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Complex Modes of Heat Shock Factor Activation

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Eucaryotic organisms respond to elevated environmental temperatures by rapidly activating the expression of heat shock genes. The transcriptional activation of heat shock genes is mediated by a conserved upstream regulatory sequence, the heat shock element (HSE). Using an HSE-binding assay, we show that a cellular factor present in a range of vertebrate species binds specifically to the HSE. This factor is presumably the transcriptional activator of heat shock genes, heat shock factor (HSF). In vertebrates, the binding of HSF to the HSE was induced when cells were subjected to heat shock at high temperatures, even in the absence of protein synthesis. Under mild heat shock conditions, HSF binding was induced to a lesser extent, but this induction required protein synthesis, suggesting that synthesis of HSF itself, or an activating factor, is necessary for response to heat shock at intermediate temperatures. The inducibility of HSF binding in higher eucaryotes is contrasted with constitutive HSF binding activity in fungi. It appears that despite conservation of the HSE in evolution, the means by which HSF is activated to bind DNA in higher and lower eucaryotes may have diverged.

In all organisms, transcription of heat shock genes is rapidly stimulated by an elevation of the ambient temperature (for reviews, see references 2, 8, and 11). The transcriptional response to heat shock is mediated in eucaryotes by a positive control element (the heat shock element [HSE]) which is present in multiple copies upstream of heat shock genes in all eucaryotes. Initial evidence for a factor that could interact with the HSE came from studies of protein-DNA interactions in *Drosophila* cell nuclei: a heat shock activator protein was found to bind specifically to the HSE only upon heat shock stimulation (21); parallel studies using DNA-binding and in vitro transcription assays also identified a heat shock transcription factor that bound specifically to the HSE (12, 20). Heat shock activator protein has been purified to homogeneity from shocked *Drosophila* cells and has a molecular size of 110 kilodaltons (kDa) on sodium dodecyl sulfate-polyacrylamide gels (23). The activator protein binds to the HSE with high affinity and specificity and can stimulate transcription of a *Drosophila* heat shock gene when microinjected along with a reporter gene into non-shocked *Xenopus* oocytes (23). HSE-binding activities in general have recently been designated as heat shock factor (HSF). We refer to heat shock activator protein as HSF hereafter.

In this study, we describe the properties of HSF in a variety of eucaryotic species. In each species, we detected a specific HSE-binding factor. HSE-binding activity was inducible in higher eucaryotes but was constitutive in fungi. The ability of HSF to bind to the HSE in higher eucaryotes could be induced, reversed, and reinduced, in the absence of protein synthesis at high heat shock temperatures, indicating a posttranslational mechanism for HSF regulation in these organisms. At intermediate heat shock temperatures, however, protein synthesis was required for activation of HSF. This requirement suggests that synthesis of HSF itself, or a factor that directly or indirectly leads to the activation of HSF, is necessary for a response by higher eucaryotes to heat shock at intermediate temperatures.

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MATERIALS AND METHODS

Growth conditions for tissue culture cells. Human HeLa S3 and mouse L cells were cultured in minimal essential medium, Eagles spinner modification (Biofluids) supplemented with glutamine and 10% fetal bovine serum. Chicken MSB cells were cultured in RPMI 1640 medium (GIBCO Laboratories) supplemented with 10% fetal bovine serum. The three cell lines mentioned above were grown in spinner culture at 37°C. *Drosophila* Schreider line 2 (SL-2) cells were grown in spinner culture at 25°C in Schneider medium (Biofluids) supplemented with 15% fetal bovine serum and 0.4% Bacto-Peptone. *Xenopus* XTC cells were grown in monolayer culture in tissue culture flasks at 25°C in Leibnovitz medium (GIBCO) supplemented with 10% fetal bovine serum.

Heat shock. All experiments were performed with logarithmically growing cells. Cells growing in spinner culture were concentrated about fivefold by centrifugation at 4°C at 540 × *g* in a GLC4 centrifuge (Ivan Sorvall, Inc.) H1000 rotor (the centrifuge was allowed to reach 2,000 rpm, at which point centrifugation was terminated and the rotor was allowed to stop with braking; this procedure was used for pelleting cells in all subsequent manipulations of cell suspensions with volumes greater than 1.5 ml). The cell pellet was resuspended in an appropriate volume of the supernatant medium and shaken at the normal growth temperature on a gyratory shaker for 1 h prior to heat shock. Samples (10 ml) of concentrated cells were then transferred to 50-ml polypropylene centrifuge tubes and immersed in a circulating water bath set at the indicated temperature. Samples (1.0 ml) of the cells were pelleted in 1.5-ml Eppendorf tubes at 10,000 × *g* for 2 s (these conditions were used for pelleting cell suspensions in Eppendorf tubes in subsequent manipulations). The supernatant was aspirated, and the cell pellets were frozen in liquid nitrogen.

The length of the heat shock treatment was recorded from the start of immersion in the water bath. Control cells were kept at the normal growth temperature in parallel with cells undergoing heat shock treatment and were harvested at the end of the heat shock experiment. The temperature indicated in the experiment is the temperature of the water bath, rather than the internal temperature of the culture. (A temperature

microprobe was used to measure the temperature during heat shock of 10 ml of 23.2°C culture placed in a 33.9°C water bath. The culture temperature was found to rise to 31.5°C after the first 5 min, to 33.0°C after 10 min, and to reach a plateau of 33.2°C 15 min after immersion. The maximum culture temperature was 0.7°C lower than the recorded temperature of the water bath, and the time required for complete equilibration to this temperature was about 15 min.

Heat shock of *Xenopus* XTC monolayer cultures was conducted by decanting the culture medium from the culture flasks and adding fresh complete medium prewarmed at 37°C. The flasks were then transferred to a 37°C incubator. Control cells were kept at 25°C. After heat shock, cell monolayers were rinsed with fresh medium (lacking serum) that was preequilibrated at the respective temperature and were detached by the addition of 3 ml of trypsin solution without EDTA (Biofluids). With cells that were heat shocked, prewarmed trypsin solution was used. At either temperature, cells detached after 2 to 3 min of trypsinization. Cell suspensions were then transferred to 50-ml Falcon polypropylene tubes, and trypsin was inhibited by the addition of fetal bovine serum to 15%. The tubes were filled completely with Leibnovitz medium (preequilibrated at the control or heat shock temperatures), and the cells were pelleted by centrifugation. The pellets were suspended gently in 500 μl of fresh complete medium, transferred to 1.5-ml Eppendorf tubes, and pelleted by centrifugation. After removal of the supernatant by aspiration, the pellets were frozen in liquid nitrogen.

Preparation of vertebrate cell extracts. Whole-cell extracts were prepared from cell pellets (containing 3×10^7 to 5×10^7 cells per tube) by the procedure described previously for *Drosophila* SL-2 cells which yielded quantitative extraction of HSF (25). Briefly, cells were disrupted by being thawed at 0°C in the presence of 5 volumes of extraction buffer (10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid] buffer [pH 7.9], 0.4 M NaCl, 0.1 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid], 0.5 mM dithiothreitol, 5% glycerol, 0.5 mM phenylmethylsulfonyl fluoride). The cell lysate was homogenized by repeated suspensions in a micropipette tip, transferred to thick-wall polycarbonate tubes (Beckman Instruments, Inc.), and centrifuged for 5 min at 100,000 × *g*. For complete disruption of XTC cells, microhomogenization was performed in a 1.0-ml Dounce homogenizer (Wheaton Industries) in extraction buffer minus NaCl. NaCl was then added to a 0.35 M final concentration, and the lysate was suspended and centrifuged at 100,000 × *g* as described above. Fresh supernatants were used for DNA-binding assays. The supernatants can be stored at −70°C for years without a significant loss of activity.

Growth of yeast, heat shock, and preparation of yeast cell extracts. The following strains of *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* were used: *S. cerevisiae* Y316 (*MATα trp1 pep4-3*), KT547 (*MATα leu2 ura3-52 his4-539 SRA1*), and DTY7 (*MATα leu2 ura3-52 cup1R-3*) and *Schizosaccharomyces pombe* SP417 (*mat1-P mat2,3 Δ::LEU2 ade6-216*). *S. cerevisiae* KT547 was used in the experiments described here; the other strains were used in work mentioned as data not shown. Liquid cultures were grown at 25°C in YPD medium (1% yeast extract, 2% Bacto-Peptone [Difco Laboratories], 2% dextrose) on a gyratory shaker at 300 rpm. A 500-ml volume of log-phase (optical density at 600 nm, 1.0) cells was concentrated 10-fold by centrifugation in 10 50-ml Falcon polypropylene tubes in a Sorvall H1000 rotor at 4°C, as described above, resuspension in an appropriate volume of the same supernatant medium, and shaking at 25°C for 1 h in an Erlenmeyer flask prior to heat shock treatment. Samples of cells in 50-ml polypropylene tubes were heat shocked by immersion of the tubes in circulating water baths. Control and heat-shocked cells were concentrated by centrifugation as described above, resuspended in about 1 ml of the supernatant medium, and pelleted in 1.5-ml Eppendorf tubes (50-μl pellet volume) by centrifugation. The supernatant was removed by aspiration, and the cell pellets were frozen in liquid nitrogen. To prepare extracts, each pellet was thawed and suspended in 2 pellet volumes of a buffer containing 15 mM Tris hydrochloride (pH 7.4), 0.1 mM EGTA, 0.5 mM dithiothreitol, and 5% glycerol. The cell suspension was transferred to a 1.5-ml Eppendorf microcentrifuge tube containing 50 μl of glass beads (type 5; Sigma Chemical Co.) that had been washed in 0.1 N HCl, rinsed extensively in water, and oven dried. Cells were broken by being vortexed at maximum speed for a total of 5 min, with an interruption every minute to cool the tube in an ice-water bath. NaCl was then added to 0.4 M (from a 4 M stock solution). After 20 min of incubation at 0°C, the lysate was centrifuged at 10,000 rpm for 30 min in an Eppendorf microcentrifuge. The supernatant was recovered on top of the glass bead-cell debris pellet and was directly assayed (yeast HSF in such extracts is unstable upon storage).

Assays for HSE-binding activity. Exonuclease III protection assays were performed as described previously, using a ^{32}P-5'-end-labeled *Drosophila hsp82* gene fragment (positions −170 to +39) (25). Gel mobility shift assays were performed as described previously (25), using a double-stranded consensus heat shock element (upper strand, 5'-GTCGACGGATCCGAGCGCGCCTCGAATGTTCTAGAA AAGG) or a natural heat shock element from the *hsp82* gene (HSE 82) (upper strand, 5'-TCCCTGGCATCCAGAA GCCTCTAGAAGTTTCTAGAGACTTCCAGTTCGGG). Nucleotides that match the alternating GAA and TTC blocks in the 5-nucleotide modular repeats (1, 24) are underlined. The DNAs were synthesized and ^{32}P labeled by primer extension, as described previously (23).

Photoaffinity labeling of HSF. Each of 30 μl of crude extract from unshocked *S. cerevisiae*, 30 μl of crude nuclear extract from heat-shocked *Drosophila* SL-2 cells (23), and 20 μl of an HSF fraction from heat-shocked human HeLa cells that was chromatographically purified on a consensus HSE column as described previously (22, 23) was bound to ^{32}P-labeled, bromodeoxyuridine-substituted HSE 82, separated from unbound DNA by native agarose gel electrophoresis, cross-linked in situ by UV irradiation, and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described previously (23).

RESULTS

Inducible HSE-binding activities in vertebrate cells. Figure 1A shows the heat shock-inducible HSE-binding activities that can be extracted from tissue culture cells derived from a range of vertebrates. The HSE-binding activities were measured by the gel mobility shift technique, using saturating amounts of a 38-base-pair synthetic ^{32}P-labeled consensus HSE sequence that was adapted from the promoter sequence of a *Drosophila hsp70* gene (23). Although the HSE was originally defined as a 14-nucleotide sequence, CT_GAA__TTC_AG (13), it has recently been revised to two or three modules consisting of alternating GAA or TTC blocks arranged at 2-nucleotide intervals (1, 24). Our con-

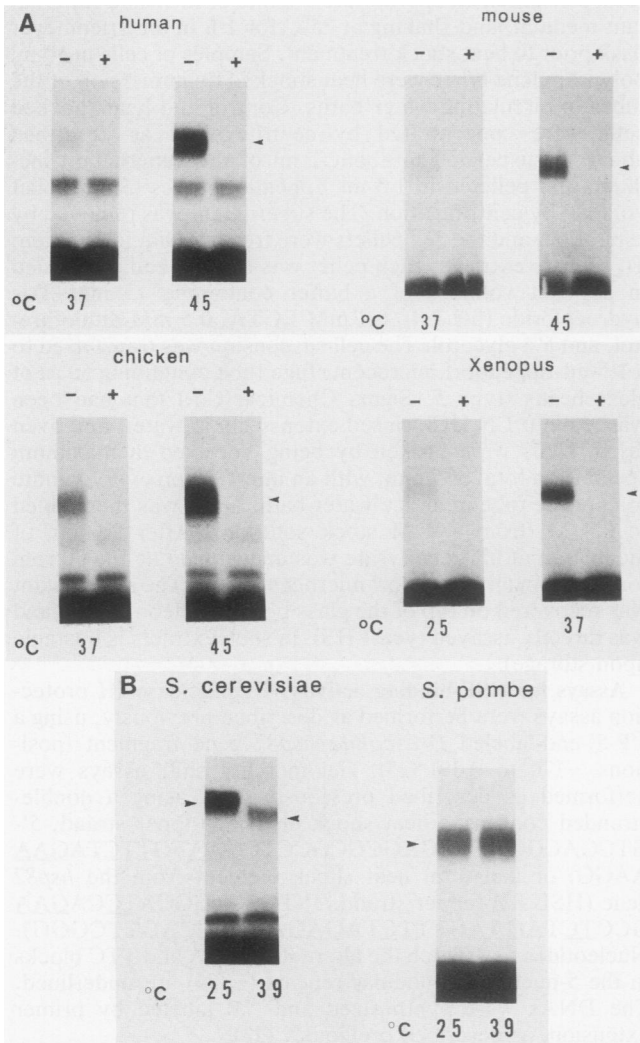


FIG. 1. (A) Inducible HSE-binding activities in vertebrate cell extracts. Logarithmically growing human (HeLa), mouse (L), chicken (MSB), and *Xenopus* (XTC) cells were heat shocked for 20 min at the indicated temperatures, and an amount of extract equivalent to 3×10^6 cells was assayed by the gel mobility shift technique, using the ^{32}P-labeled consensus HSE as the binding sequence. Positions of specific HSF-HSE complexes are indicated (◀). The presence (+) or absence (−) of a 40-fold molar excess of unlabeled HSE in the gel shift assay is indicated. The extent of induction of HSE-binding activity, as measured by densitometry of the autoradiographic signal, was 14-, (human), 9- (mouse), 5- (chicken), and 7-fold (*Xenopus*). In other experiments, the extent of induction was 8- and 13-fold in *Xenopus* cells (Fig. 3), 17-fold in chicken cells, and 14-fold in human cells (Fig. 4). (B) Constitutive HSE-binding activities in fungi. *S. cerevisiae* and *Schizosaccharomyces pombe* extracts were prepared from nonshocked (25°C) and heat-shocked (39°C) cultures and assayed by the gel mobility shift technique, using a ^{32}P-labeled synthetic DNA derived from the *Drosophila hsp82* gene promoter. This 50-nucleotide sequence (HSE 82) (23) has seven tandem repetitions of the 5-nucleotide modular unit of the heat shock regulatory region and was used because it binds more effectively to *S. cerevisiae* HSF than does the consensus HSE. The HSF-HSE complexes (◀, ▶) are specifically inhibited by a 40-fold excess of unlabeled HSE 82 (data not shown). The decrease in the level of HSE-binding activity upon heat shock of *S. cerevisiae* was threefold and was possibly due to enhanced degradation at the high temperature (V. Zimarino, unpublished observations).

sensus HSE sequence completely matches both the original and revised definitions of the HSE and was used as a standard to screen for specific HSE-binding activities in the indicated species. In all of the cell lines examined, a low level of HSE-binding activity at the normal growth temperature was indicated by the appearance of a specific gel mobility shift complex which is absent in the presence of excess unlabeled HSE. The HSE-binding activity present in nonshocked cells probably reflects a physiologically relevant basal activity, although this activity can vary, depending on the handling of the cells during the preparation of cell extracts. At elevated temperatures, however, an increase in HSE-binding activity was clearly observed for all the cell lines tested. The extent of the increase was 5- to 17-fold, depending on the cell type. Moreover, the rate of increase was rapid, reaching maximal levels after 5 min of heat shock, and the extent of induction of HSE-binding activity increases along with increasing heat shock temperatures (C. Tsai and V. Zimarino, unpublished observations). These results suggest that the temperature-dependent modulation of HSE-binding activity is a critical regulatory switch in the activation of heat shock gene transcription. The rate and extent of increase in HSE-binding activity observed in heat-shocked vertebrate cells are similar to the increase observed previously in heat-shocked invertebrate (*Drosophila* SL-2) cells (25). Inducible HSE-binding activities have also been reported by other workers for human (3, 6, 10, 14) and mouse (9) cells.

Constitutive HSE-binding activity in fungi. Unlike the inducible HSE-binding activity in *Drosophila* and vertebrate cells, a significant level of HSE-binding activity was already present at a normal temperature (25°C) in the fungi *Schizosaccharomyces pombe* and *S. cerevisiae* (Fig. 1B). Heat shock at 39°C did not increase the level of HSE-binding activity. In *S. cerevisiae*, but not in *Schizosaccharomyces pombe*, the activity actually seemed to decrease upon heat shock, and in addition, the heat shock-activated *S. cerevisiae* HSF-HSE complex migrated more quickly on the native agarose gel. A similar mobility change was observed when heat shock-activated *S. cerevisiae* HSF that had been purified by heparin-Sepharose and sequence-specific DNA affinity chromatography was used (data not shown). Sorger, Lewis and Pelham, (14) have observed a constitutive level of HSE-binding activity in *S. cerevisiae*, although they showed, by using native polyacrylamide gels, that heat shock-activated HSF had a slower instead of faster electrophoretic mobility; this difference could be attributed to the different compositions of the gel matrix, since *S. cerevisiae* HSF also migrates with a slower mobility on polyacrylamide gels in our studies (V. Zimarino, unpublished observations).

Further experiments indicate that when HSF is extracted from *S. cerevisiae* cultures that were shocked at increasing temperatures of 30, 34, and 37°C, the HSF-HSE complex in the gel shift assay migrates with a mobility that is progressively faster than the mobility observed when HSF extracted from cultures growing at 25°C was used. These changes in electrophoretic mobility upon heat shock were reversed on return to 25°C and were observed in three different strains of *S. cerevisiae*, even when protein synthesis was inhibited by cycloheximide (data not shown). The results suggest that HSF in *S. cerevisiae* may undergo multiple heat shock-induced posttranslational modifications without the requirement for protein synthesis.

Different molecular sizes of HSF from different species. Figure 2A shows a comparison of heat shock-activated HSF isolated from six species and analyzed by the gel mobility

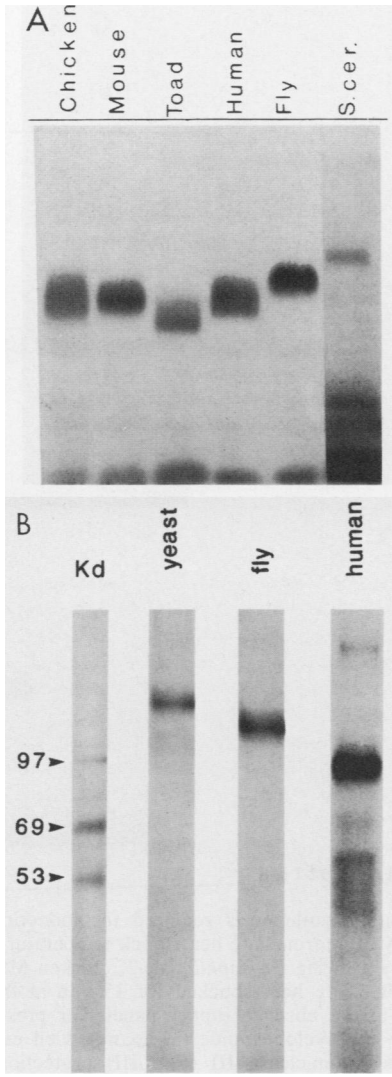


FIG. 2. (A) Comparative gel mobility shift analysis of HSF from different species. Extracts of heat shock-induced cells of the indicated species were analyzed by the gel mobility shift assay, using ^{32}P-labeled consensus HSE as the binding sequence. Assay conditions are similar to those for Fig. 1A, except that one-half of the amount of HeLa cell extract was used. The samples were electrophoresed on the same gel, but the lane containing the yeast extract was exposed for a longer time; hence, the autoradiogram shown is a composite. (B) Comparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of *S. cerevisiae*, *Drosophila*, and human HSF. HSF was photoaffinity labeled by UV cross-linking to ^{32}P-labeled, bromodeoxyuridine-substituted HSE 82 (see Fig. 1B legend). The photoaffinity labeling of the polypeptides observed in the autoradiogram is inhibited by the presence of a 40-fold molar excess of unlabeled binding site (data not shown). Molecular sizes of labeled HSF were estimated by comparing the gel mobility relative to those of the indicated ^{14}C-labeled marker proteins that were electrophoresed on the same gel.

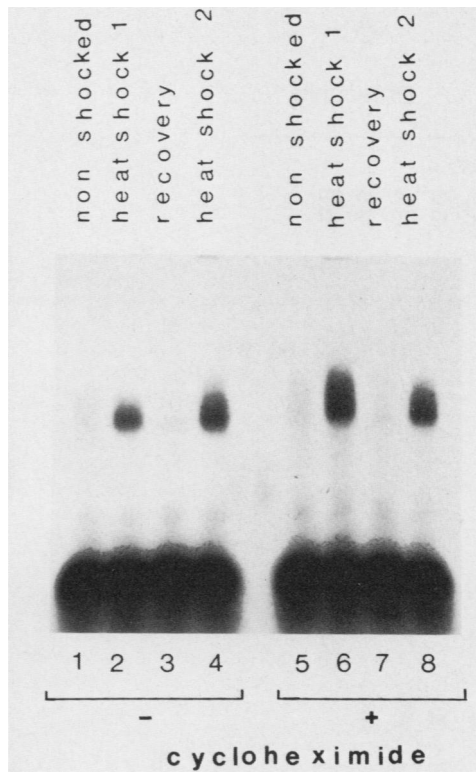
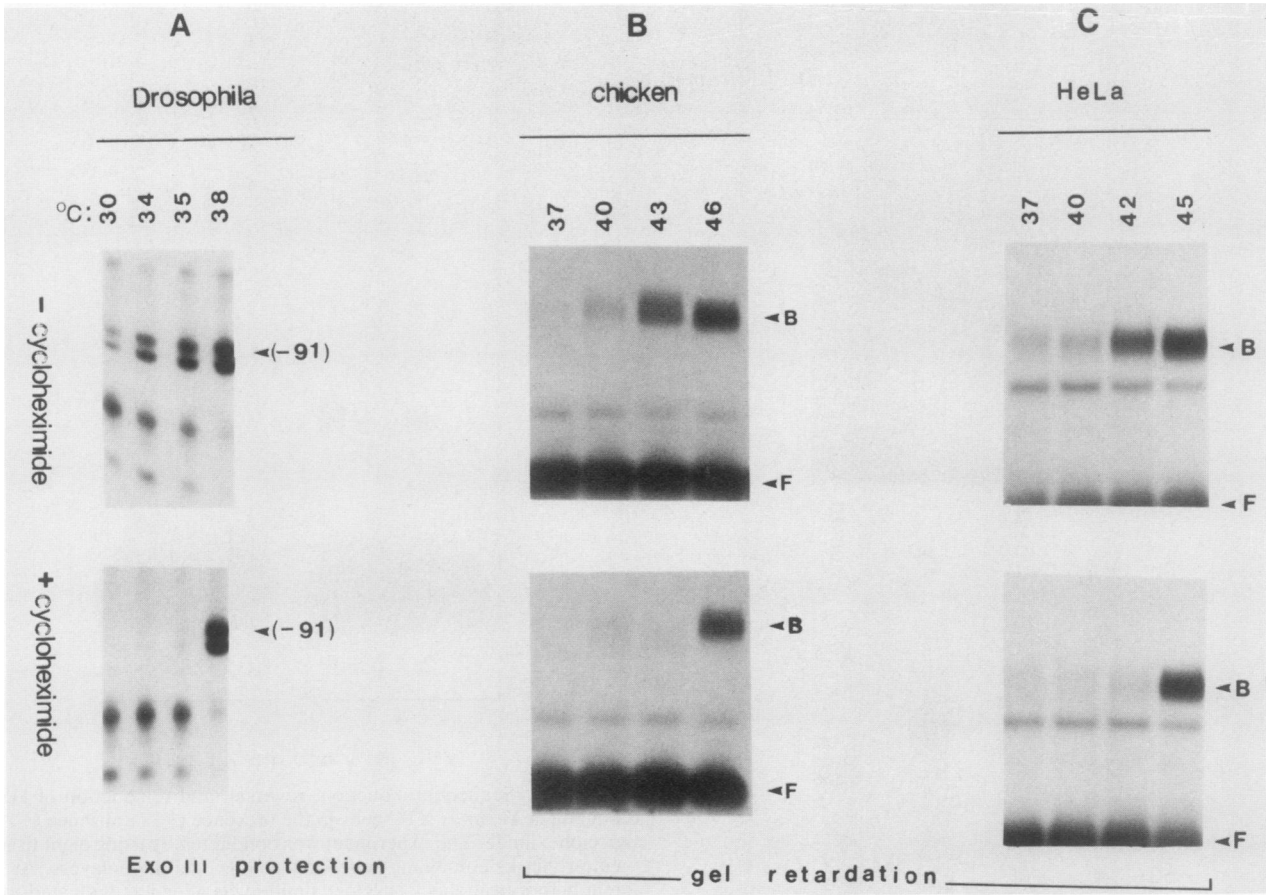


FIG. 3. Sequential induction, reversal, and reinduction of HSF binding in *Xenopus* XTC cells in the presence (+) or absence (−) of cycloheximide. The experiment was conducted by using eight tissue culture flasks containing subconfluent XTC cell monolayers. At 30 min before heat shock, cycloheximide was added as indicated to a final concentration of 118 μM (33 μg/ml). The first heat shock was administered at 37°C for 20 min. After heat shock, the flasks were recovered at 25°C with gentle shaking, before administration of the second heat shock. Cells were harvested, and extracts were prepared and assayed for HSE-binding activity at the end of heat shock and recovery, as indicated.

shift technique. The side-by-side analysis on one gel shows clearly that there are significant differences in the mobilities of the HSF-HSE complex in the species examined. We also used a photoaffinity-labeling technique (23) to determine the molecular size of HSF covalently cross-linked to ^{32}P-labeled HSE. In Fig. 2B, sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of photoaffinity-labeled *S. cerevisiae* HSF (from a nonshocked culture) and *Drosophila* and human HSF from heat-shocked cells shows major cross-linked polypeptides of 130, 110, and 90 kDa, respectively. Our comparison confirms the reported molecular size differences of purified HSF from *S. cerevisiae* (150 kDa [15]), *Drosophila* SL-2 cells (110 kDa [23]), and human HeLa cells (83 kDa [3]; 93-kDa, photoaffinity-labeled HSF [7]). The interspecies differences suggest that some divergence in the size and/or posttranslational modification of HSF may have occurred during evolution.

Protein synthesis not required for induction, reversal, and reinduction of HSF binding at high heat shock temperatures. We used the inducibility of HSF binding in higher eucaryotes as a measure of HSF activation when protein synthesis is inhibited by cycloheximide treatment. Treatment of *Xenopus* tissue culture cells with cycloheximide had no effect on the induction of HSF binding when the ambient temperature was raised from 25°C to a high heat shock temperature of 37°C (Fig. 3); there was also no effect on subsequent reversal and reinduction of HSF binding. A similar experiment using HeLa cells heat shocked at a high temperature of 45°C provided identical results (data not shown). These findings concur with previous observations on HeLa and *Drosophila* cells (6, 25) and indicate that a posttranslational mechanism



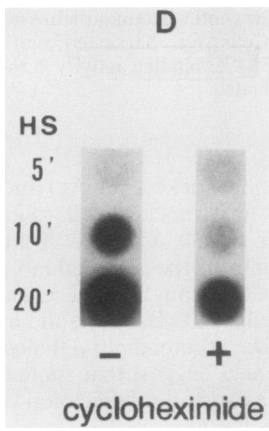


FIG. 4. Protein synthesis is required for induction of HSE-binding activity at intermediate heat shock temperatures. (A to C) Logarithmically growing *Drosophila* SL-2, chicken MSB, and human HeLa cells were heat shocked for 15 min at the indicated temperatures, in the absence (upper panels) or presence (lower panels) of 118 μM cycloheximide (33 μg/ml). Cell extracts were assayed by the exonuclease III (Exo III) protection technique (*Drosophila* cells) or by the gel mobility shift technique (chicken and human cells). (D) RNA dot blot analysis of control and cycloheximide-treated *Drosophila* SL-2 cells after 5, 10, and 20 min of heat shock (HS) at 34°C. RNA from 8×10^5 cells were applied to each dot and hybridized to ^{32}P-radiolabeled *hsp70* coding sequences.

is likely to be the general pathway by which HSF is induced to bind to the HSE when stimulated by a severe heat shock.

Protein synthesis required for induction of HSF binding at intermediate heat shock temperatures. Although protein synthesis is not required for the induction of HSF binding at high heat shock temperatures, protein synthesis is required for HSF binding at intermediate heat shock temperatures. When *Drosophila*, chicken, and human cells were stimulated in the absence of cycloheximide for 15 min at progressively higher temperatures (normal temperature in our experiments is 25°C for *Drosophila* cells and 37°C for chicken and human cells), HSE-binding activity in cell extracts progressively increased above the basal level, as assayed by exonuclease III protection (Fig. 4A, top) or by gel mobility shift analysis (Fig. 4B and C, top). The levels of HSE-binding activity stimulated by intermediate heat shock temperatures remain fixed when the cells are held constant at the intermediate temperature for prolonged periods (data not shown; Fig. 5A). In the presence of cycloheximide, however, no induction of HSE-binding activity could be observed at intermediate heat shock temperatures, whereas heat shock at the high temperature for each species resulted in a high level of HSE-binding activity (Fig. 4A, B, and C, bottom), which is comparable to the level found in cells not treated with cycloheximide. Similar results obtained with *Drosophila* cells treated with emetine, a different protein synthesis inhibitor, argue that the effects observed in the presence of these drugs are due to the inhibition of protein synthesis and not to other side effects (J. T. Westwood and C. Wu, unpublished observations). The inability to show inducible HSE-binding activity at intermediate temperatures in the presence of cycloheximide is correlated with a decreased

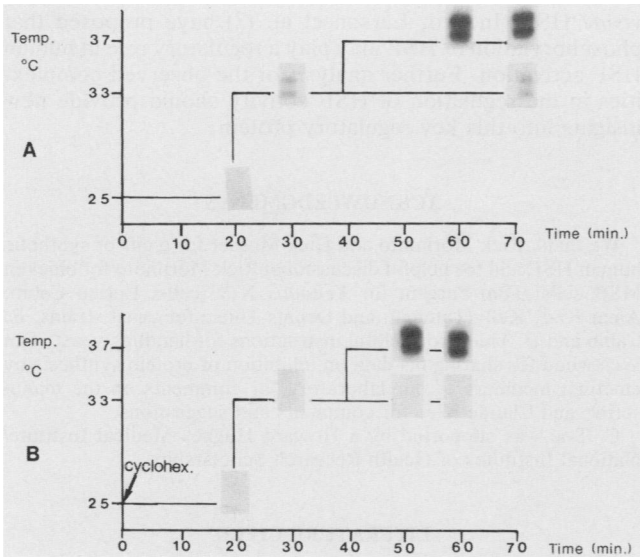


FIG. 5. Full induction of HSE-binding activity at a high heat shock temperature subsequent to a heat shock at an intermediate temperature in the presence of cycloheximide. Logarithmically growing *Drosophila* SL-2 cells were not treated (A) or were treated for 20 min (B) with 118 μM cycloheximide before the first heat shock at 33°C. At 20 min after the first heat shock, a portion of the cells were heat shocked more severely at 37°C. At the indicated times of the experiment, samples of cells heat shocked at 33 or 37°C were pelleted and frozen. Whole-cell extracts were prepared, and HSE-binding activity was assayed by the exonuclease III protection technique. The characteristic doublet fragments indicating exonuclease III protection at the upstream border of the *hsp82* gene heat shock element are shown in the insets on the schematic diagram.

level of transcription of a heat shock gene, *hsp70*, under these conditions (Fig. 4D).

Is HSF degraded in cells heat shocked at intermediate temperatures? The requirement for protein synthesis in the induction of HSF binding at intermediate heat shock temperatures could be explained if HSF was degraded under these conditions. We tested this possibility in *Drosophila* cells by questioning whether HSF binding could be induced in cells which had been heat shocked in the presence of cycloheximide, first at an intermediate temperature and then at a high temperature. Heat shock of *Drosophila* cells in the absence of cycloheximide at an intermediate, temperature (33°C) induced a low level of HSE-binding activity (Fig. 5A). A subsequent heat shock at a high temperature (37°C) increased the HSE-binding activity significantly (no further increase was observed in cells held continuously at 33°C). In the presence of cycloheximide, the first heat shock at 33°C did not induce a measurable increase of HSE-binding activity; however, the subsequent heat shock at 37°C induced the HSE-binding activity to the same high level as the activity measured without cycloheximide treatment (Fig. 5B). Hence, we conclude that the bulk of intracellular HSF is not degraded in *Drosophila* cells subjected to cycloheximide treatment and heat shock at the intermediate temperature. The inability to induce HSF binding to the HSE under such conditions might instead be due to a requirement for newly synthesized HSF itself or to an activating component that is labile at intermediate heat shock temperatures.

DISCUSSION

We have detected specific HSE-binding factors in a survey of vertebrate and yeast cell extracts. In our laboratory and others, HSE-binding activities have been purified to homogeneity or near homogeneity from *S. cerevisiae*, *Drosophila* SL-2 cells, and human HeLa cells. In all three cases, the factor, HSF, has been shown to activate transcription of heat shock genes in vivo or in vitro. It is likely that the HSE-binding activities we have observed in other species also correspond to HSF. Although all HSFs are able to bind to the same DNA sequence, there appears to be considerable variation in the protein structure in different species, and this variation may presage new complexities that may emerge in future studies of the heat shock activation pathway. In this regard, it is interesting that polyclonal antibodies raised against *Drosophila* HSF react only with the *Drosophila* factor and not with HSF from other species (S. Wilson and V. Zimarino, unpublished observations). Whether the differences reflect a fundamental divergence of HSF protein structure or merely accumulated evolutionary changes compatible with a common underlying structure and function awaits a comparative analysis of the HSF gene and protein structure in these species, when the cloning and sequence analysis of the HSF genes have been completed. The *S. cerevisiae* HSF gene has been successfully cloned recently (16, 19).

In higher eucaryotes, HSF undergoes a heat shock-inducible activation of its HSE-binding ability. To a first approximation, the extent of HSF binding that is induced directly correlates with the severity of heat shock, suggesting that the temperature-dependent modulation of HSF binding is a critical regulatory switch in the activation of heat shock gene transcription. Moreover, the lack of a requirement for protein synthesis in the induction, reversal, and reinduction of HSF binding at high heat shock temperatures suggests that a posttranslational mechanism, general to higher eucaryotes, may regulate the HSF binding under these conditions. The posttranslational mechanism may be a direct temperature-dependent induction of HSF binding, as was recently suggested from the ability to induce HSF binding in vitro with HeLa cytosol (7) and confirmed by using *Drosophila* cytosol (V. Zimarino, unpublished observations). However, by contrast with the induction in vivo, the induction in vitro is irreversible, and there is as yet no demonstration of the reversibility of HSF binding in vitro. Alternatively, the regulatory mechanism may involve additional factors that act on HSF stoichiometrically or catalytically to regulate its binding activity. To distinguish between these possible mechanisms, which are not mutually exclusive, further analysis of HSF purified from nonshocked cells will be necessary.

The requirement for new protein synthesis in the induction of HSF binding at intermediate heat shock temperatures in vertebrate and invertebrate cells is intriguing. The full induction of HSF binding in *Drosophila* cells that were first subjected to mild heat shock in the presence of cycloheximide and then to a severe heat shock demonstrates clearly that the bulk of intracellular HSF is not degraded under mild heat shock conditions. The inability to induce HSF binding under such conditions might instead be due to a component of the HSF activation pathway that is labile at intermediate heat shock temperatures but not at high heat shock temperatures. An alternative explanation might be that the newly synthesized and preexisting pools of HSF have distinct properties: the preexisting pool of HSF is stable and respon-

sive only to a severe heat shock, while the newly synthesized HSF pool has a lower temperature threshold at which HSF binding is induced.

The inducibility of HSF binding has been reported by other workers for human and mouse cells (3, 6, 9, 10, 14). However, in one study on HeLa cells, significant levels of an activity that binds specifically to a synthetic 35-base-pair human *hsp70* gene segment (human HSE) was found in extracts of nonshocked HeLa cells (10). In gel mobility shift assays, this activity from nonshocked HeLa cells forms a complex with DNA which migrates with a mobility that is distinctly faster than the mobility observed when extracts of heat-shocked HeLa cells were analyzed. Using the same synthetic human HSE, we have observed a similar binding activity in nonshocked HeLa cell extracts; however, the activity in the nonshocked extract did not interact with our consensus HSE, either in a direct gel mobility shift experiment or by competition with the binding to the human HSE (unpublished observations). By contrast, the activity in heat-shocked HeLa cell extracts binds to both the consensus HSE and to the human HSE, and the binding can be inhibited by an excess of either HSE (unpublished observations). Since the consensus HSE was derived from the *Drosophila hsp70* gene and matches completely with the original and expanded definitions of the HSE, while the human HSE is incompletely matched with either definition of the HSE and shows considerable divergence in flanking sequences, the human HSE-binding activity observed in nonshocked HeLa cell extracts could be due to an unrelated human factor that binds specifically to a sequence that overlaps with the human HSE or could be due to an inactive form of human HSF itself, provided that the factor undergoes changes in both sequence specificity of DNA binding and electrophoretic mobility upon heat shock.

The inducibility of HSF binding in response to heat shock in higher eucaryotes is also in concordance with studies of in situ protein-DNA interactions on *Drosophila* heat shock promoters, which show that the HSE in nuclear chromatin is freely accessible to nuclease cleavage under normal conditions but is protected from cleavage after heat shock (18, 21).

By contrast, HSF binding in fungal cell extracts is constitutive. Indeed, in unstressed *S. cerevisiae*, the HSE is apparently bound to HSF in vivo (4, 5, 17). Hence, the regulation of HSF in *S. cerevisiae* must occur subsequent to HSE binding. Our results concur with those of Sorger et al. (14, 16), who reported further that phosphatase treatment of heat shock-activated *S. cerevisiae* HSF results in a progressive return to the normal gel mobility, suggesting that multiple phosphorylation may be the heat shock-induced modification of HSF in *S. cerevisiae*. These workers have proposed that the addition of negatively charged phosphates to the HSE-bound factor enhances its transcriptional activity during heat shock. Whether a similar heat-induced phosphorylation modifies *Schizosaccharomyces pombe* HSF is presently unknown. There is apparently no signficiant change in the mobility of the *Schizosaccharomyces pombe* HSF-HSE complex, although modifications which are not revealed by gel mobility changes could possibly have occurred.

With regard to the properties of HSF in *S. cerevisiae*, it is conceivable that HSF in higher eucaryotes is regulated at two levels. At the first level, specific binding to the HSE could be mediated by a direct temperature sensitivity of HSF and/or by additional regulatory factors. At the second level, the transcriptional ability of HSF could be activated in a manner similar to the proposed phosphorylation of *S. cerevisiae* HSF. Indeed, Larson et al. (7) have proposed that phosphorylation of HSF may play a regulatory role in human HSF activation. Further analysis of the observed complexities in the regulation of HSF activity should provide new insights into this key regulatory protein.

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