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Activation of Human Heat Shock Genes Is Accompanied by Oligomerization, Modification, and Rapid Translocation of Heat Shock Transcription Factor HSF1

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Transcriptional activity of heat shock (hsp) genes is controlled by a heat-activated, group-specific transcription factor(s) recognizing arrays of inverted repeats of the element NGAAN. To date genes for two human factors, HSF1 and HSF2, have been isolated. To define their properties as well as the changes they undergo during heat stress activation, we prepared polyclonal antibodies to these factors. Using these tools, we have shown that human HeLa cells constitutively synthesize HSF1, but we were unable to detect HSF2. In unstressed cells HSF1 is present mainly in complexes with an apparent molecular mass of about 200 kDa, unable to bind to DNA. Heat treatment induces a shift in the apparent molecular mass of HSF1 to about 700 kDa, concomitant with the acquisition of DNA-binding ability. Cross-linking experiments suggest that this change in complex size may reflect the trimerization of monomeric HSF1. Human HSF1 expressed in *Xenopus* oocytes does not bind DNA, but derepression of DNA-binding activity, as well as oligomerization of HSF1, occurs during heat treatment at the same temperature at which hsp gene expression is induced in this organism, suggesting that a conserved *Xenopus* protein(s) plays a role in this regulation. Inactive HSF1 resides in the cytoplasm of human cells; on activation it rapidly translocates to a soluble nuclear fraction, and shortly thereafter it becomes associated with the nuclear pellet. On heat shock, activatable HSF1, which might already have been posttranslationally modified in the unstressed cell, undergoes further modification. These different processes provide multiple points of regulation of hsp gene expression.

When cells experience adverse changes in their environment, they react by increasing the expression of a small number of heat shock or stress protein (hsp) genes. This up-regulation of hsp genes has both transcriptional and posttranscriptional components. Transcriptional regulation is dependent on the presence of, typically, multiple arrays of inverted repeats of the sequence element NGAAN in the promoters of the genes (3, 67). These sequences are binding sites for a group-specific transcription factor activity commonly referred to as heat shock transcription factor (HSF), whose presence, in both unstressed and stressed cells (32, 49, 68), was demonstrated initially in *Drosophila* cells (37, 57, 65) and subsequently also in several other organisms (44; see reference 68 for a review). HSF activity purified from stressed *Drosophila* (37, 57, 66) and other (22, 48) cells was shown to be capable of specifically activating transcription from hsp genes in vitro (22, 37, 57) as well as when microinjected into *Xenopus laevis* oocytes (66).

Two related questions regarding the mechanism of transcriptional activation of hsp genes are presently under intense investigation. The first concerns the nature of the pathway by which stress experienced by cells is converted to a signal for the activation of the transcription-enhancing activity of HSF. Guided by earlier observations demonstrating activation of hsp gene transcription following incorporation of amino acid analogs into newly synthesized polypeptides (27, 31) or treatment with agents causing protein denaturation (17; reference 4 and references therein), more recent studies have demonstrated by microinjection of native and denatured model proteins into *Xenopus* oocytes that activation of transcription of hsp genes is indeed mediated by denatured proteins (4), suggesting protein denaturation as the common denominator of many of the disparate treatments inducing the response. Earlier studies have also suggested that hsp gene expression may be subject to autoregulation (15). In concordance with findings that members of the hsp70 family of proteins are capable of binding to denatured proteins and peptides (18, 36, 38), as well as of associating with nascent polypeptides (8), several recent studies showing down-regulation of the transcriptional stress response following overexpression of hsp70 (52) and the propensity of hsp70 to directly bind HSF (2, 7) have implicated hsp70 as the autoregulatory factor. After the cloning of HSF genes from a variety of organisms (13, 41, 43–45, 51, 63), support for a negative mode of regulation of HSF activity has come from observations of constitutive transcriptional activity of mutated *Saccharomyces cerevisiae* HSF (9, 35) and of constitutive DNA-binding activity of wild-type *Drosophila* and human HSF expressed in bacteria (13, 41, 45). Further evidence for such a regulatory mechanism has been provided by experiments showing that derepression of DNA-binding activity of human HSF can be reproduced in vitro by treatment with heat (33; see reference 13 for analogous experiments with *Drosophila* cells) and with agents affecting protein conformation (34). Surprisingly, considering the divergence of transcriptional mechanisms, quite analogous findings concerning the role of denatured proteins in triggering the stress response (21) and its autoregulation by hsp70 (DnaK) and other hsps (20, 53–56) were made with bacteria.

The second question with which this study is mainly concerned is directed toward the biochemical analysis of molecular events occurring when HSF is converted from an

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inactive to an active transcription factor and the points at which transcription of hsp genes is controlled as a consequence. Two themes have emerged so far: *Saccharomyces cerevisiae* HSF has constitutive DNA-binding activity (49, 68). Regulation occurs at the level of transcriptional activity of the factor (49). In contrast, HSF from *Schizosaccharomyces pombe* (19), tomatoes (44), *Drosophila melanogaster*, mice, and humans (7, 33, 34, 49, 65, 68, 69) is unable to bind to target DNA in unstressed cells. Stress leads to derepression of the DNA-binding capability of these factors. Activated HSF forms homotrimers (39, 50) or, possibly, hexamers (13), and in *Drosophila* cells the activation of HSF, which has an apparent subunit molecular mass of 110 kDa, is accompanied by a conversion of HSF-containing complexes of about 220 kDa to complexes of 690 kDa, suggesting that oligomerization may play a role in the activation process (62). Several studies with mammalian cells (26, 29, 40) suggest that at least in these cells the transformation of inactive HSF to an active transcription factor may involve events in addition to the acquisition of DNA-binding activity, one of them possibly involving phosphorylation of the factor.

Two human HSF genes (HSF1 and HSF2), encoding molecules whose nucleotide sequences are less that 40% identical, have been isolated (41, 45). The significance of this finding is not yet fully understood. To obtain a better understanding of the mechanism of activation of HSF relevant to heat regulation of hsp genes and to define points of regulation, we have raised antibodies to recombinant HSF1 and HSF2 and have monitored the events occurring in response to heat stress.

MATERIALS AND METHODS

Cell culture and preparation of extracts and subcellular fractions. Adherent human HeLa cells were grown in 100-mm dishes in Dulbecco modified Eagle medium–10% fetal calf serum at 37°C under a 5% CO_2 atmosphere. For our experiments we used 60% confluent cultures. Some experiments (indicated in the text) were also carried out with human K562 cells that were grown in RPMI 1640 medium plus 10% fetal calf serum at 37°C under 5% CO_2. Whole-cell extracts were prepared by quick-freezing pellets of phosphate-buffered saline (PBS)-washed cells in liquid nitrogen. The pulverized pellet material was thawed and resuspended in about 2 packed-cell volumes of buffer C (20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES; pH 7.9], 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol [DTT], 0.5 mM phenylmethylsulfonyl fluoride [PMSF], 10 μg of leupeptin per ml, 10 μg of pepstatin per ml, 0.01 U of aprotinin per ml, 25% glycerol), and the concentration of NaCl was adjusted to 0.38 M. After 10 min of incubation on ice, the extract was clarified by centrifugation at 4°C for 15 min at 10,000 × *g*. The lipid material on top of the extract was carefully removed. To prepare subcellular fractions, cells were lysed by a 10-min hypotonic treatment on ice in buffer A (10 mM HEPES [pH 7.9], 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM DTT, 0.5 mM PMSF, 10 μg of leupeptin per ml, 10 μg of pepstatin per ml, 0.01 U of aprotinin per ml) followed by Dounce homogenization (40 strokes with a loose-fitting pestle). The extract was then centrifuged at 4°C for 10 min at 10,000 × *g*. The supernatant solution was used as the cytosolic extract. The pelleted material (nuclei, organelles, and membranes) was washed once with ice-cold buffer A and resuspended in 2 volumes of buffer C. After the concentration of NaCl was adjusted to 0.38 M, the suspension was placed at −70°C for 10 min, thawed slowly on ice, and then incubated for 10 min in ice with intermittent tapping. After a 15-min centrifugation at 10,000 × *g* at 4°C, the supernatant solution representing the soluble nuclear fraction was removed. The pellet was resuspended in an equal volume of 2× sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer, boiled for 10 min, and cleared by centrifugation in a desktop centrifuge for 5 min at room temperature, and the supernatant solution (referred to below as the nuclear pellet fraction) was collected. In the time course experiment that required rapid fractionation of subcellular fractions (see Fig. 7), a different procedure was used, as follows. Cells were removed from the tissue culture dish by careful scraping (95% of the removed cells were viable as judged by trypan blue exclusion), transferred to an Eppendorf tube, and washed with PBS. The cells (1×10^6 to 2×10^6) were then taken up in a small volume of PBS and subjected to heat treatment in a 43°C water bath. At the appropriate time, the culture was removed from the water bath and cells were collected by a brief centrifugation in the cold and resuspended in 150 μl of Nonidet P-40 (NP-40)–low-salt buffer (50 mM Tris-HCl [pH 8.0], 50 mM NaCl, 0.5 mM PMSF, 10 μg of leupeptin per ml, 10 μg of pepstatin per ml, 0.01 U of aprotinin per ml, 1% NP-40). After brief agitation, the lysed cells were centrifuged at 4°C for 1 min at 10,000 × *g*. The lipid layer was discarded, and the supernatant solution containing cytosolic and solubilized membrane proteins was removed and used as the cytosolic fraction. The pellet was resuspended in 150 μl of NP-40–high-salt buffer (same as NP-40–low-salt buffer except for 450 mM NaCl), agitated briefly, and incubated on ice for 10 min. After centrifugation as above, the supernatant fraction (the soluble nuclear fraction) was removed and the pellet was solubilized in 100 μl of 2× SDS-PAGE loading buffer; this is referred to as the nuclear pellet fraction.

HSF1 cDNA, anti-HSF1, and HSF antibodies. A clone containing the HSF1-coding region was isolated by screening a human HeLa S3 cDNA library in lambda gt10 (Clontech) with two radiolabeled oligonucleotide probes made from nucleotide sequence information provided by C. Wu. The HSF1 cDNA region was recloned in Bluescript vector pVZ-1, in the direction permitting transcription by T3 RNA polymerase (pHSF1A, used in in vitro translations), or in pGEM-3Zf(+), in the direction permitting transcription by SP6 RNA polymerase (pHSF1B, used in oocyte injection experiments). Sequence analysis and extensive mapping indicated that our isolate is essentially identical to the HSF1 gene described by Rabindran et al. (41). To construct pMAL-HSF1, an *Xmn*I-*Eco*RI fragment including sequences coding for HSF1 residues 44 to 529 was isolated from pHSF1A DNA, an *Eco*RI linker was added to the *Xmn*I site, and the fragment was inserted in the *Eco*RI site of pMAL-cRI DNA (New England BioLabs). Plasmid HSF2-1 containing human HSF2 cDNA was obtained from R. Kingston (45). A *Sal*I linker was inserted at the unique *Sma*I site in the polylinker segment downstream for the HSF2 cDNA to give pHSF2-Sma. To construct pMAL-HSF2, a *Hpa*I-*Sal*I fragment from pHSF2-Sma including the entire HSF2-coding region was ligated to *Eco*RI (filled)-*Sal*I-digested pMAL-cRI DNA. MalE-HSF fusion polypeptides were expressed in *Escherichia coli* TB1, the host recommended by the supplier of the c-MAL expression system, and separated by SDS-PAGE, and regions containing fusion polypeptides were excised and used to raise antibodies in rabbits by a standard procedure (24). In vitro transcriptions (Stratagene) and

translation in rabbit reticulocyte lysate (Promega) were performed with commercially available kits as specified by the manufacturers.

Electrophoretic separation, transfer, and detection of polypeptides and peptides. Protein samples, up to 30 μg per lane, were separated by standard SDS-PAGE (3% stacking gel, 10% separation gel). Prestained molecular weight standards (high range; Bethesda Research Laboratories) were routinely run in parallel. To generate peptides, gel slices containing regions of interest were incubated in 0.2% CNBr for 30 min at 37°C or placed in the stacking gel of the second-dimension gel and digested in situ with V8 proteinase (0.016 μg/cm width of gel slice) for 45 min. Cleavage products were then separated by SDS-PAGE (3% stacking gel, 16% separation gel). Polypeptides or peptides were transferred electrophoretically at 4°C in 50 mM Tris–380 mM glycine–0.1% SDS–20% methanol onto an unsupported nitrocellulose membrane (BA-S85; Schleicher & Schuell) at 12 V for 4 h from 10% gels and at 6 V for 18 h from 16% gels. For immunodetection, membranes were washed for 10 min in TBS (25 mM Tris-HCl [pH 8.0], 0.136 M NaCl, 2.7 mM KCl), air dried, and blocked for 1 h at room temperature in TBS containing 4% nonfat dry milk (TN). The membranes were then incubated overnight with gentle rocking at 4°C with antiserum (usually at a 2,000-fold dilution) in TN containing 0.1% Tween 20. Filters were washed extensively in TBS plus 0.05% Triton X-100, reblocked as before, and incubated for 2 h at room temperature with a second antibody (alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G) at a 2,000-fold dilution in TN containing 0.1% Tween. After several washes with TBS plus 0.05% Triton X-100, blots were developed in 100 mM Tris-HCl (pH 9.0)–100 mM NaCl–5 mM $MgCl_2$ by using the BCIP/NBT color detection system (Boehringer Mannheim).

Limiting-pore-size gels, gel filtration, and cross-linking. Extract samples in DNA-binding reactions (with or without HSE DNA [see gel shift assays]) were applied to a native gradient PAGE gel (4% stacking gel, 5 to 20% separation gel) in TGE (40 mM Tris, 200 mM glycine, 2.4 mM EDTA) and separated at constant voltage (13 V/cm) for 20 h (at 4°C). Native molecular mass markers used were apoferritin (880 and 440 kDa), urease (480 and 240 kDa), β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), and hemoglobin (64 kDa). Gels containing radiolabeled HSE DNA were dried and autoradiographed. For immunochemical detection, gels were transferred as above except for a preequilibration step of 30 min at 68°C in SDS-PAGE running buffer. After the transfer, lanes containing molecular mass markers were stained with amido black.

For gel filtration, a 45- by 1.5-cm Sephacryl S300 HR column, equilibrated in 25 mM HEPES (pH 7.9)–0.1 M KCl–1.5 mM $MgCl_2$–0.2 mM EDTA–0.5 mM DTT–0.5 mM PMSF–10% glycerol, was calibrated with dextran blue (2,000 kDa), thyroglobulin (669 kDa), β-amylase (200 kDa), and hemoglobin (64 kDa). Cytosolic extract (3.5 mg of protein per ml) from unheated HeLa cells (0.4 ml) and nuclear extract (3.5 mg of protein per ml) from heat-treated cells (0.4 ml) were mixed and, along with 1.2 mg of dextran blue and 2 mg of hemoglobin, applied to the column. The flow rate was 6 ml/h, and 1-ml fractions were collected. Fractions were analyzed by gel shift assay with the HSE probe (30-μl aliquots) and anti-HSF1 Western immunoblot (40-μl aliquots). For the cross-linking experiment shown, 15-μl aliquots of cytoplasmic and nuclear extract were incubated for 10 min at room temperature with 2, 0.6, and 0.02 mM glutaraldehyde. Reactions were stopped by the addition of 1 μl of 2 M lysine and electrophoresed on a 6 to 12% polyacrylamide SDS-PAGE gradient gel. Immunoblotting was performed as described above.

Gel shift assays. Extract (2 to 4 μl) was added to 10 μl of 2× Kingston buffer (2× buffer is 24 mM HEPES [pH 7.9], 120 mM KCl, 4 mM $MgCl_2$, 0.24 mM EDTA, 0.6 mM PMSF, 0.6 mM DTT, and 24% glycerol [32]) and 1 μl of a mixture of sonicated salmon sperm DNA (1 mg/ml) and poly(dI-dC) poly(dI-dC) (1 mg/ml), and the volume was adjusted to 19 μl with water. After 15 min of preincubation on ice, 1 μl of ^{32}P-labeled probe (approximately 10,000 cpm) was added, and incubation was continued for 15 min at room temperature. Binding reactions were loaded on a 4.5% native PAGE gel in TGE, and electrophoresis was carried out for 4 h at 4°C. The gel was dried and exposed for autoradiography. Polynucleotide kinase-labeled, double-stranded HSE (22), NF-κB (5′-GATCGAGGGGACTTTCCCTAGC) or cyclic AMP response element-binding site (5′-GATTGGCTGACG TCAGAGAGCT) oligonucleotide was used as the probe. For desoxycholate activation of NF-κB, see reference 6.

Expression in *Xenopus* oocytes. *X. laevis* females were purchased from Xenopus I, and oocytes were prepared as previously described (60). HSF1 cDNA was linearized and transcribed in vitro by an SP6 RNA polymerase-based reaction. Batches of stage VI oocytes were microinjected in the cytoplasm with aliquots of transcription reactions. After 1 or 2 days of incubation at 20°C and heat treatment at 37°C when applicable, extracts from sets of five oocytes were prepared as described previously (13).

RESULTS

Anti-HSF1 and anti-HSF2 antibodies and the presence of HSF1 in lysates of human cells. Human HSF1 (residues 44 to 529) and HSF2 (entire coding region) were expressed in *E. coli* as MalE fusion proteins. Synthesis of full-length fusion proteins (with expected sizes of 95 and 100 kDa, respectively) was monitored by Western blot with antibody against MalE protein (Fig. 1A). Overexpression was efficient, and fusion proteins were readily detected by Coomassie blue staining (Fig. 1B). Gel slices containing fusion proteins were used to immunize rabbits by following a standard protocol. Antisera tested positive in Western blots of bacterially expressed MalE-HSF1 and HSF2 fusion proteins. To further characterize the antisera, HSF1 and HSF2 cDNAs were expressed by in vitro transcription with T3 or T7 RNA polymerase, respectively, and translation in rabbit reticulocyte lysate. The in vitro-translated HSF1 and HSF2 proteins were specifically recognized by the anti-HSF1 and anti-HSF2 antisera, respectively, and no cross-reactivity was observed (Fig. 1C; judging from the relative intensities of the HSF1 and HSF2 signals, the anti-HSF2 antibody has a lower titer than the anti-HSF1 antibody). To find whether the antisera would recognize HSF1 and HSF2 in lysates of human cells (this experiment was performed with both HeLa and K562 human cells, and virtually identical results were obtained with the two cell types), aliquots of whole-cell extracts from unstressed (37°C) or heat-stressed (incubated at 39 to 44°C) cells were incubated with a ^{32}P-labeled oligonucleotide probe containing the HSF recognition sequence (HSE) and either preimmune or anti-HSF sera, and HSE-containing complexes were analyzed on an 4.5% neutral gel (Fig. 2A). Heat-activated HSE DNA-binding activity was retarded specifically and quantitatively by anti-HSF1 but not by anti-HSF2 or control serum, suggesting that most of the HSF activity in these extracts was produced by HSF1.

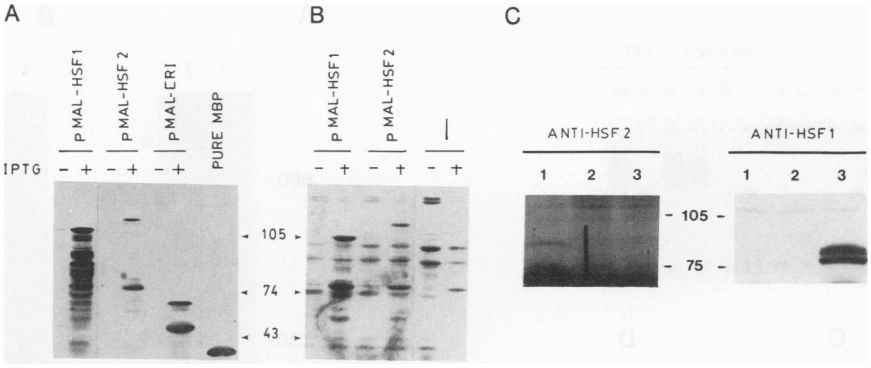

FIG. 1. Bacterial expression of MalE-HSF fusion polypeptides and characterization of anti-HSF antibodies. Panels A and B show identification of MalE-HSF fusion polypeptides in lysates of isopropyl-β-D-thiogalactopyranoside (IPTG)-induced and uninduced *E. coli* TB1 cells transformed with the constructs indicated on SDS-PAGE (10% polyacrylamide gels). (A) Western blot developed with anti-maltose-binding protein antibody (New England Biolabs). Purified maltose-binding protein was applied to the last lane. (B) Detection of fusion polypeptides by Coomassie blue staining. (C) Detection of HSF1 and HSF2 by polyclonal anti-HSF1 and anti-HSF2 antibodies. In vitro translations containing HSF2 RNA (lanes 1), no RNA (lanes 2), or HSF1 RNA (lanes 3) were solubilized in SDS-PAGE loading buffer and electrophoresed by SDS-PAGE (10% polyacrylamide). After blotting of parallel sets of samples, proteins were detected with the antisera indicated. Analysis of parallel in vitro translations performed in the presence of [^{35}S]methionine indicated that similar amounts of HSF1 and HSF2 polypeptides were synthesized in the reactions. Positions and sizes (in kilodaltons) of molecular mass markers are indicated between the panels.

Anti-HSF1 antibody detected a group of polypeptides in the 75- to 80-kDa region (Fig. 2B), where HSF was expected to be situated (22). In addition, two other polypeptides, one of about 110 kDa and the other, a minor species, of 57 kDa were recognized by the antibody. To define the similarity of the cross-reacting polypeptides, we carried out proteinase V8 digestion studies (Fig. 2C). Results indicated that the 75- to 80-kDa and the 110-kDa polypeptides are not closely related. However, a fragment pattern closely resembling that of 75- to 80-kDa polypeptides was observed for the 57-kDa polypeptide. This polypeptide may represent a precursor of the 75- to 80-kDa forms of HSF1 (the molecular mass of HSF1 calculated from the nucleotide sequence is 57 kDa [41]; see also below). Anti-HSF2 antibody did not recognize any polypeptide in the HeLa extract (Fig. 2B). Thus, either HSF2 is not made in HeLa cells or the protein is present at a concentration undetectable by the anti-HSF2 antibody. To confirm that the 75- to 80-kDa polypeptides detected by anti-HSF1 antibody corresponded to the presence of HSF1 in several different modified forms, HSF1 cDNA was transcribed in vitro and microinjected into *Xenopus* oocytes, and extracts from uninjected and injected oocytes were analyzed by Western blot with the anti-HSF1 antibody (Fig. 2D). The 110-kDa polypeptide was present both in uninjected and injected oocytes and therefore represents a ubiquitous, conserved polypeptide unrelated to HSF1. Injected but not uninjected oocytes produced 75- to 80-kDa polypeptides, identifying these polypeptides as the products of the HSF1 gene. No evidence for the presence in the oocytes of the 57-kDa polypeptide was obtained.

Change in oligomerization of HSF1 on heat treatment. To test the state of oligomerization of HSF1, we fractionated whole-cell extracts from heat-treated and unheated HeLa cells on a limiting-pore-size gel (5). After electrophoresis, proteins were blotted onto nitrocellulose and probed with anti-HSF1 antibody (Fig. 3A). In the lane of the blot containing extract from unstressed cells (Fig. 3A, lane 1), complexes of about 200 kDa were detected by the anti-HSF1 antibody. In some experiments, additional HSF1-containing complexes in the 400-kDa range were observed (data not shown). On heat treatment, the 200-kDa complexes (as well as the 400-kDa complexes where seen) disappeared and new complexes in the 700-kDa range were formed (lane 2). The 700-kDa complexes were stabilized in some experiments when extract was preincubated with HSE DNA (data not shown). To test the DNA-binding activity of the different complexes, we incubated extracts with radiolabeled HSE oligonucleotide probe and then fractionated them on a parallel gel. Radioactive protein-HSE DNA complexes were detected by autoradiography of the dried gel (Fig. 3B). Radiolabeled 700-kDa HSF1-HSE DNA complexes were formed in extract from heat-treated cells, but no HSF1-HSE complexes were observed in reactions with extract from unheated cells. Thus, in heat-treated cells HSF1 is in the form of 700-kDa complexes capable of binding HSE DNA, whereas in unstressed cells HSF1 is in 200-kDa (and 400-kDa) complexes that may be unable to bind HSE DNA.

Since it analyzed DNA-binding reactions prior to fractionation, the above experiment was not capable of directly proving that the 200-kDa (400-kDa) complexes were inactive in DNA binding. It was conceivable that gel electrophoresis disrupted larger, inactive complexes present in extract, resulting in the appearance of 200-kDa (400-kDa) core complexes that might well have DNA-binding ability. To test this possibility and to corroborate other aspects of the above experiment, extracts from heat-treated and unheated HeLa cells were mixed and fractionated on a Sephacryl S300 HR gel filtration column. Analysis of fractions by anti-HSF1 Western blot detected HSF1 in complexes with apparent molecular masses of about 700 kDa and above and in those of 200 kDa (Fig. 4A). The origin of HSF1 in the two types of complexes could be readily ascertained on the basis of the mobility difference of HSF1 in extracts from heat-treated and unheated cells (compare extract samples on the right). Parallel gel shift assays (Fig. 4B) with the HSE DNA probe indicated that only the 700-kDa (but not the 200-kDa) HSF1 complexes were capable of DNA binding, eliminating the above interpretation of the previous experiment. Since size estimation of complexes by limiting-pore-size electrophoresis or gel filtration may be influenced to a considerable extent

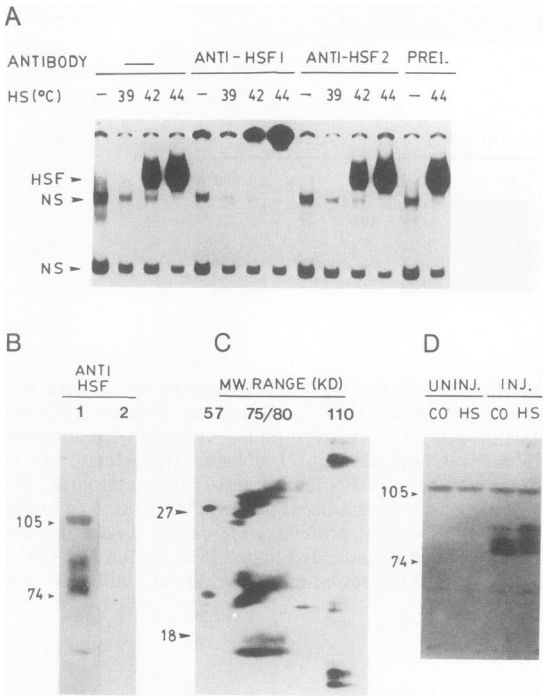

FIG. 2. Detection of HSF1 in human cells and *Xenopus* oocytes. (A) HSE DNA-binding assays were performed with whole-cell extracts prepared from human cells (K562 cells in the particular experiment shown) after exposure of the cells to the indicated temperatures for 1 h. At the end of the binding reactions, 1 μl of anti-HSF1, anti-HSF2, or preimmune serum (PREI.) was added to the reactions; after 20 min of further incubation at 4°C, the reactions were subjected to SDS-PAGE. Gels were dried and autoradiographed. The positions of HSF-HSE DNA complex (HSF) and of nonspecific complexes (NS) are indicated. Unbound probe DNA was run out of the gel in this experiment as well as in all other DNA-binding experiments shown in this paper. (B) Western blot of whole-cell extract from unheated HeLa cells (30 μg per lane) developed with anti-HSF1 (lane 1) or anti-HSF2 (lane 2) antibody. The positions and sizes (in kilodaltons) of prestained molecular mass markers are indicated on the left of each of panels B to D. (C) Anti-HSF1 Western blot of partial V8 proteinase digests of gel-isolated protein fractions. Regions containing the 110, 75- to 80-, and 57-kDa polypeptides were excised from preparative SDS-PAGE gels (10% polyacrylamide) of whole-cell extracts from unheated HeLa cells. The excised gel pieces were placed on top of a second SDS-PAGE gel (3% stacking gel, 16% separation gel) with an overlay of V8 proteinase (0.016 μg per cm of lane width). Following partial digestion, fragments were separated, blotted onto nitrocellulose, and detected with anti-HSF1 antibody. (D) Anti-HSF1 Western blot with whole-cell lysates from uninjected (UNINJ.) or HSF1 RNA-injected (INJ.) *Xenopus* oocytes. Oocytes (in groups of five), incubated for 1 day after injection at 20°C, were either kept at this temperature (lanes CO) or heat treated at 37°C for 45 min (lanes HS) immediately prior to extract preparation. See Materials and Methods for details of microinjection, extract preparation, DNA-binding assays, and Western blots.

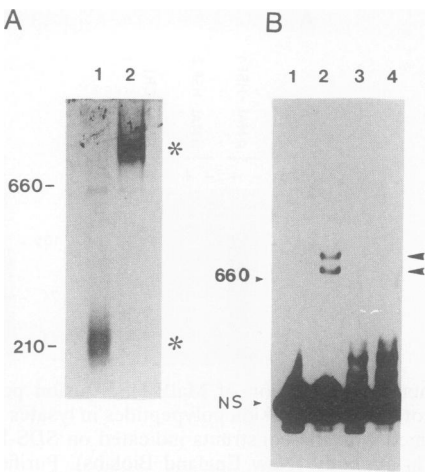

FIG. 3. Oligomerization states of HSF1 assessed by electrophoresis on native limiting-pore-size gels. (A) Anti-HSF1 Western blot of whole-cell extracts in binding buffer from heat-treated (1-h heat treatment at 43°C; lane 2) or unheated (lane 1) HeLa cells separated on a PAGE gel (5 to 20% polyacrylamide). The 700- and 200-kDa HSF1 complexes are indicated by asterisks, and molecular masses (in kilodaltons) calculated from a standard curve are indicated on the left. (B) DNA-binding reactions with whole-cell extracts from heat-treated (lanes 2 and 4) or untreated (lanes 1 and 3) HeLa cells containing radiolabeled HSE (lanes 1 and 2) or cyclic AMP-binding protein (lanes 3 and 4) DNA probes were separated on a similar gel that was dried and analyzed by autoradiography. Arrowheads indicate specific HSF-HSE DNA complexes. NS, nonspecific complexes.

by the shapes of the complexes analyzed, we resorted to cross-linking assays to characterize the oligomeric states of HSF1. (Indeed, HSF1 from heat-treated cells appeared to be considerably smaller in glycerol gradient centrifugation [data not shown] than in limiting-pore-size gel electrophoresis or gel filtration. This may suggest that HSF1 may have a rod-like structure.) Extracts were incubated with different concentrations of glutaraldehyde. Proteins were separated by SDS-PAGE, and HSF1 was detected by anti-HSF1 Western blot (Fig. 4C). At a high concentration of cross-linker, HSF1 in extract from heat-treated cells was quantitatively cross-linked and migrated at about 280 kDa, close to the expected molecular mass of HSF1 trimers. A major cross-linked species of 165 kDa, presumably representing HSF1 dimers, was found at lower concentrations of the cross-linker. HSF1 in extract from unheated cells was not cross-linked, suggesting that it was in monomeric form. Analogous results were obtained with gel filtration fractions containing isolated 700- and 200-kDa HSF1 complexes, respectively, and in experiments with several other cross-linkers (not shown).

HSF1 may undergo two subsequent modification events. The calculated molecular mass of HSF1 is 57 kDa (41). HSF1 in unstressed HeLa cells as well as when synthesized in *Xenopus* oocytes migrates on SDS-PAGE as a group of bands with apparent molecular masses of 75 to 80 kDa, suggesting that the factor may be undergoing posttranslational modification in unstressed cells. On heat treatment, the apparent molecular mass of the factor is increased further to 80 to 85 kDa (Fig. 5A). To provide evidence in addition to their antigenic relatedness that the 80- to 85-kDa polypeptides are derived from the 75- to 80-kDa forms of HSF1, the polypeptides isolated from the appropriate regions in an SDS-PAGE gel were subjected to CNBr cleavage and polypeptide fragments were analyzed by Western blot with anti-HSF1 antibody (Fig. 5B). Closely similar patterns of cleavage products were observed, confirming the relationship between the proteins. The occurrence of posttranslational modification was further documented by immunoprecipitation of HSF1 from extracts prepared from heat-treated and untreated cells. Precipitated HSF1 was then separated

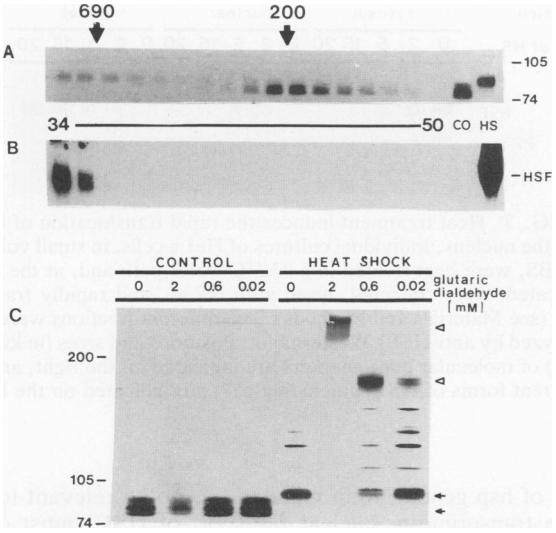

FIG. 4. Gel filtration of HSF1 complexes and analysis of complexes by cross-linking. Nuclear extract fraction from heat-treated (HS) and cytosolic extract fraction from unheated (CO) HeLa cells were mixed in a 1:1 ratio and fractionated on a Sephacryl S300 HR column. The column had been precalibrated with mixtures of native molecular mass markers (see Materials and Methods). Fractions in which marker proteins peaked are indicated for two of the marker proteins by the arrows above the figure. (A) Anti-HSF1 Western blot of fractions 34 to 50. Aliquots of extract fractions (CO, HS) were also tested. Positions and sizes (in kilodaltons) of molecular mass markers are on the right. (B) Gel shift assay of the same fractions with the HSE oligonucleotide probe. Only the region containing specific HSF-HSE DNA complexes is shown. (C) SDS-PAGE analysis of the nuclear extract fraction from heat-treated HeLa cells (HEAT SHOCK) and the cytosolic extract fraction from unheated HeLa cells (CONTROL), following incubation without or with glutaraldehyde at different concentrations. Proteins were blotted to nitrocellulose, and HSF1 was detected by anti-HSF1 antibody. Positions and sizes (in kilodaltons) of molecular mass markers are on the left. The solid arrows indicate the positions of the 75- to 80- and 80- to 85-kDa forms of HSF1, and the open arrowheads point to the major products of HSF1 cross-linking.

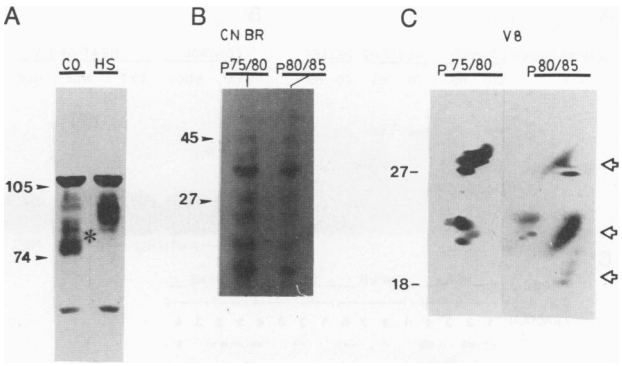

FIG. 5. Posttranslational modifications of HSF1. (A) Anti-HSF1 Western blot of whole-cell extract (30 μg) from unheated (lane CO) or heat-treated (lane HS; 43°C for 1 h) HeLa cells. HSF1 (75- to 80-kDa forms) is indicated by an asterisk. (B) Whole-cell extracts from heat-treated and unheated HeLa cells were fractionated on a preparative SDS-PAGE gel (10% polyacrylamide), and the 75- to 80-kDa (from lanes with extract from unheated cells) and the 80- to 85-kDa (from lanes with extract from heat-treated cells) regions were excised and treated with CNBr. Polypeptide fragments were run out on an SDS-PAGE gel (16% polyacrylamide), blotted onto nitrocellulose, and detected with anti-HSF1 antibody. (C) Anti-HSF1 Western blot of V8 proteinase fragments of the 75- to 80-kDa and 80- to 85-kDa forms of HSF1. The 75- to 80-kDa HSF1 forms were immunoprecipitated from a cytosolic extract fraction from unheated HeLa cells, and the 80- to 85-kDa forms were immunoprecipitated from a nuclear extract fraction from heat-treated HeLa cells. Immunoprecipitated HSF1 forms were electrophoresed on a preparative SDS-PAGE gel (10% polyacrylamide), appropriate regions were excised from the gel and placed on top of an SDS-PAGE gel (16% polyacrylamide), and the polypeptides were partially digested with proteinase V8 in situ. Following electrophoresis, fragments were blotted onto nitrocellulose and detected with anti-HSF1 antibody. The open arrows point to characteristic sets of fragments. Positions and sizes (in kilodaltons) of prestained molecular mass markers are indicated on the left side of each panel.

by SDS-PAGE (10% polyacrylamide), and regions containing HSF1 were excised, rotated clockwise by 90°, and placed on top of a second SDS-PAGE gel (16% polyacrylamide). HSF1 was partially digested in situ with V8 proteinase, and the resulting polypeptide fragments were resolved by electrophoresis in the second dimension. Fragments were detected by anti-HSF1 antibody following blotting (Fig. 5C). Similar nested sets of fragments were generated by digestion of HSF1 from heat-treated (80- to 85-kDa forms) and unheated (75- to 80-kDa forms) cells. Careful examination reveals heat-induced changes in the positions of individual fragments: for example, the two major fragments in the lower set of p75/80 fragments (Fig. 5C [left panel, and on the left of the right panel]) are depleted as a consequence of heat treatment, and they appear in a higher-molecular-mass region in the p80/85 digest.

Heat-induced nuclear import of HSF1. To localize HSF1 in heat-stressed and unstressed cells, we prepared extracts by hypotonic lysis of cells. Cytosolic fractions were obtained after removal of nuclei by centrifugation, and soluble nuclear fractions were obtained by extraction of nuclei with 0.38 M NaCl. Fractions were analyzed by anti-HSF1 Western blot either directly (Fig. 6A) or following immunoprecipitation with anti-HSF1 antibody (Fig. 6B). The 75- to 80-kDa forms of HSF1 were present exclusively in the cytosolic fraction of unstressed cells. On heat treatment these forms were converted to the larger forms of 80 to 85 kDa that resided in the soluble nuclear fraction. The different fractions were also tested for HSE DNA-binding activity (Fig. 6C). As expected, no binding activity was found in the cytosol of unstressed cells (lanes 1), and only little activity was found in the soluble nuclear fraction (lanes 2). In extracts from heat-treated cells, essentially all DNA-binding activity resided in the soluble nuclear fraction (lanes 4) and only minor amounts were observed in the cytosolic fraction (lanes 3). To determine whether a significant exchange of proteins between cytosolic and nuclear fractions had occurred in the course of their preparation, we monitored the distribution of enhancer-binding protein NF-κB. This protein is known to be present in a non-DNA-binding form in the cytosolic fraction of HeLa cells but acquires DNA-binding activity and relocates to the nucleus following stimulation by phorbol esters (6). The inactive, cytoplasmic form of NF-κB can be converted in vitro to the DNA-binding form by incubation with desoxycholate and NP-40 (6). Aliquots of cytosolic and nuclear fractions from heat-treated and untreated cells were analyzed in DNA-binding reactions in the presence or absence of the above detergents by using an end-labeled, double-stranded oligonucleotide containing a consensus NF-κB-binding site as the probe (Fig. 6C, NFκB lanes). NF-κB–

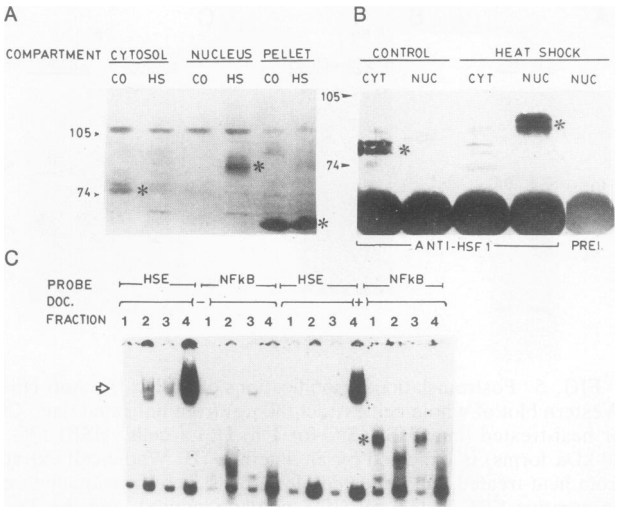

FIG. 6. Subcellular localization of HSF1. Cytosolic, soluble nuclear, and nuclear pellet (resuspended to 70% of the volume of the other fractions) fractions prepared from heat-treated (HS; 1 h at 43°C) or unheated (CO) HeLa cells lysed by hypotonic shock and Dounce homogenization as described in Materials and Methods were analyzed by anti-HSF1 Western blot (A) or by immunoprecipitation with anti-HSF1 antibody and analysis of precipitated proteins by anti-HSF1 Western blot (B). Different HSF1 forms (including the putative 57-kDa HSF1 polypeptide in the pellet fraction) are identified by asterisks, and positions and sizes (in kilodaltons) of molecular mass markers are indicated on the left side of each panel. PREI: preimmune serum. The abundant protein at the bottom of each lane in panel B represents immunoglobulin heavy chains at 55 kDa. (C) DNA-binding assays were performed with cytosolic (lanes 1 and 3) and soluble nuclear (lanes 2 and 4) fractions from unheated (lanes 1 and 2) or heat-treated (lanes 3 and 4) HeLa cells. After the addition of the oligonucleotide probes (HSE; NF-κB), binding reactions were incubated in the presence (lanes +) or absence (lanes −) of 0.2% desoxycholate (DOC.) and 0.8% NP-40 for 15 min at room temperature. After native PAGE, the gel was dried and autoradiographed. The position of the specific HSF1-HSE DNA complex is indicated by the arrow, and that of the NF-κB–DNA complex is indicated by the asterisk.

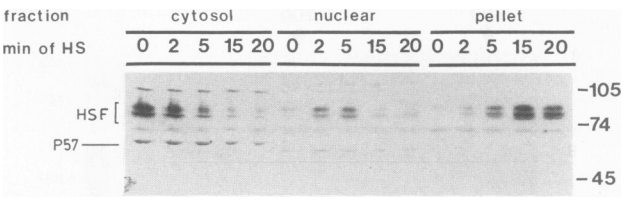

FIG. 7. Heat treatment induces the rapid translocation of HSF1 into the nucleus. Individual cultures of HeLa cells, in small volumes of PBS, were heat treated in a 43.5°C water bath and, at the times indicated, were collected, lysed with NP-40, and rapidly fractionated (see Materials and Methods). The different fractions were then analyzed by anti-HSF1 Western blot. Positions and sizes (in kilodaltons) of molecular mass markers are indicated on the right, and the different forms of HSF1 (including p57) are indicated on the left.

DNA complex was identified in the gel by comparison of untreated and detergent-treated fractions. Detergent-activatable NF-κB activity was found to be quantitatively located in cytosolic fractions from heat-treated (lanes 3) and unheated (lanes 1) cells, indicating that our fractionation procedure properly separated cytosolic and nuclear proteins.

To complete the survey, the nuclear pellet fractions, resulting from the clearance of nuclear fractions by centrifugation, that contain membrane fragments and chromatin were also analyzed by Western blot (Fig. 6A, pellet [HS plus CO]). Anti-HSF1 antibody detected a 57-kDa protein. Proteinase V8 analysis (data not shown) revealed the same fragment pattern as was found for the minor 57-kDa polypeptide present in whole-cell extract (Fig. 2C). This polypeptide may represent an unmodified or only slightly modified form of HSF1. Although this polypeptide was not present in cytosolic fractions from hypotonically lysed cells (Fig. 6A), lysis of cells by NP-40 instead of by hypotonic shock resulted in its quantitative appearance in the cytosolic fraction and absence from the pellet fraction (Fig. 7), suggesting that it may normally be associated with membranes.

On heat treatment HSF1 translocates rapidly to the soluble nuclear fraction and subsequently to the nuclear pellet fraction. Heat treatment is known to lead to increased transcription of hsp genes within minutes (1). To be relevant to hsp gene transcription, nuclear transport of HSF1 must occur within the same time frame. In the experiment in Fig. 7 the time course of the nuclear import of HSF1 was determined. Cells were harvested either immediately before or at different times after the onset of heat treatment at 43.5°C; events were frozen by rapid NP-40 lysis and separation of cytosolic, soluble nuclear, and nuclear pellet fractions; and fractions were analyzed by Western blot developed with anti-HSF1 antibody. At 2 min after the onset of heat treatment the quantity of HSF1 in the cytosolic fraction was already diminished and the protein had begun to appear in the soluble nuclear fraction. No HSF1 remained in the cytosolic fraction at 15 min. By 5 min HSF1 became abundant in the nuclear pellet fraction, presumably reflecting association of the protein with its DNA targets. At 15 min most of the HSF1 had disappeared from the soluble nuclear fraction and was found in the pellet fraction. These results indicate that HSF is rapidly transported to the nucleus upon heat treatment with a time course consistent with the rapid onset of hsp gene transcription. If it reflected binding to target DNA, the association of the 80- to 85-kDa forms of HSF1 with the nuclear pellet might be reversible. This appears to be the case since we found only a small fraction of these polypeptides in the nuclear pellet when extract was prepared by the time-consuming, standard fractionation procedure (Fig. 6A). No translocation of the putative 57-kDa HSF1 precursor polypeptide was observed.

Heat regulation of human HSF1 in *Xenopus* oocytes. To test whether some of the events accompanying the activation of HSF1 in HeLa cells also occur when human HSF1 is expressed in *Xenopus* oocytes, which would provide us with a convenient experimental system to correlate these events with molecular features of HSF1, we prepared HSF1 RNA by in vitro transcription in a reaction with SP6 RNA polymerase. Following microinjection of this RNA into oocytes and 1 or 2 days of incubation at 20°C, extract was prepared and analyzed by Western blot with anti-HSF1 antibody. Multiple bands in the 75- to 80-kDa area were detected, suggesting that HSF1 synthesized in oocytes was modified in a similar fashion as in unstressed HeLa cells (Fig. 2D). Next, the DNA-binding properties of HSF1 made in oocytes were assessed by gel shift assays with the HSE oligonucleotide probe (Fig. 8B, lane INJ., 0 MIN HS). No specific binding to HSE DNA could be detected. Hence, in contrast to bacterial cells (41), oocytes were capable of synthesizing and maintaining human HSF1 in an inactive form.

Transcription of hsp genes injected into oocytes has been

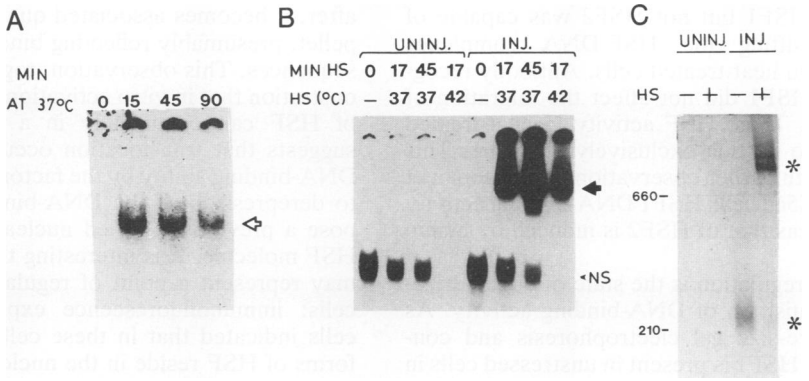

FIG. 8. Human HSF1 expressed in *Xenopus* oocytes. (A) Endogenous HSF. Groups of five uninjected oocytes were either heat treated at 37°C for the times indicated or not heat treated (lane 0). Gel shift assays to detect the weak oocyte HSF activity (open arrow) in whole-cell lysates with radiolabeled HSE probe were carried out in the presence of a fivefold-reduced concentration of nonspecific competitor DNA. This gel has been exposed 10 times longer than that in panel B. (B) HSE binding assays with whole-cell extracts from uninjected (UNINJ.) or HSF1 RNA-injected (INJ.) oocytes exposed to different levels of heat stress. The position of the specific HSF1-HSE DNA complex is indicated by the solid arrow. (C) Analysis of HSF1 complexes by limiting-pore-size gel electrophoresis. Whole-cell extracts from HSF1 RNA-injected (INJ.) and uninjected (UNINJ.) oocytes that had been heat treated at 37°C for 45 min (lanes +) or kept at 20°C (lanes –) were separated as in Fig. 3, and HSF1 complexes were detected by anti-HSF1 Western blot. Positions of complexes are marked with asterisks, and molecular masses (in kilodaltons) were calculated from a standard curve and are indicated on the left.

shown previously to be heat activated at 36 to 37°C (60). In close agreement with this observation, endogenous HSF is converted from a non-DNA-binding to a DNA-binding form at the same temperature (Fig. 8A) (68). The endogenous HSE DNA-binding activity is relatively weak even when assayed in the presence of a reduced amount of nonspecific competitor DNA (Fig. 8A; the autoradiographic exposure was 10 times longer than that in Fig. 8B) and is suppressed further when the standard amount of nonspecific competitor is used in binding reactions (Fig. 8B, UNINJ.). To find whether heat treatment leads to derepression of the DNA-binding activity of human HSF1 in oocytes, HSF1 RNA-injected and uninjected oocytes were heat treated at 37°C and an HSE DNA-binding assay was performed on extracts prepared immediately following heat treatment (Fig. 8B). High levels of HSE-binding activity were recorded in the injected but not in the uninjected oocytes, indicating that the DNA-binding activity of human HSF1 is heat regulated in the oocytes at the heat response temperature characteristic for *Xenopus* oocytes but not for human cells. No further increase in HSE DNA-binding activity occurred when oocytes were heat treated at 42°C, the heat shock temperature of human cells.

If the heat-induced changes in oligomerization of HSF1 observed in HeLa cells were essential for the acquisition of DNA-binding ability of HSF1, this event should also occur in the oocytes. Extracts from HSF1 RNA-injected (Fig. 8C, INJ.) and uninjected (Fig. 8C, UNINJ.), 37°C-heat-treated (lanes +) or untreated (lanes –) oocytes were subjected to electrophoresis on a limiting-pore-size gel, and the blot of the gel was probed with anti-HSF1 antibody. HSF1 complexes of about 200 kDa (and of 400 kDa in some experiments) were present in extract from unheated injected but not unheated uninjected oocytes. On heat treatment, new HSF1 complexes at about 700 kDa were formed, and a concurrent reduction in the intensity of the smaller HSF1 complexes was observed (Fig. 8C, lane INJ., +). These results are strikingly similar to those obtained with HeLa cells and suggest that the heat-induced change in oligomerization of HSF1 may be linked to the acquisition of DNA-binding activity.

DISCUSSION

Consistent with the many divergent roles of hsps in unstressed as well as in stressed cells, which include various aspects of transport between intracellular compartments, assembly or dissociation of protein complexes (8, 11, 12, 14, 23, 25, 58), regulation of key proteins (10, 42, 46), and rescue of denaturing proteins (8, 28, 30, 36, 38), genes encoding different hsps as well as those encoding related hsps are subject to precise and rapid regulation that can occur at both transcriptional and posttranscriptional levels. For example, in human cells hsp73 is synthesized constitutively in stressed and unstressed cells (61), whereas expression of hsp72 encoded by the hsp70A gene (64) is increased severalfold by heat treatment. The hsp70B gene (59) encoding yet another member of the hsp70 family of proteins is entirely silent in nonstressed cells but highly active in stressed cells. Experiments with reporter genes driven by the promoter of this gene showed a 500- to 1,200-fold differential between expression levels in heat-shocked and unheated cells (16). It follows from these considerations that the activities of regulatory factors (HSF being the only factor of this kind known to date) involved in the control of these genes must be tightly regulated. One obvious way to ensure such tight regulation would be to control the transcriptional capability of HSF at multiple levels. Indeed, several previous studies with mammalian cells have provided evidence for the existence of multiple layers of control (26, 29, 40) but have stopped short of establishing individual points at which control is exerted except for the regulation of DNA-binding activity of HSF (7, 33, 34, 49, 68). Given the considerable degree of evolutionary conservation of certain regions in the HSF sequences, one would reasonably expect that at least some of the mechanisms of regulation of HSF activity that have already been established in lower organisms, especially in *S. cerevisiae* and *D. melanogaster*, may also be used by mammalian cells, and comparisons should be useful.

By monitoring the changes in HSF occurring upon heat treatment of human cells, we have identified several levels at which HSF activity may be regulated. The first is differential activation of HSF1 and HSF2. In gel shift experiments,

antibody recognizing HSF1 but not HSF2 was capable of quantitatively supershifting the HSF-DNA complexes formed by extracts from heat-treated cells. Antibody recognizing HSF2 but not HSF1 did not affect the migration of HSF-DNA complexes. Thus, HSF activity in heat-treated human cells appears to be due exclusively to HSF1. This finding is consistent with earlier observations by Sistonen et al. (47) that in human K562 cells HSF1 DNA-binding activity is heat activated whereas that of HSF2 is induced by hemin but not by heat.

The second level of regulation is the state of oligomerization of HSF1 and acquisition of DNA-binding activity. As shown by limiting-pore-size gel electrophoresis and confirmed by gel filtration, HSF1 is present in unstressed cells in complexes of about 200 kDa (and of 400 kDa in some experiments). On heat treatment these complexes disappear and 700-kDa complexes are formed that, unlike the smaller complexes, have DNA-binding ability. Realizing that the accuracy of the above estimations of the molecular sizes of HSF1 complexes was influenced by the unknown shape of the complexes, we attempted to obtain independent information about the oligomeric states of HSF1 in stressed and unstressed cells by in vitro cross-linking of isolated HSF1 complexes and SDS-PAGE analysis of the products of cross-linking. This analysis suggested that the 700-kDa complexes may contain HSF1 trimers and the 200-kDa complexes may contain HSF1 monomers. Analogous observations have recently been made for *D. melanogaster*, revealing an inactive HSF complex of 220 kDa and an active complex of 690 kDa (62). One study concluded that *Drosophila* HSF is in the form of a homotrimer in stressed cells (39). However, the nature of the oligomeric state of *Drosophila* HSF is still under discussion since a second study reported the detection of hexameric HSF (13). Although it is tempting to associate changes in the oligomerization state of HSF with the acquisition of DNA-binding activity, this conclusion is premature since conversion of non-DNA-binding to DNA-binding forms of HSF is also accompanied by posttranslational modification, perhaps phosphorylation (26), of the factor.

The third level of regulation is posttranslational modification in the cytoplasm of unstressed cells. We found that HSF1 in the cytoplasm of unstressed HeLa cells or in HSF1 RNA-injected *Xenopus* oocytes appears as several polypeptides with SDS-PAGE molecular masses between 75 and 80 kDa, which are considerably larger than predicted from the nucleotide sequence of the HSF1 cDNA gene. Analysis of this group of polypeptides following V8 proteinase digestion revealed nested sets of polypeptide fragments. These observations suggest that HSF1 is modified posttranslationally in the cytoplasm in the absence of stress.

NP-40 lysis releases a 57-kDa polypeptide that appears to be related to the 75- to 80-kDa forms of HSF1. Although still preliminary, this finding suggests that unstressed cells also contain an unmodified or only slightly modified form of HSF1. Although the 75- to 80-kDa forms of HSF1 rapidly translocate to the nucleus on heat treatment of the cells, no transport of the 57-kDa polypeptide could be observed. Since it may suggest the existence of a precursor pool of unmodified or slightly modified HSF1 that should be modified before it can participate in translocation and regulation of gene expression, it is important to conduct a more thorough study of this finding.

The fourth level of regulation is the nuclear import of HSF1. On heat treatment, modified HSF1 translocates within minutes to the soluble nuclear fraction. Shortly thereafter, it becomes associated quantitatively with the nuclear pellet, presumably reflecting binding to chromosomal target sequences. This observation, together with the earlier demonstration that in vitro activation of the DNA-binding ability of HSF can occur only in a cytoplasmic fraction (33), suggests that translocation occurs after the acquisition of DNA-binding ability by the factor. The same events that lead to derepression of the DNA-binding activity may also expose a previously buried nuclear localization signal in the HSF molecule. It is interesting that this translocation event may represent a point of regulation unique to mammalian cells: immunofluorescence experiments with *Drosophila* cells indicated that in these cells both inactive and active forms of HSF reside in the nucleus (62).

The final level of regulation is the heat-induced modification of HSF1. HSF1 present in the nucleus of heat-treated cells migrates more slowly than the modified forms present in the cytoplasm of unstressed cells, indicating that HSF1 is further modified on oligomerization or derepression of DNA-binding activity or on transport to the nucleus.

Future experiments will attempt to define the functional relationship between these findings. Of particular interest will be to determine the role of oligomerization of HSF1 in derepression of the DNA-binding activity as well as that of the posttranslational modifications. We speculate that activation of transcriptionally competent HSF may involve minimally two triggering events. First, HSF1 is modified in the absence of stress. Second, heat treatment and, presumably, any other treatment that causes activation of hsp genes may bring about a conformational change in HSF (33, 34; see also below). As a consequence, the several leucine zippers present in HSF (41) may be exposed, resulting in a change in the oligomerization state, and target region(s) for additional modification and a nuclear translocation signal may become available as well. Independent of whether a single "unmasking" event is sufficient to permit oligomerization, acquisition of DNA-binding activity, modification, and nuclear transport, the existence of these different points of control should serve to maximize the difference in active HSF available for transcription activation in unstressed versus stressed cells.

Human HSF1 expressed in *Xenopus* oocytes kept at 20°C is unable to bind to DNA. Derepression of the DNA-binding activity of HSF1 occurs when oocytes are heat treated at 37°C, and incubation at higher temperatures does not result in higher levels of HSE DNA-binding activity. HSF1 is posttranslationally modified in the oocytes, and changes in the state of oligomerization similar to the ones observed in HeLa cells occur on heat treatment. Several conclusions can be drawn from these results. First, the ability of HSF1 to oligomerize is inherent in the structure of the molecule. The observation of heat-induced derepression of HSF1 DNA-binding activity suggests that the underlying mechanism either is independent of other cellular factors or is well conserved evolutionarily. Analogous results have been obtained with *Drosophila* HSF (13). Second, *Drosophila* or human hsp genes microinjected into *Xenopus* oocytes are inactive at 20°C and maximally active following heat treatment at 36 to 37°C (4, 60). DNA-binding activity of *Xenopus* HSF is also observed at 37°C but not at the lower temperature (68) (Fig. 8A). Human HSF1 expressed in the oocytes also showed maximal DNA-binding activity at 37°C, even though in human cells it is essentially devoid of DNA-binding activity at the same temperature. Temperatures between 42 and 44°C are required for maximal activation in the latter cells. This result implicates a *Xenopus* protein(s) in the regulation of human HSF1 and suggests that heat regu-

lation of HSF activity is not an inherent property of HSF but is linked to the state of the cells via an evolutionarily conserved cellular factor(s). Third, our experiments extend earlier findings that bacterially expressed human HSF1 encodes an HSE DNA-binding protein capable of enhancing transcription in vitro of hsp genes (41) by showing that the HSE DNA-binding activity of the products of the cloned human HSF1 gene is heat regulated in eukaryotic cells.

The *Xenopus* experiments presented herein, as well as several earlier studies showing that hsp genes are activated by exposure of cells to amino acid analogs (27, 31) or following microinjection of denatured but not native proteins (4) or that activation is prevented when protein synthesis is arrested (7), all point to the involvement of a cellular protein, possibly hsp70, in the stress activation of HSF. Hsp70 may directly bind to HSF (2, 7), locking the factor in an inactive conformation. Stress will lead to the accumulation of denatured proteins that may bind all available hsp70 (18, 36, 38), including that loosely associated with HSF. Consequently, HSF, unrestrained by association with hsp70, may undergo conformational changes, resulting in oligomerization and the acquisition of DNA-binding ability. Alternatively, conformational changes in HSF may be prompted by a posttranslational modification event. The modifying activity may be directly or indirectly regulated by the level of free hsp70. Our cross-linking experiments that tentatively identified inactive HSF as a monomer seem to provide support for the latter but not the former mechanism. It is important to note, however, that this interpretation of the negative results of the cross-linking experiments may be misleading. Complexes of HSF and the putative repressor may be very labile and may therefore not be readily amenable to biochemical analysis. Furthermore, extrapolating from the observation that in vitro activation of HSF DNA-binding ability is exceedingly concentration dependent (33), HSF stripped of repressor protein in the course of biochemical fractionation may not be able to oligomerize efficiently and to acquire DNA-binding ability. Clearly, direct, positive evidence is needed to distinguish between the two alternative mechanisms.

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REFERENCES

1. **Abravaya, K., B. Phillips, and R. I. Morimoto.** 1991. Attenuation of the heat shock response is mediated by the release of bound HSF and is modulated by changes in growth and heat shock temperatures. Genes Dev. **5**:2117–2127.
2. **Abravaya, K. A., M. Myers, S. P. Murphy, and R. I. Morimoto.** 1992. The human heat shock protein hsp70 interacts with HSF, the transcription factor that regulates heat shock gene expression. Genes Dev. **6**:1153–1164.
3. **Amin, J., J. Ananthan, and R. Voellmy.** 1988. Key features of heat shock regulatory elements. Mol. Cell. Biol. **8**:3761–3769.
4. **Ananthan, J., A. L. Goldberg, and R. Voellmy.** 1986. Abnormal proteins serve as eukaryotic stress signals and trigger the activation of heat shock genes. Science **232**:522–524.
5. **Andersson, L. O., H. Borg, and M. Mikaelson.** 1972. Molecular weight estimations of proteins by electrophoresis in polyacrylamide gels of graded porosity. FEBS Lett. **20**:199–201.
6. **Baeuerle, P. A., and D. Baltimore.** 1988. Activation of DNA-binding activity in an apparently cytoplasmic precursor of the NF-kB transcription factor. Cell **53**:211–217.
7. **Baler, R., W. J. Welch, and R. Voellmy.** 1992. Heat shock gene regulation by nascent polypeptides and denatured proteins: hsp70 as a potential autoregulatory factor. J. Cell Biol. **117**:1151–1159.
8. **Beckmann, R. P., L. A. Mizzen, and W. J. Welch.** 1990. Interaction of hsp70 with newly synthesized proteins: implications for protein folding and assembly. Science **248**:850–854.
9. **Bonner, J. J., S. Heyward, and D. L. Fackenthal.** 1992. Temperature-dependent regulation of a heterologous transcriptional activation domain fused to yeast heat shock transcription factor. Mol. Cell. Biol. **12**:1021–1030.
10. **Catelli, M. G., N. Binart, I. Jung-Testas, J. M. Renoir, E. E. Baulieu, J. R. Feramisco, and W. J. Welch.** 1985. The common 90-kd protein component of non-transformed '8S' steroid receptors is a heat shock protein. EMBO J. **4**:3131–3135.
11. **Chappell, T., W. J. Welch, D. M. Schlossman, K. B. Palter, M. J. Schlesinger, and J. E. Rothman.** 1986. Uncoating ATPase is a member of the 70 kilodalton family of stress proteins. Cell **45**:3–13.
12. **Chirico, W. J., G. Waters, and G. Blobel.** 1988. 70K heat shock related proteins stimulate protein translocation into microsomes. Nature (London) **333**:805–810.
13. **Clos, J., T. Westwood, P. B. Becker, S. Wilson, K. Lambert, and C. Wu.** 1990. Molecular cloning and expression of a hexameric *Drosophila* heat shock factor subject to negative regulation. Cell **63**:1085–1097.
14. **Deshaies, R. J., B. D. Koch, M. Werner-Washburne, E. A. Craig, and R. Schekman.** 1988. A subfamily of stress proteins facilitates translocation of secretory and mitochondrial precursor polypeptides. Nature (London) **333**:800–805.
15. **DiDomenico, B. J., G. E. Bugaisky, and S. Lindquist.** 1982. The heat shock response is self-regulated at both the transcriptional and posttranscriptional levels. Cell **31**:593–603.
16. **Dreano, M., J. Brochot, A. Myers, C. Cheng-Meyer, D. Rungger, R. Voellmy, and P. Bromley.** 1986. High-level, heat-regulated synthesis of proteins in eukaryotic cells. Gene **49**:1–8.
17. **Edington, B. V., S. A. Whelan, and H. E. Hightower.** 1989. Inhibition of heat shock (stress) protein induction by deuterium oxide and glycerol: additional support for the abnormal protein hypothesis of induction. J. Cell. Physiol. **139**:219–228.
18. **Flynn, G. C., T. G. Chappell, and J. E. Rothman.** 1989. Peptide binding and release by proteins implicated as catalysts of protein assembly. Science **245**:385–390.
19. **Gallo, G. J., T. J. Schuetz, and R. E. Kingston.** 1991. Regulation of heat shock factor in *Schizosaccharomyces pombe* more closely resembles regulation in mammals than in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **11**:281–288.
20. **Gamer, J., H. Bujard, and B. Bukau.** 1992. Physical interaction between heat shock proteins DnaK, DnaJ, and GrpE and the bacterial heat shock transcription factor σ^{32}. Cell **69**:833–842.
21. **Goff, S. A., and A. L. Goldberg.** 1985. Production of abnormal proteins in *E. coli* stimulates transcription of lon and other heat shock genes. Cell **41**:587–595.
22. **Goldenberg, C. J., Y. Luo, M. Fenna, R. Baler, R. Weinmann, and R. Voellmy.** 1988. Purified human factor activates heat shock promoter in a HeLa cell-free transcription system. J. Biol. Chem. **263**:19734–19739.
23. **Haas, I. G., and M. Wabl.** 1983. Immunoglobulin heavy chain binding protein. Nature (London) **306**:387–389.
24. **Harlow, E., and D. Lane.** 1988. Antibodies: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
25. **Hendershot, L., D. Bole, G. Koehler, and J. F. Kearney.** 1987. Assembly and secretion of heavy chains that do not associate translationally with immunoglobulin heavy chain-binding protein. J. Cell Biol. **104**:761–767.
26. **Hensold, J. O., C. R. Hunt, S. K. Calderwood, D. E. Housman, and R. E. Kingston.** 1990. DNA binding of heat shock factor to the heat shock element is insufficient for transcriptional activation in murine erythroleukemia cells. Mol. Cell. Biol. **10**:1600–1608.
27. **Hightower, L. E.** 1980. Cultured animal cells exposed to amino acid analogues or puromycin rapidly synthesize several polypeptides. J. Cell. Physiol. **102**:407–427.

28. **Hurtley, S. M., D. G. Bole, H. Hoover-Litty, A. Helenius, and C. S. Copeland.** 1989. Interactions of misfolded influenza virus hemagglutinin with binding protein (BiP). J. Cell Biol. **108:**2117–2126.
29. **Jurivich, D. A., L. Sistonen, R. A. Kroes, and R. I. Morimoto.** 1992. Effect of sodium salicylate on the human heat shock response. Science **255:**1243–1245.
30. **Kassenbrock, C. K., P. D. Garcia, P. Walter, and R. B. Kelly.** 1988. Heavy-chain binding protein recognizes aberrant polypeptides translocated *in vitro*. Nature (London) **333:**90–93.
31. **Kelly, P. M., and M. J. Schlesinger.** 1978. The effect of amino acid analogues and heat shock on gene expression in chicken embryo fibroblasts. Cell **15:**1277–1286.
32. **Kingston, R. E., T. J. Schuetz, and Z. Larin.** 1987. Heat-inducible human factor that binds to a human hsp70 promoter. Mol. Cell. Biol. **7:**1530–1534.
33. **Larson, J. S., T. S. Schuetz, and R. E. Kingston.** 1988. Activation *in vitro* of sequence-specific DNA binding by a human regulatory factor. Nature (London) **335:**372–375.
34. **Mosser, D. D., P. T. Kotzbauer, K. D. Sarge, and R. I. Morimoto.** 1990. In vitro activation of heat shock transcription factor DNA-binding by calcium and biochemical conditions that affect protein conformation. Proc. Natl. Acad. Sci. USA **87:**3748–3752.
35. **Nieto-Sotelo, J., G. Wiederrecht, A. Okuda, and C. S. Parker.** 1990. The yeast heat shock transcription factor contains a transcriptional activation domain whose activity is repressed under nonshock conditions. Cell **62:**807–817.
36. **Palleros, D. R., W. J. Welch, and A. L. Fink.** 1991. Interaction of hsp70 with unfolded proteins: effects of temperature and nucleotides on the kinetics of binding. Proc. Natl. Acad. Sci. USA **88:**5719–5723.
37. **Parker, C. S., and J. Topol.** 1984. A *Drosophila* RNA polymerase II transcription factor binds to the regulatory site of an hsp70 gene. Cell **37:**273–283.
38. **Pelham, H. R. B.** 1986. Speculations on the functions of the major heat shock and glucose-regulated proteins. Cell **46:**959–961.
39. **Perisic, O., H. Xiao, and J. T. Lis.** 1989. Stable binding of *Drosophila* heat shock factor to head-to-head and tail-to-tail repeats of a conserved 5 bp recognition unit. Cell **59:**797–806.
40. **Price, B. D., and S. K. Calderwood.** 1991. Ca^{2+} is essential for multistep activation of the heat shock factor in permeabilized cells. Mol. Cell. Biol. **11:**3365–3368.
41. **Rabindran, S. K., G. Giorgi, J. Clos, and C. Wu.** 1991. Molecular cloning and expression of a human heat shock transcription factor, HSF1. Proc. Natl. Acad. Sci. USA **88:**6906–6910.
42. **Sanchez, E. R., D. O. Toft, M. J. Schlesinger, and W. B. Pratt.** 1985. Evidence that the 90-kDa phosphoprotein associated with the untransformed L-cell glucocorticoid receptor is a murine heat shock protein. J. Biol. Chem. **260:**12398–12401.
43. **Sarge, K. D., V. Zimarino, K. Holm, C. Wu, and R. I. Morimoto.** 1991. Cloning and characterization of two mouse heat shock factors with distinct inducible and constitutive DNA-binding ability. Genes Dev. **5:**1902–1911.
44. **Scharf, K.-D., S. Rose, W. Zott, F. Schoff, and L. Nover.** 1990. Three tomato genes code for heat stress transcription factors with a remarkable degree of homology to the DNA-binding domain of the yeast HSF. EMBO J. **9:**4495–4501.
45. **Schuetz, T. J., G. J. Gallo, L. Sheldon, P. Tempst, and R. E. Kingston.** 1991. Isolation of a cDNA for HSF2: evidence for two heat shock factor genes in humans. Proc. Natl. Acad. Sci. USA **88:**6910–6915.
46. **Schuh, S., W. Yonemoto, J. Brugge, V. J. Bauer, R. M. Riel, W. P. Sullivan, and D. O. Toft.** 1985. A 90,000-dalton binding protein common to both steroid receptors and the Rous sarcoma virus transforming protein, $pp60^{v\text{-}src}$. J. Biol. Chem. **260:**14292–14296.
47. **Sistonen, L., K. D. Sarge, B. Philips, K. Abravaya, and R. I. Morimoto.** 1992. Activation of heat shock factor 2 (HSF2) during hemin-induced differentiation of human erythroleukemia cells. Mol. Cell. Biol. **12:**4104–4111.
48. **Sorger, P. K., and H. R. B. Pelham.** 1987. Purification and characterization of a heat-shock element binding protein from yeast. EMBO J. **6:**3035–3041.
49. **Sorger, P. K., M. J. Lewis, and H. R. B. Pelham.** 1987. Heat shock factor is regulated differently in yeast and HeLa cells. Nature (London) **329:**81–84.
50. **Sorger, P. K., and H. C. M. Nelson.** 1989. Trimerization of a yeast transcriptional activator via a coiled-coil motif. Cell **59:**807–813.
51. **Sorger, P. K., and H. R. B. Pelham.** 1988. Yeast heat shock factor is an essential DNA-binding protein that exhibits temperature-dependent phosphorylation. Cell **54:**855–864.
52. **Stone, D. E., and E. A. Craig.** 1990. Self-regulation of 70-kilodalton heat shock proteins in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **10:**1622–1632.
53. **Straus, D. B., W. A. Walter, and C. A. Gross.** 1989. The activity of σ^{32} is reduced under conditions of excess heat shock protein production in *Escherichia coli*. Genes Dev. **3:**2003–2010.
54. **Straus, D. B., W. A. Walter, and C. A. Gross.** 1990. DnaK, DnaJ, and GrpE heat shock proteins negatively regulate heat shock gene expression by controlling the synthesis and stability of σ^{32}. Genes Dev. **4:**2202–2209.
55. **Tilly, K., N. McKittrick, M. Zylicz, and C. Georgopoulos.** 1983. The *dnaK* protein modulates the heat shock response of *Escherichia coli*. Cell **34:**641–646.
56. **Tilly, K., J. Spence, and C. Georgopoulos.** 1989. Modulation of stability of the *Escherichia coli* heat shock regulatory factor σ^{32}. J. Bacteriol. **171:**1585–1589.
57. **Topol, J., D. M. Ruden, and C. S. Parker.** 1985. Sequences required for *in vitro* transcriptional activation of a *Drosophila* hsp70 gene. Cell **42:**527–537.
58. **Ungewickel, E.** 1985. The 70-kd mammalian heat shock proteins are structurally and functionally related to the uncoating protein that releases clathrin triskelia from coated vesicles. EMBO J. **4:**3385–3391.
59. **Voellmy, R., A. Ahmed, P. Schiller, P. Bromley, and D. Rungger.** 1985. Isolation and functional analysis of a human 70,000-dalton heat shock protein gene segment. Proc. Natl. Acad. Sci. USA **82:**4949–4953.
60. **Voellmy, R., and D. Rungger.** 1982. Transcription of a *Drosophila* heat shock gene is heat-induced in *Xenopus* oocytes. Proc. Natl. Acad. Sci. USA **79:**1776–1780.
61. **Welch, W.** 1990. The mammalian stress response, cell physiology and biochemistry of proteins, p. 223–278. *In* R. I. Morimoto, A. Tissieres, and C. Georgopoulos (ed.), Stress proteins in biology and medicine. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
62. **Westwood, J. T., J. Clos, and C. Wu.** 1992. Stress-induced oligomerization and chromosomal relocalization of heat-shock factor. Nature (London) **353:**822–827.
63. **Wiederrecht, G., D. Seto, and C. S. Parker.** 1988. Isolation of the gene encoding the *S. cerevisiae* heat shock transcription factor. Cell **54:**841–853.
64. **Wu, B., C. Hunt, and R. I. Morimoto.** 1985. Structure and expression of the human gene encoding major heat shock protein hsp70. Mol. Cell. Biol. **5:**330–341.
65. **Wu, C.** 1984. Activating protein factor binds *in vitro* to upstream control sequences in heat shock gene chromatin. Nature (London) **311:**81–84.
66. **Wu, C., S. Wilson, S. Walker, I. Dawid, T. Paisley, V. Zimarino, and H. Ueda.** 1987. Purification and properties of *Drosophila* heat shock activator protein. Science **238:**1247–1253.
67. **Xiao, H., and J. T. Lis.** 1988. Germline transformation used to define key features of heat shock response elements. Science **239:**1139–1142.
68. **Zimarino, V., C. Tsai, and C. Wu.** 1990. Complex modes of heat shock factor activation. Mol. Cell. Biol. **10:**752–759.
69. **Zimarino, V., and C. Wu.** 1987. Induction of sequence-specific binding of *Drosophila* heat shock activator protein without protein synthesis. Nature (London) **327:**727–730.