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The DNA-Binding Activity of the Human Heat Shock Transcription Factor Is Regulated In Vivo by hsp70

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The human heat shock transcription factor (HSF) is maintained in an inactive non-DNA-binding form under nonstress conditions and acquires the ability to bind specifically to the heat shock promoter element in response to elevated temperatures or other conditions that disrupt protein structure. Here we show that constitutive overexpression of the major inducible heat shock protein, hsp70, in transfected human cells reduces the extent of HSF activation after a heat stress. HSF activation was inhibited more strongly in clones that express higher levels of hsp70. These results demonstrate that HSF activity is negatively regulated in vivo by hsp70 and suggest that the cell might sense elevated temperature as a decreased availability of hsp70. HSF activation in response to treatment with sodium arsenite or the proline analog azetidine was also depressed in hsp70-expressing cells relative to that in the nontransfected control cells. As well, the level of activated HSF decreased more rapidly in the hsp70-expressing clones when the cells were heat shocked and returned to 37°C. These results suggest that hsp70 could play an active role in the conversion of HSF back to a conformation that does not bind the heat shock promoter element during the attenuation of the heat shock response.

All organisms react to hyperthermia and other protein-damaging conditions by increasing the synthesis of a group of stress-induced or heat shock proteins (hsp) (reviewed in references 32 and 37). hsp synthesis enables the organism to resist the deleterious effects that these conditions have on cells. Under physiological conditions, these proteins and their constitutively synthesized cognates play essential roles in modulating protein-protein interactions. Members of the hsp70 family participate in the folding, assembly, and translocation of intracellular proteins (reviewed in references 16 and 19). hsp70 interacts with numerous proteins in vivo, and this interaction often stabilizes an unfolded and functionally inactive or immature form. hsp70 binds to unfolded proteins in vitro (38) and to proteins unfolded as a consequence of metabolic stress in vivo (5). The binding and release of hsp70, which requires ATP hydrolysis, facilitates protein folding and assembly and perhaps also the disassembly of protein aggregates that formed during conditions of cellular stress (16, 39).

In eukaryotic cells heat shock gene expression is dependent upon the activation of a preexisting heat shock factor (HSF) (reviewed in references 24, 31, 47, and 59). HSF is bound constitutively to the heat shock promoter element (HSE) at all temperatures in the yeast *Saccharomyces cerevisiae*, and its transcriptional activity is correlated with changes in phosphorylation (22, 36, 49, 50). In higher eukaryotes HSF activation involves conversion of the inactive factor to a form that is capable of specifically binding to the HSE (25, 48, 61). In human cells the abundance of the HSE-binding form of the factor is correlated with hsp70 transcriptional activity during heat shock and recovery or treatment with cadmium or amino acid analogs (2, 34). Human HSF can acquire specific DNA-binding ability by incubation of extracts from nonstressed cells at elevated temperatures in vitro (26). HSF can also be activated in vitro without temperature elevation by conditions that alter protein structure, such as reduced pH or treatment with nonionic detergents or urea, indicating that the active form of the factor results from a conformational change in the molecule (33). The observation that *Drosophila* HSF can be induced to gain DNA-binding ability in vitro by incubation with antibodies raised against the active form of the factor provides additional evidence that a conformational change is involved (60).

The activated form of *Drosophila* HSF exists as a trimer, and this oligomerization step is negatively regulated under nonstress conditions (8, 42, 57). HSE-binding ability is temperature regulated when the cloned *Drosophila* HSF gene is expressed in *Xenopus* oocytes (8) or when the human (41) or mouse (45) HSF1 gene is transcribed and translated in vitro. However, expression in *Escherichia coli* results in constitutive DNA-binding ability (8, 41, 45), and this has led to the suggestion that negative regulation is mediated by a cellular factor present in eukaryotic cells (8). The involvement of hsp70 as a negative regulator of the HSE-binding ability of HSF has been proposed as a mechanism for autoregulation (reviewed in references 9, 31, and 47). This is based on the following observations: (i) treatments that increase the abundance of substrates for hsp70 are able to trigger the heat shock response; (ii) activation of HSF involves a conformational change in the molecule, allowing trimerization to occur; and (iii) the proposed function of hsp70 is to maintain or alter the conformational state of cellular proteins. Further evidence to support this model has come from the observation that activated HSF can be found in a complex with hsp70 by gel mobility shift analysis of cell extracts that had been incubated with anti-hsp70 antibodies (1, 4). However, the functional significance of these in vitro interactions remained to be examined in vivo.

To directly address whether hsp70 negatively regulates the activity of HSF in vivo, we examined HSF activation in human cells that constitutively overexpress hsp70 by stable transfection with a β-actin promoter–hsp70 construct. We

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show here that the extent of HSF activation in the hsp70-expressing transfectants is directly related to their level of hsp70 expression. As well, activated HSF is converted back to a non-DNA-binding conformation more rapidly in the transfected cell lines during a recovery from heat shock. These results provide evidence that hsp70 plays an active role in modulating the DNA-binding ability of HSF in vivo.

MATERIALS AND METHODS

Transfection and screening. The human acute lymphoblastic leukemia T-cell line PEER (43) was transfected with a plasmid containing the human hsp70 gene under the control of the human β-actin promoter. The plasmid (βAPrhsp70) was constructed by subcloning the 2.3-kb *Bam*HI-*Hin*dIII fragment of pH2.3 (20) into the *Hin*dIII-*Sal*I cloning sites in pHβAPr-1-neo (18). Each plasmid was blunt ended by digestion with either *Bam*HI (pH2.3) or *Sal*I (pHβAPr-1-neo) followed by treatment with the Klenow fragment of DNA polymerase (all restriction and modification enzymes were from New England Biolabs Ltd.). The plasmids were then digested with *Hin*dIII, and the appropriate fragments were gel purified and extracted with phenol-chloroform prior to ligation.

PEER cells were transfected by electroporation. Cells (2×10^7) in log-phase growth were washed with phosphate-buffered saline (PBS) and resuspended in 0.8 ml of PBS containing 25 μg of the βAPr hsp70 plasmid. The plasmid was linearized by digestion with *Eco*RI prior to electroporation. The cells were incubated on ice in an electroporation cuvette for 10 min and then exposed to an electric pulse (250 V/cm at a capacitance of 960 μF) with a Bio-Rad Gene Pulser (Bio-Rad Laboratories Ltd.). The electroporated cells were returned to ice for 10 min and then incubated in a humidified 5% CO_2 atmosphere in a 37°C incubator with 20 ml of Iscove's modified Dulbecco's medium supplemented with 10% (vol/vol) fetal bovine serum, 500 U of penicillin per ml, and 500 μg of streptomycin per ml (GIBCO BRL). Forty-eight hours after electroporation, the cells were pelleted, resuspended in fresh medium containing 500 μg of G418 (GIBCO BRL) per ml, and transferred to 96-well culture dishes at a concentration of 1,000 viable cells per well.

Neomycin-resistant clones were expanded and screened for hsp70 expression by immunoblotting. Cells were washed with PBS, centrifuged, and frozen at −80°C. Pellets were resuspended in 20 μl of lysis buffer (10 mM Tris [pH 7.4], 5 mM $MgCl_2$, 0.5 mM phenylmethlsulfonyl fluoride, 50 μg of RNase per ml, 50 μg of DNase per ml, 0.5% Nonidet P-40), vortexed, and incubated on ice for 15 min. Protein in these samples was quantitated with the Bradford dye reagent (Bio-Rad) with bovine serum albumin (BSA) as a standard. The lysates were mixed 1:1 with 2× Laemmli buffer and boiled for 5 min, and then equivalent amounts of protein (50 μg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred onto nitrocellulose, and the filters were then incubated in PBS containing 5% milk powder for 1 h at 22°C before being reacted with a 1:1,000 dilution of a monoclonal antibody specific for hsp70 (clone C92F3A-5; StressGen, Victoria, Canada). The filter was washed three times for 10 min each with PBS–0.05% Tween 20 and then incubated for 1 h with a 1:1,000 dilution of an alkaline phosphatase-conjugated goat anti-mouse antibody (Jackson Immunoresearch Laboratory Inc.). Complexes were visualized by development with nitroblue tetrazolium (12). Clones that had elevated levels of hsp70 were recloned by limiting dilution and rescreened by immunoblotting. Two independently isolated clones (F2 and F3) having different levels of constitutive hsp70 expression were used in the experiments shown here. Similar results were obtained with three other clones. The F3 cell line, which has the highest level of hsp70, grows more slowly than the nontransfected cell line, PEER. However, the F2 cell line, which has an intermediate level of hsp70, grows at approximately the same rate as the PEER cells. Overexpression of hsp70 has also been observed to reduce the rate of cell proliferation in transfected *Drosophila* and rodent cell lines (13, 21, 27). The hsp70-expressing clones were subcultured by dilution with fresh medium containing 200 μg of G418 per ml three times weekly. Cells were pelleted and resuspended in fresh medium without G418 at least 24 h prior to all experiments.

Protein labeling, gel electrophoresis, and immunoblotting. hsp synthesis was examined in cells that were heated at 43°C for 30 min and then returned to 37°C for periods of 1, 3, 5, 7, and 9 h. For the heat shock treatments the cells were seeded into flasks at a concentration of 10^6 cells per ml and submerged in a circulating water bath regulated at ±0.1°C of the set temperature. The cells were labeled during the final hour of incubation in 0.5 ml of Dulbecco's modified Eagle medium without L-methionine (GIBCO BRL) containing 10% dialyzed fetal bovine serum, and 50 μCi of [^{35}S]methionine (Trans[^{35}S]Label; ICN Biochemicals Inc.) per ml. Cells were washed with PBS, centrifuged, and prepared for electrophoresis as described above. Equivalent amounts of protein were electrophoretically separated on SDS-polyacrylamide gels. The gels were stained with Coomassie blue to visualize the molecular weight standards (Bio-Rad), destained in 50% ethanol–7.5% acetic acid (vol/vol), soaked in Amplify (Amersham Ltd.), dried, and exposed to Kodak XAR-5 film with intensifying screens.

Parallel cultures were heat shocked and collected after the same time periods for immunoblot analysis. Extracts containing equivalent amounts of protein (25 μg) were separated by SDS-PAGE. Following transfer to nitrocellulose, the blots were stained with Ponceau red to ensure equal loading and to visualize the molecular weight markers. The nitrocellulose membranes were blocked and then incubated with monoclonal antibodies to hsp70 (clone C92F3A-5) or hsp27 (clone G3.1; StressGen). The filters were washed and incubated for 1 h with 5 μg of rabbit anti-mouse immunoglobulin G (Jackson Immunoresearch) per ml and then for 2 h with a 1:1,000 dilution of ^{125}-protein A (Amersham). The filters were then washed twice with PBS and once with PBS containing 1 M NaCl and exposed to film. Multiple autoradiographic exposures were obtained, and those within the linear range of the film response were quantitated by using the Jandel Video Analysis system (Jandel Scientific, Corte Madera, Calif.).

The levels of HSF1 in the PEER cell line and the transfected cell lines overexpressing hsp70 were examined by immunoblotting. Whole-cell extracts containing equivalent amounts of protein (5 μg per lane for the hsp70 blot and 20 μg per lane for the HSF1 blot) were separated on SDS-polyacrylamide minigels and transferred onto nitrocellulose. An antiserum specific for HSF1, provided by R. I. Morimoto (Northwestern University), was used as described by Sarge et al. (44). hsp70 was detected with the monoclonal antibody C92F3A-5 (StressGen). The blots were incubated with a 1:20,000 dilution of horseradish peroxidase-conjugated goat anti-rabbit (for HSF1) or anti-mouse (for hsp70) immunoglobulin G secondary antibodies (Amersham) for 1 h at 22°C. Blots were washed six times with PBS–0.1% Tween 20, and

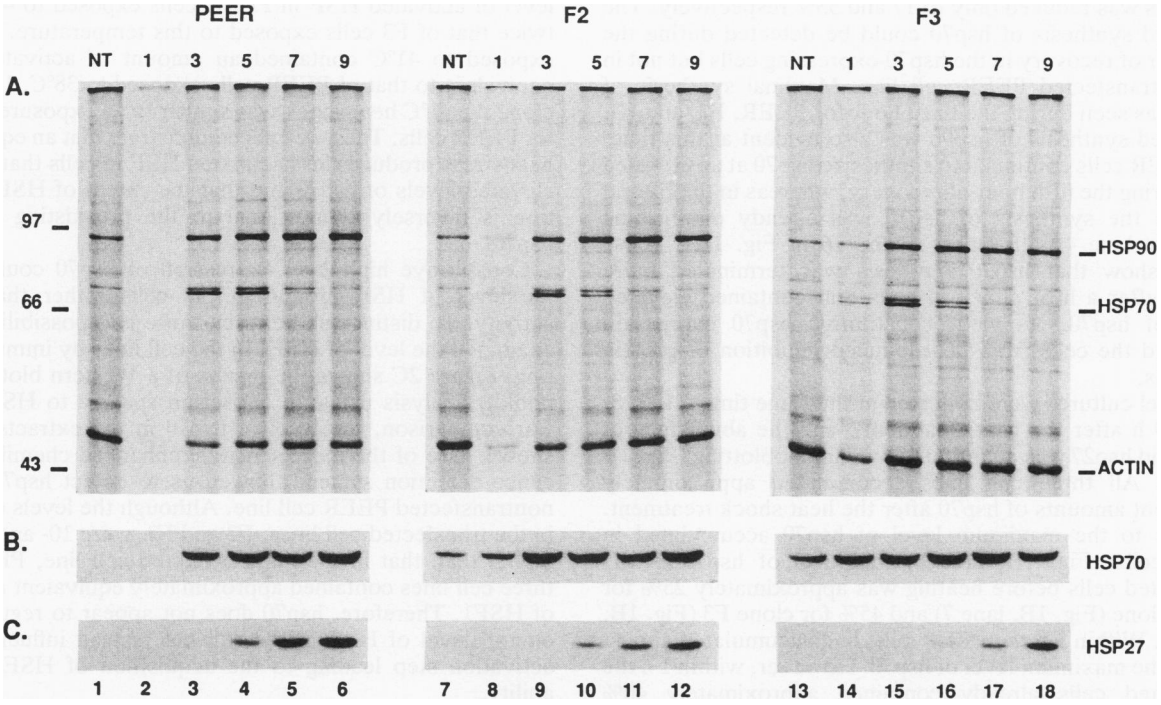


FIG. 1. Heat shock response of PEER cells (lanes 1 to 6) and the hsp70-expressing transfectants, F2 (lanes 7 to 12) and F3 (lanes 13 to 18). Cells were heat shocked at 43°C for 30 min and then returned to 37°C for periods ranging from 1 to 9 h. The length of time (hours) at 37°C is denoted above each lane. Control, nontreated cells (NT) were maintained at 37°C. (A) SDS-PAGE of proteins synthesized during recovery from heat shock. Newly synthesized proteins were labeled with [^{35}S]methionine during the final hour of incubation. Molecular mass markers (in kilodaltons) are indicated on the left of the fluorograph, and the positions of actin and the 90- and 70-kDa hsp are shown on the right. Levels of hsp70 (B) and hsp27 (C) were analyzed by immunoblotting in parallel cultures.

then the complexes were detected by enhanced chemiluminescense (Amersham).

Preparation of cell extracts and gel shift assay. For each experiment the nontransfected parental cell line, PEER, and the hsp70-expressing clones were adjusted to 10^6 cells per ml with fresh medium prior to treatment. Cells were heat treated as described above or incubated at 37°C with 0.5 to 100 μM sodium arsenite or 5 mM L-azetidine-2-carboxylic acid (Sigma Chemical Co.). At the end of each treatment, 1-ml aliquots were transferred to 1.5-ml microcentrifuge tubes, and the cells were immediately pelleted in a microcentrifuge. Cells were washed in ice-cold PBS, repelleted, and rapidly frozen in a dry ice-ethanol bath. The cell pellets were then transferred to −80°C. Whole-cell extracts were prepared in a buffer containing 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (pH 7.9), 25% (vol/vol) glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5 mM dithiothreitol, as described previously (34).

For the gel mobility shift assay, whole-cell extracts containing 10 μg of protein were mixed with 0.1 ng of a ^{32}P-HSE oligonucleotide probe and 0.5 μg of poly(dI-dC) · poly(dI-dC) (Pharmacia LKB Inc.) in 20 mM Tris (pH 8.0)–50 mM NaCl–1 mM EDTA–0.5 mM dithiothreitol–10% glycerol–0.3 mg of BSA per ml in a final volume of 15 μl. The HSE probe was prepared by annealing the self-complementary oligonucleotide (5′-CTAGAAGCTTCTAGAAGCTTCTAG-3′) and end labeling with T4 polynucleotide kinase (New England Biolabs) and [γ-^{32}P]ATP (ICN Biomedicals Canada Ltd.) as described previously (34). Binding-reaction mixtures were incubated on ice for 30 min, and then free probe was separated from HSE-HSF complexes by electrophoresis through 4% polyacrylamide gels (acrylamide-bisacrylamide, 40:1) containing 50 mM Tris (pH 8.5), 400 mM glycine, 2 mM EDTA, and 2.5% glycerol. Electrophoresis was carried out at room temperature at 200 V for the first 10 min and then for 2 h at a constant current of 160 mA. Gels were dried at 80°C and then exposed to Kodak XAR film without intensifying screens. The level of activated HSF was determined by quantification of the radiographic images with a Jandel Video Analysis system and was plotted relative to the maximum level for each experimental treatment.

RESULTS

Effect of constitutive hsp70 overexpression on hsp synthesis. The response of PEER cells to heat shock was compared with that of the hsp70-expressing transfectants (clones F2 and F3). The proteins synthesized in response to a 30-min exposure to 43°C were radiolabeled with [^{35}S]methionine for periods of 1 h starting immediately after the heat shock and following a recovery of 2, 4, 6, and 8 h at 37°C. Extracts containing equivalent amounts of protein were analyzed by SDS-PAGE and fluorography (Fig. 1A). Total protein synthesis was drastically reduced in the PEER cells after the heat shock treatment (Fig. 1A, lane 2) but then recovered to control levels during the incubation period at 37°C (lanes 3 to 6). In contrast, protein synthesis was not as severely inhibited in the hsp70-expressing clones (compare lane 2 with lanes 8 and 14). During the first hour of recovery, the synthesis of actin was reduced to 7% of the control level in the PEER cells. However, in the F2 and F3 cells actin

synthesis was reduced only to 17 and 53% respectively. The enhanced synthesis of hsp70 could be detected during the first hour of recovery in the hsp70-expressing cells but not in the nontransfected PEER cell line. Maximal synthesis of hsp70 was seen during the third hour for PEER, F2, and F3. Enhanced synthesis of hsp90 was also evident at this time. The PEER cells continued to synthesize hsp70 at an elevated level during the fifth hour of recovery, whereas in the F2 and F3 cells the synthesis of hsp70 was already diminishing (compare lane 4 with lanes 10 and 16 in Fig. 1A). These results show that hsp70 synthesis was terminated more rapidly after a heat shock in cells that contained elevated levels of hsp70. As well, constitutive hsp70 expression protected the cells from heat-induced inhibition of protein synthesis.

Parallel cultures were collected at the same times (1, 3, 5, 7, and 9 h after the heat treatment), and the abundance of hsp70 and hsp27 was quantitated by immunoblotting (Fig. 1B and C). All three cell lines accumulated approximately equivalent amounts of hsp70 after the heat shock treatment. Relative to the maximum level of hsp70 accumulated in PEER cells (Fig. 1B, lane 6) the level of hsp70 in the transfected cells before heating was approximately 25% for the F2 clone (Fig. 1B, lane 7) and 45% for clone F3 (Fig. 1B, lane 13). Within 3 h the PEER cells had accumulated almost 70% of the maximum level of hsp70. However, within 1 h the transfected cells already contained approximately 40% (clone F2) and 75% (clone F3) of the maximum level of hsp70 accumulated by PEER cells. The fold increase in hsp70 after 9 h was only about 3.5 and 1.5 for the F2 and F3 cells, respectively. Therefore, constitutive hsp70 expression reduced the amount of hsp70 that needed to be synthesized after a heat shock and allowed the cells to acquire this level much sooner. This differed from the pattern of hsp27 accumulation (Fig. 1C). hsp27 was first detectable 5 h after the heat treatment. At this time and over the subsequent 4 h, the PEER cells had a substantially higher level of hsp27 than either of the hsp70-expressing clones. The F3 cell line, which contains the highest level of hsp70, synthesized less hsp27 than either the PEER or F2 cell line.

Constitutive overexpression of hsp70 reduced the extent of HSF activation in heat-shocked cells. The results shown in Fig. 1 revealed that the duration of hsp70 synthesis after heat shock was directly dependent upon the amount of hsp70 present in the cell prior to the heat treatment. This attenuation of the heat shock response could be the result of a reduction in the level of HSF activation in the hsp70-expressing cells. We therefore examined whether constitutive overexpression of hsp70 influences HSF activation. The level of activated HSF was measured in whole-cell extracts prepared from cells that received a 15-min exposure to temperatures ranging from 37 to 43°C. Figure 2A shows the results of a gel mobility shift assay using a [^{32}P]-labeled oligonucleotide probe containing four repeats of the nGAAn heat shock element. A quantitation of the results is presented in Fig. 2B. Increasing the incubation temperature brought about an increase in the amount of activated HSF for each cell line until a maximum level was reached. For the PEER cells maximum HSF activation occurred at 40 to 41°C. However, in the hsp70-expressing clones maximal levels of activated HSF were achieved at a slightly higher temperature range of 41 to 42°C. What is most striking is that at each exposure temperature the F2 and F3 cells contained less activated HSF than did the PEER cells. Moreover, the extent to which HSF activation was reduced is proportional to the level of hsp70 expression in the transfected cells. The level of activated HSF in PEER cells exposed to 41°C was twice that of F3 cells exposed to this temperature. F3 cells exposed to 41°C contained an amount of activated HSF equivalent to that of PEER cells exposed to 38°C. In the F2 clone the 41°C heat shock was similar to an exposure of 39°C for PEER cells. These results demonstrate that an equivalent heat stress produces less activated HSF in cells that contain elevated levels of hsp70 and that the extent of HSF activation is inversely proportional to the preexisting level of hsp70.

Constitutive high-level expression of hsp70 could affect the level of HSF protein in the cells rather than HSF activity. To distinguish between these two possibilities, we examined the level of HSF1 in the cell lines by immunoblotting. Figure 2C shows the results of a Western blot (immunoblot) analysis using an antiserum specific to HSF1 (44). For comparison, the level of hsp70 in the extracts is also shown. Use of the more sensitive enhanced chemiluminescence detection system allowed us to detect hsp70 in the nontransfected PEER cell line. Although the levels of hsp70 in the transfected cell lines, F2 and F3, were 10- and 20-fold higher than that in the nontransfected cell line, PEER, all three cell lines contained approximately equivalent amounts of HSF1. Therefore, hsp70 does not appear to regulate the overall level of HSF in the cell but instead influences the activation step leading to the acquisition of HSE-binding ability.

Levels of activated HSF in cells continuously exposed to elevated temperature. HSF is only transiently activated during a continuous exposure to 40°C (Fig. 3A). Levels of activated HSF reached a peak within 15 to 30 min (lanes 2 and 3) and then subsided. After this time, a low level of activated HSF was maintained until the end of the experiment (lanes 5 to 7). The hsp70-expressing cells acquired less activated HSF than the PEER cell line during the initial phase of the heat shock treatment. In the second phase of the response, the PEER cells maintained an elevated level of HSF that was approximately fourfold higher than that of the F3 cells (Fig. 3B). The level of activated HSF in the F3 cells after 60 min of exposure was only slightly higher than that seen in the extracts prepared from nontreated cells (Fig. 3A, lane 1). The F2 cells maintained an intermediate level of activated HSF during this terminal phase.

Activation of HSF by sodium arsenite. The degree to which HSF is activated in response to chemical treatments is also dependent upon the cellular content of hsp70. HSF levels were measured in cells that were given a 90-min exposure to various concentrations of sodium arsenite (Fig. 4A). The lowest concentration that resulted in the activation of HSF was approximately 1.0 to 2.5 μM for the PEER cells. In the hsp70-expressing cells activation of HSF required concentrations of sodium arsenite greater than 5 μM. Maximum activation of HSF occurred at 50 to 100 μM sodium arsenite in the PEER cells. The concentration that brought about one-half of this maximum level of activation was about 5 μM (Fig. 4B). Achievement of this amount of activated HSF required a threefold-higher concentration of sodium arsenite in the F2 cell line, and a sevenfold-higher concentration was needed in the F3 cells.

HSF activation in azetidine-treated cells. Incorporation of the proline analog azetidine into newly synthesized proteins alters their secondary structure and leads to the activation of HSF (34). Incorporation of azetidine into HSF could alter its conformation in a manner that would allow it to acquire HSE-binding ability. Alternatively, activation of HSF could occur by a mechanism in which the cell senses an increase in

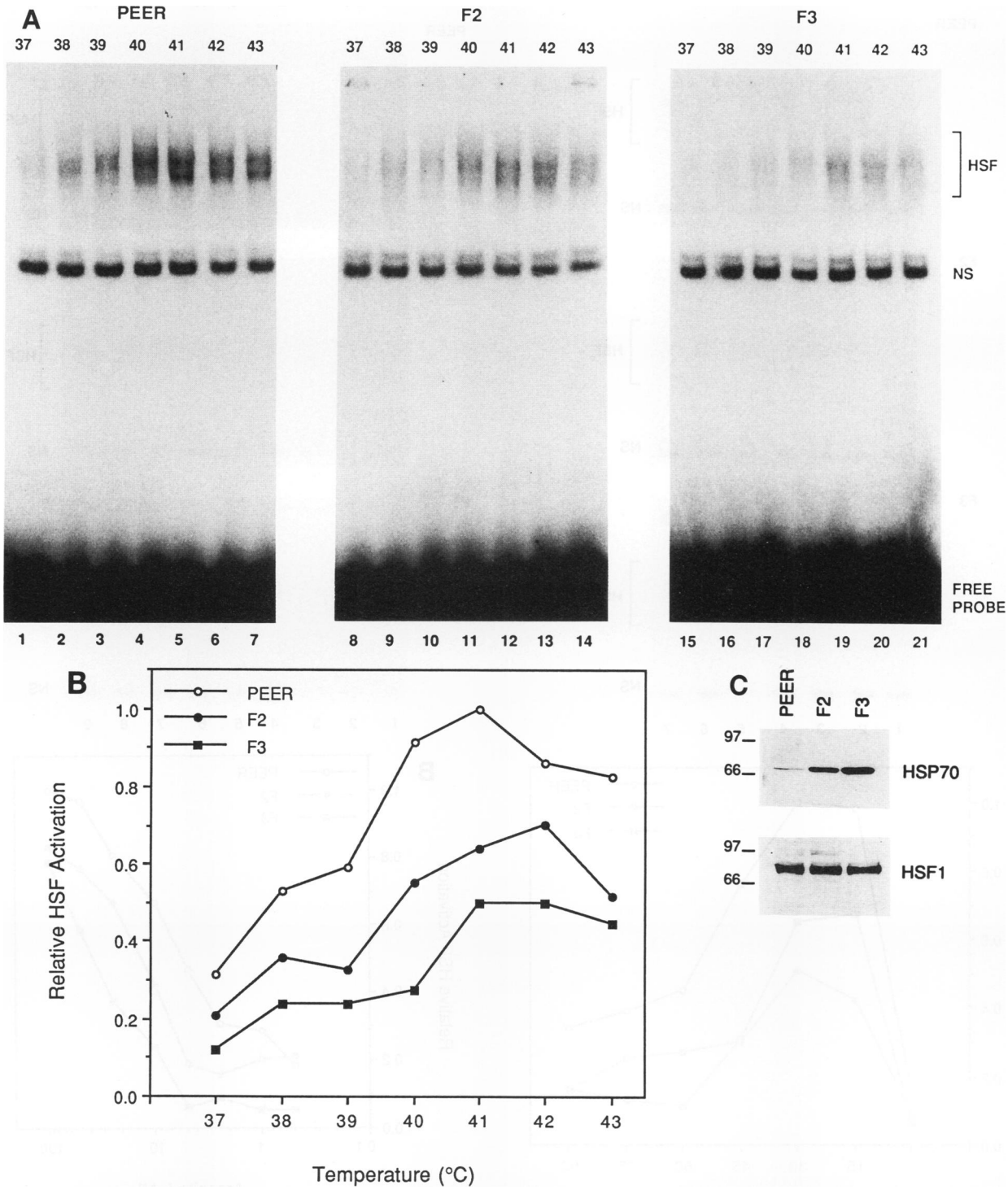


FIG. 2. Activation of HSF by elevated temperature. (A) Gel mobility shift analysis of the level of activated HSF in whole-cell extracts prepared from PEER (lanes 1 to 7), F2 (lanes 8 to 14), and F3 (lanes 15 to 21) cells that were heated for 15 min at the temperatures indicated above the lanes. The locations of the free HSE oligonucleotide probe, a nonspecific complex (NS), and the specific HSF-HSE complexes are indicated at the right of the autoradiogram. (B) The relative levels of activated HSF were quantitated by video densitometric analysis of the radiographic images and plotted. (C) Immunoblot showing the levels of hsp70 and HSF1 in whole-cell extracts prepared from the PEER, F2, and F3 cell lines. The positions of molecular mass markers (in kilodaltons) are indicated on the left.

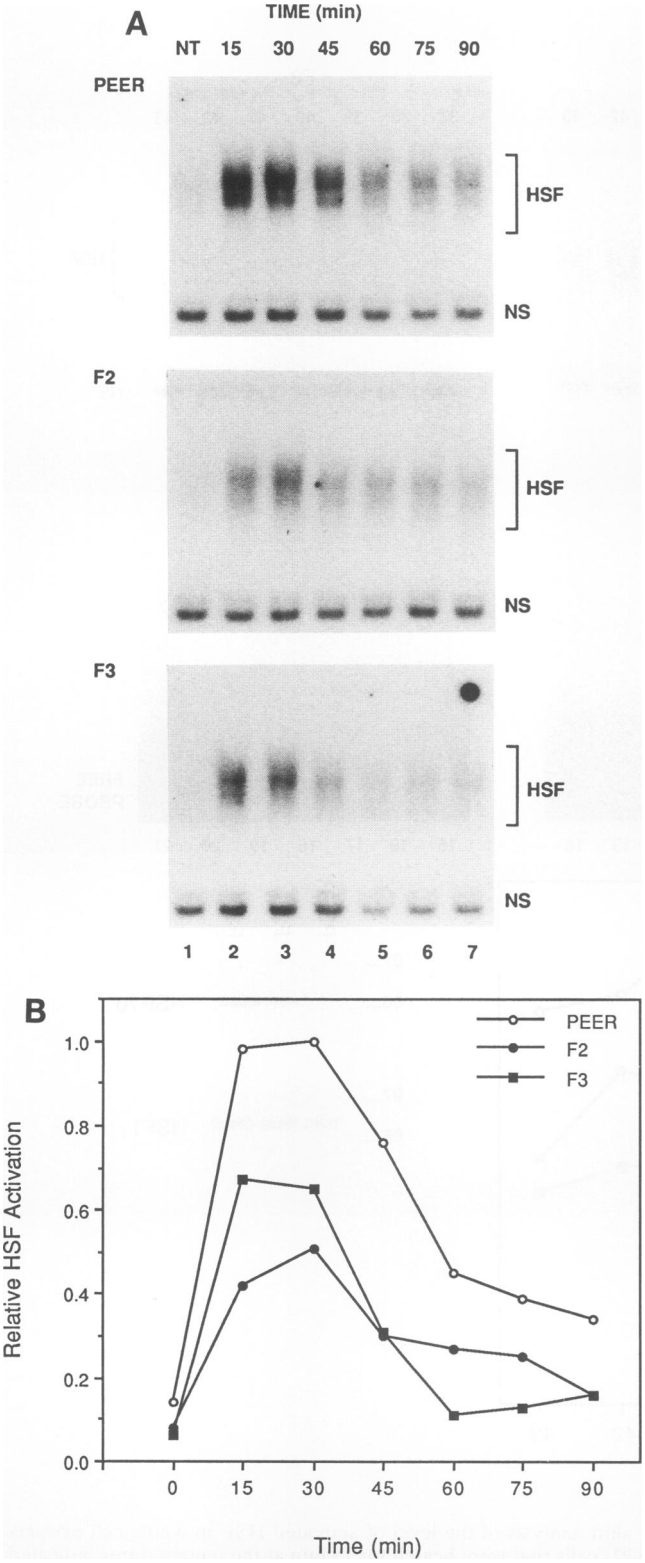


FIG. 3. Levels of activated HSF in cells continuously exposed to 40°C for periods ranging from 15 to 90 min. (A) Gel mobility shift assay of HSE-binding activity in whole-cell extracts prepared from PEER, F2, and F3 cells. Control, nontreated cells (NT) were maintained at 37°C (lane 1). The locations of the nonspecific complex (NS) and the specific HSF-HSE complexes are indicated at the right of the autoradiograms. (B) Plot showing the relative levels of activated HSF.

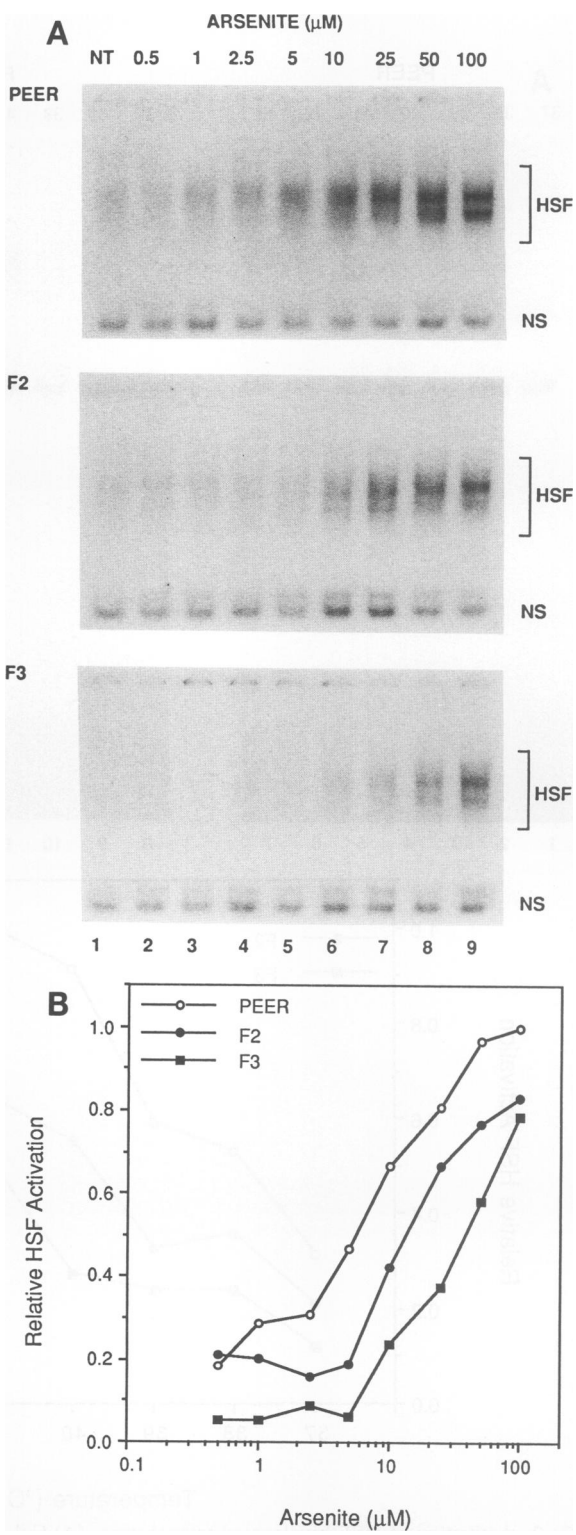
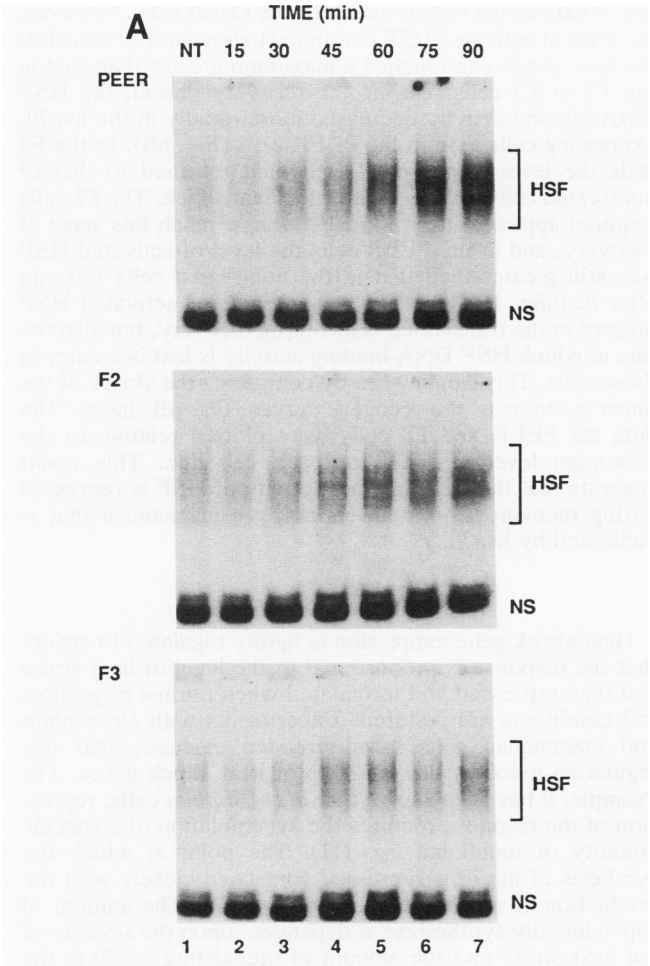


FIG. 4. Levels of activated HSF in sodium arsenite-treated cells. PEER, F2, and F3 cells were exposed to sodium arsenite at concentrations ranging from 0.5 to 100 μM for 90 min. (A) Gel mobility shift assay of HSE-binding activity in whole-cell extracts prepared from the treated cells. Control cells (NT) were maintained at 37°C (lane 1). The locations of the nonspecific complex (NS) and the specific HSF-HSE complexes are indicated at the right of the autoradiograms. (B) Plot showing the relative levels of activated HSF.



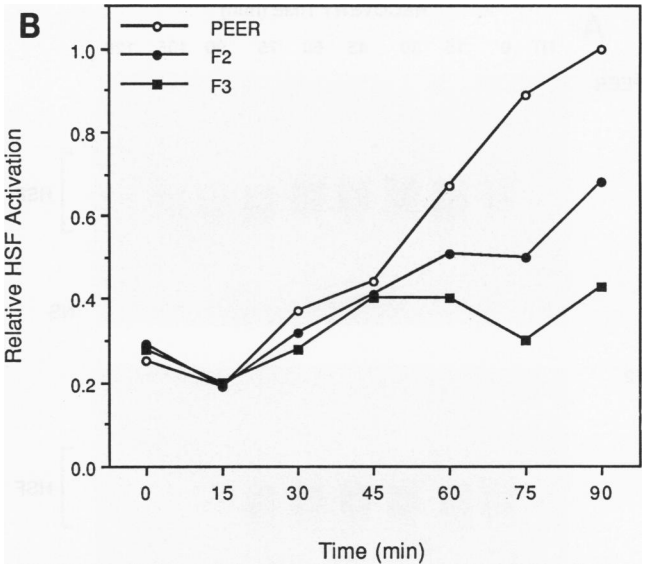
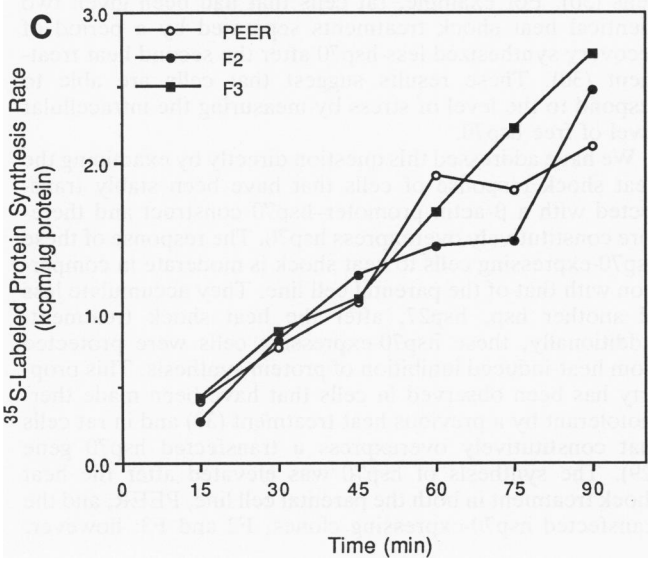


FIG. 5. Levels of activated HSF in cells incubated with the proline analog azetidine. (A) Gel mobility shift assay of HSE-binding activity in whole-cell extracts prepared form PEER, F2, and F3 cells grown in the presence of 5 mM azetidine for periods ranging from 15 to 90 min. NT, control (nontreated) cells (lane 1). The locations of the nonspecific complex (NS) and the specific HSF-HSE complexes are indicated at the right of the autoradiograms. (B) Plot showing the relative levels of activated HSF. (C) The rate of protein synthesis in each cell line was measured by [^{35}S]methionine labeling. Cells were incubated with [^{35}S]methionine for periods ranging from 15 to 90 min. [^{35}S]methionine incorporation (counts per minute) was measured in cell extracts by precipitation with trichloroacetic acid and is expressed relative to the amount of protein (micrograms) in the extract.



the abundance of misfolded azetidine-substituted proteins. If the incorporation of azetidine into HSF, rather than other cellular proteins, is required for HSF to become active, then the amount of activated HSF in azetidine-treated cells should be independent of the hsp70 concentration. However, this was not the case. PEER cells exposed to 5 mM azetidine accumulated more activated HSF during a 90-min incubation than did either the F2 or F3 cells (Fig. 5A and B). This was not because of a lower rate of protein synthesis in the transfected cells. The hsp70-expressing cells and the nontransfected PEER cells incorporated [^{35}S]methionine at similar rates and would be expected to have accumulated an equivalent level of azetidine-substituted proteins (Fig. 5C). Therefore, HSF activation requires a higher concentration of misfolded azetidine-substituted proteins in cells that contain an increased cellular content of hsp70.

HSF DNA-binding activity is down-regulated more rapidly in the hsp70-expressing cells. Heat shock genes are expressed transiently when cells are either briefly exposed to an elevated temperature or continuously subjected to a moderate increase in incubation temperature. This down-regulation is associated with a rapid loss in HSF DNA-binding ability (2, 34). The release of HSF from the hsp70 promoter in vivo occurs more rapidly than the dissociation rate measured for the HSF-HSE oligonucleotide complex in vitro, indicating that a cellular factor(s) facilitates this process in heated cells (2).

The involvement of hsp70 in facilitating the down-regulation of HSF DNA-binding ability was examined. Cells were given a 15-min exposure to 42°C and then returned to 37°C for up to 2 h. Levels of activated HSF were measured in cells collected immediately after the heat shock and at intervals of 15 min (Fig. 6A). Maximum levels of activated HSF were attained at the end of the heat shock treatment in the

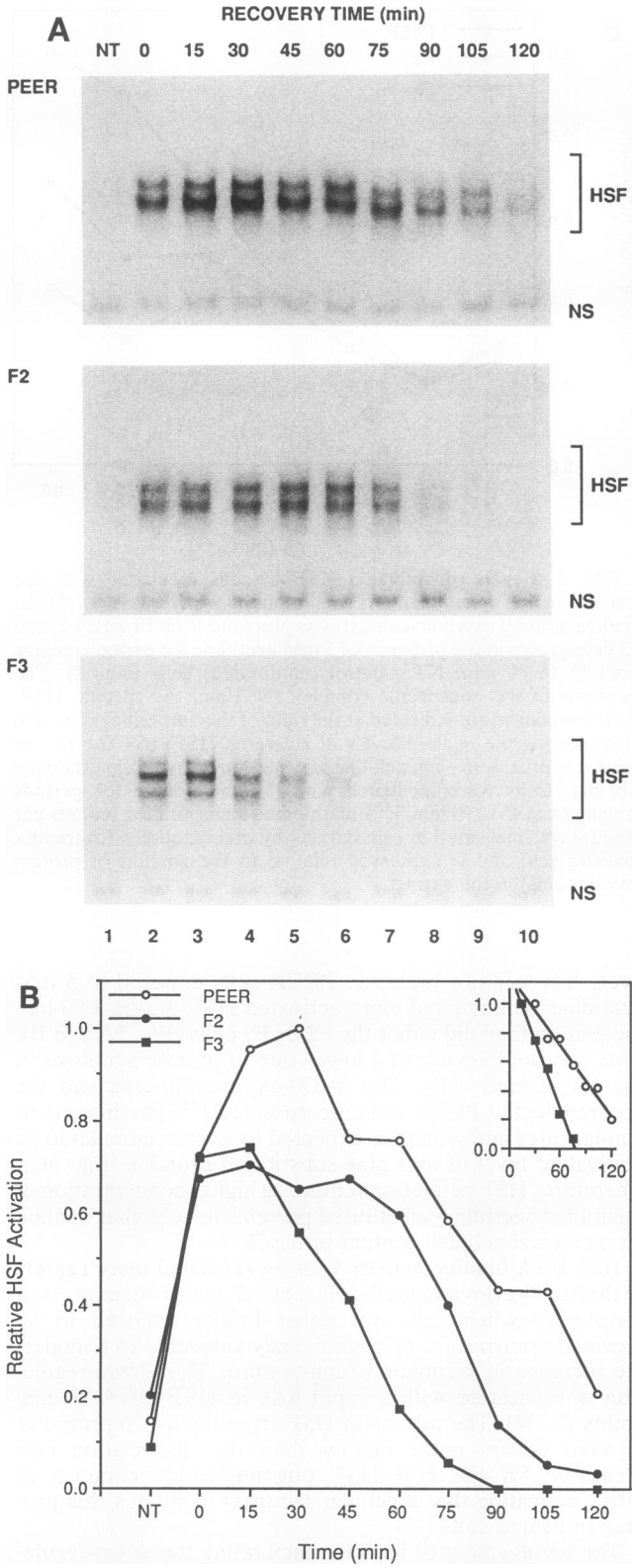


FIG. 6. Analysis of HSF levels in cells recovering from heat shock. (A) Gel mobility shift analysis of levels of activated HSF. PEER, F2, and F3 cells were exposed to 42°C for 15 min and then returned to 37°C. Whole-cell extracts were prepared immediately after the heat shock (time zero, lane 2) and after recovery periods of 15 to 120 min. The control, nontreated cells (NT) were maintained at 37°C (lane 1). The locations of the nonspecific complex (NS) and the specific HSF-HSE complexes are indicated at the right of the autoradiograms. (B) Plot showing the relative levels of activated HSF. The inset shows a plot of the data for PEER and F3 cells expressed relative to the maximum amount of HSF attained by each cell line. Only the linear portion of the plot is shown.

hsp70-expressing cells (lane 2). In the PEER cells, however, the level of activated HSF continued to rise until 30 min after the heat shock and reached a maximum greater than that in the F2 or F3 cells. During the recovery period, the HSF DNA-binding activity decreased more rapidly in the hsp70-expressing cells than in the PEER cells (Fig. 6B). In the F3 cells the level of activated HSF had returned to that of nontreated cells by 75 min after the heat shock. The F2 cells required approximately 90 to 105 min to reach this stage of recovery, and in the PEER cells the level of activated HSF was still greater than that in the nontreated cells 120 min after heating. Not only are lower levels of activated HSF present in the transfected cells during recovery, but also the rate at which HSF DNA-binding activity is lost is greater in these cells. This can be seen by comparing the slopes of the linear portions of the recovery curves (Fig. 6B, inset). The data for PEER and F3 cells were plotted relative to the maximum level of HSF for each cell line. This result suggests that the HSE-binding activity of HSF is repressed during recovery from heat shock by a mechanism that is facilitated by hsp70.

DISCUSSION

Heat shock gene expression is tightly regulated to ensure that the response is proportional to the level of heat stress and then repressed and terminated when normal physiological conditions are restored. Experiments with *Drosophila* and mammalian cells have provided evidence that this regulation involves the products of heat shock genes. For example, it has been shown that in *Drosophila* cells, repression of the response requires the accumulation of a specific quantity of functional hsp (11). The point at which the synthesis of hsp70 is repressed correlated closely with the production of a specific quantity of hsp70. The amount of hsp70 initially synthesized is dependent upon the severity of the heat stress and the amount of preexisting hsp70 in the cells (30). For example, rat cells that had been given two identical heat shock treatments separated by a period of recovery synthesized less hsp70 after the second heat treatment (30). These results suggest that cells are able to respond to the level of stress by measuring the intracellular level of free hsp70.

We have addressed this question directly by examining the heat shock response of cells that have been stably transfected with a β-actin promoter–hsp70 construct and therefore constitutively overexpress hsp70. The response of these hsp70-expressing cells to heat shock is moderate in comparison with that of the parental cell line. They accumulate less of another hsp, hsp27, after the heat shock treatment. Additionally, these hsp70-expressing cells were protected from heat-induced inhibition of protein synthesis. This property has been observed in cells that have been made thermotolerant by a previous heat treatment (30) and in rat cells that constitutively overexpress a transfected hsp70 gene (29). The synthesis of hsp70 was elevated after the heat shock treatment in both the parental cell line, PEER, and the transfected hsp70-expressing clones, F2 and F3; however,

this elevated level of synthesis was terminated earlier in the F2 and F3 cells. Both the PEER and the transfected cell lines accumulate the same quantity of hsp70 in response to heat shock treatment, indicating that the total amount of hsp70 required by the cell is dependent upon the severity of the stress. However, the hsp70-expressing cells are able to attain this level sooner than the parental cell line because of their increased constitutive levels, and for the F3 clone this requires only a marginal increase in the total amount of hsp70. Our results provide direct evidence that the response of cells to heat shock is dependent upon the preexisting level of hsp70.

The rapid transcriptional activation of heat shock genes that occurs in response to environmental stress is mediated by the binding of HSF to a conserved HSE located in the promoters of heat-inducible genes. A key question is how the cell senses temperature elevation and conveys this information to HSF. In higher eukaryotes HSF exists in a non-DNA-binding state under physiological conditions and responds to stress by undergoing a conversion to a form that has specific HSE-binding ability (reviewed in references 24, 31, 47, and 59). Conversion to the active form involves a conformational change, and this could be mediated by the disruption of a complex containing HSF and a regulatory protein (8, 33, 60). This conformational change allows oligomerization of HSF to occur, yielding active DNA-binding trimers (8, 42, 57). An attractive model that has emerged is that negative regulation of HSF is mediated by hsp70, the major heat-induced protein (1, 4, 33; reviewed in references 9, 31, and 47). This negative regulation would be relieved when substrates for hsp70 are elevated as a result of cellular stress.

The heat shock response is autoregulated by hsp70 in *E. coli* and the yeast *S. cerevisiae*. Mutations in the hsp70 gene result in elevated hsp synthesis at control temperatures and a reduced ability to shut off the response after a heat stress (10, 53, 55). Constitutive overproduction of hsp70 diminishes the heat shock response in *E. coli* (55) and the activity of an hsp70 promoter-driven reporter gene in *S. cerevisiae* (51). Transcriptional activation of bacterial heat shock genes is regulated by the abundance of an alternate σ factor, σ^{32} (17, 52). DnaK, the *E. coli* homolog of hsp70, together with DnaJ and GrpE, physically interacts with σ^{32} (15, 28) and modulates its synthesis and stability (54, 56). Negative regulation in *S. cerevisiae* involves HSF, since the overexpression of heat shock genes that occurs in yeast cells harboring hsp70 mutations can be abolished by mutating the HSE (7).

hsp70 has also been implicated in the negative regulation of HSF activity in mammalian cells. The amount of activated HSF generated in response to heat shock can be reduced by experimentally manipulating the cellular hsp level. Activation of HSF is diminished in human cells that were given a previous heat shock and allowed to accumulate hsp (4). Inhibiting hsp synthesis with the RNA polymerase II inhibitor DRB (5,6-dichloro-1-D-ribofuranosylbenzimidazole) extends the period of HSF activation in cells given a second heat stress (40). Also, the activation of HSF is reduced in human cells that are preincubated with cycloheximide prior to heat shock treatment (3, 4, 34). Cycloheximide-treated cells would likely contain an elevated pool of free hsp70 and the cognate hsc72, since nascent polypeptides are a major substrate for the 70-kDa family of stress proteins (6).

Our results demonstrate that the HSE-binding activity of human HSF is regulated in vivo by hsp70. Constitutive overexpression of hsp70 results in a reduction in the level of HSF activation in response to temperature elevation. The extent of inhibition was greater in those cells that express higher levels of hsp70. This indicates that with respect to activation of the heat shock response, the cell senses temperature elevation as a decrease in the level of available hsp70. Furthermore, the effects of other stress conditions, such as sodium arsenite exposure or amino acid analog incorporation, are assessed in the same way. That is, activation of HSF occurs as a consequence of hsp70 being diverted away from HSF in response to an increased pool of damaged or misfolded protein substrates. Relieving the influence that hsp70 normally exerts on HSF would allow oligomerization and HSE binding to occur. The idea that the heat shock response is self-regulated and tied to the level of free hsp70 was first proposed on the basis of an analysis of hsp70 mRNA accumulation and hsp70 synthesis in *Drosophila* cells (11). Activation of the stress response is likely triggered by a shift in the equilibrium between free and substrate-bound forms of hsp70, since treatments that increase substrates for hsp70 result in increased hsp synthesis (6, 27; reviewed in reference 9). A reduction in free hsp70 has previously been proposed to be the key step leading to the activation of HSF (1, 4, 33; reviewed in references 9, 31, and 47).

Activated HSF exists as a trimer formed by the interaction of hydrophobic heptad repeats located at the NH_2-terminal end of the molecule (8, 49). A fourth zipper motif is located at the COOH termini of all HSF molecules that have heat-inducible HSE-binding ability (8, 14, 41, 45, 46). This COOH-terminal hydrophobic heptad repeat has been postulated to play a role in regulating the HSE-binding activity of HSF (8). *S. cerevisiae* and *Kluyveromyces lactis* HSF, both of which possess constitutive HSE-binding ability, lack this domain (23, 50, 58). Deletion of this domain from human HSF allows constitutive HSE-binding ability when HSF is transiently expressed in human cells (42). Under normal conditions, an interaction between this domain and the NH_2-terminal heptad repeats could maintain the protein in a folded conformation, and the disruption of these intramolecular interactions by temperature elevation could allow trimerization to occur (42). hsp70 could negatively regulate HSF by stabilizing the factor in this inactive folded conformation. hsp70 might form a stable complex with the inactive form of HSF and thereby prevent trimerization. This complex would then be disrupted by hyperthermia or other conditions of cellular stress. Alternatively, hsp70 could transiently interact with HSF and influence its state of folding such that the inactive folded form predominates in nonstressed cells. When the level of free hsp70 becomes limiting, activation of HSF would then occur as a consequence of hsp70 being diverted away from the inactive form of HSF.

Complexes containing active HSF and hsp70 have been detected in extracts from heat-shocked human cells (1, 4). Although it is not known whether hsp70 interacts with the inactive form of HSF, excess exogenous hsp70 does prevent the activation of HSF in vitro (1). hsp90, which negatively regulates the transcriptional activity of steroid hormone receptors, could also interact with and repress the activity of HSF. hsp90 is capable of interacting with HSF, since immobilized hsp90 specifically retains HSF from a crude extract of rat liver (35). The hsp90-HSF interaction appears to be specific for the inactive form of the factor, since antibodies to hsp90 do not interact with the active form of HSF bound to DNA (4). However, hsp90 has not been shown to regulate the activity of HSF in vivo. The fact that the interaction of hsp70 with HSF does not interfere with its HSE-binding

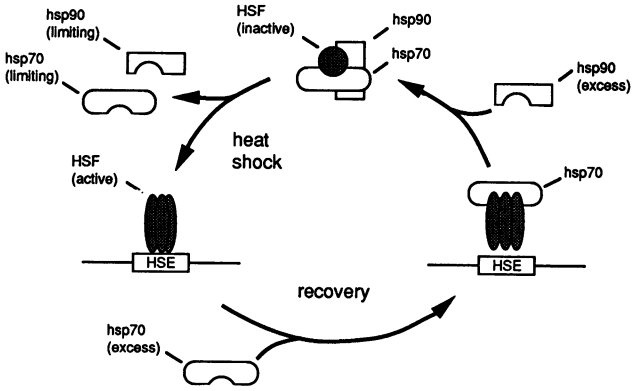


FIG. 7. Model for regulation of HSF activity by hsp70. HSF exists in three possible configurations. The inactive form is a monomer that either transiently interacts with or is stably associated with hsp70 (and/or hsc70 or hsp90). The active form is a trimer that is capable of binding to the HSE. HSF trimers associate with hsp70 during recovery from heat shock. hsp70 disrupts these trimers and refolds the monomers. See Discussion for details. Similar models have been presented elsewhere (1, 4, 33; reviewed in references 9, 31, and 47).

ability (1, 4) could indicate that the release of hsp70 from HSF is not essential for HSF to gain DNA-binding ability. Activated HSF can be detected in three complexes when bound to an HSE oligonucleotide and separated in the gel mobility shift assay. hsp70 was found to be present in only the two more slowly migrating complexes (1, 4). The complexes with hsp70 might contain HSF molecules that are being targeted for conversion to the inactive form. In the hsp70-expressing cell line, F3, these two more slowly migrating complexes are abundant during recovery from heat shock, whereas in the nontransfected parental cell line, PEER, the faster-migrating complex is the predominant form (Fig. 6A). The rapid attenuation of HSE-binding activity in the hsp70-expressing cell lines suggests that hsp70 could facilitate the conversion of active HSF trimers to inactive folded monomers.

A model for the regulation of HSF activity by hsp70 is shown in Fig. 7. In this model HSF is maintained in an inactive monomeric form by association with hsp70. This association need not be stable but instead could constitute a transient interaction in which hsp70 aids in the conversion of HSF to the folded and inactive form. The constitutively synthesized hsc70 and hsp90 might also assist in this function. During periods of cellular stress, the concentration of competing substrates for hsp70 (and hsp90) increases. As the cellular level of these proteins becomes limiting, the influence that they normally exert on HSF is relieved, allowing oligomerization of HSF to occur. The transcriptional activation of heat shock genes subsequently provides the cell with an amount of hsp70 that exceeds its cellular demand. hsp70 then facilitates the conversion of the active form of HSF to inactive monomers during recovery, leading to repression of the heat shock response. Whether hsp70 remains associated with inactive HSF or is released in the refolding process remains to be determined. Another possibility is that inactive HSF is bound to hsp90 but not hsp70. In this model the function of hsp70 is to bind to active HSF and refold it, while hsp90 acts to maintain HSF in this inactive form. This is consistent with the observation that hsp90 interacts with the inactive form of HSF (35) but not with the active form (4), whereas hsp70 has been shown to bind only to the active form (1, 4). This is also consistent with our results demonstrating that constitutive overexpression of hsp70 reduces the level of HSF activation. Disruption of an hsp90-HSF interaction would require a higher level of cellular damage because this would only occur when hsp70 becomes limiting.

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