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Different Thresholds in the Responses of Two Heat Shock Transcription Factors, HSF1 and HSF3*

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Avian cells express three HSF genes encoding a unique factor, HSF3, as well as homologues of mammalian HSF1 and HSF2. HSF1 is the major factor that mediates the heat shock signal in mammalian cells. We reported previously that cHSF3, as well as cHSF1, is activated by heat shock in chicken cells. In this study, we examined the functional differences between cHSF1 and cHSF3. Comparison of the heat-inducible DNA binding activity of cHSF1 with cHSF3 at various temperatures revealed that the latter was activated at higher temperatures than the former. At a mild heat shock, such as 41 °C, only cHSF1 was activated, whereas both cHSF1 and cHSF3 were activated following a severe heat shock at 45 °C. Heat-inducible nuclear translocation and trimerization were accompanied by DNA binding activity. We also observed that cHSF3 was activated by treating cells with higher concentrations of sodium arsenite compared to cHSF1. The DNA binding activity of cHSF3 by severe heat shock lasted for a longer period than that of cHSF1. Interestingly, the total amount of cHSF3 increased only upon severe heat shock, whereas that of HSF1 decreased. Substantial amounts of cHSF3 remained in the soluble fraction under severe heat shock, whereas cHSF1 rapidly moved to the insoluble fractions in that conditions. Comparison of transcriptional activity of the activation domains of cHSF1 and cHSF3 revealed that the activity of cHSF3 was as strong as that of cHSF1. These findings indicate that there are different thresholds for cHSF1 and cHSF3 and that cHSF3 is involved in the persistent and burst activation of stress genes upon severe stress in chicken cells. Pretreatment of cycloheximide elevated the threshold concentrations of arsenite of both factors. This suggests that denaturation of nascent polypeptides could be the first trigger for the activation of both factors, and the pathways for activation of cHSF1 and cHSF3 may be identical, or at least share some common mechanisms.

When cells are exposed to elevated temperature and to certain chemical and physiological stresses, the synthesis of a set of proteins called heat shock proteins (HSPs)[1] or stress proteins is rapidly induced. These polypeptides are well conserved between bacteria and mammals and function as molecular chaperones to prevent and repair the protein damage caused by various stresses (1–4). Heat shock gene expression is regulated mainly at the level of transcription.

Heat shock factor (HSF) is a sequence-specific DNA binding protein that binds tightly to multiple copies of a highly conserved sequence motif (nGAAn) in the promoter regions of heat shock genes, termed the heat shock element (HSE). Heat-inducible activation of pre-existing HSF causes a rapid and transient increase of heat shock gene transcription (5, 6). In the budding yeasts *Saccharomyces cerevisiae* and *Kluyveromyces lactis*, HSF constitutively binds to HSE as a trimer and stimulates transcription upon heat shock (7–10). In contrast, HSF does not bind to HSE under normal growth conditions in the fusion yeast *Schizosaccharomyces pombe*, *Drosophila melanogaster*, and higher eukaryotes but acquires HSE binding activity by trimerization upon exposure to stress (10–16).

In vertebrate cells, members of the HSF gene family, which includes HSF1, HSF2, HSF3, and HSF4, are thought to respond differently to various forms of stresses (17–21). Preexisting HSF1 is rapidly activated in response to heat and other physiological stresses (22–24). In contrast, HSF2 is activated in human K562 cells during hemin-induced erythroid differentiation (25, 26). During this differentiation, the amount of HSF2 increased post-transcriptionally. This signaling pathway, which leads to the activation of HSF1 or HSF2, is not yet clear, although it is speculated that HSP70 is a sensor for heat stress (27, 28).

cHSF3 was originally cloned from a chicken erythrocyte cDNA library (18). We reported recently that both cHSF1 and cHSF3 are activated by heat shock and sodium arsenite in chicken cells (29). Activation of cHSF3 after heat shock is accompanied by nuclear translocation, acquisition of DNA binding activity, and conversion from an inert dimeric form to a DNA-binding trimeric form. The kinetics of stress-induced DNA binding and nuclear translocation of cHSF1 and cHSF3 are different. cHSF1 responds to stress rapidly, whereas cHSF3 is converted to a trimer and moves to the nucleus slowly. However, the functional differences between cHSF1 and cHSF3 are still unclear.

Here we report that the threshold temperatures required to activate cHSF1 and cHSF3 are different. cHSF3 was activated by heat shock at a higher temperature than was cHSF1. Upon severe heat shock, the total amount of cHSF3 increased whereas that of cHSF1 decreased. Whereas cHSF1 rapidly moved to insoluble fraction, much of cHSF3 remained in the soluble fraction. The ability of cHSF3 to activate transcription

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[1] The abbreviations used are: HSP, heat shock protein; HSE, heat shock element; HSF, heat shock factor; CEF, chicken embryo fibroblast(s); PAGE, polyacrylamide gel electrophoresis.

was as strong as that of cHSF1. These results suggest that cHSF3 has a role in persistent and burst activation of stress genes under conditions of severe stress in chicken cells.

EXPERIMENTAL PROCEDURES

Cell Culture and Preparation of Whole Cell Extracts, Cytoplasmic Extracts, and Nuclear Extracts—Chicken embryo fibroblasts (CEF) were maintained in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum, 2% chicken serum, and 2% tryptose phosphate broth. The avian leukosis virus-induced chicken B cell line DT40 (30) was provided by Dr. S. Takeda (Kyoto University) and cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 1% chicken serum, 0.01 mM 2-mercaptoethanol. Cells were incubated in 5% CO_2 at 37 °C. To prepare whole cell extracts, cells were rapidly frozen in liquid nitrogen and suspended in buffer C containing 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, pH 7.9), 25% (v/v) glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 1 μg/ml leupeptin, and 1 μg/ml pepstatin A. The lysates were kept on ice for 5 min and centrifuged at 100,000 × *g* for 5 min at 4 °C. The supernatants were frozen in liquid nitrogen and stored at −80 °C until use. The pellet fractions were suspend in Laemmli's sample buffer and sonicated.

Subcellular fractionation was performed as described previously (31). The protein concentration was determined using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, CA).

Gel Mobility Shift Assay—The antibody supershift experiments were performed by incubating 2.0 μl of 1:10 diluted antiserum in PBS with 1–2 μl of cell lysates in a total volume of 10 μl. After incubation on ice for 20 min, a nucleotide probe mixture containing 0.1 ng of ^{32}P-labeled self-complementary ideal HSE oligonucleotides (20) and 0.5 μg of poly(dI-dC) (Pharmacia Fine Chemicals, Piscataway, NJ) in 10 mM Tris-HCl (pH 7.8) and 1 mM EDTA was added to a final volume of 25 μl. The binding reactions were incubated on ice for 20 min, mixed with 2 μl of a dye solution (0.2% bromphenol blue, 0.2% xylene cyanol, and 50% glycerol) and then separated on 4% native polyacrylamide gels. Gels were run in 0.5 × TBE buffer at room temperature for 1.7 h at 140 V, dried, and exposed to x-ray film at −80 °C with intensifying screens.

Gel Filtration—Whole cell extracts (200 μl containing 300–500 μg of protein) were applied to a Superdex 200HR column attached to a fast protein liquid chromatography apparatus (Pharmacia). The samples were eluted at 0.3 ml/min with a buffer containing 1% glycerol, 20 mM Tris-HCl (pH 7.9), 200 mM KCl, and 1.5 mM $MgCl_2$. Each fraction (0.5 ml) was precipitated with trichloroacetic acid (final concentration, 10%), washed with acetone, dried, suspended in gel sample buffer, and analyzed by SDS-PAGE followed by Western blotting. The peak positions of HSFs were determined as described previously (29).

Immunofluorescence Study—CEF were cultured on glass coverslips for 16 h, fixed with 50% ethanol-50% acetone at −20 °C for 20 min, and washed with PBS. After blocking with 10% normal goat serum in PBS, cells were incubated with 1:200-diluted αHSF1 and αHSF3 serum in 2% normal goat serum in PBS and then with fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin G antibody (1:200 dilution) (Cappel). The coverslips were washed and mounted in 80% glycerol in 10 mM Tris-HCl (pH 7.5) on a coverglass. Immunostained cells were observed by fluorescence microscopy (Nicon, Tokyo, Japan).

Western Blot Analysis—Proteins were fractionated by SDS-PAGE and transferred onto nitrocellulose filters. The filters were blocked with 5% dry milk in PBS for 1 h at room temperature and incubated with a 1:500 dilution of rabbit antiserum against each HSF in PBS with 2% dry milk. After washing with PBS, the filters were incubated with a 1:1000 dilution of horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (Cappel) for 30 min at room temperature and washed with 0.1% Tween 20 in PBS. Signals were detected using the ECL system (Amersham Corp.).

Northern Blot Analysis—Total RNA was isolated by the acid guanidium thiocyanate/phenol/chloroform method (32). 10 μg of RNA was fractionated on 1% agarose gel containing formaldehyde and capillary transferred onto GeneScreen Plus membrane (DuPont NEN). The filter was baked at 80 °C for 2 h and hybridized in a solution containing 50% formamide, 5 × SSC, 6% PPBF (2% polyvinyl pyrrolidone, 2% bovine serum albumin, 2% ficoll 400), 1% SDS, and 0.1 mg/ml salmon sperm DNA at 42 °C for 6 h. The filters were then incubated with radiolabeled chicken HSP70 cDNA (0.5 kb *Hin*dIII-*Sma*I fragment of pC1.8, a gift from Dr. R. I. Morimoto, Northwestern University) (33), human HSP90α cDNA (0.7 kb *Sac*I fragment of pC1–11R, a gift from Dr. K. Yokoyama, RIKEN, Tsukuba, Japan) (34), human HSP27 cDNA (0.4 kb *Pst*I fragment of pHS208, a gift from Dr. L. A. Weber, South Florida University) (35), or human β-actin cDNA (2.0 kb *Bam*HI fragment of pHFβA-1, a gift from Dr. L. Kedes, Stanford University) (36) as probes.

Transfection and Luciferase Assay—Co-transfection and luciferase assay were performed described previously (17, 29).

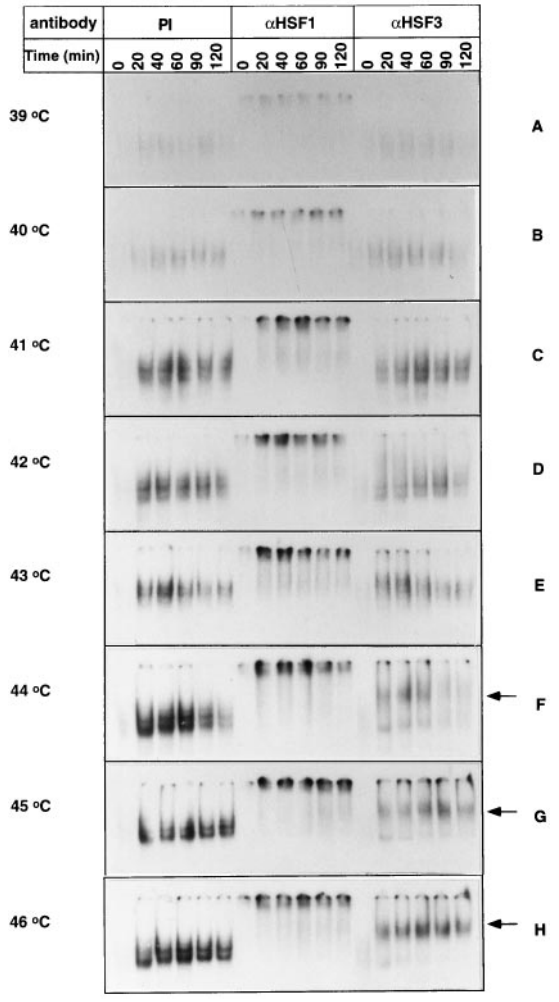

FIG. 1. **DNA binding activities of cHSF1 and cHSF3 induced at various temperatures.** CEF were heat shocked at 39–46 °C for the indicated times, and whole cell extracts were prepared. Five-μg aliquots of cell extracts were incubated with preimmune serum (*PI*) or antisera specific for the carboxyl-terminal regions of cHSF1 (*αHSF1*) or cHSF3 (*αHSF3*; 2 μl of 1:10 diluted serum to a final volume of 10 μl in PBS) on ice for 20 min and then with ^{32}P-labeled HSE. The mixtures were separated on 4% native gels. The *arrows* on the *right side* indicate the positions of supershifted bands of cHSF3·HSE complex, detected after addition of anti-cHSF3 antiserum.

RESULTS

Different Effects of Various Temperatures on the Acquisition of cHSF1 and cHSF3 DNA Binding Activities—We previously reported that the DNA binding activities of both cHSF1 and cHSF3 were induced by incubating chicken HD6 erythroblastic cells at 45 °C and that the induction of cHSF3 DNA binding activity was delayed compared with that of cHSF1 (29). CEF cultured at 37 °C were heat shocked for up to 120 min at a series of temperatures from 39 to 46 °C, and then whole cell extracts were prepared for gel shift assay. Very weak but distinct heat-inducible HSE binding activity was observed when cells were incubated at 39 °C, and this heat-inducible DNA binding activity became clearer with the elevation of the heat shock temperature (Fig. 1, *left*). Supershift experiments using specific antisera against cHSF1 or cHSF3, which recognized the carboxyl regions of the respective HSF, showed that only anti-cHSF1 serum supershifted the DNA binding activity

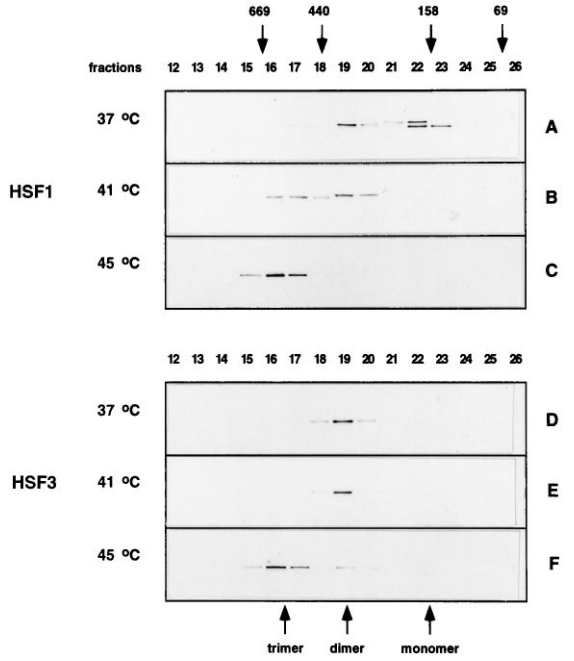

FIG. 2. **Oligomeric structures of cHSF1 and cHSF3 in cells treated at low or high temperature.** Whole cell extracts of control CEF or cells incubated at 41 or 45 °C for 30 min were prepared. These lysates were fractionated by gel filtration using a Superdex 200 HR column (Pharmacia). Proteins from each fraction were precipitated with trichloroacetic acid, fractionated by SDS-PAGE, and analyzed by Western blotting using antisera against cHSF1 or cHSF3. The positions of monomeric, dimeric, and trimeric forms of cHSF1 and cHSF3 are indicated at the *bottom*. The elution positions of protein standards are indicated at the *top* (669 kDa, thyroglobulin; 440 kDa, ferritin; 158 kDa, aldolase; 69 kDa, bovine serum albumin).

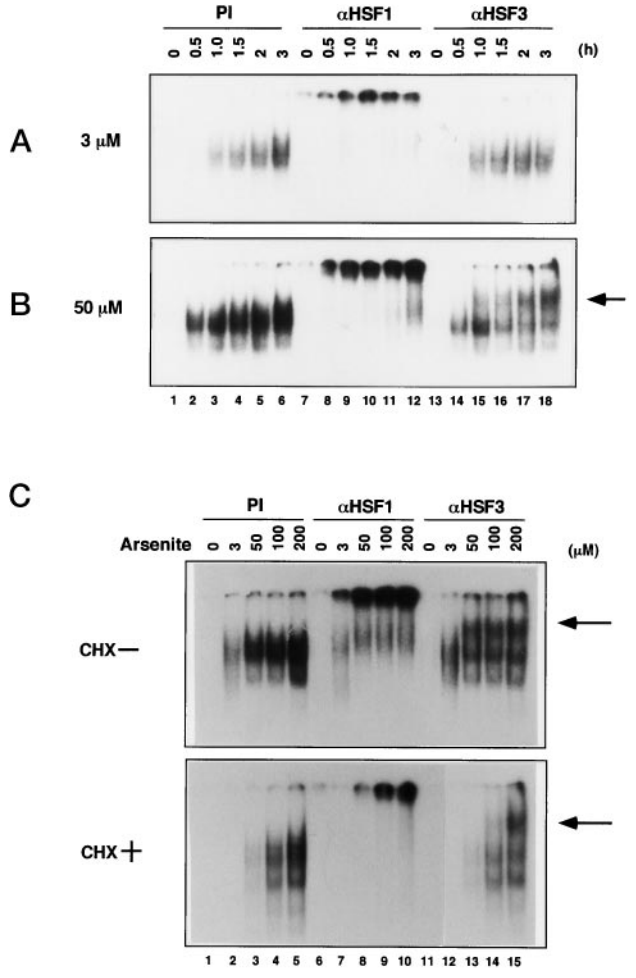

FIG. 3. **DNA binding activity of cHSF1 and cHSF3 induced by sodium arsenite.** CEF were treated with 3 μM (*A*) or 50 μM (*B*) sodium arsenite for the indicated periods, and whole cell extracts were prepared. Gel shift assays and supershift experiments were performed as described in the legend to Fig. 1. The *arrow* in *B* indicates the position of the supershifted band of cHSF3·HSE complex. *C*, CEFs were exposed to the indicated concentrations of sodium arsenite for 2 h without cycloheximide (*CHX−, upper panel*). In the lower panel, cells were pretreated with 100 μM of cycloheximide for 1 h and then with sodium arsenite in the presence of cycloheximide for 2 h (*CHX+*). Gel shift assays were performed using 5-μg aliquots of whole cell extracts. The *arrow* indicates the supershifted band of cHSF3·HSE complex.

at mild heat shock temperatures, such as 39, 40, and 41 °C, whereas anti-cHSF3 did not at these temperatures (Fig. 1, *A–C*). At higher temperatures, such as 44, 45, and 46 °C, anti-cHSF3 clearly supershifted a part of the DNA binding activity (Fig. 1, *F-H, arrows*). The DNA binding activity of cHSF1, which was not supershifted by the addition of anti-cHSF3 serum, gradually decreased at 44 °C and rapidly disappeared at 46 °C. These results suggest that the DNA binding activity of cHSF1 and cHSF3 were both induced in CEF by heat shock, but the threshold temperatures for their activation are different. DNA binding activity of cHSF3 is induced by treatment of cells at higher temperatures compared with cHSF1.

We next examined the heat shock-induced changes in the oligomeric form of each HSF. Whole cell extracts from control and heat-shocked cells were fractionated on a Superdex 200HR column, and the fractions containing cHSF1 and cHSF3 were analyzed by immunoblot analysis as described under "Experimental Procedures" (Fig. 2). The inert monomeric form of HSF1 is reported to be converted to a DNA-binding trimeric form after heat shock (15, 29). In the control CEFs, cHSF1 was eluted as a major peak of 160 kDa and a minor peak of 360 kDa (Fig. 2*A*). After cells were incubated at 45 °C for 30 min, all of the cHSF1 was eluted at a peak of approximately 600 kDa (Fig. 2*C*). Even when cells were mildly heat shocked at 41 °C for 30 min, most of the monomeric cHSF1 was converted to the trimeric form (Fig. 2*B*). In contrast, the dimeric form of cHSF3, which was eluted at a major peak of 390 kDa, was not converted to the trimer after a mild heat shock at 41 °C for 30 min (Fig. 2, *D* and *E*). Most of the cHSF3 was converted to the trimeric form when cells were exposed to severe heat shock at 45 °C for 30 min (Fig. 2*F*). These observations were consistent with the DNA binding activities of cHSF1 and cHSF3, shown in Fig. 1 after treatment of the cells at various temperatures.

Different Thresholds for the Activation of cHSF1 and cSHF3 by Chemical Stress—The heat shock response is also induced by chemical stresses. Previously, we reported that both cHSF1 and cHSF3 are activated when cells are exposed to sodium arsenite (29). To examine whether there are different thresholds for activation of cHSF1 and cHSF3 by chemical stress, whole cell extracts from CEF exposed to various concentrations of sodium arsenite were prepared and HSE binding activity was examined by gel shift assay (Fig. 3). When cells were treated with a low concentration of sodium arsenite (3 μM), HSE binding activity was induced and increased up to 3 h (Fig. 3*A, lanes 1–6*). Supershift experiments showed that the induced activity was due solely to cHSF1 (*lanes 7–12*); no activity of cHSF3 was induced (*lanes 13–18*). When cells were treated with a high concentration of sodium arsenite (50 μM), a burst of HSE binding activity was induced even at 30 min (Fig. 3*B, lanes 1–7*). Supershift experiments using specific antisera revealed that these HSE binding activities were composed of both cHSF1 and cHSF3 (Fig. 3*B, lanes 8–18*). These results indicate that a high concentration of sodium arsenite is needed for activation of cHSF3. Again, the DNA binding activity of cHSF1,

which was not supershifted with anti-cHSF3 serum, clearly decreased (Fig. 3*B, lanes 14–18*).

Protein Synthesis Inhibitor Elevates the Thresholds for the Activation of Both cHSF1 and cHSF3—Pretreatment with cycloheximide, an inhibitor of protein synthesis, was reported to suppress the stress response at mild heat shock (37), and this suppression was shown to be coupled with down-regulation of HSE binding activity (38). In contrast, cells exposed to severe stress showed induction of stress proteins even when they were pretreated with cycloheximide (37). Thus, it has been considered that the first cellular target of mild stress might be newly synthesized polypeptides. We examined the effect of a protein synthesis inhibitor upon activation of cHSF1 and cHSF3. CEF were exposed to various concentrations of sodium arsenite in the presence or absence of cycloheximide, and the whole cell extracts were subjected to gel shift assay (Fig. 3*C*). In the absence of cycloheximide, treatment with sodium arsenite for 2 h induced HSE binding activity in a dose-dependent manner (Fig. 3*C, upper panel, lanes 1–5*). Supershift experiments showed that cHSF1 was activated at 3 μM sodium arsenite, whereas cHSF3 was not (*lanes 7* and *12*). Activation of cHSF3 was detected at concentrations of 50 μM or higher (Fig. 3*C, lanes 13–15*), which is consistent with the results shown in Fig. 3*B*. However, when cells were pretreated with cycloheximide for 1 h, the relative induction of HSE binding activity was significantly reduced at concentrations of 3–100 μM of arsenite (Fig. 3*C,* compare *upper* and *lower panels, lanes 2–5*). In the presence of cycloheximide, cHSF1 and cHSF3 activation were observed at arsenite concentrations as low as 50 μM and 100 μM respectively (Fig. 3*C, lower panel, lanes 8* and *14*). These concentrations were higher than those in cells exposed to arsenite without cycloheximide treatment.

We further analyzed the oligomeric state of cHSF3. After treatment of the cells with 50 μM sodium arsenite, cHSF3 was mainly eluted corresponding to the position of trimeric form. In contrast, no trimeric form of cHSF3 was detected following treatment with sodium arsenite at a concentration of 50 μM in the presence of cycloheximide (data not shown). These results indicate that activation of cHSF3, as well as that of cHSF1, was affected by cycloheximide treatment, suggesting that the first trigger for activation of these factors might be the denaturation of nascent polypeptides.

Subcellular localization of cHSF1 and cHSF3 by a Mild and Severe Heat Shock—We further studied the subcellular localizations of cHSF1 and cHSF3, both before and after mild and severe heat shock, by biochemical fractionation followed by Western blot analysis (Fig. 4*A*). Most cHSF1 and cHSF3 existed in the cytoplasmic fraction in control cells (Fig. 4*A, lanes 1* and *11*), and all of both molecules was translocated to the nuclear fraction after cells were incubated at 45 °C for 60 min (Fig. 4*A, lanes 8* and *18*). When cells were stressed with a mild heat shock at 41 °C, about one-half of the cHSF1 was observed in the nuclear fraction (Fig. 4*A, lanes 3–6*), whereas none of the cHSF3 moved to the nuclear fraction even after heat treatment for 60 min (Fig. 4*A, lanes 13–16*). The decrease in the amount of cHSF1 after heat shock at 45 °C for 2 h was due to its insolubilization (Fig. 4*A, lane 10*; see Fig. 7). The difference in subcellular localization between cHSF1 and cHSF3 was also examined by indirect immunofluorescence analysis (Fig. 4*B*). cHSF1 staining was observed mainly in the cytoplasm, and very weak staining was observed in the nucleus in control cells (Fig. 4*B, a*). Upon heat shock at 41 °C, the intensity of nuclear staining increased and cHSF1 was detected mainly in the nucleus at 45 °C (Fig. 4*B, b* and *c*). In contrast to cHSF1 staining, diffuse cytoplasmic staining of cHSF3 was observed in cells heat shocked at 41 °C as well as in control cells (Fig. 4*B, d* and *e*). Severe heat shock at 45 °C caused a punctate nuclear staining of cHSF3 (Fig. 4*B, f*). The results obtained by indirect immunofluorescence analysis are consistent with those obtained by biochemical cell fractionation.

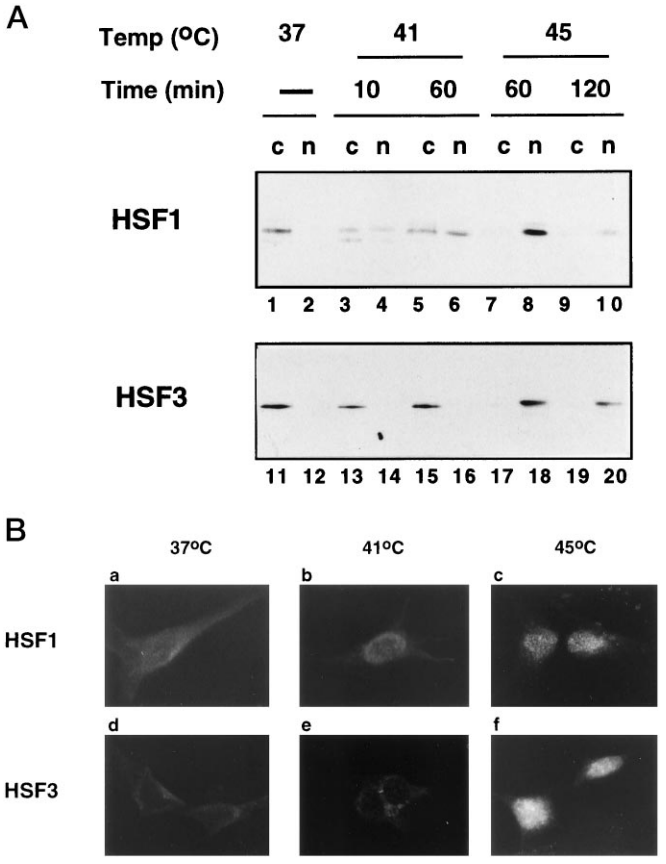

FIG. 4. **Subcellular localization of cHSF1 and cHSF3 in cells heat shocked at low or high temperature.** *A*, nuclear (*n*) and cytoplasmic (*c*) extracts were prepared from control CEF and cells that were heat shocked at 41 or 45 °C for the indicated periods. Four-μg aliquots of extracts were analyzed by SDS-PAGE and Western blotting as described in the legend to Fig. 2. *B*, nuclear translocation of cHSF1 (*a–c*) and cHSF3 (*d–f*) upon heat shock. Control CEF (*a* and *d*) and cells heat shocked at 41 °C (*b* and *e*) or 45 °C (*c* and *f*) were subjected to indirect immunofluorescence analysis using specific antisera against cHSF1 or cHSF3 as a first antibody and fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (Cappel) as the second antibody. Immunolabeled cells were photographed by fluorescence microscopy (Nicon, Tokyo, Japan).

Taken together, these results strongly suggest that there are different threshold temperatures for activation of each HSF; a higher temperature was needed for cHSF3 to acquire DNA binding activity, to form a trimer, and to move to the nucleus than for cHSF1.

mRNA Accumulation of Stress Genes Induced by Mild and Severe Heat Shock—The results described above led us to an idea that cHSF3 may be involved in the induction of stress proteins in response to severe heat shock. To determine whether there are any differences in the responses to mild and severe heat shock, we performed Northern analysis using cDNA probes for various heat shock genes. In both CEF and chicken B lymphocyte DT40 cells, induction of HSP70 and HSP90 mRNA was observed following heat shock even at 41 °C (Fig. 5, *lanes 2, 3, 7,* and *8*). The levels of HSP70 mRNA were markedly increased when cells were continuously exposed to a temperature of 45 °C (Fig. 5, *lanes 4, 5, 9,* and *10*), whereas the HSP90 mRNA level after 45 °C heat shock was similar to that observed at 41 °C (Fig. 5, *HSP90*α). The expression of HSP90 may be differentially regulated post-transcriptionally (39).

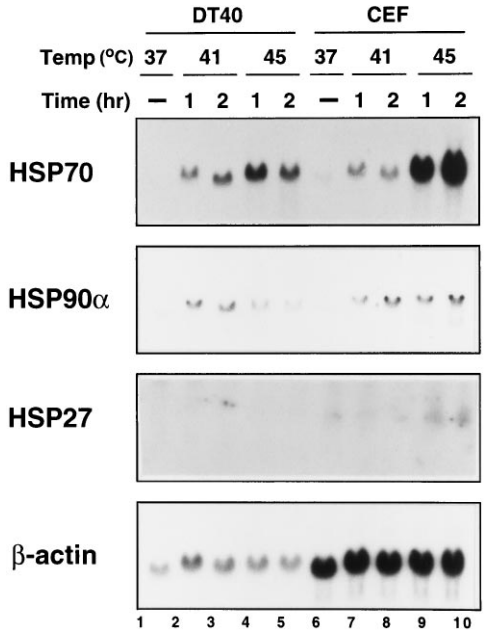

FIG. 5. **Accumulation of HSP mRNA induced by low or high temperature.** CEF and chicken B lymphocyte DT40 cells were heat shocked at 41 and 45 °C for 1 or 2 h, and then total RNAs were prepared. Ten-μg aliquots of total RNA were electrophoresed, and Northern blot analysis was performed using cDNA probes for chicken HSP70, human HSP90α, human HSP27, and human β-actin as described under "Experimental Procedures."

HSP27 mRNA was detected only in CEF, and its accumulation was similar to that of HSP70.

Stability of cHSF1 and cHSF3 at Higher Temperatures—To examine the total amount of cHSF1 and cHSF3, the same numbers of DT40 cells were heat shocked at 41 or 45 °C for the indicated periods and directly suspended in Laemmli's sample buffer by sonication. These samples were run on SDS-10% polyacrylamide gels and subjected to Western blot analysis with cHSF1 or cHSF3 antiserum. At a mild heat shock, such as 41 °C, the protein levels of both factors were unchanged (Fig. 6, *lanes 1–5*). However, when cells were exposed to severe heat shock, such as 45 °C, the total amounts of cHSF1 and cHSF3 were changed in different ways. Remarkably, the amount of cHSF3 increased about 2-fold after heat shock at 45 °C for 1–2 h and then decreased slowly, whereas that of cHSF1 decreased rapidly (*lanes 6–10*).

We wondered whether HSF1 could act as a transcriptional activator even at higher temperatures at which HSF3 is activated. To test this, we next examined the stability of cHSF1 and cHSF3 under heat shock conditions. Cells were heat shocked at a temperature of 39–46 °C for 1 h. Proteins in whole cell extracts and insoluble pellet fractions were separated by SDS-PAGE, and the amounts of each HSF contained in these fractions were quantified by Western blotting using anti-cHSF1 or anti-cHSF3 serum. At mild heat shock temperatures, such as 39, 40, 41, 42, or 43 °C, the amounts of both factors in the soluble fractions were unaffected (Fig. 7*A, lanes 1–6*). When the heat shock temperature was shifted to 44, 45, or 46 °C, cHSF1 was markedly decreased in the soluble fractions (*lanes 7–9*) and appeared in the insoluble pellet fractions (*lanes 16–18*). We could hardly detect soluble cHSF1 after heat shock at 46 °C for 1 h (*lane 9*). In contrast to cHSF1, a substantial amount of cHSF3 remained in the soluble fraction even after the heat shock at 46 °C for 1 h (Fig. 7*A, lane 9*), although cHSF3 appeared also in the insoluble fraction after heat shock at 44, 45 and 46 °C as cHSF1 did.

The kinetics of aggregate formation during mild or severe heat shock were then examined. When cells were incubated at 41 °C, both cHSF1 and cHSF3 were stable in the soluble fractions (Fig. 7*B, lanes 1–5*). When cells were incubated at a higher temperature, such as 45 °C, cHSF1 disappeared rapidly from the soluble fraction, and no cHSF1 could be detected after 90 min (Fig. 7*B, lanes 11–15*). On the other hand, a substantial amount of cHSF3 remained in the soluble fractions even after 90 min (*lane 15*). The time course of aggregate formation of cHSF1 is different among cell types. For example, in CEF cells, it took 2 h for cHSF1 to move to the insoluble fraction almost

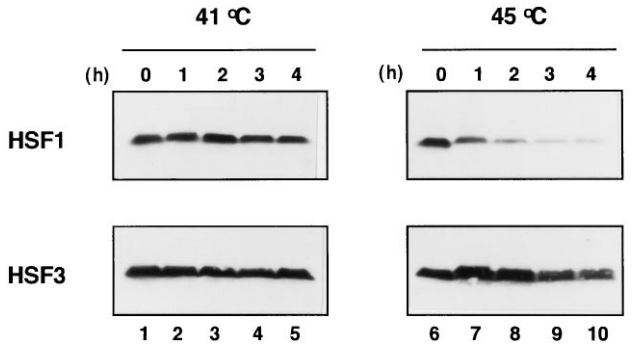

FIG. 6. **The protein levels of cHSF1 and cHSF3 under mild or severe heat shock.** DT40 cells were heat shocked at 41 or 45 °C for the indicated periods. After being washed with PBS, cells were directly suspended in Laemmli's sample buffer by sonication. Aliquots extracted from equal numbers of cells were subjected to SDS-PAGE and Western blotting as described in the legend to Fig. 2.

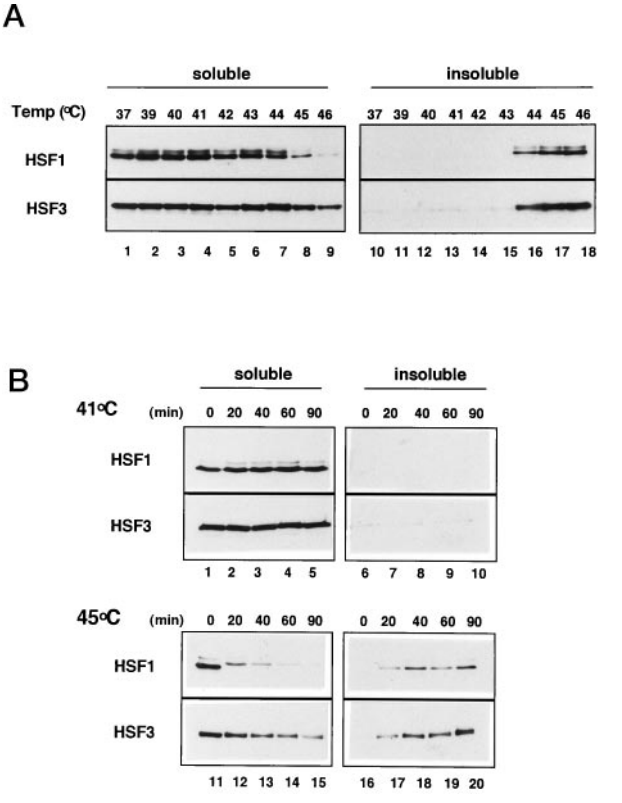

FIG. 7. **The levels of soluble and insoluble cHSF1 and cHSF3 at various temperatures.** *A*, DT40 cells maintained at 37 °C were heat shocked at the indicated temperatures for 1 h. Whole cell extracts (soluble fractions) were prepared by a standard procedure. Pellet fractions were suspended in Laemmli's sample buffer by sonication (insoluble). Aliquots extracted from an equal number of cells were subjected to SDS-PAGE and Western blotting as described in the legend to Fig. 2. *B*, DT40 cells were heat shocked at 41 or 45 °C for the indicated times. The protein levels of cHSF1 and cHSF3 in soluble and insoluble fractions were analyzed as described above.

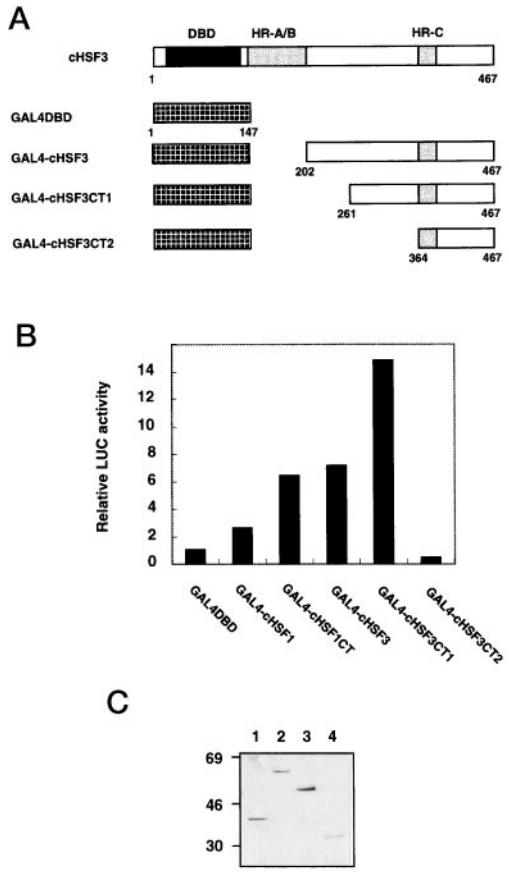

FIG. 8. **Comparison of the transcriptional activity of cHSF1 and cHSF3.** *A*, schematic representation of cHSF3 deletions fused to GAL4 DNA binding domain. The numbers indicate the positions of amino acids. *DBD*, DNA binding domain; *HR-A/B*, heptad repeat of hydrophobic amino acids for trimer formation; *HR-C*, heptad repeat necessary for negative regulation. *B*, Each expression vector for the GAL4 DNA binding domain (147 amino acids), GAL4-cHSF1 (amino acids 209–491 of cHSF1), GAL4-cHSF1CT (amino acids 313–491), GAL4-cHSF3 (amino acids 202–467 of cHSF3), GAL4-cHSF3CT1 (amino acids 261–467), and GAL4-cHSF3CT2 (amino acids 364–467) (20 μg each) was co-transfected into COS7 cells together with 5 μg of a reporter plasmid ptk-gal p3-luc and 1 μg of pSV CAT as an internal control. After 36 h of transfection, cells lysates were prepared and luciferase activities were assayed. Relative luciferase activities are shown after being normalized by chloramphenicol acetyltransferase activities. *C*, the fusion proteins expressed in COS7 cells were analyzed by Western blotting. *1*, GAL4-cHSF1CT; *2*, GAL4-cHSF3; *3*, GAL4-cHSF3CT1; *4*, GAL4-cHSF3CT2.

completely (data not shown; see Fig. 4*A*), whereas cHSF3 was relatively stable as was in DT40 cells.

Because we observed rapid decrease of cHSF1 under severe heat shock (Fig. 6) and the aggregated cHSF1 could not bind to DNA any more (Fig. 1), cHSF1 was assumed not to act at severe heat shock. Instead, cHSF3 is activated and is thought to act as an activator under severe heat shock.

Comparison of Transcriptional Activity of cHSF1 and cHSF3—We have previously demonstrated that cHSF3 has a potential to activate reporter genes (29). Recently, a carboxyl-terminal transactivation domain and its negative regulatory domain were revealed in human HSF1 (40–42). Chicken HSF1 is highly identical to human HSF1, and in chicken HSF1, the transactivation domain, related to that of human HSF1, revealed the same activity as in human HSF1 (data not shown). To compare the activity of transactivation domain of cHSF3 with that of cHSF1, we constructed deletion of cHSF3 fused to the GAL4 DNA binding domain (Fig. 8*A*). The GAL4-driven luciferase reporter gene was co-transfected to COS7 cells to measure the transcriptional activities of each chimeric proteins. As was shown in human HSF1, cHSF1 lacking the regulatory domain (GAL4-cHSF1CT) revealed 2–3-fold induction of the luciferase activity relative to GAL4-cHSF1, which has the regulatory domain (Fig. 8*B*). The activities of GAL4-cHSF3 and GAL4-cHSF3CT1 are similar to or higher than that of GAL4-cHSF1CT. The expressed protein level of GAL4-cHSF1CT, GAL4-cHSF3, GAL4-cHSF3CT1, and GAL4-cHSF3CT2 were comparable (Fig. 8*C*). This result indicates that the activity of transactivation domain of cHSF3 is as strong as that of cHSF1.

DISCUSSION

In this study, we clearly showed that the threshold temperatures that activate HSF1 or HSF3 are different in chicken embryo fibroblasts. This is the first demonstration that differences in severity of stress activate different sets of HSFs in higher eukaryotes.

Since the isolation of three chicken heat shock factors, HSF1, HSF2, and HSF3, all of which can bind to the consensus HSE (18), our aim has been to understand the respective roles of each HSF. Many previous studies revealed that HSF1 responds to the classical inducers including heat shock, whereas HSF2 is induced during hemin-induced differentiation of erythroleukemia cells (22, 24–26). All three HSFs exist as non-DNA-binding forms under normal growth conditions in many cell types. Our previous study showed that the DNA binding activities of both cHSF3 and cHSF1 are induced by heat shock as well as by sodium arsenite treatment (29). Both factors are simultaneously activated by various stresses, such as amino acid analogue, prostaglandin A_1, superoxide, and osmotic shock, in addition to heat shock[2] and are potentially transcriptional activators (29). These findings raised the question of the functional differences between cHSF1 and cHSF3 in the stress response.

After heat shock at lower temperatures, such as 41 °C, only the DNA binding activity of cHSF1 was induced in chicken embryo fibroblasts, whereas the DNA binding activities of both cHSF1 and cHSF3 were clearly induced at relatively high temperatures, such as 45 °C (Fig. 1). Sodium arsenite treatment showed similar results; a low concentration of sodium arsenite, such as 3 μM, activated only cHSF1, whereas a high concentration, such as 50 μM, activated both cHSF1 and cHSF3 (Fig. 3). Remarkably, we showed that under severe heat shock condition, the level of cHSF1 decreased fast, whereas some increase of cHSF3 level was observed (Fig. 6). The mRNA level of cHSF3 was not induced even at severe heat shock (data not shown; Ref. 18). It is interesting to note that the protein level of HSF2 increased when HSF2 was activated during the differentiation of K562 cells (25). Our previous study showed that cHSF1 is activated immediately after heat shock or arsenite treatment, whereas activation of cHSF3 is delayed and sustained for a much longer period (29). Taken all together, cHSF1 may be involved in rapid response to mild and severe stress, whereas cHSF3 may play a role in persistent and burst activation of stress genes upon severe stress.

We encountered another question: whether cHSF1 could act as strong activator upon severe shock with cHSF3 or whether cHSF1 could become inactivated by severe shock. We showed that most cHSF1 moves to the insoluble pellet fraction rapidly after severe heat shock, whereas a substantial amount of cHSF3 remains in the soluble fraction (Fig. 7). It is interesting to note that the incubation temperature (44 °C) at which cHSF1 began to move to the pellet fraction is the same as that at which cHSF3 started to be activated. Once cHSF1 forms an insoluble aggregate, it cannot bind to DNA anymore, and consequently, it

[2] Y. Kawazoe, M. Tanabe, K. Nagata, and A. Nakai, unpublished observation.

cannot act as a transcriptional activator. It was reported that HSF2, which is not functional for heat shock response, becomes insoluble immediately after heat shock (43). This observation is consistent with the idea that insoluble cHSF1 does not act anymore. We think that relatively stable cHSF3 may make up the lost activity of cHSF1 upon severe heat shock.

Although the pathway that leads to HSF1 activation has not yet been clarified, heat shock causes denaturation of cellular proteins, and this would trigger the activation of HSF1 (6, 28). Stress proteins like HSP70 interact transiently with nascent polypeptides even in unstressed cells (38, 44). Under stressed conditions, HSP70 binds to newly synthesized proteins that cannot fold properly and to misfolded mature proteins (37). Depletion of free HSP70 has been reported to cause the activation of HSF1 (45, 46). There are several possible mechanisms by which cHSF1 and cHSF3 may respond to the same heat shock differently. First, there might be different targets of heat stress for activation of each HSF. Second, the same intracellular signal might differentially trigger the conformational changes of cHSF1 and cHSF3 necessary for binding to DNA. To address these, we first used the protein synthesis inhibitor cycloheximide, which increases the heat shock threshold temperature by depleting newly synthesized proteins, which are thought to be the major targets of heat stress (37, 38). Treatment with cycloheximide increased the threshold concentrations and the temperatures of both cHSF1 and cHSF3 (Fig. 3*C* and data not shown). Although we did not examine other signals, this suggests that both cHSF1 and cHSF3 activations may be triggered by the same signal, the denaturation of newly synthesized proteins. In reference to the second possibility, we clearly showed differences in the oligomeric forms of cHSF1 and cHSF3 under nonstressed conditions (Ref. 29; see Fig. 2). Monomeric cHSF1 is converted to the trimeric form by mild stress, whereas dimeric cHSF3 responds only to severe stress. Moreover, a portion of cHSF1 remains in the dimeric form after mild heat shock (Fig. 2*B*). These results indicate that the second possibility is more likely and suggest that the oligomeric form might be responsible for the differences in heat shock threshold.

In *Escherichia coli*, the heat shock response is controlled by two σ factors, σ^{32} and σ^{E}(σ^{24}) (47–50). σ^{32} regulates the classical heat shock response, whereas σ^{E} responds to extracytoplasmic stress and is necessary for maintenance of the heat shock response at high temperatures by inducing the σ^{32} factor (51, 52). In higher eukaryotes, the level of HSF1 is not controlled by heat shock (19, 24, 49). Although the transcriptional machinery is different between bacteria and higher eukaryotes, there might be similar systems for responding to stresses of varying severity in common.

In this study, we showed that cHSF3 was activated by heat shock not only in HD6 cells but also in CEF, although we previously detected no cHSF3 activation in CEF (29). The intensity of heat shock response is not determined by the absolute temperature of heat shock, but rather by the difference in temperature between culture conditions and heat shock stimulus (53). Thus, the reason for the above discrepancy may have been that in previous experiment the heat shock temperature was not sufficient to activate cHSF3 in CEF, or the antiserum we previously used to screen for cHSF3 activation in various cells may not have recognized the activated native cHSF3 well.

We have a working hypothesis that cHSF3 may play a role in persistent and burst activation of stress genes upon severe stress in chicken cells. In mammalian cells, no HSF3 activity has yet been detected, and HSF1 is known to cause burst induction of stress genes after heat shock. Interestingly, superphosphorylation of chicken HSF1 could not be well detected after heat shock (29), whereas mammalian HSF1 is heavily phosphorylated (22, 24, 54). It is necessary to investigate whether mammalian HSF1 can be replaced by chicken HSF1 in future experiments.

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