electrophoretic mobility shift assay. As a reference point for the HSF−HSE complex, S100 heat activated in vitro (heated S100) and a partially pure active HSF preparation (HSF) were included in the mobility shift assay. Sedimentation coefficients ($S_{20,w}$) were determined from the $\omega^2 t$ integration function (Beckman Instruments, Inc.) and by cosedimentation of protein standards with known $S_{20,w}$ values. The $S_{20,w}$ values of representative fractions are indicated.

Analytical gel filtration of purified active HSF (Figure 4A) was compared to the elution profile of known standards (Figure 4B) to determine a Stokes radius of 94 Å (Table 1). Sedimentation velocity analysis of purified active HSF was done using 10−30% sucrose gradients, and a sedimentation coefficient ($S_{20,w}$) of 5.9 was determined by the $\omega^2 t$ integration function (Beckman Instruments, Inc.) and by cosedimentation with known standards (Figure 4C and Table 1). From the Stokes radius and $S_{20,w}$ a native molecular mass for purified active HSF was calculated to be 240 kDa (Table 1).

To determine the native molecular weight of inactive HSF, active HSF was purified from nuclear extracts of heat shocked cells and then deactivated in vitro. This would also help determine if deactivation causes the formation of an anomalous form of HSF by comparing deactivated purified HSF here to natural inactive human HSF in extracts (Sistonen et al., 1994). Columns and gradients were assayed by ELISA. To minimize loss of HSF protein during deactivation, HSF was only partially deactivated and, therefore, retained some active HSF as determined by electrophoretic mobility shift assays (EMSA; data not shown). This also

Table 1: Physical Properties of HSF[a]

| complex | | Stokes radius[b] | $D_{20,w}$[c] | $S_{20,w}$[d] | $m$ (kDa)[e] | $f/f_{sph}$[f] |
|---|---|---|---|---|---|---|
| active HSF | trial 1 | 98 | $2.2 \times 10^{-7}$ | 5.9 | 250 | 2.1 |
| | trial 2 | 90 | $2.4 \times 10^{-7}$ | 5.9 | 230 | 1.9 |
| inactive HSF | trial 1 | 58 | $3.7 \times 10^{-7}$ | 3.0 | 76 | 1.8 |
| | trial 2 | 54 | $3.9 \times 10^{-7}$ | 3.6 | 86 | 1.6 |

[a] As described in Materials and Methods. [b] Stokes radius ($r$) in Å, Figures 4A,B and 5A,B. [c] Diffusion coefficient, from the Stokes–Einstein equation. [d] Sedimentation coefficient ($10^{-13}$), Figures 4C and 5C. [e] Molecular mass, from the Svedberg equation. [f] Perrin factor.

provided an internal control for the difference between active and inactive HSF. Two peaks of HSF activity eluted from the gel filtration column (Figure 5A,B). Peak A represented active HSF because it had the same Stokes radius as active HSF. We therefore inferred that peak B represented inactive HSF. The Stokes radius of peak B was 56 Å (Table 1). When partially deactivated HSF was analyzed by sucrose gradients, three peaks were observed, X, Y, and Z (Figure 5C, dashed line). To determine which peak contained inactive HSF, we compared deactivated HSF (Figure 5C, dashed line) to pure HSF prior to deactivation (Figure 5C, solid line). Peaks X and Y were present in both active and deactivated HSF. On the basis of the sedimentation profile of active HSF as measured by EMSA, we conclude that peak Y represented active HSF present in the deactivated sample because of incomplete deactivation. The slight change in $S$ value of active HSF in this experiment (compare Figures 4C and 5C) might reflect the different solution conditions used in the two experiments. We believe peak X, which migrated with a very high sedimentation coefficient, represented aggregates of HSF present in both the purified and the deactivated fractions. Inactive HSF would then be represented by peak Z, which had an $S_{20,w}$ value of 3.3 (Table 1). This is supported by the observation that the amount of peak Z was substantially increased by deactivation. The conclusion that peak Z is inactive HSF is also supported by analysis of HSF1 in crude extracts where an identical $S_{20,w}$ was obtained (Sistonen et al., 1994). The calculated native molecular mass of inactive HSF was 81 kDa (Table 1).

The molecular mass of HSF deduced from the amino acid sequence of the HSF1 gene product is 57 kDa (Rabindran et al., 1991), while the molecular mass of HSF activated in HeLa cells in SDS–PAGE analysis is 87 kDa (Schuetz et al., 1991). HSF is also post-translationally modified in human cells (Larson et al., 1988; and data not shown). Our results indicate deactivated HSF (81 kDa, Table 1) is a monomer while active HSF (240 kDa, Table 1) is a trimer. These results are similar to those found with *Drosophila* HSF in extracts (Westwood & Wu, 1993) and human HSF1 in extracts (Sistonen et al., 1994). This suggests that purified, deactivated HSF undergoes a change in oligomeric status identical to naturally inactive HSF upon heat activation. These data further demonstrate that the deactivation procedure used in these experiments produces inactive HSF with similar physical properties as naturally inactive HSF.

These experiments also provide insight into the shape of HSF. The Perrin factor is a ratio of the frictional coefficient to the frictional coefficient of a hydrated sphere. A Perrin ratio of 1.0 would indicate a spherical shape. The Perrin factors calculated from these data indicated that both active and deactivated HSF were not spherical, which may explain

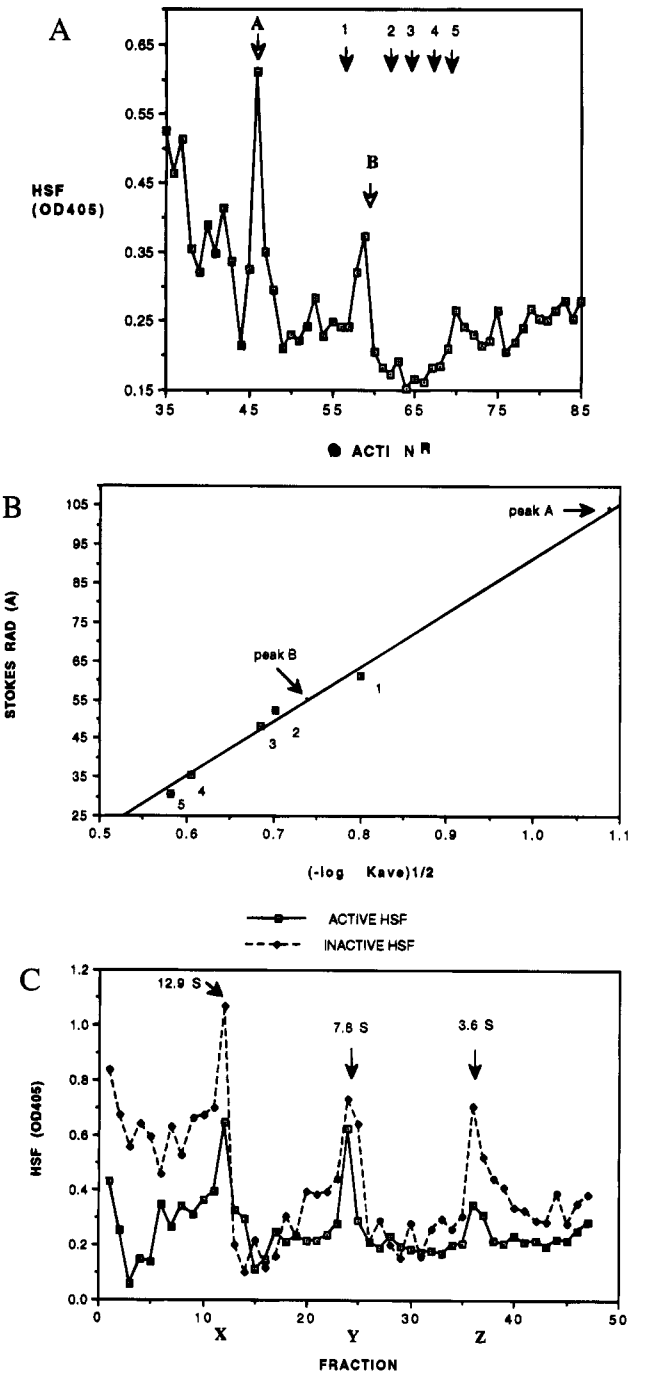


FIGURE 5: Gel filtration and sedimentation velocity analysis of purified inactive heat-inducible HSF. (A) Gel filtration analysis of deactivated HSF. Purified, active HSF was subject to the deactivation protocol (see main text) to yield partially inactive, heat-inducible pure HSF. Approximately 5 ng of this material was loaded on a Sephacryl S300 HR column. Fractions were collected and analyzed by ELISA with anti-HSF antibodies (Schuetz et al., 1991). The elution profile of protein standards used to calibrate the column is depicted above the corresponding fractions. Protein standards were as follows: 1, ferritin, 440 kDa, 61.0 Å; 2, catalase, 232 kDa, 52.2 Å; 3, aldolase, 158 kDa, 48.1 Å; 4, albumin, 67 kDa, 35.5 Å; and 5, ovalbumin, 43 kDa, 30.5 Å. A, B: HSF peaks. (B) Determination of the Stokes radius of deactivated HSF. A plot of $K_{av}$ versus Stokes radius was performed as in Figure 4. Locations of HSF peaks A and B are shown. (C) Sedimentation velocity analysis of deactivated HSF. Approximately 5 ng of purified, partially deactivated, heat-inducible pure HSF was made and analyzed by sucrose gradients. Fractions were collected starting with the bottom of the gradient. Active, purified HSF was analyzed in parallel. HSF peaks were detected by ELISA and denoted X, Y, and Z. The sedimentation coefficients ($S$) of these peaks are noted.

the aberrant mobilities in the pore exclusion analysis (Figure 3). Active HSF had a Perrin factor of 2.0, and inactive HSF had a Perrin factor of 1.7. These values are comparable to those obtained with fibrous proteins, such as human fibrinogen (Caspary & Keckwick, 1957), and would indicate axial ratios of 20:1 (active HSF) and 15:1 (inactive HSF), assuming that HSF is a prolate ellipsoid (Eisenberg & Crothers, 1979). Thus, both active and inactive homogeneous HSF have extended structures. The structural parameters for homogeneous inactive and active HSF found here are similar to those found for *Drosophila* HSF (Westwood & Wu, 1993) and human HSF1 (Sistonen et al., 1994) when crude extracts from normal and heated cells were analyzed. There are slight differences in these structural parameters when these studies are compared that could be caused by the different methodologies or by the use of purified reagents in our assays. Our results suggest that deactivated homogeneous HSF undergoes a change in conformation and oligomerization status similar to natural inactive HSF upon heat activation.

## DISCUSSION

*Human HSF Can Directly Sense Temperature in Vitro.* The mechanism whereby cells sense a heat shock and translate this into activation of HSF has remained elusive. Activation of the DNA binding ability of HSF appears to be caused by trimerization of HSF monomers. Our results here represent a biochemical characterization of HSF to determine if HSF itself might play an active role in sensing a heat shock. To do this, we purified HSF from human cells in an active form and deactivated it in vitro. We found that homogeneous deactivated HSF can respond to increased temperatures by increasing its DNA binding capabilities (Figure 2) and altering its oligomeric status (Figure 3 and Table 1). No other factors were required to maintain purified HSF in an inactive conformation or to activate HSF upon heat shock in vitro. These biochemical properties of pure HSF suggest HSF may play an active role in sensing a heat shock in human cells.

Several lines of evidence imply that non-HSF factors are required to establish the characteristic temperature of activation in an intact cell (Mezger et al., 1989; Abravaya et al., 1991; Gallo et al., 1993) and that these factors may be hsps (reviewed in Craig & Gross, 1991; Abravaya et al., 1992; Baler et al., 1992; Mosser et al., 1993; Nadeau et al., 1993). When human HSF is expressed in *Drosophila*, it is induced to bind DNA at *Drosophila* heat shock temperatures, temperatures that do not activate HSF in humans (Clos et al., 1993). This suggests non-HSF factors regulate HSF by functioning as biological thermometers. Our data support this model; the temperature profiles of activation of homogeneous HSF are not identical to activation of HSF in human cell extracts (Figure 2). Specifically, some activation is observed at non-heat shock temperatures which is not observed in human cell extracts. The loss in temperature specificity occurs when HSF in S100 extracts is excised from SDS−PAGE and deactivated (Figure 2) but not by deactivation alone (Figure 1). This suggests a regulatory factor is lost during excision from SDS−PAGE and this factor functions by negatively regulating the DNA binding activity of HSF.

It is possible, however, that the properties of HSF we describe here contribute to heat-induced activation in vivo. Maximal activation of purified, deactivated HSF occurred at the heat shock temperature, 43 °C, with less activation observed at temperatures above or below 43 °C (Figure 2). This temperature profile was observed with two separate forms of purified HSF. This suggests heat shock temperatures may cause direct changes in HSF that promote activation. This is supported by experiments where *Drosophila* HSF was expressed in human cells (Clos et al., 1993). *Drosophila* HSF is constitutively active in human cells, which grow normally at temperatures that stress *Drosophila*. These data are consistent with the hypothesis that HSF itself can sense temperature. Several lines of evidence suggest that hsps might be involved in regulating HSF, possibly by acting as a temperature sensor (reviewed in Craig & Gross, 1991; Abravaya et al., 1992; Baler et al., 1992; Mosser et al., 1993; Nadeau et al., 1993); however, recent data indicate that hsps may not be sufficient to regulate HSF activity (Rabindran et al., 1994). Taken together, these studies suggest that appropriate regulation of HSF in response to temperature requires multiple factors. The biochemical properties of HSF that we describe here suggest that HSF might be one of the factors that plays a role in sensing temperature.

It is important to consider whether the deactivation procedure used in this research produces a biologically relevant form of HSF. Although this is impossible to determine absolutely, we have compared the biochemical properties of homogeneous deactivated HSF with natural inactive HSF. Deactivation causes the formation of HSF species similar in both the number of complexes and the migration patterns to natural inactive HSF in native gels (Figure 3 and Westwood et al., 1991; Baler et al., 1993; Sarge et al., 1993). Pure deactivated HSF is also a monomer of similar shape as natural inactive HSF (Figures 4 and 5, Table 1, and Westwood & Wu, 1993; Sistonen et al., 1994). The effect of heat incubation on pure deactivated HSF is similar to natural inactive HSF in vivo and in vitro. Activation occurs maximally at heat shock temperatures (Figure 2), is concurrent with oligomerization (Figure 3), and is extremely concentration sensitive (data not shown). Thus, the deactivation procedure produces an inactive HSF with properties similar to those of natural inactive HSF.

*Oligomerization of HSF.* We have also analyzed the changes in oligomerization that occur to homogeneous HSF following heat activation. We observe a change in mobility on native polyacrylamide gels and a change in size and Perrin factor on glycerol gradients and sizing columns (Figures 3−5 and Table 1). These results are similar to those found for *Drosophila* HSF (Westwood et al., 1991; Westwood & Wu, 1993), mouse HSF (Sarge et al., 1993), and human HSF (Baler et al., 1993; Sistonen et al., 1994) when crude extracts were examined. The similarity between purified deactivated HSF and HSF in human cell extracts in these assays suggests other factors are not stably bound to HSF at normal temperatures. Activation appears to occur via homo-trimerization, and trimerization can occur in solution without other factors or DNA.

Many observations support the correlation between homotrimerization and activation of DNA binding of HSF. First, dilution of S100 before heat incubation severely decreases activation potential, and the dilution profile is consistent with a third order reaction mechanism (Larson et al., 1988; J. S. Larson, Nai Wen Chi, and R. E. Kingston, unpublished data).

Second, denaturation and renaturation of HSF without dilution yields active HSF (data not shown). Third, deactivated HSF that has been diluted but not concentrated is inactive but cannot be reactivated by heat (data not shown). Finally, overexpression of genes of HSF proteins known to be heat inducible (*Drosophila* HSF, mouse HSF1, and human HSF1) either in bacteria or in vitro yields constitutively active HSF (Clos et al., 1990; Rabindran et al., 1991; Sarge et al., 1993). Expression of heat-inducible HSF1 in human cells is also apparently concentration dependent (Rabindran et al., 1993). The correlation between concentration and activity of HSF argues that the factor can be driven into the active (multimeric) state by mass action.

The above data then argue that HSF has an intrinsic ability to respond to temperature. Heat apparently promotes a direct conformational change in HSF that allows trimerization to occur and subsequent DNA binding. Trimerization of HSF is believed to be mediated by a hydrophobic repeat structure adjacent to the DNA binding domain. Perhaps inactive HSF exists in a conformation that shields the trimerization domain and heat induces a conformational change that makes this domain accessible (Lis & Wu, 1993; Morimoto, 1993; Rabindran et al., 1993; Sheldon & Kingston, 1993). In the intact cell, factors that modulate the ability of HSF to respond to temperature would be expected to affect the change in oligomeric status of HSF.

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## REFERENCES

Abravaya, K., Phillips, B., & Morimoto, R. I. (1991) *Mol. Cell. Biol. 11*, 586−592.
Abravaya, K., Myers, M. P., Murphy, S. P., & Morimoto, R. I. (1992) *Genes Dev. 6*, 1153−1164.
Amin, J., Ananthan, J., & Voellmy R. (1988) *Mol. Cell. Biol. 8*, 3761−3769.
Ananthan, J. Goldberg, A. L., & Voellmy, R. (1986) *Science 232*, 522−524.
Baler, R., Welch, W. J. & Voellmy, R. (1992) *J. Cell Biol. 117*, 1151−1159.
Baler, R., Dahl, G., & Voellmy, R. (1993) *Mol. Cell. Biol 13*, 2486−2496.
Beckman Instruments Inc., *Applications Data* $\omega^2 t$ , DS-528.
Caspary, E. A., & Kekwick, R. A. (1957) *Biochem J. 67*, 41−48.
Clos, J., Westwood, J. T.. Becker, P. B., Wilson, S., Lambert, K., & Wu, C. (1990) *Cell 63*, 1085−1097.
Clos, J., Rabindran, S., Wisniewski, J., & Wu, C. (1993) *Nature 364*, 252−255.
Craig, E. A., & Gross, C. A. (1991) *Trends Biochem. Sci. 16*, 135−140.
Dignam, J. D., Lebovitz, R. M., & Roeder, R. G. (1983) *Nucleic Acids Res. 11*, 1475−1489.
Eisenberg, D., & Crothers, D. M. (1979) *Physical Chemistry*, pp 718−722, Benjamin/Cummings, Menlo Park, CA.
Gallo, G. J., Schuetz, T. J., & Kingston, R. E. (1991) *Mol. Cell. Biol. 11*, 281−288.
Gallo, G. J., Prentice, H., & Kingston, R. E. (1993) *Mol. Cell. Biol. 13*, 749−761.
Harrison, C. J., Bohm, A. A., & Nelson, H. C. M. (1994) *Science 263*, 224−227.
Hightower, L. E. (1991) *Cell 66*, 191−197.
Hornbeck, P. (1991) in *Current Protocols in Molecular Biology* (Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., & Struhl, K., Eds.) pp 11.2, John Wiley and Sons, New York.
Jakobsen, B. K., & Pelham, H. R. B. (1991) *EMBO J. 10*, 369−375.
Kingston, R. E., Schuetz, T. J., & Larin, Z. (1987) *Mol. Cell. Biol. 7*, 1530−1534.
Larson, J. S., Schuetz, T. J., & Kingston, R. E. (1988) *Nature 335*, 372−375.
Lindquist, S., & Craig, E. A. (1988) *Annu. Rev. Genet. 22*, 631−677.
Lis, J., & Wu, C. (1993) *Cell 74*, 1−4.
Mezger, V., Bensaud, O., & Morange, M. (1989) *Mol. Cell. Biol. 9*, 3888−3896.
Morimoto, R. I. (1993) *Science 259*, 1409−1410.
Mosser, D. D., Kotzbauer, P. T., Sarge, K. D., & Morimoto, R. I. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 3748−3752.
Mosser, D. D., Duchaine, J., & Massie, B. (1993) *Mol. Cell. Biol. 13*, 5427−5438.
Nadeau, K., Das, A., & Walsh, J. T. (1993) *J. Biol. Chem. 268*, 1479−1487.
Nakai, A., & Morimoto, R. I. (1993) *Mol. Cell. Biol. 13*, 1983−1997.
Pelham, H. R. B., & Bienz M. (1982) *EMBO J. 1*, 1473−1477.
Perisic, O., Xiao, H., & Lis, J. T. (1989) *Cell 59*, 797−806.
Peteranderl, R., & Nelson, H. C. M. (1992) *Biochemistry 31*, 12272−12276.
Rabindran, S. K., Giorgi, G., Clos, J., & Wu, C. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 6906−6910.
Rabindran, S. K., Haroun, R. I., Clos, J., Wisniewski, J., & Wu, C. (1993) *Science 259*, 230−234.
Rabindran, S. K., Wisniewski, J., Li., L., Li., G. C., & Wu, C. (1994) *Mol. Cell. Biol. 14*, 6552−6560.
Sarge, K. D., Zimarino, V., Holm, K., Wu, C., & Morimoto, R. I. (1991) *Genes Dev. 5*, 1902−1911.
Sarge, K. D., Murphy, S. P., & Morimoto, R. I. (1993) *Mol. Cell. Biol. 13*, 1392−1407.
Scharf, K.-D., Rose, S., Zott, W., Schoff, F., & Nover, L. (1990) *EMBO J. 9*, 4495−4501.
Schuetz, T. J., Gallo, G. J., Sheldon, L., Tempst, P., & Kingston, R. E. (1991) *Proc. Natl. Acad. Sci. USA 88*, 6911−6915.
Sheldon, L. A., & Kingston, R. E. (1993) *Genes Dev. 7*, 1549−1558.
Sistonen, L., Sarge, K. D., & Morimoto, R. I. (1994) *Mol. Cell. Biol. 14*, 2087−2099.
Smith, J. A. (1991) in *Current Protocols in Molecular Biology* (Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., & Struhl, K., Eds.) pp 10.9.2−10.9.11, John Wiley and Sons, New York.
Smith, M. H. (1966) *J. Theor. Biol. 13*, 261−282.
Sorger, P. K. (1991) *Cell 65*, 363−366.
Sorger, P. K., & Pelham, H. R. B. (1988) *Cell 54*, 855−864.
Sorger, P. K., & Nelson, H. C. M. (1989) *Cell 59*, 807−813.
Sorger, P. K., Lewis, M. J., & Pelham, H. R. B. (1987) *Nature 329*, 81−84.
Weber, K., & Osborn, M. (1969) *J. Biol.. Chem. 244*, 4406−4412.
Westwood, J. T., & Wu, C. (1993) *Mol. Cell. Biol. 13*, 3481−3486.
Westwood, J. T., Clos, J., & Wu, C. (1991) *Nature 353*, 822−827.
Wiederrecht, G., Seto, D., & Parker, C. C. (1988) *Cell 54*, 841−853.
Wu, C. (1984) *Nature 311*, 81−84.
Xiao, H., & Lis, J. T. (1988) *Science 239*, 1139−1142.
Zimarino, V., & Wu, C. (1987) *Nature 327*, 727−730.
Zimarino, V., Tsai, C., & Wu, C. (1990a) *Mol. Cell. Biol. 10*, 753−759.
Zimarino, V., Wilson, S., & Wu, C. (1990b) *Science 249*, 546−549.

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