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Stable Overexpression of Human HSF-1 in Murine Cells Suggests Activation Rather Than Expression of HSF-1 to be the Key Regulatory Step in the Heat Shock Gene Expression

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Abstract Transcription of the heat shock genes is regulated by the activation of the heat shock transcription factor (HSF-1). After heat shock, HSF-1 forms oligomers and binds to the heat shock element (HSE), which consists of several repeats of NGAAN located in the promoter region of the heat shock genes. HSF-1 is then phosphorylated, leading to the enhanced transcription of the heat shock genes likely by transactivation. We have stably overexpressed the human heat shock transcription factor-1 (HSF-1) in murine cells to investigate whether the regulation of the expression of the heat shock genes may partly reside at the level of HSF-1 expression. Human HSF-1 cDNA was cloned into a retroviral vector (pvhhsf-1) and was overexpressed in a murine fibroblast cell line. The overexpressed human HSF-1 is found in both the cytoplasm and nucleus of control cells but is translocated into the nucleus upon heat shock. Electrophoretic mobility shift analysis suggests that the human HSF-1 has constitutive DNA binding ability and its DNA binding ability is increased upon heat shock. Cross-linking experiments indicate that the overexpressed human HSF-1 is mainly a monomer under control conditions and forms oligomers upon heat shock. Immunoblotting shows that the human HSF-1 is phosphorylated upon heat shock and its apparent molecular weight is shifted up by at least 10 kDa. In spite of both the DNA binding ability and phosphorylation, the overexpression of human HSF-1 does not increase the transcription of murine HSP-70 mRNA or increase the synthesis of other HSPs after heat shock beyond that observed in control untransfected cells. An exception is the enhanced synthesis of a 47–50 kDa protein after heat shock and an apparent lack of induction of one HSP-70 kDa species when the protein pattern is analyzed by isoelectric focusing. Interestingly, cells overexpressing human HSF-1 show a 4-fold increase in the basal expression of luciferase when the plasmids containing the human HSP-70 promoter ligated to the luciferase reporter gene are transiently expressed in these cells. Murine cells overexpressing human HSF-1 are more resistant to the cytotoxic effects of heat when compared to the control untransfected cells, but the kinetics of thermotolerance development and decay is similar between HSF-1 transfected and untransfected cells. In conclusion, human HSF-1 protein in murine fibroblasts is modified in a similar fashion as the endogenous mouse HSF-1 after heat shock. However, the overexpression of HSF-1 does not result in overproduction of heat shock proteins after heat shock, perhaps because these cells contain abundant amounts of endogenous HSF-1.

Key words: heat shock genes, HSF-1, murine cells, retroviral vector, monomer, murine fibroblasts

There is extensive heterogeneity in the responses of various tissues to thermal stress. This heterogeneity has been attributed to be at the level of expression of heat shock transcription factor in different tissues [Sarge et al., 1991; Mivechi et al., 1992]. Mammalian cells respond to stress by enhanced transcription and translation of a variety of heat shock genes [Pelham, 1990; Morimoto and Milarski, 1990; Li and Werb, 1982; Schlesinger et al., 1982; Mivechi et al., 1991]. Transcription of heat shock genes is mediated by the heat shock transcription factor (HSF) that acquires the ability to bind to a control element (HSE) that is found in multiple copies in the promoter region of the heat shock genes after heat shock [Abravaya et al., 1991; Westwood et al., 1991; Sorger et al., 1987; Kingston et al., 1987]. Eukaryotic cells possess multiple HSFs, suggesting that they

Abbreviations: HSF-1, heat shock factor-1; HSP, heat shock protein.

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mediate response to variety of stresses with possible overlapping functions [Rabindran et al., 1991; Sarge et al., 1991; Schuetz et al., 1991]. HSF-1 protein is approximately 69 kDa as a monomer. HSF-1 contains four leucine zippers; three of the leucine zippers are near the DNA binding domain in the NH_2-terminal region of the molecule. The fourth leucine zipper is located near the C-terminal region and is believed to prevent oligomerization and DNA binding under unstressed growth conditions [Rabindran et al., 1993; Westwood and Wu, 1993]. HSF-1 becomes phosphorylated upon heat shock but the manner in which phosphorylation affects HSF-1's ability to activate transcription is not yet known [Sarge et al., 1993]. Under unstressed growth conditions, HSF-1 may be under the negative regulation complexed by an as yet unknown molecule because both the recombinant and the overexpressed HSF-1 have constitutive DNA binding ability, suggesting that the negative regulator can be outnumbered [Clos et al., 1990; Sarge et al., 1993; Rabindran et al., 1993].

The current hypothesis of HSF-1 regulation of heat shock gene transcription is the following: at 37°C, HSF-1 protein is folded by the interaction of the C-terminal leucine zipper and the N-terminal leucine zipper perhaps in a complex with a negative regulator, such as HSP-70 or another heat shock protein [Sorger, 1991; Mosser et al., 1993; Morimoto, 1993; Craig and Gross, 1991]. On the onset of the stress, HSPs bind to the denatured proteins, allowing HSF-1 to unfold and form oligomers. Since HSF-1 is also phosphorylated to some extent under control conditions [Sarge et al., 1993], it is conceivable that HSF-1 may not be bound to a negative regulator, but it becomes unfolded upon stress by a change of conformation due to dephosphorylation of one of its phosphorylated residues. HSF-1 oligomers can then bind to DNA and become more extensively phosphorylated. Although HSF-1 is heavily phosphorylated on serine residues, both tyrosine as well as ser/thr protein kinases have been implicated in its activation [Mivechi, et al., 1994; Cheng, et al., 1993]. All the modifications in HSF-1 protein upon heat shock leads to the transactivation and expression of the heat shock genes. When enough HSPs, including HSP-70, have accumulated, HSP-70 may then bind to the activated HSF-1, rendering it inactive. The interaction of HSP-70 with the oligomeric form of HSF-1 have been demonstrated by electrophoretic mobility shift analysis showing supershift of HSF-HSE complex in the presence of antibody specific to HSP-70 [Abravaya et al., 1991; Mosser et al., 1993]. Recent evidence from our laboratory suggests that HSP-70 can activate ser/thr protein phosphatases in vitro [Mivechi et al., 1993]. It is possible that HSP-70/HSF-1 complex also contains ser/thr protein phosphatases in order to dephosphorylate and inactivate HSF-1.

In these studies we have stably overexpressed the human HSF-1 cDNA in murine cells to determine if the increased levels of expression of HSF-1 results in an increase in the HSPs mRNA expression after heat shock. Our results show that in a murine fibroblast cell line that contains sufficient amounts of HSF-1 for a maximum heat shock response, the overexpression of human HSF-1 does not increase the levels of heat shock gene expression after heat shock, but nevertheless renders cells more resistant to cytotoxic effects of heat.

MATERIALS AND METHODS

Plasmids

The plasmid pHUHSF-1 was kindly provided by Dr. Carl Wu, National Institute of Health, Bethesda, MD. This plasmid contains the entire human HSF-1 cDNA inserted into the EcoRI site in the plasmids pBluescript II SK(−) [Rabindran et al., 1991]. To construct the expression plasmids containing the human HSF-1 cDNA under the control of the retroviral promoters, the HSF-1 cDNA was digested with XhoI at the position (−)6 from the ATG transcription start site. The XhoI site was blunt ended using Klenow polymerase and an EcoRI linker (Stratagene, La Jolla, CA) was ligated at the XhoI site. The HSF-1 cDNA fragment was then digested from pHUHSF-1 by EcoRI, leaving the entire coding sequence and the 3′-end untranslated region of the HSF-1 cDNA intact. The 1932 bp human HSF-1 fragment was then inserted into the EcoRI site of the plasmids pLgNL-586δϵ (gift of Dr. I. Weissman, Stanford University) containing murine molony leukemia viral LTRs and polyoma viral enhancers. Figure 1 shows the map of the pvhhsf-1 construct. The retroviral vector pLgNL-586δϵ does not carry the neomycin phosphotransferase gene as a selectable marker and to select the permanent neomycin resistant cells containing HSF-1 cDNA, the plasmids pSV_2-neo and pvhhsf-1 were cotransfected into Xi-2 cells and neomycin resistant clones

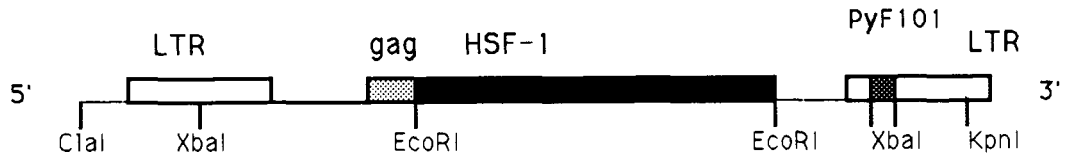

Fig. 1. *Map of pvhhsf-1. The pvhhsf-1 plasmids containing human HSF-1 cDNA. "LTR" indicates the long terminal repeats of the Molony murine leukemia virus. Gag is the envelope glycoprotein. pYF101 is polyoma viral enhancer. HSF-1 contains the entire human HSF-1 cDNA as described in Materials and Methods.*

were selected following the incubation in Geneticin (Gibco/BRL, Gaithersburg, MD)-containing medium.

Procedure for Stable Transfection

With 20 μg of the plasmids pvhhsf-1 and 1 μg of pSV_2-neo with electroporation, 4×10^6 exponentially growing cells were transfected. Cells were then plated into tissue culture dishes and incubated at 37°C for 24 h at which time fresh medium was added and cells were incubated at 37°C for an additional 24 h. Cells were then replated at 10^5 cells/100 mm dish in medium supplemented with 10% FCS and 400 μg/ml of Geneticin. Individual colonies were selected on day 8 and expanded. To test the clones for the expression of human HSF-1 mRNA, total RNA was isolated from several clones and 15 μg of total RNA was analyzed by Northern blotting using ^{32}P-labeled HSF-1 cDNA as a probe. The size of the human HSF-1 mRNA transcripts is approximately 2.0 kb. The size of the human HSF-1 cDNA transcript produced from the pvhhsf-1 is approximately 2.8 kb.

Procedure for Transient Transfection

Transient transfection studies were performed by calcium phosphate method followed by glycerol shock [Sambrook et al., 1989]. Briefly, the plasmids pHSP-70-luciferase plus pBR322 (carrier DNA) were added dropwise onto 2×10^6 cells in 60 mm tissue culture dish containing 1 ml of serum-free growth medium for 5 h. After incubation, cells were fed with 3 ml of growth medium supplemented with 10% FCS and were allowed to incubate overnight at 37°C. On the following day, cells were glycerol shocked (15%, v/v) for 1 min at room temperature, washed once with PBS, re-fed with medium, and were incubated at 37°C for an additional 24 h. Cells were then either kept as control or heat shocked at 42°C for 1 h and further incubated at 37°C for 6 h to permit luciferase accumulation before cell lysis. Luciferase activity following transient transfections was measured as follows: Cells were lysed in 100 μl of 50 mM Tris-HCl, pH 7.8, and frozen and thawed 3 times. The lysates were then microfuged and an aliquot was added to the luciferase assay reagent (20 mM tricine, 1 mM $(MgCO_3)$ Mg $(OH)2.5\ H_2O$; 2.67 mM $MgSO_4$; 0.1 mM EDTA; 530 mM ATP, final pH 7.8). Luciferase activity was measured by luminometer for a period of 20 s [Leuhrsen et al., 1992]. In transfection studies, 1 μg of plasmids pHSP-70-luciferase (gift of Dr. Richard Morimoto, Northwestern University) [Williams et al., 1989] was transfected together with 15 μg of pBR322 as a carrier DNA.

Cell Culture and Cell Survival

Xi-2 is a packaging cell line and was originally derived from NIH3T3 cells [Mann et al., 1983]. Xi-2 cells were maintained in Iscove's (Gibco/BRL, Gaithersburg, MD) medium plus 15% FCS. Transfected cells described below were also maintained in the same medium plus 100 μg/ml of Geneticin. The transfected cells were as follows: Neo-2 cells were transfected with pSV2-neo (plasmids containing neomycin phosphotransferase gene under the control of SV-40 promoter) and were resistant to neomycin; hsf-1-1 cells were the pool of several neomycin resistant positive and negative (for the expression of HSF-1 protein as assessed by immunofluorescence analysis using anti-human HSF-1 antibody) clones which was selected after the initial transfection of Xi-2 cells with pSV2-neo and pvhhsf-1. The clones hsf-1f and hsf-1e were individual clones expressing human HSF-1 protein and were selected for further analysis. Permanent transfected clones were stable over a period of 2 months in culture; however, after this time the levels of expression of HSF-1 protein varied between cells as determined by immunofluorescence analysis.

Cell survival studies were performed by colony formation as previously described [Mivechi et al., 1991].

Northern Blotting

Total RNA was isolated by RNAzol (Tel-Test, Friendswood, Texas) according to the manufacturer's instructions. Fifteen micrograms of total RNA was fractionated in 0.8% agarose/formaldehyde gel and transferred onto nitrocellulose membrane as described by Sambrook et al. [1989]. The blots were hybridized to the ^{32}P-labeled BamHI-HindIII fragments of the human HSP-70A from the plasmids phhsp-70 that contains the entire 2.3 kb of the HSP-70A gene (gift of Dr. R. Morimoto, Northwestern University, Evanston, IL) [Hunt and Morimoto, 1985]. To detect the human HSF-1 mRNA, the ^{32}P-labeled EcoRI digest of pvhhsf-1 was used. To detect the mouse actin mRNA, the 2 kb fragments of human actin was used (Clontech, Palo Alto, CA).

Immunofluorescence Analysis

Cells were grown in multichannel tissue culture slides and were either left untreated or were heat shocked. Cells were then fixed in cold 100% methanol for 20 min. Fixed cells were blocked with 5% goat serum plus 5% BSA in phosphate buffered saline (PBS) for 1 h at 37°C. Cells were then exposed to 1:1,000 dilution of antihuman HSF-1 antibody (a gift of Dr. Carl Wu, NIH) for 1 h at 37°C. After rinsing with PBS, cells were exposed to FITC-labeled goat antirabbit IgG for 1 h at 37°C, rinsed again with PBS, and analyzed. The antihuman HSF-1 antibody recognizes the human HSF-1 and does not recognize the murine HSF-1.

Immunoblotting

Approximately 1×10^6 cells were lysed in SDS sample buffer [Laemmli, 1975]. Equal number of cells (1×10^5) or 30–50 μg of protein from each sample were electrophoresed on a 10% polyacrylamide gel (unless otherwise indicated) and transferred onto a nitrocellulose membrane. The blots were blocked with 5% nonfat dry milk in PBS for 1 h and were exposed to a 1:500 dilution of anti-HSP-70 antibody (C92, Amersham, Arlington Heights, IL). The blots were rinsed with PBS supplemented with 0.1% Tween 20 and 1% BSA and exposed to alkaline phosphatase (AP) goat antimouse IgG for 1 h at room temperature. After rinsing, blots were stained. In experiments where the chemoluminiscent (ECL, Amersham) was used, after the blots were incubated in the presence of the primary antibody as described above, the blots were rinsed and were incubated in the presence of biotin-labeled goat antirabbit IgG for 1 h at room temperature. After rinsing, the blots were incubated in the presence of HRP-streptavidin for 20 min and were exposed to X-ray film after rinsing and incubation in the ECL solution for 1 min.

The unphosphorylated and phosphorylated forms of HSF-1 in mammalian cells were originally detected and reported by Sarge et al. [1993], using antibody prepared to mouse HSF-1. In unstressed cells, the HSF-1 protein has an apparent molecular weight of 67–72 kDa. In heat shocked cells, the phosphorylated form of HSF-1 has an apparent molecular weight of 80–85 kDa. The "broadness" of the HSF-1 band suggests the degree of its phosphorylation, as the potato acid phosphatase treatment of the cell lysates results in a band at 67–72 kDa [Mivechi et al., 1994].

Isoelectric Focusing

For two-dimensional isoelectric focusing (IEF), control or heat shocked cells were labeled with 20 μCi/ml of ^{3}H-leucine for 6 h at 37°C in growth medium plus 10% FCS. Cells were then rinsed with PBS and lysed in isoelectric focusing sample buffer (urea, 9.5 M; NP40, 2%; pH 5–7 ampholine 1.6%; pH 3.5–10 ampholine, 0.4%; 2-mercaptoethanol, 5%) and an equal number of cells were analyzed by IEF gel containing urea, 9.5M; 30% acrylamide, 3.5%; 10% NP-40, 2%; pH 5–7 ampholine, 1.33%; pH 3.5–10 ampholine 0.67% [O'Farrel, 1975]. After the analysis of the samples on the first dimension, samples were analyzed on the second dimension on a 10% polyacrylamide SDS-PAGE as described by Laemmli [1970]. Gels were then stained with Coomassie blue R-250, destained in 10% acetic acid: 40% methanol, and soaked in En^{3}hance solution (Biotechnology Systems, Boston, MA) for 1 h. Gels were subsequently rinsed with water, dried and exposed to X-ray film at −70°C for 4 days.

Cross-Linking of HSF-1 Protein

Cross-linking experiments were performed essentially as described by Rabindran et al. [1993]. Briefly, control or heated cells were lysed by homogenization in extraction buffer (10 mM 4(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.9; 0.4 M NaCl; 0.1 mM ethyleneglycol

bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid; 0.5 mM dithiotreitol (DTT); 5% glycerol; 0.5 mM phenylmethylsulfonyl fluoride (PMSF)). The cell lysates were made to 250 μl with the following buffer: 150 mM NaCl; 25 mM $NaPO_4$, pH 7.8; 10% glycerol. Ten microliters of EGS (ethylene glycol bis(succinimidylsuccinate)) (Pierce Biochemical Co., Rockford, IL) that had been dissolved in DMSO was added to the samples to concentrations of 0.2 or 1 mM. The mixture was then incubated at 25°C for 20 min and the reaction was quenched by the addition of glycine to 75 mM. The lysate was TCA precipitated by the addition of 350 μl of 10% TCA and the pellet was dissolved in 2× SDS sample buffer and equal amounts of protein were resolved on a 5% polyacrylamide gel. The gels were then transferred to nitrocellulose filters and analyzed by immunoblotting as described above.

Electrophoretic Mobility Shift Analysis

For quantifying the binding of the activated HSF to the HSE, the procedure of Zimarino and Wu [1987] was used: 10–20 μl of the cell extracts (equivalent to 20–30 μg of protein) in the extraction buffer [(10 mM 4(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.9; 0.4 M NaCl; 0.1 mM ethyleneglycol bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid; 0.5 mM dithiotreitol (DTT); 5% glycerol; 0.5 mM phenylmethylsulfonyl fluoride (PMSF)] were added to 4 μl of the binding buffer (37.5 mM NaCl; 15 mM Tris-HCl, pH 7.4; 0.1 mM ethyleneglycol bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid; 0.5 mM DTT; 5% glycerol) which also contained 1 μl yeast tRNA (10 μg), 1 μg of sheared *E. coli* DNA, polydeoxyinosine-8-polydeoxycytidylate (*10 μg*), *and 1* ng of ^{32}P-labeled HSE. The mixture was incubated at 25°C for 15 min and ^{32}P-labeled HSE. The mixture was incubated at 25°C for 15 min and electrophoresed on a nondenaturing 4.5% polyacrylamide gel. After electrophoresis gels were fixed in 7% (v/v) acetic acid for 5 min, rinsed once with distilled water, dried under vaccum, and exposed to the x-ray film. The nucleotide sequence for HSE was the same as that originally described by Zimarino and Wu [1987] and was as follows: 5′-GTCGACGGATCC-GAGCGCCTCGAATGTT-CTAGAAAAAGG-3′. The double-stranded oligonucleotide was labeled by the fill in reaction using Klenow fragment of the DNA polymerase I and α-^{32}P-dCTP.

All the experiments described in the results section have been performed at least two times and the results were consistent.

RESULTS

Expression of Human HSF-1 mRNA in Stably Transfected Mouse Cells

Figure 2 shows the Northern blot analysis of the mRNA from the pool (hsf-1-1) containing neomycin resistant cells which were positive or negative for the expression of HSF-1 as assessed by immunofluorescence analysis using antihuman HSF-1 antibody and two of the colonies that were selected from the pool and were positive for the expression of human HSF-1 cDNA. The parent cell line (Xi-2) and cells stably transfected with neomycin resistant gene (neo-2) that were used as control groups in the subsequent studies are also shown. The location of the HSF-1 mRNA in 293, a human adenovirus transformed embryonic kidney cell line, is also shown as positive control. The size of the human HSF-1 cDNA is approximately 2.0 kb. The size of the human HSF-1 cDNA transcript produced from pvhhsf-1 is approximately 2.8 kb. The band above

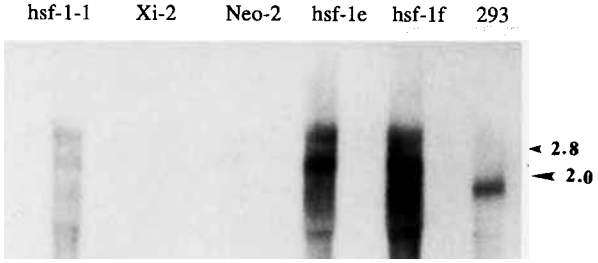

Fig. 2. Northern blot analysis of total RNA obtained from cells overexpressing human HSF-1 cDNA. Total RNA was prepared from cells and analyzed by Northern blotting using the ^{32}P-labeled human HSF-1 cDNA as a probe. hsf-1-1 is a pool of several neomycin resistant clones positive or negative for the expression of human HSF-1 cDNA. Xi-2 is the parent cell line. Neo-2 are cells resistant to neomycin. hsf-1e and hsf-1f are cells overexpressing the human HSF-1 cDNA. 293 is a human embryonic kidney cell line showing the position of the human HSF-1 mRNA at 2 kb. The overexpressed HSF-1 cDNA is at 2.8 kb.

the 2.8 kb transcript is present in some human as well as mouse cells tested in our laboratory.

Human HSF-1 is Distributed in the Cytoplasm and Nucleus of Transfected Cells Under Normal Growth Conditions and Translocates Into the Nucleus After Heat Shock

To assess the intracellular distribution of the overexpressed human HSF-1 in hsf-1f cells, immunofluorescence analysis was performed using FITC-conjugated IgG against human HSF-1 antibody (Fig. 3). Heat shocked (42°C for 1 h) untransfected cells are shown for comparison (Fig. 3A). In the absence of stress, the overexpressed human HSF-1 is distributed diffusely in both the cytoplasm and in the nucleus (Fig. 3B). HSF-1 is only faintly visible when it is in monomeric form and distributed in the cytoplasm. However, all cells show nuclear staining immediately after a heat shock of 42°C for 1 h (Fig. 3C). These results are consistant with the earlier observations regarding the distribution of the endogenous mouse HSF-1 under control or heat shocked conditions [Sarge et al., 1993], suggesting that the overexpressed human HSF-1 distributes in a similar fashion as the endogenous mouse HSF-1.

Human HSF-1 Contains Constitutive DNA Binding Ability in vitro

Heat shock stimulus is thought to facilitate the monomer to trimer transition of the HSF-1 protein that is required for DNA binding. To determine if the overexpressed human HSF-1 in murine cells is capable of binding to the DNA, control or heat shocked cells were analyzed by electrophoretic gel mobility shift analysis. As Figure 4 shows, under unstressed growth conditions the heat shock transcription factor does not exhibit an ability to bind to the heat shock element (HSE) in control neo-2 cells. The HSF, however, acquires DNA binding ability immediately after a heat shock of 42°C for 1 h. The DNA binding ability of HSF-1 is reduced following a 2 h recovery period at 37°C. In both hsf-1e and hsf-1f clones that overexpress human HSF-1, however, HSF shows constitutive HSE binding ability under control conditions and HSF-HSE binding is increased after heat shock.

Why the overexpressed HSF-1 can bind DNA constitutively is not clear. However, HSF-1 is capable of binding to the DNA without heat shock both when it is expressed in *E. coli* and when it is transiently transfected into mammalian cells. A negative regulatory element normally present in cells and possibly bound to HSF-1 has been suggested to prevent HSF-HSE binding under normal growth conditions. This negative regulator may be outnumbered when

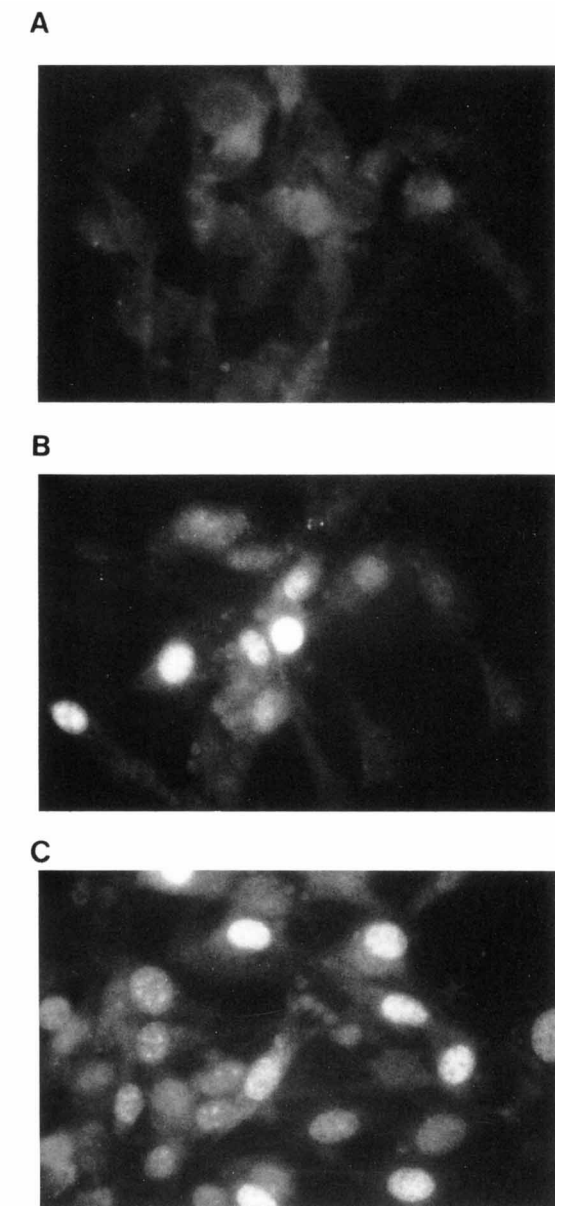

Fig. 3. Immunofluorescence analysis of transfected cells showing the intracellular location of human HSF-1. Control or heat shocked cells were analyzed following immunofluorescence staining using anti-human HSF-1 antibody. **A:** Untransfected Xi-2 cells immediately after a 42°C, 1 h heat shock. **B:** hsf-1f cells overexpressing human HSF-1 under control unstressed growth conditions. **C:** hsf-1f cells immediately after a 42°C, 1 h heat shock.

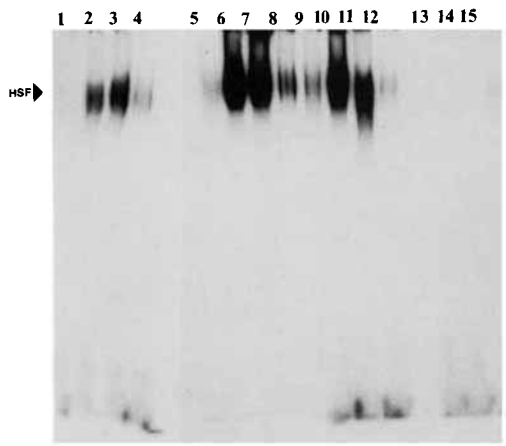

Fig. 4. Gel retardation assays of cells overexpressing human HSF-1. Whole cell extracts of control or heat shocked cells were analyzed by gel retardation assays. *Lanes 1–4* are neo-2 cells under control conditions (lane 1) or heated at 42°C for 1 h and were analyzed either immediately (lane 2) or after 1 or 2 h recovery time at 37°C (lanes 3, 4). *Lanes 5–8* are hsf-1f cells under control conditions (lane 5) or heated at 42°C for 1 h and were analyzed either immediately (lane 6) or after 1 or 2 h recovery time at 37°C (lanes 7, 8). *Lanes 9–12* are hsf-1e under control conditions (lane 9) or heated at 42°C for 1 h and were analyzed either immediately (lane 10) or after 1 or 2 h recovery time at 37°C (lanes 11, 12). *Lanes 13–15* are the same cell lysates as lanes 2, 6, and 10 but were incubated in the presence of 200-fold excess cold HSE as a competitor to show specific HSF-HSE binding.

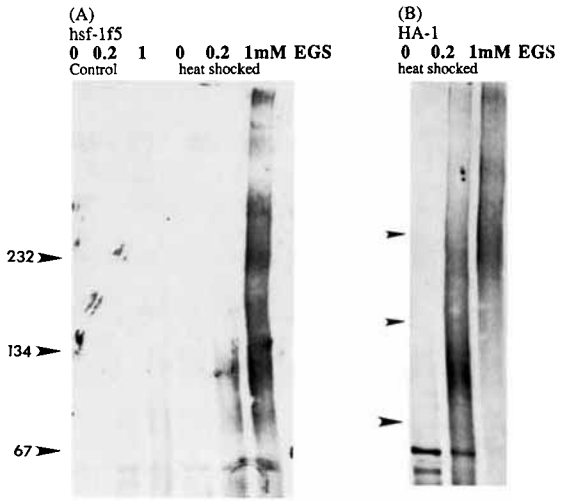

Fig. 5. The overexpressed human HSF-1 forms a trimer after heat shock. **A:** Control or heat shocked (43°C, 1 h) hsf-1f5 cells were lysed and incubated in the presence of 0, 0.2, or 1 mM EGS for 20 min at room temperature. After the addition of glycine to 75 mM, samples were precipitated with TCA and lysed in SDS sample buffer [Rabindran et al., 1993]. Samples were electrophoresed on a 5% polyacrylamide gel and blotted onto a nitrocellulose membrane and analyzed by Western blotting using anti-human HSF-1 antibody as described in methods. **B:** The Chinese hamster ovary HA-1 cells were heat shocked at 45°C, 15 min and immediately after heat shock cell extracts were treated with EGS as described in A. Blots were analyzed using anti-mouse HSF-1 antibody.

HSF-1 is overexpressed [Clos et al., 1990; Rabindran et al., 1993; Sarge et al., 1993].

Overexpressed HSF-1 Forms Oligomers and is Phosphorylated Upon Heat Shock

Upon the onset of stress, HSF-1 forms oligomers and its apparent molecular weight is increased from 67–70 kDa to approximately 210 kDa. After heat shock, HSF-1 is also phosphorylated and its apparent molecular weight increases by as much as 11 kDa. To determine if the overexpressed human HSF-1 forms oligomers after heat shock, cross-linking experiments were performed using increasing concentrations of cross-linking reagent, EGS. For these experiments, control or heat shocked cells (43°C for 1 h) were lysed and treated with 0, 0.2, or 1 mM EGS for 20 min at room temperature [Rabindran et al., 1993]. Cell extracts were then subjected to immunoblot analysis using anti-human HSF-1 antibody. Figure 5A shows the cross linking experiments performed with control or heat shocked hsf-1f cells. Under control conditions, the majority of the human HSF-1 does not appear to be in a trimeric form in hsf-1f cells treated with 1 mM EGS (Fig. 5A, lane 3). However, the dimer and trimer forms of HSF-1 are observed at 0.2 and 1 mM EGS concentrations after heat shock. Perhaps due to the increase in levels of HSF-1 molecule in hsf-1f cells, both the dimer and trimer forms of HSF-1 are visible at 1 mM EGS. Higher concentrations of EGS may be required to shift all the HSF-1 molecule to appear as trimers in hsf-1f cells. For comparison, cross-linking experiments of HSF-1 in Chinese hamster HA-1 cells are also shown (Fig. 5B). Heating doses that were used resulted in equal amounts of damage in terms of cellular cytotoxicity in both hsf-1f and HA-1 cells. To detect the HSF-1 in HA-1 cells, antibody to mouse HSF-1 was used (a gift of Dr. Morimoto, Northwestern University). It should be noted that the cross-linking experiments of HSF-1 in control HA-1 cells resulted in a similar observation as shown for control hsf-1f cells and the dimer and the trimer forms of HSF-1 are observed only in the presence of 0.2 and 1 mM EGS after heat shock.

To find out if the human HSF-1 expressed in murine cells is phosphorylated after heat shock, control or heat shocked cells were analyzed by immunoblotting using anti-human HSF-1 anti-

body. Figure 6 shows that the overexpressed HSF-1 is phosphorylated upon heat shock (42°C for 1 h) in both hsf-1f and hsf-1e cells (Fig. 6, lanes 5–8). As expected, the murine HSF-1 in untransfected or neo-2 cells can not be detected by the anti-human HSF-1 antibody (Fig. 6, lanes 1–4). The overexpressed HSF-1 is shifted up in its apparent molecular weight by approximately 10 kDa, results of modifications which at least in part are due phosphorylation [Sarge et al., 1993; Mivechi et al., 1994].

The conclusions reached from above studies are that the overexpressed human HSF-1 has an ability to form oligomers after heat shock. Further, the protein kinases in murine cells are able to phosphorylate and modify the human HSF-1 protein as they do the endogenous mouse HSF-1.

Effect of the Overexpression of Human HSF-1 on Transcriptional Activation of Heat Shock Genes

To investigate whether the overexpression of HSF-1 alters the HSP-70 mRNA expression in control or heat shocked cells, untransfected or transfected cells were heat shocked at 42°C for 1 h. Cells were then incubated at 37°C for up to 24 h to allow time for the HSP-70 mRNA accumulation. Total RNA was isolated and analyzed by Northern blotting. The samples have been normalized to approximately equal levels for actin mRNA. HSP-70 mRNA expression was detected using the ^{32}P-labeled BamHI-HindIII fragments of the human HSP-70A gene as the probe. As shown in Figure 7, there is a 10–15-fold increase in HSP-70 mRNA levels in untransfected or neo-2 cells. Maximum HSP-70 accumulation is observed 4 h post heat treatment and the HSP-70 mRNA levels return to the control levels by 24 h. As the lower panel of Figure 7 shows, cells expressing human HSF-1 (hsf-1e and hsf-1f)

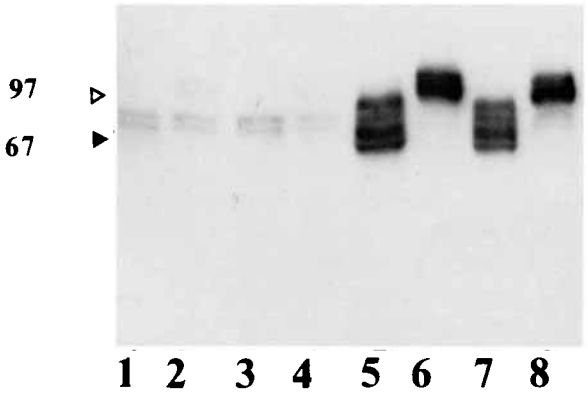

Fig. 6. Western blot analysis showing the phosphorylation of overexpressed human HSF-1 protein upon heat shock. Control or heat shocked cell extracts were analyzed by SDS-PAGE and Western blotting using anti-human HSF-1 antibody. *Lanes 1 and 2* are heat shocked or control Xi-2 cells, *lanes 3 and 4* are heat shocked or control neo-2 cells, *lanes 5 and 6* are heat shocked or control hsf-1f cells, *lanes 7 and 8* are control or heat shocked hsf-1e cells. Cells received 42°C for 1 h heat shocked and immediately analyzed. The arrows indicate the position of the unphosphorylated (67 kDa) and phosphorylated human HSF-1.

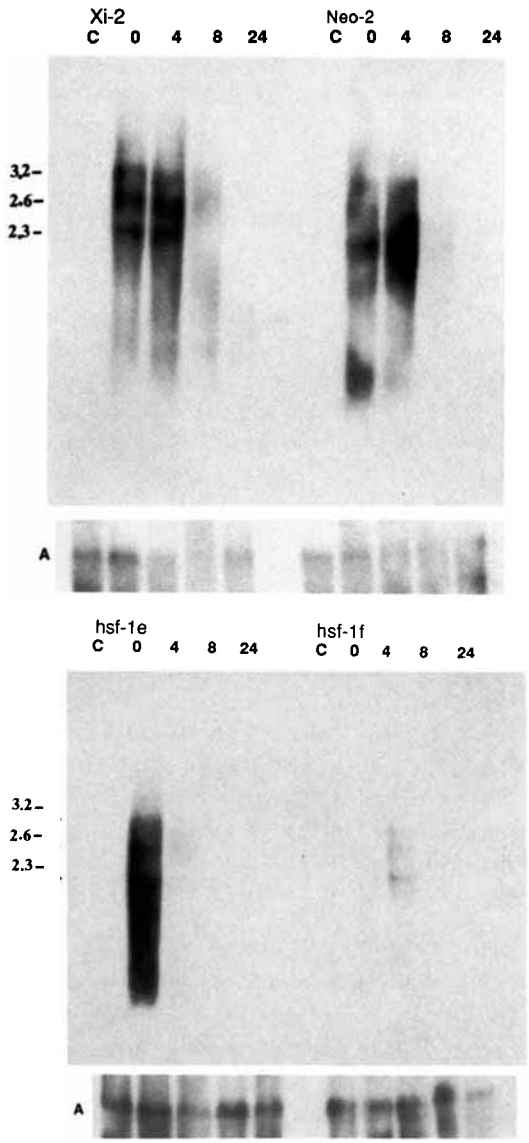

Fig. 7. Northern blot analysis of HSP-70 mRNA expression in control untransfected or cells neo-2 (**upper panel**) and cells overexpressing human HSF-1 cDNA (**lower panel**). Total RNA were isolated from control or heat shocked cells and analyzed by Northern blotting using ^{32}P-labeled HSP-70 as the probe. Cells were heat shocked at 42°C, 1 h and were incubated at 37°C for 0, 4, 8, or 24 h. C is control. A is actin. 2.3, 2.6, and 3.2 are the positions of the two heat inducible HSP-70 mRNA transcripts in kb. The level of actin in each sample is shown at the bottom of the Figure.

also express elevated levels of HSP-70 mRNA after heat shock. However, there is a 3–5-fold reduction in the expression of the heat inducible HSP-70 mRNA after heat shock in hsf-1e and hsf-1f cells when compared to untransfected or neo-2 cells as determined by the ^{32}P analysis of blots by AMBIS Scanner (Atomated Microbiology Systems, San Diego, CA) [Mivechi et al., 1992]. Contrary to hsf-1f, the hsf-1e cells show HSP-70 mRNA induction immediately after heat shock of 42°C for 1 h. However, the HSP-70 mRNA decayed by 4 h, suggesting that there was no further mRNA synthesis after heat shock in hsf-1e cells and by 4 h post heating most of it had decayed. This rapid turnover of HSP-70 mRNA in hsf-1e cells may be the reason why the levels of induction of HSP-70 protein in hsf-1f and hsf-1e cells are not very different from each other, as shown in Figure 8.

Note that due to the use of the human HSP-70 as the probe and the fact that there are several species of heat inducible mouse HSP-70 mRNA species present in heat shocked cells, perhaps with different levels of heat inducibility, the Northern blots appear as an area rather than as individual bands. However, the three major HSP-70 mRNA bands at 2.3, 2.6, and 3.2 kb are visible in heat shocked cells [Hunt and Calderwood, 1990].

Such a reduction in the accumulation of HSP-70 mRNA after heat shock in hsf-1f and hsf-1e cells also resulted in the reduction in the accumulation of 70 kDa heat shock proteins in these cells when compared to untransfected or neo-2 cells (Fig. 8). Immunoblot analysis of control or heat shocked cells (42°C for 1 h) indicate

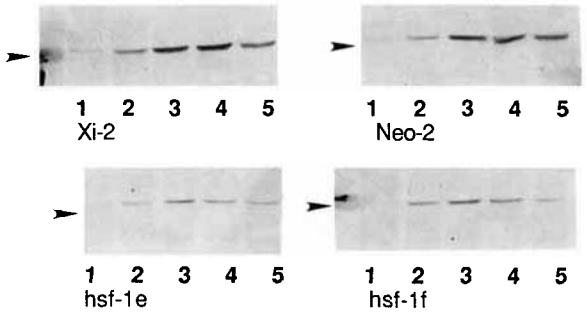

Fig. 8. Western blot analysis of HSP-70 kDa in control untransfected cells and cells overexpressing human HSF-1 protein. Cells were heat shocked at 42°C for 1 h and after incubation at 0, 4, 8, or 24 h at 37°C samples were lysed in SDS-sample buffer and analyzed by gel electrophoresis and Western blotting using anti-HSP-70 antibody (C92) as a probe. *Lanes 1–5* are, respectively, control and 0, 4, 8, or 24 h recovery time at 37°C after heat shock. The arrow indicates the position of the 67 kDa mol. wt. marker.

that the level of accumulation of HSP-70 kDa are reduced by approximately 2-fold in hsf-1e and hsf-1f cells when compared to the untransfected or neo-2 cells.

The effects of HSF-1 overexpression on the accumulation of several HSP-70 species were also analyzed using isoelectric focusing. Cells were labeled with ^{3}H-leucine for 6 h at either 37°C or after a heat dose of 42°C for 1 h. The patterns of protein synthesis in neo-2 and hsf-1f cells are shown in Figure 9. The upper panel of Figure 9 shows the isoelectric focusing of neo-2 and hsf-1f cells under control conditions. Under control conditions, the hsf-1f cells show elevated levels of several HSP-70 kDa species (upper right panel of Fig. 9) due to slight differences in loading the samples, since the levels of action are also higher in hsf-1f cells when compared to neo-2 cells. However, as it is apparent in the lower panel of Figure 9, there is an increase in the synthesis of several HSP-70 kDa species (shown by arrow) in heat shocked neo-2 and hsf-1f cells when compared to the control neo-2 and hsf-1f cells. The hsf-1f cells show reduction in the overall synthesis of HSP-70 kDa proteins and they appear not to accumulate one species of HSP-70 (shown by clear arrow). As the lower panel of Figure 9 also shows, there is a dramatic induction of a 47–50 kDa protein after heat shock in hsf-1f cells. The pI of the 47–50 kDa protein is 7–7.5, and this protein is most likely not the collagen-binding glycoprotein HSP-47 kDa that is known to have a pI of 9.0 and is not normally detectable by IEF-PAGE [Nagata et al., 1986; Nakai et al., 1992].

To further investigate the effect of the overexpressed HSF-1 on the expression of HSP-70 in neo-2 and hsf-1f cells, transient transfection studies were performed using plasmids containing the human HSP-70-luciferase reporter gene. Two days after transfection, cells were either left untreated or heated at 42°C for 1 h and incubated at 37°C for 6 h to allow time for the accumulation of luciferase. Cells were then lysed and luciferase activity was determined. As the data in Figure 10A suggests, hsf-1f cells show a 4-fold increase in the basal expression of luciferase when compared to neo-2 cells, while the luciferase activity was less in hsf-1f cells after heat shock (Fig. 10B). The human HSP-70 promoter in plasmids pHSP-70-luciferase contains elements other than HSE and, therefore, the increase in the basal expression of luciferase may not be directly the results of the overex-

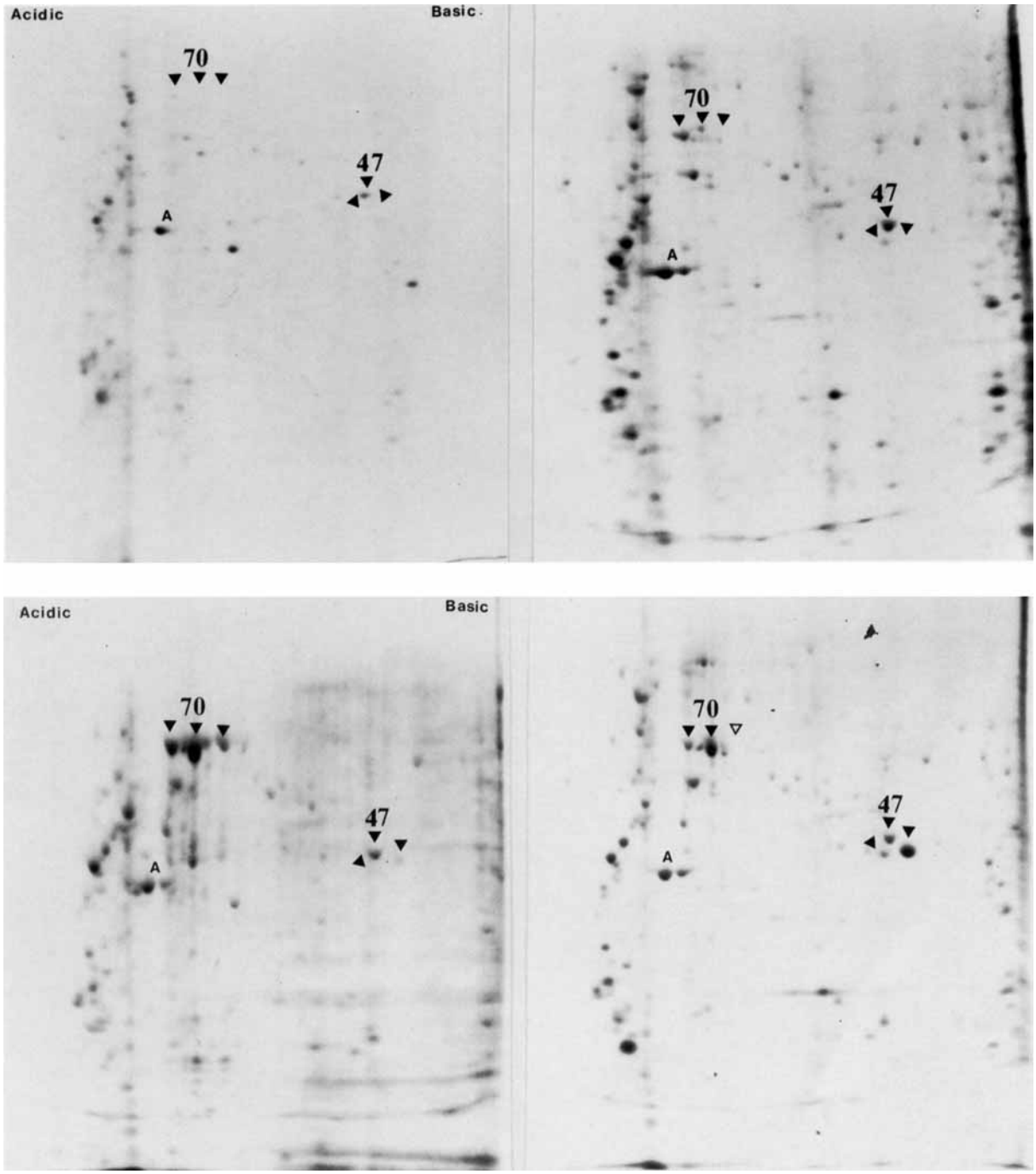

Fig. 9. Two-dimensional gel electrophoresis of neo-2 and hsf-1f cells under control or heat shock conditions. Upper panel shows control neo-2 (**upper left panel**) and control hsf-1f (**upper right panel**) cells. Cells were labeled with medium containing 20 μCi/ml of ^{3}H-leucine for 6 h. Lower panel shows heat shocked neo-2 (**lower left panel**) and hsf-1f (**lower right panel**) cells. Cell were heat shocked at 42°C for 1 h and then labeled immediately for 6 h at 37°C with medium containing 20 μCi/ml of ^{3}H-leucine. After labeling, cells were rinsed with PBS, lysed in isoelectric focusing sample buffer [O'Farrel, 1975], and analyzed by two-dimensional gel electrophoresis. The solid arrows show the 68–70 kDa and 46–47 kDa heat shock proteins. The clear arrow shows the 70 kDa species which is missing in heat shocked hsf-1f cells when compared to neo-2 cells. The acidic and basic marks show the pH range of 3.8 to 9.5 from left to right.

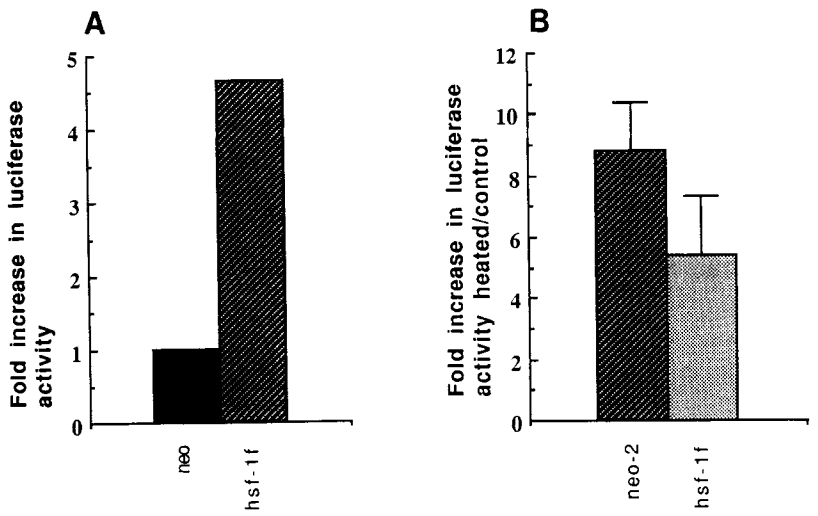

Fig. 10. HSF-1 overexpression increases the basal expression of HSP-70-luciferase reporter gene. Neo-2 and hsf-1f cells were transiently transfected with plasmids pHSP-70-luciferase. Forty-eight hours post transfection cells were either left untreated or heat shocked at 42°C for 1 h and kept at 37°C for 6 h. After this period, cells were lysed and luciferase activity was determined. **A:** Fold increase in luciferase activity in control hsf-1 cells relative to neo-2 cells. **B:** Luciferase activity of transfected cells after heat shock.

pressed HSF-1. The fact that the basal expression of HSP-70-luciferase is high in hsf-1f cells, however, is interesting and may explain the reason why the cells overexpressing HSF-1 are more resistant to the cytotoxic effects of heat as it is described below.

Cells Overexpressing Human HSF-1 Are More Resistant to the Cytotoxic Effects of Heat

One of the consequences of HSPs accumulation after heat shock is the development of transient thermotolerance [Gerner and Schneider, 1975; Hahn and Li, 1990; Li and Werb, 1982; Mivechi et al., 1991]. Permanent overexpression of HSP-70 and HSP-28 kDa proteins has been shown to render cells more resistant to killing by heat shock [Li et al., 1991; Landry et al., 1989]. To determine if cells overexpressing human HSF-1 show alteration in their thermal sensitivity, cells were heat shocked at 43°C or 45°C for various lengths of time and cell survival was assessed by colony formation. As shown in Figure 11, both hsf-1f and hsf-1e show resistance to the cytotoxic effects of heat by approximately 10-fold below 10% survival levels when compared to neo-2 and Xi-2 cells. The differences in the expression of HSF-1 in different cells may partly explain the reason for the increase in the slope rather than increase in both the shoulder and the slope of the heat survival curve that is observed in hsf-1f and hsf-1e cells. However, hsf-1e, hsf-1f, Xi-2, and neo-2 cells show exactly the same kinetics for the development and decay of thermotolerance (Fig. 12). For thermotolerance experiments, cells were heat shocked at 42°C for 1 h to induce thermotolerance and cells were then incubated at 37°C for 6, 24, 48, 72, or 96 h before they were challenged with a heat shock of 45°C for 1 h. The kinetics of the development and decay of thermotolerance were similar for all cell lines.

DISCUSSION

We have stably overexpressed the human HSF-1 in murine cells. This human HSF-1 protein is functional in mouse cells by all the criteria that have been reported for endogenous mouse HSF-1. The overexpressed HSF-1 has the apparent molecular weight of approximately 67–72 kDa (Fig. 6). Under normal growth conditions, HSF-1 is present in the cytoplasm and nucleus and it is translocated into the nucleus after heat shock (Fig. 3). The immunostained HSF-1 is not easily observed when it is distributed in the cytoplasm; however, the overexpressed HSF-1 is easily detectable after heat shock, possibly due to the oligomerization of HSF-1. The double immunofluorescent staining of HSP-70 using C92 antibody (recognizes the heat inducible HSP70) or N27 antibody (recognizes both the constitutive and the heat inducible HSP-70) and antibody to HSF-1 showed that HSP-70 is mainly in the cytoplasm under control growth conditions (data not shown).

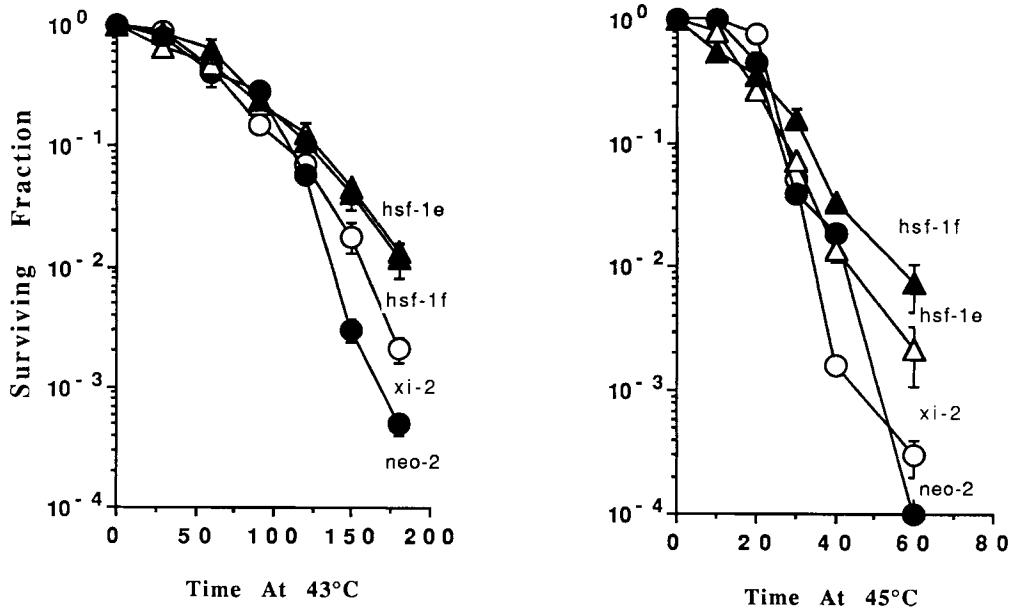

Fig. 11. Heat responses of control untransfected cells and cells overexpressing human HSF-1. Cells were heat shocked at 43°C (**left panel**) or 45°C (**right panel**) for various lengths of time. Cellular survival were analyzed by colony formation.

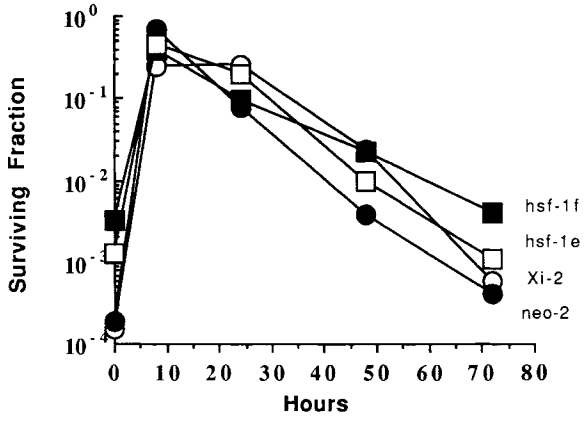

Fig. 12. Thermotolerance response of control and cells overexpressing human HSF-1. Cells received an initial heat shock of 42°C for 1 h to induce thermotolerance. Cells were then incubated at 37°C for increasing length of time before receiving a heat shock of 45°C for 1 h. Cells were then assayed by colony formation. "0" point in the x-axis indicates cell killing for each cell line after a 45°C, 1 h heat shock without the initial 42°C, 1 h heat shock.

HSP-70 localized in the nucleus and nucleolus after heat shock. Both HSF-1 and HSP-70 remained in the nucleus when cells were incubated at 37°C for 8 h after heat shock. HSP-70 returned to the cytoplasm 24 h after heat shock (data not shown). The human HSF-1 translocated into the nucleus immediately after heat shock and remained in the nucleus even 24 h after heat shock. These immunofluorescent studies suggest that the mouse HSP-70 is distributed in the cytoplasm or nucleus in similar kinetics to what has been shown previously [Welch and Framisco, 1984; Pelham, 1984; Welch and Mizzen 1988; Milarski et al., 1989] and seemed to be rather independent of the overexpressed HSF-1 presence or distribution (data not shown).

Under normal growth conditions, the overexpressed HSF-1 has constitutive DNA binding ability (Fig. 4) and its DNA binding ability is increased after heat shock. The constitutive DNA binding of HSF-1 is consistent with the earlier hypothesis that HSF-1 is normally under negative regulation and a negative regulatory factor can be outnumbered when HSF-1 is overexpressed. Cross-linking experiments show that the overexpressed HSF-1 forms oligomers after heat shock, suggesting that only a small fraction of overexpressed HSF-1 may be in oligomeric form under unstressed growth conditions. The overexpressed HSF-1 is phosphorylated upon heat shock as determined by the shift in its apparent molecular weight (Fig. 6). The phosphorylation of the overexpressed human HSF-1 in mouse cells suggests that an abundant amount of human HSF-1 can be phosphorylated by protein kinases in mouse cells.

Surprisingly, the overexpressed human HSF-1 does not increase the transcription of the mouse

HSP-70 genes after heat shock. In fact, cells overexpressing human HSF-1 accumulate lower levels of the mouse HSP-70 mRNA and protein after heat shock (Fig. 7). Such results were also obtained when cells were transiently transfected with HSP-70-luciferase reporter plasmids, although there was an increase in the basal expression of the human HSP-70-luciferase in hsf-1f cells. These results are interesting, since HSF-1 overexpressing cells were found to be resistant to heat shock. It is possible, therefore, that there is an overall increase in the levels of several HSPs in transfected cells under control conditions.

There may be several reasons that murine cells overexpressing human HSF-1 protein do not show increased levels of endogenous HSP-70 mRNA after heat shock: One, there may be sufficient mouse HSFs available in these cells to induce the transcription of HSPs mRNA, and overexpression of the human HSF-1 cannot enhance the synthesis of mRNA for HSPs beyond that observed in control untransfected cells. Two, there is a possibility that human HSF-1 cannot bind to the HSE of the mouse HSP-70 genes (or other HSPs) in vivo, and, therefore, it cannot induce enhanced synthesis of HSPs, although studies have shown that the human and mouse HSE have as much as 85% homologous regions [Kroeger et al., 1993]. Further, the molecular weights and the critical sequences for the DNA binding and multimerization domains of the human and mouse HSF-1 are similar and therefore such differences most likely are not the reasons for the lack of HSF-1's ability to promote binding to HSE in vivo. Three, it is conceivable that one of the regulatory steps in HSF-1 activation is at the level of its phosphorylation and, therefore, due to the overexpression of HSF-1, not all the HSF-1 molecules are phosphorylated, including the endogenous mouse HSF-1. Since all the mouse and human HSF-1 molecules may have DNA binding ability after heat shock, there will be a competition therefore between phosphorylated and unphosphorylated forms of HSF-1 and some may remain bound to HSE, but may not be phosphorylated and therefore, not transcriptionally active. In fact, that may lead to a decrease in the levels of HSP-70 mRNA accumulation which is observed in cells overexpressing HSF-1. Four, it is possible that there are variations in the pathway leading to HSF-1 activation between human and mouse cells. As a result, although human HSF-1 undergoes all the known modifications after heat shock in the mouse cells, the overexpressed HSF-1 remains inactive in the mouse cells. For example, mouse HSF-1 may not become as heavily phosphorylated as HSF-1 usually is in human cells. It is also possible that HSF-1 is normally present in a complex together with other factors, and such interactions positively regulate transcription of heat shock genes, and that there are insufficient amounts of such factors present in order to activate all the HSFs present in cells overexpressing the human HSF-1.

Cells overexpressing human HSF-1 are approximately 10-fold more resistant to the cytotoxic effects of heat, which is manifested more in the slope rather than the shoulder region of the heat survival curves. As suggested from the data shown in Fig. 10, it is possible that under normal growth conditions, there may be a small overall increase in the level of several HSPs, making HSF-1 overexpressing cells more resistant to heat shock. The two-dimensional gel electrophoresis experiments were not conclusive in showing whether there is an enhanced synthesis of HSPs under control growth conditions. A 47–50 kDa protein was found to be enhanced in cells overexpressing human HSF-1, the identity of which is not known at this time. Other possibilities as to why cells overexpressing HSF-1 are resistant to heat may be due to their accumulation in G1 phase of the cell cycle [Westra and Dewey, 1971]. Our flow cytometric analysis indicates that cells overexpressing HSF-1 accumulate in G1 phase of the cell cycle by as much as 25% more when compared to the parent line (data not shown). Cells in G1 are more resistant to heat than cells in S phase of the cell cycle.

Other observations regarding HSF-1 overexpression is that transfected cells with relatively high levels of HSF-1 show slower growth characteristics when compared to the parent line. Cells overexpressing human HSF-1 are in general slower in growth and contain reduced levels of the human HSF-1 expression with continuous passaging in vitro.

In conclusion, the overexpression of human HSF-1 does not increase the levels of heat shock proteins in mouse cells after heat shock. This may indicate that human HSF-1 may have higher levels of complexity in terms of its activation following heat shock that may be absent in mouse cells, or it may be that the regulation of heat shock response does not reside at the level

of HSF-1 expression but rather at the level of HSF-1 activation.

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