

## An Alteration in Molecular Form Associated with Activation of Human Heat Shock Factor

Yoko Kimura<sup>\*,\*\*</sup>, Tadatsugu Taniguchi<sup>\*\*</sup>, and Ichiro Yahara<sup>\*1</sup>

<sup>\*</sup>Department of Cell Biology, The Tokyo Metropolitan Institute of Medical Science, Tokyo 113, and <sup>\*\*</sup>Institute for Molecular and Cellular Biology, Osaka University, Osaka 565, Japan

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**ABSTRACT.** In higher eucaryotes, heat shock factor (HSF) exists in a cryptic form in unstressed cells. We investigated molecular forms of human HSF before and after activation by sucrose density gradient centrifugation and by gel mobility shift assay using a <sup>32</sup>P-labeled heat shock element (HSE). We found that the *in vivo* or *in vitro* activated HSF, which is capable of binding to HSE, and its inactive form present in unstressed cells have different sedimentation coefficient; the former is 8 S whereas the latter is 4–5 S. Both the 8 S and 4–5 S forms contain the HSF polypeptide which has the ability to bind to HSE upon activation. The inactive 4–5 S form acquires HSE-binding ability when activated by heat shock or other stimuli. This HSF activity was greatly reduced, however, during recentrifugation in sucrose density gradient and, in addition, the residual activity was not recovered in 8 S fractions. Transformation of the inactive 4–5 S form of HSF to the stable, active 8 S form was achieved when the inactive form was activated and mixed with cytosols of unstressed cells.

When cells are exposed to high temperature or to certain forms of stress, a small set of proteins called heat shock proteins are expressed (9, 10, 18). Their expressions are activated at both the transcriptional and translational levels. In eucaryotes, this activation in the transcriptional level is mediated by a specific DNA motif known as the heat shock element (HSE) in the promoter region of heat shock genes (15, 16). A positive transcription factor, the heat shock factor (HSF) which binds HSE, has been shown to play a key role in this activation (7, 11, 26).

The gene encoding the yeast HSF has been cloned and extensively characterized, and it was demonstrated that the yeast HSF oligomerizes *in vitro* (14, 21–24). The yeast HSF has been shown to bind DNA even in unstressed cells (20). By contrast, in higher eucaryotes, the HSE binding activity of HSF is induced in response to various forms of stress (20). The *in vitro* induction of the DNA binding activity of HSF does not require *de novo* protein synthesis and the *in vitro* induction occurs in the absence of ATP (8, 12, 29). Moreover, HSF is activated *in vitro* in response not only to heat shock but also to treatment with nonionic detergents, Ca<sup>2+</sup>-ion or anti-HSF antibodies, or at low pH (13, 30). Many if not all of these agents cause conformational changes of proteins, suggesting that HSF itself is the sensory machinery which recognizes various forms of stress and

thereby induces the conformational changes of HSF responsible for the activation (13, 30). Recently, the cDNA of *Drosophila* HSF has been cloned and expressed in *Escherichia coli* (5). The recombinant HSF produced in *E. coli* was active without heat shock, strongly suggesting that *Drosophila* HSF is intrinsically active in DNA binding and that the activity is repressed *in vivo* (5).

In this paper, we provide evidence that the inactive form and the active HSE-binding form of human HSF can be separated on the basis of different sedimentation coefficients estimated by sucrose density gradient centrifugation. The active form sediments at 8 S and the inactive form at 4–5 S. Although the HSE-binding activity can be induced on the 4–5 S HSF by heat shock or other stimuli, it cannot be transformed to the stable 8 S form unless it is incubated with unstimulated cytoplasmic extracts. These results will be discussed in the light of the importance of the alterations in molecular structures and assemblies of HSF during the activation.

### MATERIALS AND METHODS

**Cell culture.** HeLa cells were cultured using spinner culture bottles at 37°C in Eagle's MEM medium (Nissui) supplemented with 5% or 10% Tissue plus (Sanko Junyaku, Co. Ltd). When cells were subjected to heat shock, the bottles containing cultures were placed in a water bath at 43°C for 1 hr during which time the cell suspensions were constantly stirred.

<sup>1</sup> To whom correspondence should be addressed.

**Preparation of extracts.** Cytoplasmic extracts of HeLa cells were prepared as described previously (2) with a slight modification in the presence of PMSF, leupeptin, and aprotinin. The  $1 \times$  buffer D was modified to 20 mM Hepes (pH 7.9), 50 mM KCl, 0.5 mM dithiothreitol (DTT), 0.2 mM EDTA and 10% glycerol; NP40 was omitted throughout the preparation. The cell extracts were concentrated using Omega Cells (Filtron) until the protein concentrations reached about 50 mg/ml. Nuclear extracts were prepared as described elsewhere (6), and dialyzed against  $1 \times$  buffer D.

**Sucrose density gradient centrifugation.** Five milliliters of linear sucrose density gradients (10–30 (w/v) %) were prepared in 20 mM Hepes (pH 7.9), 50 mM KCl, 10 mM sodium molybdate, 0.5 mM DTT and 0.2 mM EDTA. Aliquots (200  $\mu$ l) of cell extracts were applied onto the gradients and centrifuged at  $190,000 \times g$  for 16 h, after which fractions of 350  $\mu$ l were successively collected from the bottom of each tube.

**Gel mobility shift analysis.** DNA-binding reaction mixtures contained, in final volumes of 10  $\mu$ l, fractionated or unfractionated HeLa cell extracts, 3.2  $\mu$ g of poly (d[I-C]) (Pharmacia), 0.25  $\mu$ g of pUC19, 4 fmole of  $^{32}$ P end-labeled heat shock element (HSE) oligonucleotide (upper strand, 5'-CTA GAAGCTTCTAGAAGCTTCTAGAGGATCCCCG-3') and a buffer composed of 20 mM Hepes (pH 7.9), 1 mM  $MgCl_2$ , 60 mM KCl, 12% glycerol and 1 mM DTT (19). For competition experiments, the above specific and a nonspecific competitor HSE (5'-CTAGAGATCTCTAGAGATCTCTAGAGGATCCCCG-3') were separately added to the reaction mixtures to 40-fold molar excess of the labeled probe (19). The mixtures were incubated for 30 min at room temperature and analyzed on native 4% polyacrylamide gels. The gels were dried and autoradiographed.

**Cross-linking experiments.** Formaldehyde cross-linking experiments were performed as described elsewhere (25). Protein-HSE complexes were separated from free HSE by gel mobility shift analysis, and were excised after the gels were dried. The excised bands were immersed in 1% formaldehyde for 1 hr at 4°C, rinsed with distilled water and finally immersed in a sample buffer (0.125 M Tris/HCl, pH 6.8, 0.1% SDS, and 1 mM EDTA) (4) at room temperature for 30 min. Each piece of the immersed gels was placed in a well of SDS (sodium dodecyl sulfate)-polyacrylamide gel. The gel system was 2 mm thick, 8% SDS-polyacrylamide gel with 6% stacking gel, onto which 20  $\mu$ l of the sample buffer containing 20% glycerol was applied.

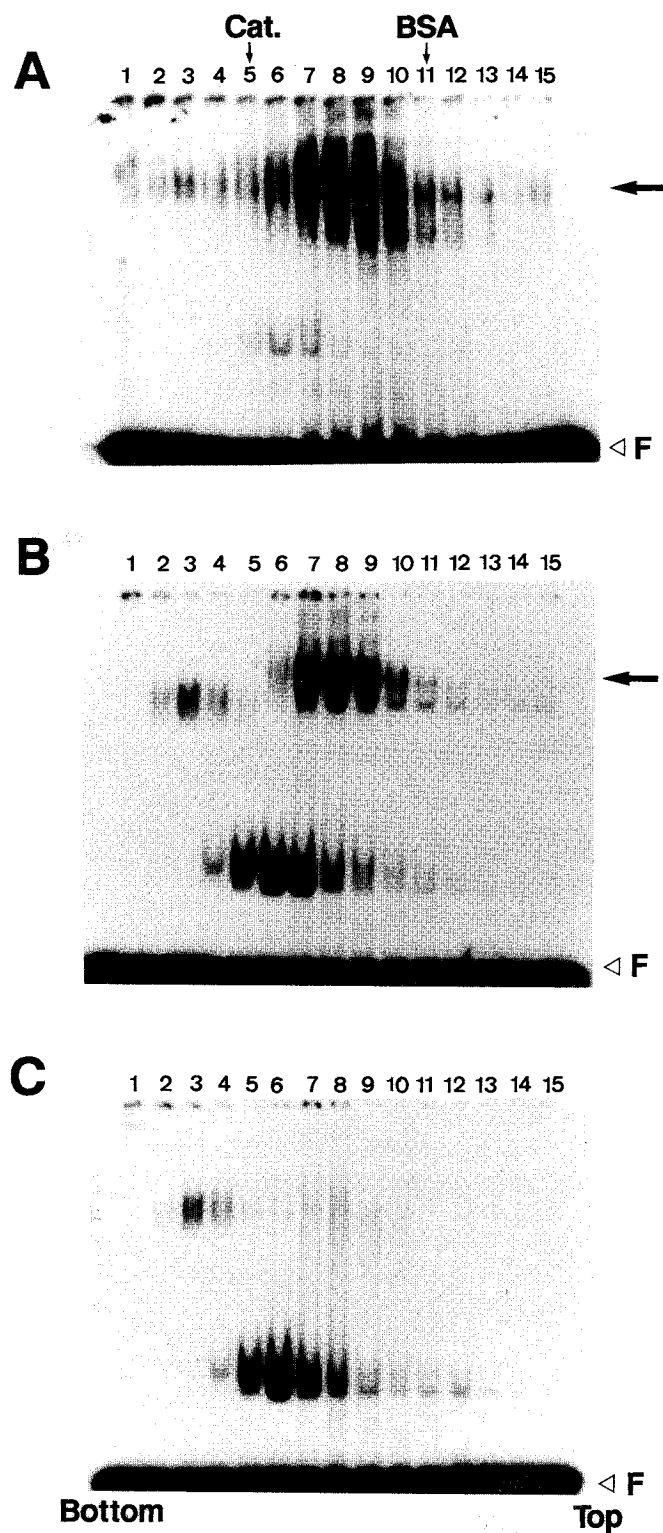
## RESULTS

***In vivo* activated HSF has a sedimentation coefficient of 8 S.** To estimate the molecular size of the activated HSF complex, the nuclear extracts and cytoplasmic extracts prepared from heat shocked HeLa cells, and the cytoplasmic extracts from unshocked cells were separately resolved by sucrose density gradient centrifugation. Following fractionation, the HSE-binding activity

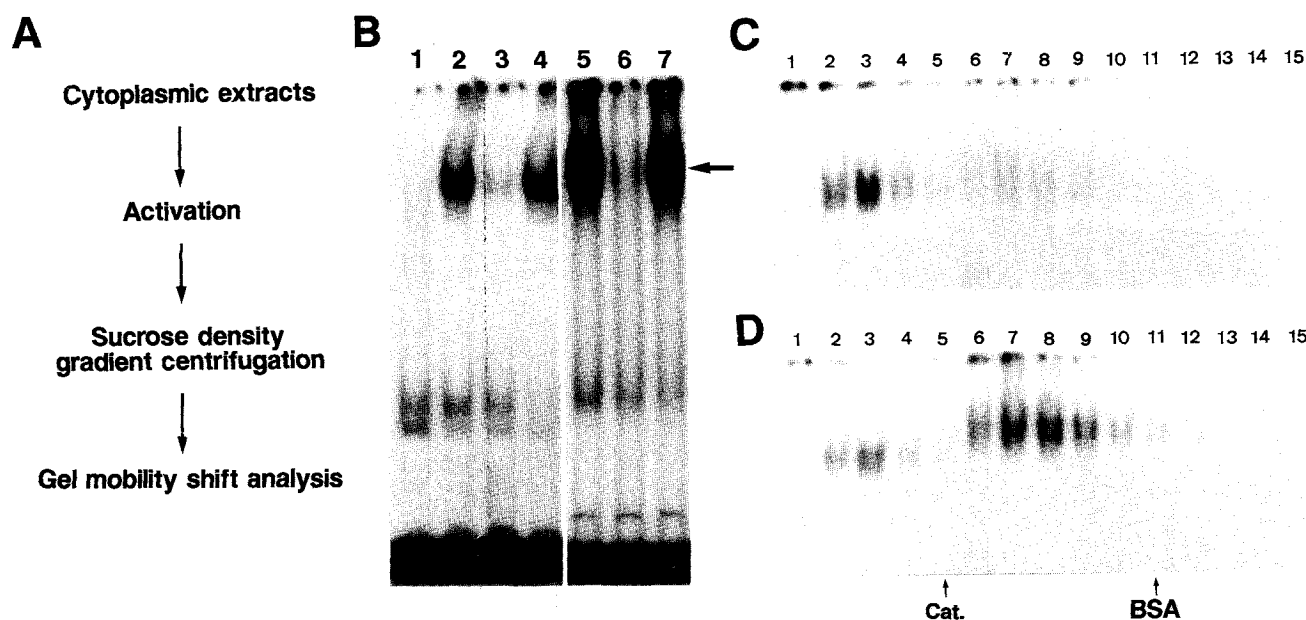
was examined for each fraction by gel mobility shift analysis using a  $^{32}$ P-labeled synthetic oligonucleotide which contains four times repeated HSE modules (NGAAN) (1, 16, 27). The results clearly revealed that the HSE-binding activity sedimented at a peak of about 8 S for both the nuclear extracts (Fig. 1A) and the cytoplasmic extracts (Fig. 1B) of heat shocked cells. Only a low level of the HSE-binding activity was detected in the unshocked cytoplasmic extracts (Fig. 1C). This protein-DNA complex formation was greatly reduced in the presence of 40-fold molar excess of the unlabeled HSE oligonucleotide, but not in that of an altered HSE oligonucleotide (data not shown). In cytoplasmic extracts of both unheated and heated cells, two other HSE binding activities were detected; one near the bottom (around fraction 3) of the gradient and the other around fraction 6 (Fig. 1B and 1C), but neither of them was specific for HSE (data not shown).

***In vitro* activated HSF exists also as an 8 S form.** In higher eucaryotes, HSF exists as a cryptic form in unshocked cytoplasmic extracts and the HSE-binding activity is induced *in vitro* by heat shock (Fig. 2B) or by other reagents such as NP40 (8, 13, 30). Unshocked HeLa cytoplasmic extracts were incubated at 43°C for 45 min and subjected to sucrose density gradient centrifugation. The HSE-binding activity was determined by gel mobility shift assay for each fraction, and was again found to sediment as a molecular form of 8 S (Fig. 2B and 2D). Essentially the same result was obtained with cytoplasmic extracts treated with 2% NP40 at 37°C (data not shown). Thus, HSF activated *in vitro* shows essentially the same sedimentation profile as active HSF present in heat-shocked cells.

***Inactive HSF shows a different sedimentation profile.*** To determine the molecular size of inactive HSF, each of the fractions recovered from sucrose density gradient centrifugation of cytoplasmic extracts of unshocked cells was subjected to heat activation followed by gel mobility shift analysis using  $^{32}$ P-labeled HSE. As observed by others (8), we have noted that the *in vitro* activation of HSE binding using cytoplasmic extracts of unshocked cells was less efficient when the extracts were diluted (data not shown). We then decided to concentrate each fraction about to 5–6 fold before activation, during which sucrose contained in the fractions was removed by adding buffer D. As shown in Fig. 3, we found that the HSE-binding activity was induced in fractions corresponding to 4–5 S after incubation at 43°C for 45 min. The bands revealing the HSE binding activity shown in Fig. 3C disappeared when an excess of unlabeled HSE was added to the binding mixture but not when that of unlabeled altered HSE was added (Fig. 3D). The HSF activity was also induced significantly when the fractions were incubated at 37°C for 45 min with 2% NP40, 1.9 M urea or 10 mM  $CaCl_2$  or at pH



**Fig. 1.** Sucrose density gradient centrifugation patterns of *in vivo* activated human HSF. Nuclear extracts (A) and cytoplasmic extracts (B) prepared from HeLa cells that had been incubated at 43°C for 1 h, and cytoplasmic extracts from unstressed cells (C) were subjected separately to sucrose (10–30% (w/v)) density gradient centrifugation. Bovine serum albumin (BSA) (4.4 S) and catalase (11–11.3 S) were used as sedimentation markers. Each fraction was assayed for its HSE-binding activity by gel mobility shift analysis. Specific complexes of HSF and HSE are indicated by arrows. Bands of free  $^{32}\text{P}$ -labeled HSE are indicated by F.



**Fig. 2.** Sucrose density gradient centrifugation patterns of *in vitro* activated human HSF. (A) An outline of the experiment. (B) Specificity of the complex formation of  $^{32}\text{P}$ -labeled HSE and proteins on gel mobility shift assay. 70  $\mu\text{g}$  of total proteins were loaded onto each lane. Cytoplasmic extracts prepared from unstressed HeLa cells (lane 1), and the cytoplasmic extracts heated *in vitro* at 43°C for 45 min (lane 2) were analyzed. Forty-fold amounts of the unlabeled HSE (lane 3) and of the unlabeled altered HSE (lane 4) were added as competitors to the mixtures of the heated cytoplasmic extracts and the  $^{32}\text{P}$ -labeled HSE. Fraction 8 recovered from centrifugation of the heated cell extracts (see Fig. 2D) was examined for the specific HSE-binding (lanes 5–7). No competitor (lane 5), a 40-fold amount of unlabeled HSE (lane 6), and a 40-fold amount of unlabeled altered HSE (lane 7) were added to the mixtures of fraction 8 and  $^{32}\text{P}$ -labeled HSE. (C) Cytoplasmic extracts of unstressed HeLa cells were subjected to sucrose density gradient centrifugation and gel mobility shift analysis. (D) The cytoplasmic extracts were heated at 43°C for 45 min, and resolved by sucrose density gradient centrifugation.

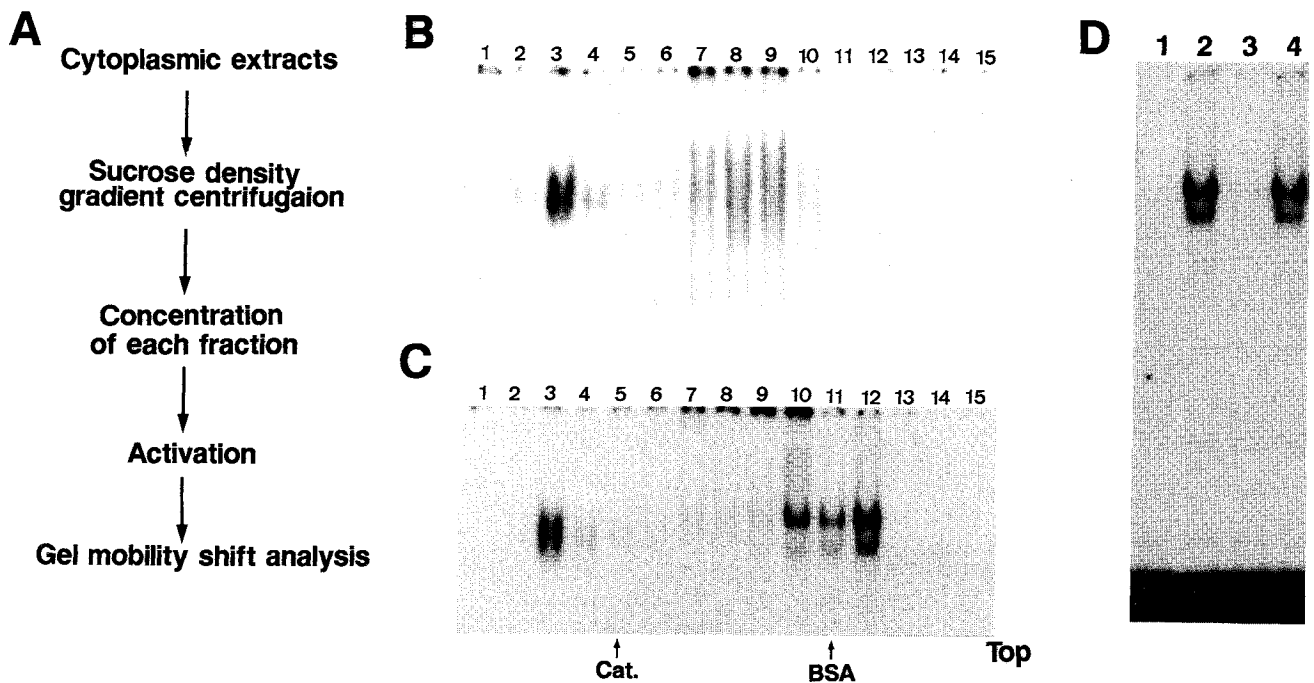
6.0 (Fig. 4) (13). These results indicate that the active form of HSF (8 S) and the inactive form of HSF (4–5 S) are different in molecular size.

**Cross-linking to  $^{32}\text{P}$ -labeled HSE probe of proteins contained in the inactive and active forms of HSF.** Chemical cross-linking experiments using formaldehyde were performed to estimate the molecular mass of the specific HSE-protein complexes observed on gel shift assay. The complex band formed on gel mobility shift assay of *in vitro* heat-activated cytoplasmic extracts, NP-40-activated 4–5 S fractions from cytoplasmic extracts of unshocked cells and the 8 S fractions obtained after centrifugation of *in vitro* activated cytoplasmic extracts, was respectively treated with 1% formaldehyde, and analyzed by SDS-polyacrylamide gel electrophoresis. It yielded a band corresponding to about 100 K (Fig. 5A). When the formaldehyde treatment was omitted, the band was not detected (Fig. 5B). We interpreted these results as indicating that the Mr 100 K band contains one  $^{32}\text{P}$ -labeled HSE oligonucleotide (Mr 22 K) and one HSF molecule whose molecular weight was estimated to be 83 K (7).

**Transition of inactive 4–5 S form to stable, active 8 S form of HSF requires an additional factor(s) in cyto-**

**plasmic extracts.** The above results suggest that the inactive 4–5 S form of HSF senses stress either directly or indirectly to become the 8 S active DNA binding form. To test this hypothesis, fractions containing 4–5 S form of HSF (fractions 11 and 12) were concentrated, heated at 43°C for 45 min, and sedimented in a sucrose density gradient. Contrary to expectations, the HSE-binding activity was not recovered in the 8 S regions. Instead, only 20% of the HSF activity was recovered between 8 S and 4–5 S fractions (Fig. 6 B).

By contrast, recentrifugation of the 8 S active form of HSF sedimented in 8 S fractions again and the recovery of the activity was more than 60% (Fig. 7). When the 4–5 S fractions of unheated cell extracts were activated at 43°C for 45 min and kept at 4°C for 3 days, the HSF activity was not reduced at all (data not shown). The loss of the HSF activity associated with the activated 4–5 S form may be attributable to a possible instability of the activity which is vulnerable to dilution during recentrifugation. If so, one may assume the presence of a factor(s) in crude cytosols of unstressed cells but not in 4–5 S fractions, which may be necessary to constitute the stable, active 8 S form. In order to test this possibility, we added cytoplasmic extracts of un-



**Fig. 3.** Sucrose density gradient centrifugation patterns of inactive HSF. (A) An outline of the experiment. Cytoplasmic extracts were prepared from unstressed cells and fractionated by sucrose density gradient centrifugation. Each fraction was concentrated, heated at 43°C for 45 min, and subjected to gel mobility shift assay. (B) Control; the activation process shown in (A) was omitted. (C) The fractions were concentrated and activated. (D) The mixture of concentrated fractions 11 and 12 (corresponding to sedimentation coefficient of 4–5 S) and subjected to gel mobility shift assay before (lane 1) and after (lane 2) activation by heating at 43°C for 45 min. The specificity of the HSE/HSF complex formation seen in lane 2 was examined in lanes 3 and 4. Forty-fold amounts of the unlabeled HSE oligonucleotides (lane 3) and the altered HSE (lane 4) were added to the gel shift mixtures.

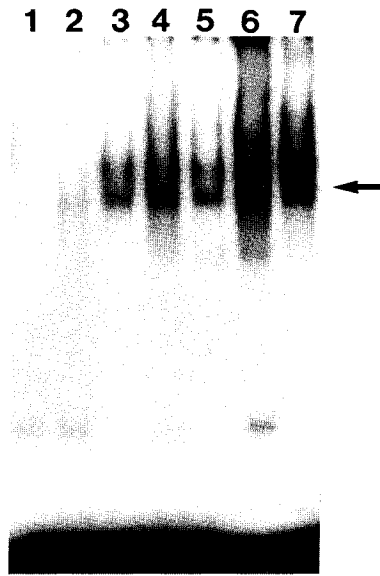
heated cells to 4–5 S fractions, which had been activated at 43°C for 45 min, incubated at 0°C for 1 h, and centrifuged again. The result shown in Fig. 6 D revealed that the HSE-binding activity recovered in 8 S fractions was greatly increased by adding the cytoplasmic extracts to be activated 4–5 S fractions. The recovery of the HSE-binding activity was increased to 43%. We also noted that the addition of the cytoplasmic extracts does not increase the HSE-binding activity contained in the mixture of the activated 4–5 S fractions and the cytoplasmic extracts (data not shown).

#### DISCUSSION

In higher eucaryotic cells, HSF is present in the cytosols in an inactive form in terms of HSE-binding and responds either directly or indirectly to heat shock or other forms of stress, converting to its active form which is capable of binding to HSE (8, 13, 30). Although the active form of HSF has been purified from heat shocked *Drosophila* and mammalian cells and has been characterized to some extent (7, 16, 26), the molecular properties of its inactive form have not been fully investigated. For this reason, the mechanism of

heat shock-induced conversion of the inactive HSF into its active form remains unclear. In this study, we determined the sedimentation coefficient of inactive human HSF using sucrose density gradient centrifugation and gel mobility shift assay. Our results clearly revealed that HSF has different sedimentation coefficients before and after activation. In fact, the *in vivo* and *in vitro* activated HSF has a sedimentation coefficient of 8 S whereas the inactive HSF in unstressed cell cytosol exists as a 4–5 S form.

The following two observations strongly suggest that the active 8 S form and inactive 4–5 S form of human HSF contain the same polypeptide that is directly involved in the binding to HSE upon activation. Firstly, cross-linking experiments using formaldehyde indicated that both the activated form derived from the inactive 4–5 S form and the active 8 S form are constituted by components with the same molecule mass that bind HSE. As mentioned previously by others (25), the conditions employed for cross-linking experiments would be more favorable for the formation of protein-DNA complexes than the formation of protein-protein complexes. It is likely, therefore, that the 100 K band on SDS-polyacrylamide gel electrophoresis yielded by the

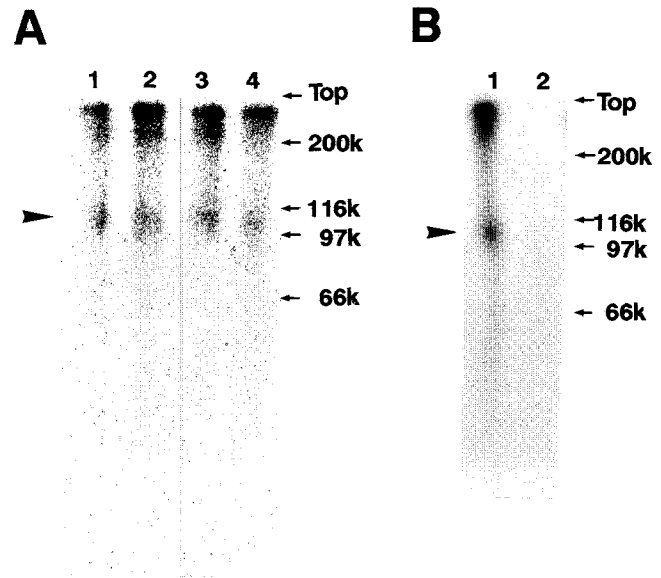


**Fig. 4.** Activation of the inactive 4–5 S form of HSF by various treatments. Cell extracts were prepared from unstressed cells and fractionated by sucrose density gradient centrifugation. Fractions corresponding to 4–5 S were concentrated to about 5–6 fold, and washed with buffer D. The fractions were kept on ice (lane 1), incubated separately at 37°C (lane 2), at 43°C (lane 3), with 2% NP40 at 37°C (lane 4), with 1.9 M urea at 37°C (lane 5), in 0.2 M phosphate buffer (pH 6.0) at 37°C (lane 6), and with 10 mM  $\text{CaCl}_2$  at 37°C (lane 7). All the treatments were allowed to stand for 45 min. Gel mobility shift assay was performed as described above. The specific complexes formed are indicated by an arrow.

HSE-protein complexes on gel mobility shift assay consists of one HSE and one polypeptide. Since the HSE oligonucleotide used in the present study is 22 K, the above interpretation of the result is compatible with the report of others that the molecular weight of human HSF is approximately 83 K (7).

Secondly, recentrifugation of the activated 4–5 S form in sucrose density gradient resulted in a significant increase of the active 8 S form when mixed with unstressed cell cytosols. Since the level of the HSE-binding activity associated with the activated 4–5 S fractions was not increased by mixing with unstressed cytosols, we think it unlikely that the inactive HSF molecules contained in the cytosols are activated by mixing with the heat-treated 4–5 S fractions. It is thus plausible that the 4–5 S inactive form of HSF is induced to transform to the stable, active 8 S form by heat treatment or other stimuli with the aid of an unknown factor(s) contained in the cytosols of unstressed cells.

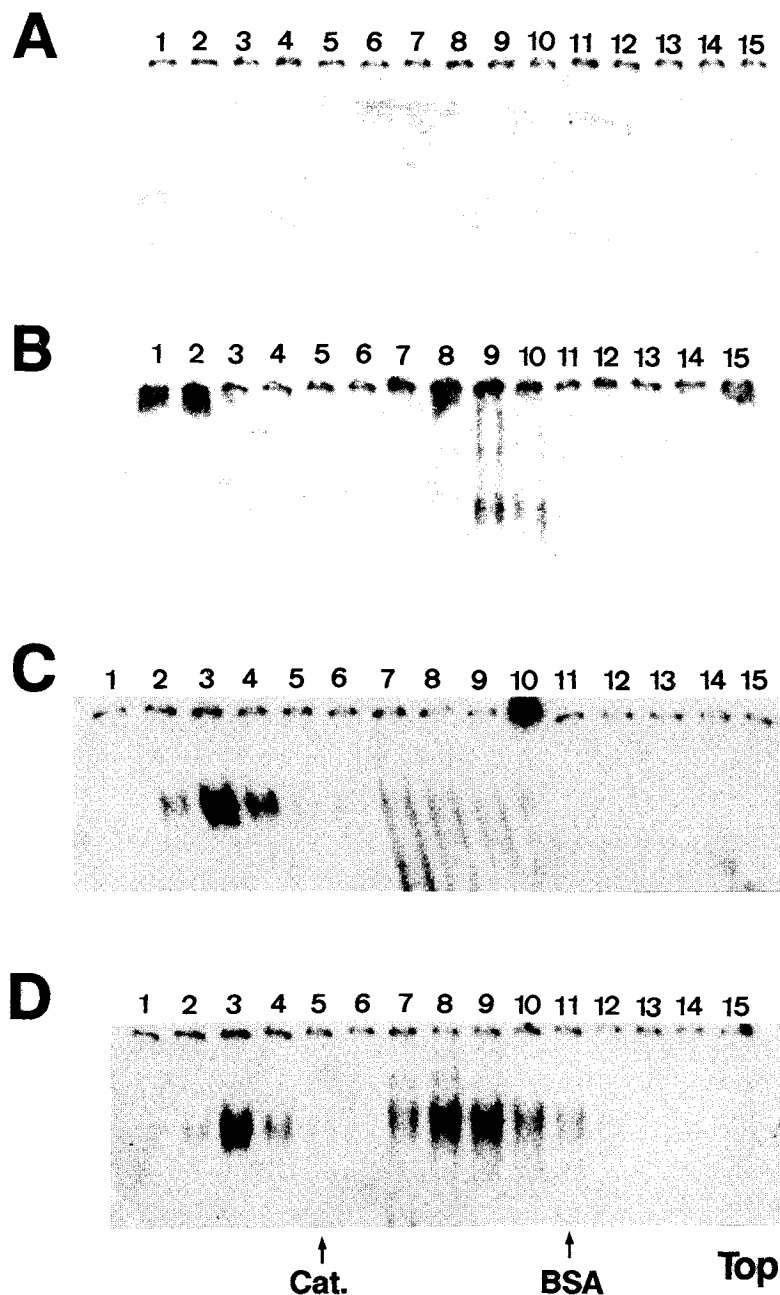
What then is the nature of the active and inactive HSF? In view of the fact that the yeast HSF oligomerizes *in vitro* (22) and the *Drosophila* HSF forms a hexamer (5), the 8 S active form might be such an oligomeric form of the human HSF. As mentioned before



**Fig. 5.** Chemical cross-linking of HSF to HSE. After gel mobility shift analysis, the specific HSE-protein complexes were excised, cross-linked with 1% formaldehyde and examined by SDS-polyacrylamide gel electrophoresis. (A) The complexes yielded by the heat-activated (lane 1), by the 2% NP-40-activated unfractionated cytoplasmic extracts of unstressed cells (lane 2), by the NP-40-activated 4–5 S fractions of unstressed cells (lane 3), and by the 8 S fractions of the *in vitro* heat-activated cytoplasmic extracts (lane 4) were separately treated with formaldehyde. Visualization of radioactive bands were performed using an image analyzer, BAS2000 (Fujix). (B) The HSE-containing complexes yielded by the heat-activated 4–5 S fractions of unstressed cells were treated with (lane 1) or without 1% formaldehyde (lane 2). Visualization of radioactive bands were performed by autoradiography. Arrow-heads indicate the radio-labeled HSE-protein complexes. The migrating-positions of size markers are indicated by arrows.

and described by others (8), the *in vitro* activation of HSF by heat shock using cytosol of unstressed cells is strictly concentration-dependent. This is consistent with the possibility that oligomerization of HSF is a key event of the activation. Recently, it has been suggested that the inactive HSF senses various forms of stress including heat shock and alters its conformation, possibly for oligomerization (13, 30). Although our results showed that the inactive HSF has a sedimentation coefficient of 4–5 S, it is not clear whether it is a monomer of HSF or, alternatively, a complex of HSF with another component(s) as shown in the NF- $\kappa$ B/I $\kappa$ B complex (2) or the steroid hormone receptor/HSP90 complexes (3, 17).

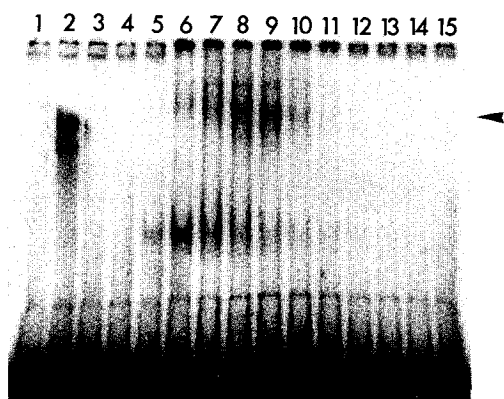
We have shown that neither heat activation nor treatment with other stimuli of the inactive 4–5 S form of HSF alone is sufficient to yield the stable, active 8 S form and that an additional factor(s) contained in unstressed cell cytosols is required for the conversion of HSF from the 4–5 S form to its stable 8 S form. There



**Fig. 6.** Transition from the inactive 4–5 S form of HSF to the stable, active 8 S form. Cytoplasmic extracts (about 50 mg protein) of unstressed cells were divided into 5 aliquots, and fractionated by sucrose density gradient centrifugation. Fractions 11 and 12 recovered from each tube were combined, washed with buffer D and concentrated to about 7–8 fold. The combined fractions were then divided into four aliquots (125  $\mu$ l each). Two of them were placed on ice (A and C) and the others were heated at 43°C for 45 min and then chilled on ice (B and D). Sixty microliters of buffer D (A and B) and unfractionated cytoplasmic extracts of unstressed cells (about 1.8 mg proteins) (C and D) were added, respectively, and then incubated on ice for 1 h. All the mixtures were subjected to sucrose density gradient centrifugation. Fractions recovered from the mixtures were analyzed by gel mobility shift assay.

are at least two possible explanations for these results. First, an additional factor may be necessary for the formation of the 8 S form of HSF that is stable *per se*. The ability to bind HSE can be induced on the 4–5 S form,

to produce an intermediate state, but the HSE-binding ability associated with this state is lost during recentrifugation. Second, the formation of the 8 S form, which is associated with the acquisition of the HSE-binding ac-



**Fig. 7.** Recentrifugation of the 8 S fractions recovered from the *in vitro* activated cytoplasmic extracts. Cytoplasmic extracts of unstressed cells were activated *in vitro* at 43°C for 45 min and fractionated by sucrose density gradient centrifugation. Fraction 8 (corresponding to 8 S) was washed with buffer D, concentrated to about 3–4 fold and applied to the sucrose gradient analysis again. Fractions from the second centrifugation were examined by gel mobility shift analysis. An arrow-head indicates the HSE/HSF complexes.

tivity, is achieved by activation of the 4–5 S inactive form. The stabilization of the 8 S form requires an additional factor(s) which is present in crude cell cytosols but not in 4–5 S fractions. Further work is needed to critically examine these two possibilities in order to clarify this unidentified factor(s).

It is pertinent to mention that protein synthesis is required for the occurrence of the HSE-binding activity in both invertebrate and vertebrate cells upon mild heat shock (29). This can be interpreted as an indication that newly synthesized HSF differs from mature HSF in their sensitivity to stimuli. In addition, it was demonstrated that purified human HSF which had been denatured in SDS regained the ability to bind HSE when transferred to a nitrocellulose membrane (7). Furthermore, a recent report has clearly demonstrated that the recombinant *Drosophila* HSF produced in *E. coli* has the ability to bind HSE without activation (5). These results suggest that the pure unmodified polypeptide of HSF is essentially active. Taken together, we assume that the inactive 4–5 S form of HSF is a modified (e.g. phosphorylated) HSF polypeptide and/or a complex form with another protein which interferes with the formation of the active form. The activation by heat shock or other forms of stress may remove the modification (e.g. dephosphorylation) of HSF or induce dissociation of the complex. In any case, the activation renders HSF into a stable, active 8 S form with the aid of unknown factor(s) present in the cytosol of unstressed cells.

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