

# Heat Shock Gene Regulation by Nascent Polypeptides and Denatured Proteins: hsp70 as a Potential Autoregulatory Factor

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**Abstract.** Heat shock genes encode proteins (hsp's) that play important structural roles under normal circumstances and are essential to the cells' ability to survive environmental insults. Evidence is presented herein that transcriptional regulation of hsp gene expression is linked with the regulation of overall protein synthesis as well as with the accumulation of proteins

denatured by stressful events. The factor that connects the three processes appears to be one of the hsp's, presumably a member(s) of the hsp70 family. Biochemical experiments demonstrate that complexes containing hsp70 and heat shock transcription factor, the specific regulator of hsp gene activity, are formed in the cells.

WHEN cells experience adverse changes in their environment, they react by increasing the expression of stress or heat shock proteins (hsp's<sup>1</sup>; references 3, 30). An important role of these proteins may be to provide the cells with a mechanism to handle increased amounts of abnormally folded proteins (31, 52). Many of the agents and treatments that enhance the activity of hsp genes are known to cause unfolding of preexisting proteins or to induce synthesis of nonfolded polypeptides (12, 15, 22, 26, 48). Thus, the accumulation of nonfolded polypeptides could be the signal for increased expression of hsp genes. Support for this hypothesis has been obtained by coinjection in *Xenopus laevis* oocytes of reporter genes driven by hsp gene promoters and individual purified proteins either in their native state or after denaturation (2). Reporter gene activity is much higher in oocytes that received denatured proteins than in oocytes injected with native proteins. The findings that the quantity of hsp's synthesized in response to a stress is directly proportional to the severity of the stress and that the return to normal of the rate of hsp70 mRNA synthesis after heat shock is much slower in cells treated with an inhibitor of protein synthesis than in untreated cells led to the hypothesis that this response was autoregulated by one of the hsp's (10). This idea has been carried furthest in experiments that suggested self-regulation of one of the hsp70 genes in yeast at least some of which occurred at the transcriptional level (45). A number of hsp genes are not only active in stressed but also in unstressed cells. Consequently, some of the hsp's

are present in substantial concentrations in unstressed cells where they participate in numerous processes involving intracellular protein transport, assembly or dissociation of protein complexes (6, 7, 9, 20, 21, 47), or modulation of the activity of key proteins (5, 39, 41).

Nascent polypeptides are almost certainly the major class of nonfolded proteins present in cells. Since accumulation of proteins denatured by stress leads to an enhancement of hsp gene expression, the question arises whether hsp gene activity is also regulated by changes in the concentration of nascent polypeptides, i.e., in the rate of protein synthesis. If so, control of hsp gene expression by nascent polypeptides could be mediated by a different mechanism from that used during stress or by a common mechanism that integrates the concentrations of the two different types of nonfolded proteins. More general questions are whether stress-denatured proteins and nascent polypeptides are treated the same by the cells, and, if they are, whether this can be explained based on our present understanding of the properties of hsp's.

We are presenting evidence herein that transcriptional expression of hsp genes in human cells is activated by stress denaturation of proteins but that this activation is inhibited when the concentration of nascent polypeptides is reduced. This suggests that, at least at the level of regulation of hsp genes, the cells are indeed capable of integrating changes in the concentrations of stress-denatured and of not-yet-folded, nascent polypeptides. The factor(s) involved in these regulatory events is postulated to be normally associated with nascent polypeptides and is an hsp itself that may be identical with hsp70. In experiments with nuclear extracts and with purified components, heat shock transcription factor (HSF) and hsp70 are shown to interact with some specificity.

1. *Abbreviations used in this paper:* CAT, chloramphenicol acetyltransferase; HSE, heat shock element; HSF, heat shock transcription factor; hsp, heat-shock protein.

## Materials and Methods

### Cell Culture, Labeling of Proteins, and RNase Protection Assay

Adherent human HeLa cells were grown in 100-mm dishes in DMEM and 10% FCS at 37°C and 5% CO<sub>2</sub>. Experiments used 60% confluent cultures. Many of the experiments herein were repeated with human K562 cells that were grown in suspension, in 25-cm<sup>2</sup> flasks, in RPMI medium containing 10% FCS, sodium pyruvate, and nonessential amino acids. They were kept at a density of  $\sim 7 \times 10^5$  cells/ml. To label newly synthesized proteins, cultures were washed with leucine-free MEM and were then incubated for the time indicated in the same medium containing 40  $\mu$ Ci/ml [<sup>3</sup>H]leucine (specific radioactivity 60 Ci/mmol). Cells were then washed with PBS lysed in SDS sample buffer, and aliquots of the samples (equal counts in Fig. 1 A) were applied to 10% SDS-polyacrylamide gels. After electrophoresis gels were treated for fluorography (salicylate method). Total cytoplasmic RNA was isolated from individual cultures in 100-mm dishes as described by Gough (19), and 10- $\mu$ g aliquots were hybridized overnight with riboprobe ( $\sim 5 \times 10^5$  cpm) at 45°C in 30  $\mu$ l of 40 mM Pipes, pH 4.6, 0.4 mM NaCl, 1 mM EDTA, 80% formamide. RNase A and T1 digestion and processing of samples used standard procedures (38). Analysis was on 6% polyacrylamide-urea gels. The hsp70A riboprobe contained sequences of the coding strand extending from a BamHI site located 65-bp upstream from the initiation codon of the hsp70A gene (53) to an SphI site 340 bp further upstream, and the hsp70B riboprobe from +111 to -580 (40). The plasmid used to prepare a  $\tau$ -actin riboprobe contained a 900-nt human  $\tau$ -actin cDNA fragment.

### Establishment of the D2500-CAT Cell Line, Chloramphenicol Acetyltransferase (CAT) Assays

HeLa cells ( $3 \times 10^5$ ) in a 100-mm dish were transfected by a calcium phosphate precipitation procedure (18) with a mixture of 20  $\mu$ g of pD2500-CAT (40) and 2  $\mu$ g of pRSV-neo (18). After overnight exposure to DNA, the cells were washed and incubated in fresh medium containing 400  $\mu$ g/ml geneticin (Sigma Chem. Co., St. Louis, MO). After 6 wk, colonies were transferred to 96-well plates, and cultures were expanded. For the identification of lines expressing CAT, cells were either heat-treated at 42°C or kept at 37°C, incubated for one additional day, and then assayed for CAT activity (17). One of the lines selected, referred to as D2500-CAT line, that displayed highly heat-inducible expression of the CAT gene was used for all experiments. It was maintained in the presence of 200  $\mu$ g/ml geneticin.

### Preparation of Extracts and Gel Retardation Assays

Whole cell extract was prepared by scraping cells ( $\sim 10^6$ ) off a 100-mm dish (only procedures used with HeLa cells are discussed here). Cells were then washed with cold PBS and collected by centrifugation at 3,200 g. Pellets were frozen in liquid nitrogen for 20 s and thawed; and the cells were resuspended in 50  $\mu$ l buffer C (20 mM Hepes, pH 7.9, 1.5 mM MgCl<sub>2</sub>, 0.42 mM NaCl, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT, and 25% glycerol). One more freeze-thaw cycle (dry ice/EtOH to 20°C) was followed by incubation on ice for 15 min. The cell lysate was cleared by a 20-min centrifugation at 13,000 g. Supernatant solution, referred to here as whole cell extract, was stored in aliquots at -70°C. Nuclear extract was prepared according to Dignam et al. (11). Briefly, cells were incubated for 15 min on ice in hypotonic buffer A (10 mM Hepes, pH 7.9, 1.5 mM MgCl<sub>2</sub>, 10 mM KCl, 0.5 mM DTT), collected by centrifugation at 1,400 g, resuspended in two packed-cell volumes of buffer A, and broken in a Dounce homogenizer. The lysate was centrifuged briefly at 1,400 g to pellet nuclei. After the addition of 0.1 vol buffer B (300 mM Hepes, pH 7.9, 30 mM MgCl<sub>2</sub>, 1.4 M KCl), the supernatant solution was cleared by centrifugation at 50,000 g for 50 min and was then used as cytosolic fraction for in vitro activation of HSF. The nuclear pellet was washed in buffer A, resuspended in 50  $\mu$ l/10<sup>6</sup> cells of buffer C, dounce-homogenized, and incubated on ice for 30 min. The supernatant solution of the subsequent 20-min centrifugation at 9,000 g was used as nuclear extract. All steps were at 4°C unless indicated differently. DNA binding assays that contained  $\sim 1$   $\mu$ g of extract protein or 5–20 ng of purified HeLa HSF and 2  $\mu$ g non-specific competitor DNA (mixture of poly[dIdC]  $\times$  poly[dIdC] and salmon sperm DNA) in 20  $\mu$ l binding buffer (12 mM Hepes, pH 7.9, 12% glycerol, 2 mM MgCl<sub>2</sub>, 60 mM KCl, 0.12 mM EDTA, 0.3 mM PMSF, 0.3 mM DTT) were preincubated for 15 min at room temperature. Complementary synthetic oligonucleotides were labeled by T4 polynucleotide kinase and

[<sup>32</sup>P] $\gamma$ -ATP, precipitated several times, annealed, and then used as probes (20,000 cpm/reaction). After addition of probe DNA, reactions were incubated for 15 min at 25°C and then analyzed on nondenaturing (4.5%) polyacrylamide gels in TGE buffer (40 mM Tris, 270 mM glycine, 2 mM EDTA, pH 8.0). Runs were at 13 V/cm at 4°C. OTF: 5'CTTCACCTATTTCATAGC; SPI: 5'AACTGGGCGGAGTTAGGGGCGGGATGGGCGGAGTTA; for HSE see reference 16.

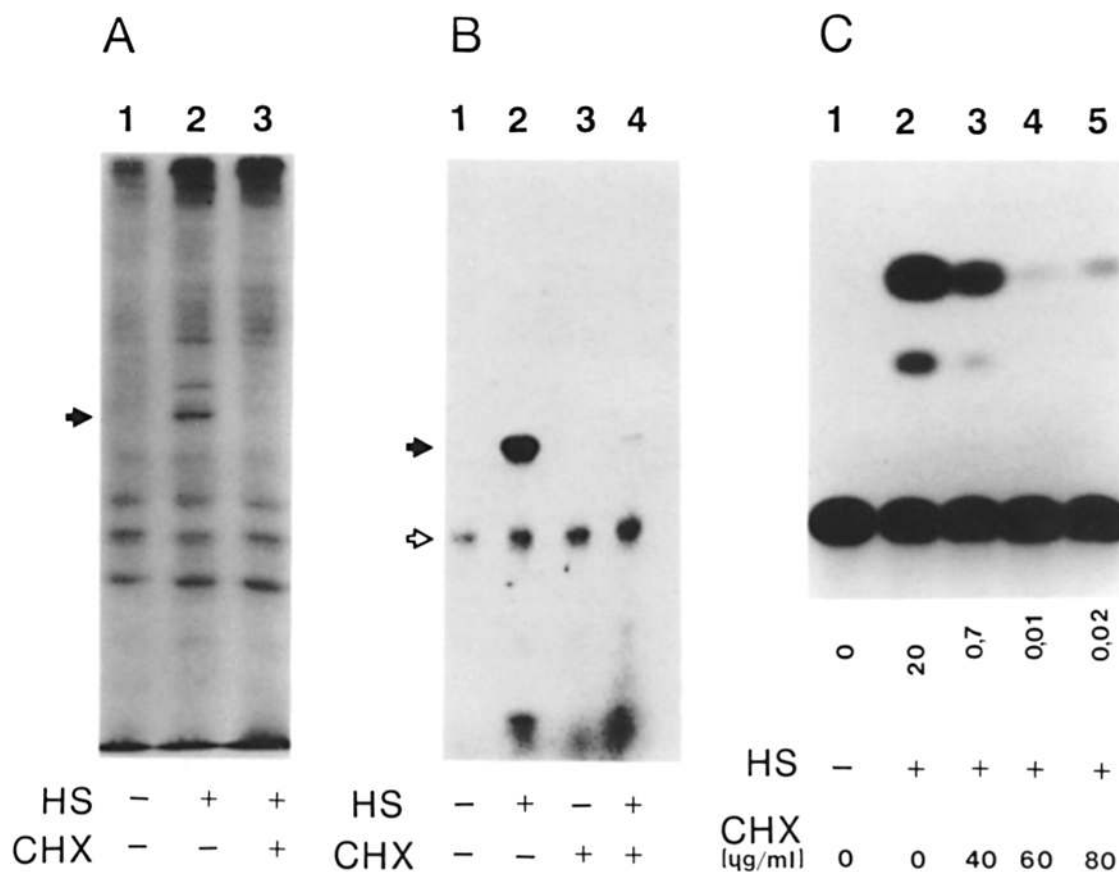
Most experiments presented in this publication were also carried out with human K562 cells, and similar results were obtained.

## Results

### Heat Induction of hsp70 Expression Is Suppressed by Prior Exposure of Cells to an Inhibitor of Protein Synthesis

In search of evidence for a regulatory influence of nascent polypeptides, human HeLa cells were preincubated for 50 min or longer with cycloheximide at a concentration (60  $\mu$ g/ml) at which inhibition of protein synthesis was virtually complete. The cells were then either exposed to the heat shock temperature of 42°C typically for 50 min or incubated at the control temperature of 37°C for the same time period. To examine the patterns of protein synthesis after the treatments, the cells were washed extensively with leucine-free medium to remove cycloheximide. They were then incubated in the same medium but containing [<sup>3</sup>H]leucine at 37°C for 2 h. Newly synthesized, <sup>3</sup>H-labeled proteins were separated by SDS-PAGE and detected by autoradiography. The results of the experiment revealed that cycloheximide pretreatment all but eliminated induction of hsp70 synthesis by a 42°C heat shock (Fig. 1 A, see also reference 4). This was not due to a general, irreversible effect of the inhibitor on protein synthesis since most other proteins were synthesized at normal rates after the removal of cycloheximide.

To find out which step of hsp gene expression was affected by cycloheximide, transcript levels of endogenous, heat-regulated hsp70 genes were estimated by RNase protection assays. Total cytoplasmic RNA was isolated from cycloheximide-treated and untreated cultures immediately after heat treatment or incubation at the control temperature. Riboprobes contained unique sequences from the 5' nontranslated and promoter regions of the human hsp70A (53) and B genes (49), respectively. Cycloheximide preincubation was found to inhibit accumulation of both hsp70A and hsp70B (not shown) mRNA during heat treatment (Fig. 1 B). The following experiment suggested that this inhibition most likely occurred at the transcriptional level, rather than at the level of RNA stability or during export. HeLa cells were cotransfected with a construct that contained a CAT gene under the control of the promoter of the human hsp70B gene (D2500-CAT; reference 40) and a construct carrying the neomycin resistance gene employed as the selectable marker gene. A stable cell line (D2500-CAT line) was selected that expressed the CAT gene in a strictly heat-regulated fashion (Fig. 1 C). Cultures of the D2500-CAT line were pretreated with cycloheximide and heat-treated as described above. After extensive washing with complete medium, the cultures were incubated overnight at 37°C, and assays of CAT activity were carried out subsequently. The results of this experiment demonstrated that preincubation with cycloheximide severely inhibited the heat-induced expression of the CAT gene (Fig. 1 C). Inhibition was dependent on the concentration of cycloheximide.



**Figure 1.** Effect of cycloheximide preincubation on heat-induced hsp70 and hsp70-CAT gene expression. (A) Protein synthesis. HeLa cultures that had been preincubated in the presence or absence of 60 µg/ml cycloheximide (CHX) for 2 h were heat-treated for 50 min at 42°C (HS). These and control cultures were then pulse-labeled with [<sup>3</sup>H]leucine for 2 h at 37°C. Newly synthesized proteins were analyzed on 10% SDS-polyacrylamide gels. The arrow in the fluorograph indicates the position of hsp70. (B) Accumulation of hsp70A gene transcripts. Treatment as in A except that cells were harvested immediately after the heat treatment, and total cytoplasmic RNA was prepared and analyzed by RNase protection assay. Riboprobes that specifically detected hsp70A (filled arrow) or r-actin transcripts (open arrow) were used. (C) Expression of human hsp70B-CAT reporter gene in the D2500-CAT line. After treatments, cells were washed free of cycloheximide and incubated for 24 h at 37°C before CAT activities were measured.

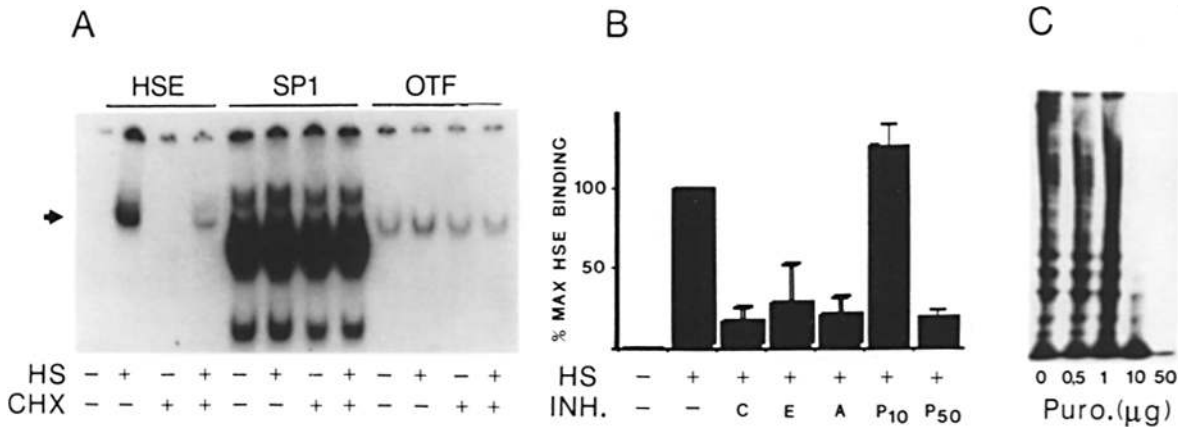
### Activation of HSF Is Suppressed by Preexposure of Cells to Inhibitors of Protein Synthesis

Transcription from heat-shock promoters is regulated by HSF (33) which is normally present in cells in an inactive form but is activated at elevated temperature. Addition of cycloheximide at the time of the temperature increase does not prevent activation of HSF (27, 44, 54). A critical early step in this activation process is the derepression of the DNA binding activity of the factor. We next tested whether cycloheximide pretreatment prevented activation of HSF at this step. Cycloheximide-pretreated or untreated HeLa cells were heated at 42°C for 50 min or kept at 37°C, and nuclear extracts (or whole cells extracts in other experiments) were prepared. Binding of HSF to a <sup>32</sup>P-end-labeled, synthetic DNA fragment that included an HSE (1, 35) sequence was analyzed by gel retardation assay. Much less HSF binding was induced by the 50-min heat treatment at 42°C in the cycloheximide-pretreated than in the control cells (70–90% reduction; Fig. 2 A). Binding of factors to oligonucleotides containing SP1 and OTF consensus binding sequences was not affected by heat shock alone or in combination with a cycloheximide pretreatment. Thus, cycloheximide pretreat-

ment selectively inhibited the stress activation of HSF. It appeared that ongoing protein synthesis was an essential ingredient to a strong response to heat stress at the transcription level.

To test the possibility that cycloheximide affected activation of HSF by a mechanism unrelated to its inhibitory effect on protein synthesis, analogous experiments were carried out with other inhibitors of protein synthesis such as emetine, anisomycin, and puromycin, whose mechanisms of action differ from cycloheximide. All inhibitors were found to suppress heat activation of HSF (Fig. 2 B).

There are several possible explanations for these observations. First, HSF or some essential component of the activation pathway may be short-lived and thus be eliminated rapidly during pretreatment with an inhibitor of protein-synthesis. Second, when protein synthesis is inhibited, factors that normally are sequestered due to their involvement in protein synthesis may become available throughout the cytosol (and the nucleus), and one of these factors may inhibit activation of HSF. Third, this factor that normally is engaged in protein synthesis is itself an hsp that is identical with or has properties similar to members of the hsp70 family. For



**Figure 2.** Protein synthesis is required for stress activation of preexisting HSF in HeLa cells. (A) Gel retardation assay on nuclear extracts prepared from HeLa cells treated with cycloheximide (60  $\mu\text{g}/\text{ml}$ , added 2 h before heat treatment) and heat-treated (50 min at 42°C; HS) as indicated using as probes radiolabeled oligonucleotides containing recognition sites for HSF (HSE), SP1, and OTF. Arrow points to the major HSF-HSE complex. Free probe was run out of the gel. (B) Effect of pretreatment with various protein synthesis inhibitors on HSF heat activation. Retardation assays were carried out, using the HSE probe, with nuclear extracts prepared from cells pretreated with 60  $\mu\text{g}/\text{ml}$  cycloheximide for 2 h (C), 5  $\mu\text{M}$  emetine for 45 min (E), 40  $\mu\text{M}$  anisomycin for 45 min (A), 10  $\mu\text{g}/\text{ml}$  (P<sub>10</sub>), or 50  $\mu\text{g}/\text{ml}$  puromycin (P<sub>50</sub>) for 1 h before the 50-min 42°C heat-shock. Results expressed as percent bound HSE were obtained by scanning autoradiographs of retardation gels. (C) Analysis by SDS-PAGE of newly made polypeptides in cells pulse-labeled with [<sup>3</sup>H]leucine during exposure (90 min) to puromycin at concentrations from 0.5 to 50  $\mu\text{g}/\text{ml}$ .

convenience, the postulated regulatory hsp is referred to below as hsp70. This name is being used here generically to include any member or groups of members of the hsp70 family of proteins. Note that although this identification seems likely to be correct, no formal proof is offered in this paper. Consistent with this view, however, is that members of the hsp70 family are capable of binding peptides and nonfolded polypeptides (12-14, 25). During stress hsp70 may associate with and stabilize proteins that are unfolding (15, 22, 26, 29, 32, 34, 42, 48). Beckman et al. (4) have recently obtained evidence that hsp70 may bind transiently to nascent polypeptides. Since preincubation with inhibitors of protein synthesis depletes the cells of nascent polypeptides, hsp70 molecules normally bound to nascent or recently synthesized polypeptides would be liberated such that the concentration of free (unbound) hsp70 would increase. Now with sufficient levels of free hsp70, there would be no need for new hsp70 synthesis in the following stress event. Thus, activation of HSF may be inhibited by a feedback mechanism that senses the concentration of free vs. bound hsp70. The following two sets of experiments favor this explanation.

#### **Suppression by Inhibitors of Protein Synthesis of the Activation of HSF during Exposure to a Modest Stress Can Be Overcome by a Severe Stress**

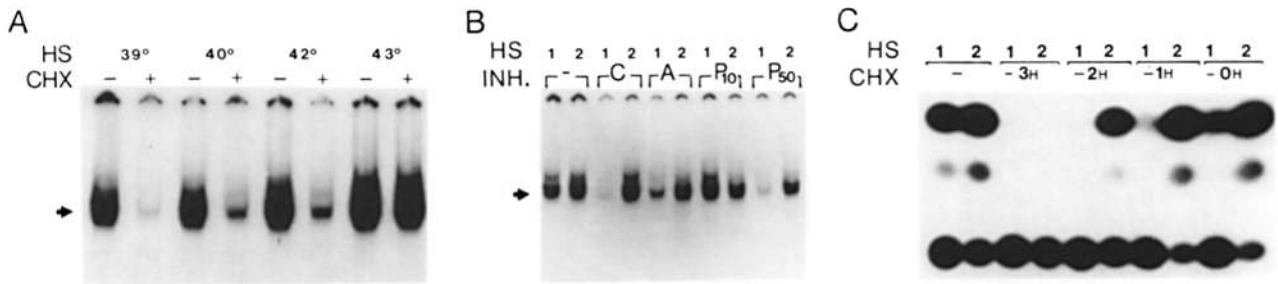
Using the third explanation as the point of departure for the discussion, the increased concentration of free hsp70 in cells treated with an inhibitor of protein synthesis was apparently adequate for dealing with a moderately severe stress such as exposure to 42°C for 1 h. A more severe stress, causing massive protein denaturation, would increase the need for hsp70 to a level that may be similar or even exceed the total amount normally engaged in and retrievable from protein synthesis. Consequently, the concentration of free hsp70 should fall to a very low level resulting in the activation of HSF and hsp gene expression. The same prediction would also be made based on the second explanation. In contrast, the first explanation that is based on the lability of HSF or a component

of the activation pathway would lead to the prediction that no amount of stress could rescue HSF activity in cells pretreated with an inhibitor of protein synthesis. In the experiment in Fig. 3 A, the intensity of stress was increased by raising the heat shock temperature. The typical inhibition of HSF activation by cycloheximide preincubation occurred at 39, 40, and 42°C. At 43°C, however, the negative effect of cycloheximide was no longer observed.

In the experiment in Fig. 3 B, stress was increased by extending the period of heat treatment in the presence of cycloheximide at 42°C from 50 to 100 min rather than by further increasing the temperature. HSF activity was rescued by the longer treatment. Analogous findings were also made (Fig. 3 B) with anisomycin and with puromycin at 50  $\mu\text{g}/\text{ml}$ . Thus, the results were as predicted by the second and third explanation but essentially ruled out the first explanation: HSF and essential components of the activation machinery were stable for the duration of the experiments.

Additional support came from experiments using puromycin instead of cycloheximide. In these experiments the level of stress in cells undergoing a 42°C heat shock was raised further by reducing the concentration of puromycin from one (50  $\mu\text{g}/\text{ml}$ ) that caused virtually complete inhibition of protein synthesis to one (10  $\mu\text{g}/\text{ml}$ ) at which essentially no large proteins were synthesized but small, prematurely terminated (nonfolded) polypeptides were made in considerable amounts (Fig. 2 C). Analogous to the above experiments, heat activation of HSF was inhibited at the higher concentration of puromycin (corresponding to a moderate stress) but was not affected at the lower concentration (corresponding to a severe stress; Fig. 2 B; see also Fig. 3 B).

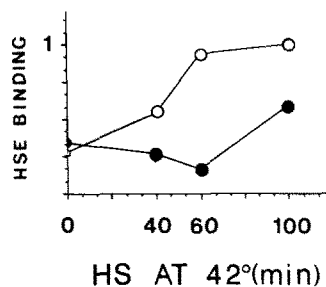
At the level of hsp gene transcription, cycloheximide pretreatment reduced CAT gene activity in the D2500-CAT cell line after a mild stress, a 50-min heat treatment, but not after a more severe stress, a 100-min heat shock (Fig. 3 C). Note that preincubation with cycloheximide for up to 2 h is tolerated, while longer exposure appears to uncover general toxic effects of the inhibitor.



**Figure 3.** Inhibition of protein synthesis increases the threshold level of stress required for HSF activation. (A) Gel retardation assays using the HSE probe are shown with whole cell extracts from cells exposed to different 1-h heat shocks after cycloheximide pretreatment (60  $\mu\text{g/ml}$ ; 2 h at 37°C). (B) Cells pretreated with different inhibitors of protein synthesis (see Fig. 2 B) were either subjected to a 50-min (lanes 1) or 100-min heat treatment (lanes 2) at 42°C. Gel retardation assays were performed on whole-cell extracts using the HSE probe. Arrow in A and B points to the major HSF-HSE complex. (C) Inhibition of protein synthesis increases the threshold level of stress required to activate the CAT gene in the D2500-CAT cell line. Cells pretreated (or not) with cycloheximide (60  $\mu\text{g/ml}$ ) for different periods were exposed to a 50- (lanes 1) or a 100-min (lanes 2) heat treatment at 42°C. Cells were washed, incubated for an additional 24 h at 37°C, and then collected for measurements of CAT activities.

### The Regulatory Factor Is an hsp Itself

If inhibition of HSF activation were mediated by one of the hsp's, it should be more difficult to heat-activate HSF in cells that contained elevated levels of the hsp because of a previous heat treatment than in naive cells. This should be true whether or not the cells were engaged in protein synthesis before and during the second heat treatment provided that there was a period after the first treatment during which hsp's could be made. HeLa cells that had been or had not been heat-treated at 43°C for 1 h were incubated further at 37°C for 4 h, with cycloheximide present during the last 2 h, and the cells were then exposed to the second heat treatment at 42°C (Fig. 4). HSF activity was then measured in nuclear extracts, and it was found that the previous heat treatment had indeed rendered HSF refractory to activation by the subsequent heat treatment. HSF activation had become "thermotolerant" (for other aspects of thermotolerance see reference 46). Similar results were obtained when cycloheximide was omitted. Tolerance did not develop when cycloheximide was added in the first heat treatment, i.e., when hsp synthesis was not allowed before the second heat treatment. These results distinguish the second and the third explanation and suggest that the latter is the correct one. It has been assumed above that the most likely hsp to play the proposed regula-



**Figure 4.** Prior heat treatment of cells raises further the stress threshold for HSF activation. HeLa cultures were either preheated for 1 h at 43°C and then allowed to recover at 37°C for 2 h or kept at 37°C during the same period. Cycloheximide was added, and 2 h later the cultures were subjected to 42°C heat treatments for different periods of

time. Gel retardation assays with the HSE probe were carried out on nuclear extracts. Areas containing HSF-HSE complexes were excised from the gel, and amounts of radioactivity present in complexes were determined by liquid scintillation counting. Shown are actual counts ( $\times 10^{-3}/\text{min}$ ). (●) Preheated cells; (○) not preheated cells.

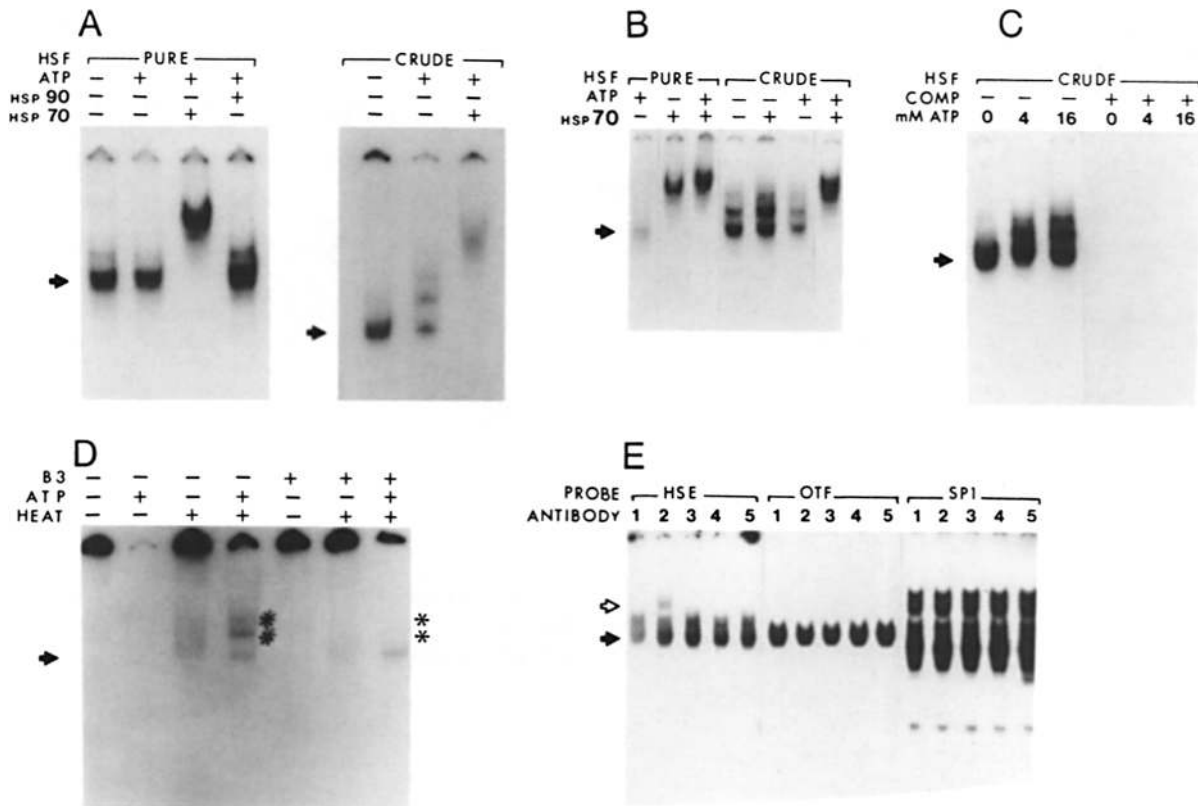
tory role was one of the hsp70 family of proteins. The following experiments were aimed at obtaining *in vitro* evidence for interactions between HSF and hsp70.

### hsp70 Interacts With HSF

Active HeLa HSF affinity purified from heat-treated cells (16) was preincubated with purified HeLa hsp70 (a mixture of hsp70 proteins; reference 51) or hsp90, and then incubated with radiolabeled HSE probe. Protein-DNA complexes were analyzed on neutral acrylamide gels. In the presence of hsp70, but not hsp90 (or BSA, not shown), a protein-DNA complex was formed that migrated much more slowly than HSF-HSE complex (Fig. 5 A, left). At lower concentrations of hsp70, several different complexes that migrated with intermediate rates were resolved (not shown, but see Fig. 5 A, right, for a parallel observation with HSF in nuclear extract). hsp70-HSF-HSE complexes were stable in the presence of ATP (Fig. 5 B) which distinguishes the interaction between hsp70 and HSF-HSE from those of hsp70 with other polypeptide substrates where ATP stimulates substrate release (4, 29, 32).

To ascertain whether hsp70-containing complexes could also form at or below the respective intracellular concentrations of hsp70 and HSF, experiments were carried out with nuclear extracts. Extracts from heat-treated HeLa cells gave rise to several differently migrating complexes, with the fastest complex (corresponding to HSF-HSE) usually being the predominant and in some instances virtually the only form (Fig. 5, B and C). Addition of ATP shifted the equilibrium in favor of the more slowly migrating complexes (Fig. 5, B and C). Assuming that the slower migrating complexes represent HSF-HSE complexes containing one or more molecules of hsp70 (see below), this observation would suggest that hsp70 has a higher affinity for HSF-HSE than for the bulk of other proteins present in the extract. A competition binding assay with nonradioactive HSE DNA confirmed that ATP addition to an extract did not result in nonspecific binding to the HSE probe (Fig. 5 C).

The slower migrating complexes were also formed in extracts prepared from unheated HeLa cells in which HSF was activated by *in vitro* heat treatment (28) in the presence of ATP (Fig. 5 D). To demonstrate that these complexes con-



**Figure 5.** Binding of hsp70 to HSF. (A–C) Complexes formed on HSE DNA upon preincubation of affinity-purified HSF (PURE; reference 16) or HSF in nuclear extract from heat-treated cells (CRUDE) with ATP (0.8 mM, except where indicated differently), hsp70 (100  $\mu$ g/ml affinity-purified HeLa hsp72/73; see reference 51), or hsp90 (500  $\mu$ g/ml purified HeLa hsp90; reference 50). Preincubations of  $\sim$ 30 min at 37°C were followed by 15-min incubations at 25°C with radiolabeled HSE probe or, in C (COMP), a mixture of radiolabeled and a 100-fold excess of unlabeled HSE DNA. Note that the presence of hsp70 or hsp90 appears to stabilize affinity-purified HSF and complexes containing the purified factor. (D) Heat activation of HSF in vitro in the presence or absence of anti-hsp 70 antibodies. Aliquots of cytosolic extract from unheated cells (60  $\mu$ g) were preincubated for 1 h on ice with B3 antibodies (200  $\mu$ g/ml; mixture of C96 and N27 anti-hsp70 mAbs [reference 36]); or with control antibodies (in all samples lacking B3 antibodies) and were then heat-treated at 43°C for 1 h in the presence or absence of 1 mM ATP. Complexes containing the HSE probe were analyzed by gel retardation assay. Asterisks highlight the slowly migrating complexes whose formation is affected by anti-hsp70 antibodies. (E) On the specificity of the hsp70–HSF interactions. Nuclear extract was prepared from cells that had been exposed to 4  $\mu$ g/ml puromycin for 1 h. Aliquots were preincubated for 1 h on ice with different mAbs or ascites fluid (200  $\mu$ g/ml): (lanes 1) N27 anti-hsp70 (reference 29); (lanes 2) C96 anti-hsp70 (reference 36); (lanes 3) 92D anti-hsp 90 mouse monoclonal antibody; (lanes 4) anti-*Escherichia coli*  $\beta$ -galactosidase mouse monoclonal antibody (Promega Corp., Madison, WI); (lanes 5) ascites fluid. This preincubation was followed by retardation assays using HSE, OTF, and SP1 probes. Open arrow indicates the position of the antibody-retarded hsp70–HSF complex. Filled arrows point to the positions of basic HSF–HSE complex.

tained hsp70, a mixture of monoclonal anti-HeLa hsp70 antibodies (B3) was included in the activation reactions. Antibody addition selectively prevented the formation of the slower migrating complexes but did not affect the rapidly migrating complex.

To determine whether the interaction between HSF and hsp70 was unusual among transcription factors, nuclear extract was prepared from HeLa cells that had been exposed for a short time to a low concentration of puromycin (22, 26). This procedure for activation of HSF, rather than a heat treatment, was employed to avoid partial denaturation of transcription factors that, in agreement with its purported role in stress, could result in the recruitment of hsp70 to the unfolding factors. Parallel DNA binding assays were carried out in the presence of different antibodies and with radiolabeled oligonucleotide probes containing binding sites for HSF, OTF, and SP1, respectively (Fig. 5 E). Addition of C96 and less efficiently of N27 anti-hsp70 monoclonal antibody

generated supershifted hsp70–HSF complexes, apparently at the expense of the slower migrating forms discussed above. No analogous changes could be observed for the factor(s) binding to the OTF and SP1 recognition sequences, or when equal amounts of ascites fluid or monoclonal antibodies against hsp90 or  $\beta$ -galactosidase were used instead of the anti-hsp70 antibodies. We conclude from these experiments that hsp70 interacts with HSF with some specificity.

## Discussion

The intensity of the response to a moderately severe stress in cells that are prevented from synthesizing new proteins is reduced dramatically when compared with the normal response. This phenomenon was observed initially by pretreating cells with cycloheximide, exposing them to a 42°C heat shock in the presence of the inhibitor, removing the inhibitor by extensive washes immediately after heat treatment, and



pulse-labeling newly synthesized hsp's by incubation in the presence of [<sup>3</sup>H]leucine. Much less hsp70 was synthesized after heat treatment in cells that had been than in cells that had not been exposed to cycloheximide (Fig. 1 A). These findings when interpreted as reflecting transcriptional regulation of hsp70 genes were, at least on their surface, against the expectation. Already in 1963 work on heat-induced puffing in *Drosophila* had suggested that heat shock gene transcription occurred in the presence of an inhibitor of protein synthesis (37). Experiments with inhibitors reappeared over the years in different incarnations, recently to demonstrate that heat shock transcription factor HSF in *D. melanogaster* and human cells preexist in an inactive form and can be activated in vitro and in vivo in the absence of protein synthesis (27, 44, 54). There exists, however, an important difference between these types of experiments and the ones described herein: cells were preincubated with cycloheximide before they were exposed to stress in the experiments shown here, whereas the inhibitor was presumably added immediately before heat treatment in the earlier experiments. Time course experiments revealed that suppression of the induction of hsp synthesis essentially does not occur when the inhibitor is added during (near the beginning) heat treatment and becomes more pronounced with increasing length of preincubation with the inhibitor until a maximal effect is reached at preincubation times of 1 to 2 h (not shown, but for an analogous experiment with the D2500-CAT line, see Fig. 3 C). Thus, our experiments do not disagree with the earlier observations by others. They are concerned with the different aspect that regulation of hsp synthesis either involves a labile component or that the concentration of nascent polypeptides is itself regulatory.

Different types of experiments were carried out to define the level at which cycloheximide interfered with hsp expression. The effect of cycloheximide could be detected at the level of hsp70 A and B mRNA accumulation (Fig. 1 B), of the activity of a reporter gene driven by the promoter of the hsp70B gene (Fig. 1 C), and, most importantly, of DNA binding activity of HSF (Fig. 2 A). An effect of cycloheximide on DNA binding activity of HSF also has been observed recently by another group (55). Presumably then, cycloheximide affected transcription of hsp genes through its effect on the activation of the group-specific transcription factor. To ascertain that the inhibitory effect of cycloheximide preincubation on HSF was specific, DNA binding activities of other factors were assayed in the same extracts and were found to be unaffected (Fig. 2 A). Analogous effects on HSF but not on other transcription factors were observed in experiments with three other inhibitors of protein synthesis. The results suggested that either inactive HSF or a component of the activation mechanism is labile or nascent polypeptides are themselves regulating negatively HSF activity.

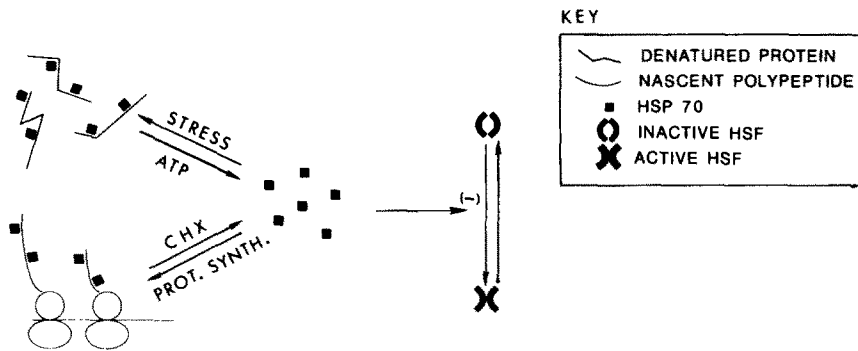
Earlier work had suggested that hsp gene expression may be autoregulated and that the kinetics with which hsp70 accumulates make it the best candidate for the presumed autoregulator (10). Recent work by Stone and Craig (45) implicates an hsp70 protein in the autoregulation of one of the hsp70 genes in yeast. Also, several lines of evidence indicate that the *E. coli* hsp70 homolog, the product of the *dnaK* gene, together with the *dnaJ* and *grpE* gene products, acts as negative regulator of hsp gene expression by controlling tightly

the synthesis and stability of the heat shock gene-specific sigma factor, sigma 32 (8). Unlike other hsp's, members of the hsp70 family are ubiquitous in the cells and are capable of binding to unfolded polypeptides and peptides (12–14, 25). The observation by Beckman et al. (4), suggesting that much if not most hsp70 is normally associated with nascent polypeptides aiding in their timely folding and processing, leads to several predictions that if fulfilled strongly suggest a role of hsp70 in the regulation of hsp transcription. First, if the concentration of nascent polypeptides is altered rapidly and dramatically, the level of free hsp70 should change as a consequence, and if hsp70 regulates HSF, a complementary change should occur in the activity of HSF. Our experiments with cycloheximide and the other inhibitors of protein synthesis tested this prediction since they brought about a dramatic reduction in the concentration of nascent polypeptides by blocking new protein synthesis and permitting preexisting nascent polypeptides to either mature or be degraded. The concentration of free hsp70 should have increased and, as a consequence, HSF could be expected to become refractory to activation by stress. This result indeed was observed (Fig. 2 A).

Second, it should be possible to reverse the inhibition of stress activation of HSF caused by the increased free hsp70 level in inhibitor-treated cells by creating a higher demand for hsp70. Since hsp70 associates with denaturing proteins, an increase in the severity of the stress applied to the cells—and hence in the amount of protein denaturing—should reduce the level of free hsp70 and restore full activation of HSF. The experiments in Fig. 3 demonstrated that this prediction was correct. Obviously, this result eliminated the possibility that the inhibitor effect could be explained by the lability of inactive HSF or a component of the activating device.

Finally, since stress events cause the concentration of hsp70 to increase, HSF in cells that have undergone a prior heat treatment should be more refractory than HSF in naive cells to activation by the next heat shock. This should be true irrespective of whether or not cycloheximide was added before the (second) heat shock. Again, the results of the experiments were as predicted (Fig. 4).

A summary of our interpretation of the results is shown in Fig. 6. In this model hsp gene activity is regulated negatively by hsp70 or another hsp with similar properties that participates in protein synthesis and interacts with nascent polypeptides as well as with stress-unfolded proteins. Changes in the total load of nonfolded polypeptides are translated into changes in the concentration of the free form of this hsp that is normally sufficiently high to prevent activation of HSF. The concentration of free hsp70 is decreased when the load of denatured proteins increases because of stress or when the rate of protein synthesis increases. This results, more or less directly, in the activation of HSF and in enhanced hsp gene expression. The concentration of the free form of hsp70 is increased and inhibits activation of HSF when the rate of protein synthesis is reduced. The model accommodates pertinent earlier observations (2, 10, 12, 15, 22, 26, 45, 48). Interactions of hsp70 with nascent polypeptides and presumably also with unfolded polypeptides are transient and involve ATP binding to hsp70 and hydrolysis. The model suggests that ATP depletion may decrease the



**Figure 6.** Possible regulatory role of hsp70 in the heat shock response. Free hsp70 may negatively regulate events leading to the activation of HSF. Levels of free hsp70 may be increased by a reduction in protein synthesis but reduced by a stress event.

concentration of free hsp70, activating HSF. Indeed, ATP depletion induces hsp gene expression (reference 24, our unpublished results).

As mentioned before, a cycloheximide effect on the activation of the DNA binding activity of HSF has been reported previously (55). Although it did not examine the phenomenon in great depth, the earlier study presented data that closely match some of the findings herein. While pointing towards subtle and complex ways in which protein synthesis might be coupled with HSF activity, the major conclusions of the earlier study differ greatly from those discussed here. The study had concluded that the cycloheximide effect on HSF activation could be explained (a) by a component of the activation machinery that is labile at the intermediate but not at the high heat shock temperature or (b) by two different populations of HSF, newly synthesized HSF molecules that can be activated at the intermediate temperature and more "mature" HSFs that can only be activated by a more severe heat shock. As discussed above (Fig. 3 B), the finding that HSF activation is suppressed by cycloheximide during the first 50 min of incubation at 42°C but occurs upon further incubation at the same temperature despite the presence of cycloheximide rules out the first possibility. That a previous heat treatment raises the stress threshold for activation of HSF similarly to cycloheximide (experiment in Fig. 4) would be unexpected in cells containing newly synthesized HSF that, according to the second explanation, is activated by low levels of stress. The result is more easily understood if one considers that what a previous heat treatment and cycloheximide pretreatment have in common is that both are expected to increase the level of free hsp70.

In the simplest version of the model discussed above (Fig. 6), a member(s) of the hsp70 family would bind to HSF and influence or participate in a mechanism that controls activity of the factor. Note that hsp70 could either play a catalytic role during inactivation of HSF or may constitute (part of) the device that keeps HSF in an inactive configuration. We have attempted to obtain direct evidence for a binding interaction between HSF and hsp70. Our initial experiments using highly purified HSF and hsp70 suggested that such an interaction could occur. However, considering the properties of hsp70, the possibility that hsp70 could nonspecifically bind to other proteins when both components are highly enriched could not be dismissed lightly. Thus, demonstration of an interaction between hsp70 and HSF in unfractionated extracts was emphasized. Different complexes in extracts of hsp70 and HSF but not of hsp70 and two other transcription factors were indeed revealed by monoclonal antibodies against hsp70 (Fig. 5).

ATP is well-known to release hsp70 from binding to peptides and stress-denatured proteins (4, 29, 32). It should be pointed out that the interaction between hsp70 and HSF appears to be of a different nature. hsp70-HSF complexes formed with purified components are resistant to ATP addition (Fig. 5, A and B). In extracts ATP enhanced the stability or formation of hsp70-HSF complexes, presumably through releasing hsp70 from other extract proteins. Thus, in the cell hsp70 may have a higher affinity for HSF than for other proteins.

Although hsp70 interacts *in vitro* with some specificity with HSF, this interaction does not lead to the inactivation of the binding activity of HSF. This absence of inactivation may be explained either by the inadequacy of the *in vitro* conditions for the formation of the correct complex (the protein concentration in the cell may be an order of magnitude higher than in the nuclear extracts) or by the complex nature of the regulatory system that may require other collaborating components. Note that active HSF in extract from heat-treated cells is not inactivated by further incubation of the extract with or without added hsp70 at low temperature. This contrasts with the *in vivo* situation where HSF is inactivated shortly after cells are returned from heat shock to control temperature.

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