Human heat shock factor 1 is predominantly a nuclear protein before and after heat stress

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SUMMARY

The induction of the heat shock genes in eukaryotes by heat and other forms of stress is mediated by a transcription factor known as heat shock factor 1 (HSF1). HSF1 is present in unstressed metazoan cells as a monomer with low affinity for DNA, and upon exposure to stress it is converted to an ‘active’ homotrimer that binds the promoters of heat shock genes with high affinity and induces their transcription. The conversion of HSF1 to its active form is hypothesized to be a multistep process involving physical changes in the HSF1 molecule and the possible translocation of HSF1 from the cytoplasm to the nucleus. While all studies to date have found active HSF1 to be a nuclear protein, there have been conflicting reports on whether the inactive form of HSF is predominantly a cytoplasmic or nuclear protein. In this study, we have made antibodies against human HSF1 and have reexamined its localization in unstressed and heat-shocked human HeLa and A549 cells, and in green monkey Vero cells. Biochemical fractionation of heat-shocked HeLa cells followed by western blot analysis showed that HSF1 was mostly found in the nuclear fraction. In extracts made from unshocked cells, HSF1 was predominantly found in the cytoplasmic fraction using one fractionation procedure, but was distributed approximately equally between the cytoplasmic and nuclear fractions when a different procedure was used. Immunofluorescence microscopy revealed that HSF1 was predominantly a nuclear protein in both heat shocked and unstressed cells. Quantification of HSF1 staining showed that approximately 80% of HSF1 was present in the nucleus both before and after heat stress. These results suggest that HSF1 is predominantly a nuclear protein prior to being exposed to stress, but has low affinity for the nucleus and is easily extracted using most biochemical fractionation procedures. These results also imply that HSF1 translocation is probably not part of the multistep process in HSF1 activation for many cell types.

Key words: Heat Shock, Heat Shock Factor, Nuclear localization, Stress response

INTRODUCTION

The regulation of specific transcription factor activity is essential to the normal development and maintenance of all organisms. A large number of transcription factors preexist in various cells in a latent inactive form and are ‘activated’ to promote transcription of specific genes once the cell has received certain stimuli. There are a wide variety of mechanisms which regulate the activity of preexisting transcription factors including: binding of a specific ligand to the factor to activate the factor’s activity (e.g. Cu^{2+} to the ACE1 factor, steroid hormones to the steroid hormone receptors); removal of an inhibitory protein from the transcription factor (e.g. hsp90 from the glucocorticoid receptor, inhibitor IkB from nuclear factor IkB (NFkB)); transcription factor modification (e.g. phosphorylation of cyclic AMP responsive element binding protein, CREB); and subcellular localization (e.g. SV40 large T antigen) (for reviews see Whiteside and Goodbourn, 1993; Latchman, 1995). Some transcription factors use various combinations of the above mechanisms to regulate their activity.

One well-studied transcription factor, which preexists in all eukaryotic cells, is a protein called heat shock factor (HSF). HSF mediates the transcription of the heat shock genes. All organisms respond to elevated temperatures and other forms of ‘stress’ such as inhibitors of oxidative respiration, sulfhydryl reagents, certain heavy metals, and the generation of abnormal proteins or oxygen radicals within cells, by inducing the transcription of a family of genes known as the heat shock (hs) genes. The products of these genes, the hs proteins (hsp), help cells recover from the effects of the stress and protect them from further trauma (Morimoto et al., 1994; Parsell and Lindquist, 1994). For reviews of HSF function see Morimoto (1998), Mager and De Kruijff (1995), Wu (1995) and Voellmy (1994).

All eukaryotes appear to possess HSF1, the major stress-inducible form of HSF. However, many species possess other HSFs in addition to HSF1. For example, mammals and chickens both probably have at least three different HSF genes each (Sarge et al., 1991; Nakai and Morimoto, 1993; Nakai et al., 1997). The other HSFs appear either to respond to more
severe forms of heat stress (Tanabe et al., 1997) or to other
types of stimuli. For example, HSF2 is active during
embryogenesis, spermatogenesis and erythroid differentiation
(Sistonen et al., 1992; Mezger et al., 1994; Sarge et al., 1994),
but its activity is not stimulated during heat shock.

The transformation of HSF1 from its inactive to active form
is believed to be a multistep process (Sarge et al., 1993; Cotto
et al., 1996; Wu, 1995). These steps have been proposed to
include: (1) conversion of HSF1 from monomer to a
hetromerimer (Westwood et al., 1991; Westwood and Wu, 1993;
Baler et al., 1993; Sarge et al., 1993); (2) secondary changes
in HSF conformation such that certain domains believed to be
involved in the transcription promoting activities of HSF1
become exposed (Chen et al., 1993; Westwood and Wu, 1993;
Wu, 1995); and (3) stress-induced hyperphosphorylation of
HSF1 (Sorger and Pelham, 1988; Larson et al., 1988; Sarge et
al., 1993; Cotto et al., 1996; Winegarden et al., 1996). It has
also been proposed that for mammalian (Sarge et al., 1993;
Baler et al., 1993), chicken (Nakai et al., 1995) and Drosophila
cells (Zandi et al., 1997), translocation of HSF1 from the
cytoplasm to the nucleus is a step that regulates HSF activity.
The homotrimerization of HSF1 has been shown to be essential
for HSF to bind the heat shock element(s) (HSEs) found
upstream of all hs genes with high affinity (Sorger and Pelham,
1988; Clos et al., 1990; Fernandes et al., 1994; Wu, 1995). The
exact roles of other changes in HSF1, including stress-induced
hyperphosphorylation, are yet to be determined for HSF1 in
metazoans.

All studies to date have been in agreement that the trimeric
‘active’ form of HSF1 is a nuclear protein. However, there has
been some controversy as to the location of HSF1 prior to
stress in metazoans. In Drosophila, a number of reports have
suggested that HSF1 is a nuclear protein before and after stress
(Westwood et al., 1991; Wu et al., 1994; Shopland and Lis,
1996; Orosz et al., 1996), but one report has suggested that it
is predominantly cytoplasmic prior to heat shock and nuclear
after heat stress (Zandi et al., 1997). Similarly, while some
studies have suggested that mammalian HSF1 is always a
nuclear protein (Wu et al., 1994; Martinez-Balbas et al., 1995),
others have suggested that mammalian HSF1 is predominantly
a cytoplasmic protein under nonshock conditions and is
translocated into the nucleus upon heat stress (Sarge et al.,
1993; Baler et al., 1993; Sistonen et al., 1994). Chicken HSF1
has been reported to be a cytoplasmic protein before heat stress
and a nuclear protein after heat stress (Nakai et al., 1995).
Xenopus HSF1 has been reported to be a nuclear protein before
and after heat stress (Mercier et al., 1997). In tomatoes,
the localization of two different HSFs has been studied (Lyck
et al., 1997). The constitutive, stress-activated HSF called HSFA1
(formerly Lp-HSF8) appears to be distributed between the
nucleus and cytoplasm before heat shock and is exclusively
nuclear after heat shock.

In this study we have reexamined the localization of
mammalian HSF1 prior to stress. Using an affinity-purified
preparation of a polyclonal antibody made against a
bacterially produced maltose binding protein + Homo sapiens
HSF1 (MBP-HsHSF1) fusion protein, we confirm that
HsHSF1 is predominantly a nuclear protein before and after
stress. This finding implies that for many cell types, the
translocation of HsHSF1 from the cytoplasm to the nucleus
is not a step involved in regulating HSF1 transcriptional
activity and that any cellular signals that are involved in the
activation/deactivation of HSF1 are probably realized in the
nucleus.

MATERIALS AND METHODS

Tissue culture

Human HeLa and A549 cells, and green monkey Vero cells were
grown in 45% Dulbecco’s Modified Eagles medium (Sigma), 45% F-12
Ham nutrient mixture, Hepes modification (Sigma) supplemented with
25 mM NaHCO3, 2% Fetal clone II (Hyclone), 8% Cool Calf 2
(Sigma), 20 μg/ml gentamycin (Sigma), pH 7.4, at 37°C in T-75 tissue
culture flasks (Sarstedt). A549 and Vero cells were generously
provided by W. Furyaya and S. Grinstein (Hospital for Sick Children,
Toronto, Ontario). Heat-shocked cells were prepared by immersion of
T-flasks in a circulating water bath at the temperatures indicated in
the Fig. legends. HeLa cells used for experiments in Fig. 3A,B were
grown in suspension Eagles Minimum Essential Medium, Joklik
modification (Sigma), supplemented with 10 mM Hepes, pH 7.4, 25
mM NaHCO3, 10% Cool Calf 2 (Sigma), 20 μg/ml gentamycin in
spinner flasks (Belco Biotechnology). Prior to these experiments, 5
ml samples of cell suspension at a concentration of 4.5×105 cells/ml
were transferred to 50 ml polypropylene tubes and aerated by shaking
at 180 rpm, 37°C, for 5 hours.

Preparation of protein extracts

Whole cell extracts

Cells were scraped into the medium and pelleted; the medium was
removed, the pellet resuspended in 3 volumes of 1× SDS sample
buffer (Ausubel et al., 1995) and then sonicated for 10 seconds with
a Heat Systems Ultrasonic sonicator.

NP-40 based fractionation

This procedure is a modification of the procedure described by Baler
et al. (1993). Briefly, 2.5×106 cells were pelleted, washed once in 1×
PBS and resuspended in 150 μl Nonidet P-40 (NP-40) low salt buffer
containing 50 mM Tris-Cl, pH 8.0, 50 mM NaCl, 1% NP-40, 1 mM
dithiothreitol (DTT), 0.5 mM phenylmethysulfonyl fluoride (PMSF).
The nuclei were pelleted by centrifugation at 7,000 g for 1 minute at
4°C. The supernatant was removed and saved as the cytoplasmic
fraction. 150 μl NP-40 high salt buffer (50 mM Tris-Cl, pH 8.0, 450
mM NaCl, 1% NP-40, 1 mM dithiothreitol, 0.5 mM PMSF) was then
added to the nuclei. After incubation for 10 minutes at 4°C the sample
was sonicated for 5 seconds and the resulting extract was the nuclear
fraction.

Dignam fractionation

The procedure used was modified from one described by Lee et al.
(1994). Briefly, 2.5×106 cells were pelleted, washed once in 1× PBS,
resuspended in 1 packed cell volume of Buffer A (10 mM Hepes,
pH 8.0, 1.5 mM MgCl2, 10 mM KCl, 1 mM DTT, 0.5 mM PMSF)
and incubated on ice for 15 minutes. The cells were then lysed by
passing them through a 28.5-gauge needle attached to a 0.5 ml
syringe 5 times and the nuclei pelleted by centrifugation for 20
seconds at 12,000 g. The supernatant was removed and saved as the
cytoplasmic fraction. The nuclei were then resuspended in one
packed cell volume of Buffer C (20 mM Hepes, pH 8.0, 1.5 mM
MgCl2, 25% glycerol, 420 mM NaCl, 0.2 mM EDTA, 1 mM DTT,
0.5 mM PMSF) and incubated on ice for 30 minutes with occasional
shaking. The sample was then centrifuged for 5 minutes at 12,000
g at 4°C to separate the nuclear soluble (supernatant), and nuclear
insoluble (pellet) fractions. To prepare cytoplasmic and nuclear
protein extracts for SDS-PAGE, 1/6 volume of 6× SDS sample
buffer (Ausubel et al., 1995) was added, and for the nuclear
insoluble fraction, two packed cell volumes of 1× SDS sample buffer
The cDNA clone of human HSF1, pBS10811 (Rabindran et al., 1995) was modified and the mixture sonicated for 10 seconds.

**Electrophoretic mobility-shift assays (EMSAs)**

HSF1 DNA binding activity was analyzed by electrophoretic mobility-shift assay. Binding reactions contained 3 μl of protein extract, 1 μl 10× buffer mix (100 mM Hepes, pH 7.9, 30% glycerol w/v) and 1 μl 10× bovine serum albumin/nucleotide mix (20 mg/ml bovine serum albumin fraction V, 0.5 mg/ml E. coli DNA, 2 mg/ml tRNA, 0.2 mg/ml poly d(N)6, 0.5 mg/ml poly(dI-dC)/poly(dI-dC), NaCl to 200 mM final concentration, and double distilled water to 9 μl. This mixture was incubated on ice for 10 minutes. 1 μl of a 32P-labelled oligonucleotide containing a heat shock element (HSE) (0.2 pmol) (Winegarden et al., 1996) was then added to the above mixture. Binding reactions were then allowed to proceed for 10 minutes at room temperature (18-21°C), after which 2 μl of 6× loading dye (0.25% Bromophenol Blue, 30% glycerol, 3× Tris-borate-EDTA buffer) was added. Samples were electrophoresed, dried and exposed to film as previously described (Winegarden et al., 1996).

**Production of anti-human HSF1 antibodies**

The cDNA clone of human HSF1, pBS10811 (Rabindran et al., 1991) was digested with EcoR1 and religated. Clones were selected that had HSF1 in the opposite orientation from the original pBS10811. The HSF1 coding sequences were removed with XbaI and HindIII and cloned into pMalc2 (New England Biolabs) digested with XbaI and HindIII. This yielded a construct which produced an in-frame fusion protein between maltose binding protein (MBP) and full-length HsHSF1. E. coli containing either MBP or MBP+HsHSF1 constructs were induced to overexpress, and induced proteins were purified essentially as per the manufacturer’s instructions (New England Biolabs). Antibodies were produced to MBP+HsHSF1 in rabbits by an initial injection of 1.5 mg of purified antigen mixed 1:1 with complete Freund’s adjuvant. Anti-HsHSF1 antiserum (PM95-1) was affinity purified following a procedure described by Harlow and Lane (1988). Briefly, crude antiserum was passed over MBP coupled to Affigel 10 beads (BioRad) until all of the anti-MBP antibodies had been removed (as tested by western blot analysis). The anti-MBP-depleted serum was passed over MBP+HsHSF1 coupled to Affigel-10. Bound anti-HsHSF1 antibodies were eluted with low pH buffer (Harlow and Lane, 1988).

**Western blot analysis**

Proteins fractionated by SDS-PAGE (8% polyacrylamide for HSF, 12% for histone H1 and IκB) were electroblotted onto nitrocellulose (Biorad NT, Gelman) and the blots blocked with 5% powdered milk in TBST (20 mM Tris, pH 7.6, 137 mM NaCl, 0.1% Tween 20). Antibodies were diluted in 2% gelatin (BioRad) in TBST. For Fig. 1, the antibody dilutions were 1:2000 for preimmune; 1:2000 for crude anti-MBP-HsHSF1 (PM95-1); 1:100 for affinity-purified anti-HsHSF1 (PM95-1); and 1:1000 for SR191 (a gift from S. Rabindran and C. Wu, National Institutes for Health, Bethesda, MD). For Fig. 2, the antibody dilutions were 1:2000 for PM95-1 antibody, the anti-histone antibody (Chemicon International Inc., cat. no. MAB052), and 1:1000 for the anti-IκB antibody (Santa Cruz Biotechnology, cat. no. SC847). Blots were incubated in the primary antibody for 1 hour at room temperature, washed with TBST, and then incubated for 45 minutes with secondary antibody, which was either alkaline phosphatase-conjugated goat anti-rabbit IgG (BioRad) diluted 1:2000 in 2% gelatin/TBST for the anti-HSF antibodies, or horseradish peroxidase-conjugated goat anti-mouse IgG (Sigma) diluted 1:40,000 in 5% powdered milk/TBST for the anti-histone and anti-IκB antibodies. Blots were then washed in TBST and developed for alkaline phosphatase activity using 5-Bromo-4-chloro-3-indolylphosphate p-Toluidine Salt (BCIP) and Nitroblue Tetrazolium Chloride (NBT) reagents (BRL Life Technologies) or for horseradish peroxidase activity using a chemiluminescence reagent kit (NEN Life Science) as per the manufacturer’s instructions.

**Immunofluorescence microscopy**

Cells, grown in chamber slides (NUNC), were either kept in non-shock conditions (37°C) or heat shocked (42°C) by placing slides in a humidified container floating in a water bath. The medium was removed and the cells were fixed in 4% paraformaldehyde in 1× PBS. The slides were either placed in 95% ethanol or stained immediately. After blocking with 0.5% BSA, 0.5% Tween-20 in 1× PBS for 20 minutes at room temperature, slides were incubated in 1:10 affinity-purified anti-MBP+HsHSF1 (PM95-1), 1:250 anti-HsHSF1 antibody (SR191), or 1:200 anti-hsp70 (Stressgen, cat. no. SPA-810) in 0.5% BSA, 0.5% Tween 20 in 1× PBS for 1 hour. Slides were then washed 3 times in 1× PBS, followed by incubation for 30 minutes with FITC-conjugated goat anti-rabbit antibody (1:200; Cappel, cat. no. 55655) or rhodamine-conjugated goat anti-mouse antibody (1:500; Jackson Immunoresearch Laboratories Inc.) diluted in 0.5% BSA, 0.5% Tween-20 in 1× PBS. The slide was washed 3 times in 1× PBS and then the DNA was stained for fluorescence with Hoechst 33342 (Sigma) diluted to 1 μg/ml in 1× PBS for 5 minutes. The slide was then rinsed once in 0.05% Tween 20 in 1× PBS. The cells were mounted in 1 drop of antifade (0.1% phenylenediamine (Sigma) in 70% glycerol) before a coverslip was applied. Photography was performed using a Nikon Microphot fluorescence microscope, a Nikon Plan 40× objective and Fujichrome Sensia 400 ISO film. Exposure times were 8 seconds for Hoechst-stained preparations, and 16 seconds (with neutral density filters) for FITC-stained samples. 35 mm transparencies and autoradiograms were digitized using an Agfa Arcus II scanner. When necessary, digitized images were adjusted for contrast and brightness using Adobe Photoshop.

**Hoffman modulation contrast microscopy**

Modulation contrast images of the cells were taken using a Zeiss Axiosvert 100 using a Hoffman HMC 20× LWD objective and corresponding condenser (Modulation Optics Inc., Greenvale NY). Images were captured using Northern Exposure software (v2.9d) (Empix Imaging; Mississauga, Ontario), a Sony black and white CCD video camera (Model XC-75) and an ATI Optimum frame grabber card.

**Quantification of immunofluorescence**

To determine the relative fluorescence (HSF1 staining) in the nucleus and cytoplasm, HeLa cells were incubated with SR191 antiserum (1:250 dilution) and processed as described above. Fluorescent images of cells were captured using Northern Exposure software and a Sony CCD video camera as described above, except that the camera was attached to a Nikon Microphot fluorescence microscope (Nikon Plan 40× objective). For nuclear images (Hoechst stain), two neutral density filters (total value 6) were used and 0.5 second integrated exposures were taken. FITC (anti-HsHSF1) images were captured in the absence of neutral density filters, using 2.5 second integrated exposures. Quantification analysis of the images was performed using Northern Exposure. In order to quantify nuclear area, the Hoechst captures were thresholded such that only nuclei were selected. Nuclei were manually traced, and the software was asked to calculate perimeter, density and area of the traced section. FITC fluorescence (anti-HsHSF1) was quantified similarly. For each condition in a single experiment, three different slides were used, and 10 to 20 cells from each slide were quantified. Each set of conditions was performed in three separate experiments. Cells stained with secondary antibody alone were also thresholded to select the fluorescent areas. Since the fluorescence across the cells was uniform, small sections in the center of the image were selected for density and area quantification.
RESULTS

Production of polyclonal antibodies specific to human HSF1

We produced polyclonal antibodies to a maltose binding protein-human HSF1 (MBP+HsHSF1) fusion protein. The cDNA for HsHSF1 was cloned behind an MBP expression construct to facilitate rapid large-scale preparation. Using this construct under control of the lac repressor, MBP+HsHSF1 was overexpressed and purified (Fig. 1A). During purification, approximately half of the full-length MBP+HsHSF1 protein degraded to yield a product that is presumed to contain, on the basis of its apparent molecular size, 15 kDa of HsHSF1 fused to the MBP moiety as well as other degradation products. The eluate from the purification was used to raise polyclonal antibodies by injection into rabbits. Crude antiserum raised against MBP+HsHSF1 was affinity purified to yield antibodies specific to HsHSF1. The ability of the crude and affinity-purified antibodies (PM95-1) to recognize HsHSF1 in HeLa cell extracts was tested by western blot analysis (Fig. 1B). A different polyclonal antiserum made against HsHSF1 produced in bacteria (SR191) (provided by S. Rabindran and C. Wu; Rabindran et al., 1991) was also tested for comparison (Fig. 1B). Both the affinity-purified PM95-1 antibodies and SR191 recognized an 82 kDa band corresponding to HsHSF1 in nonshocked cells (37°C) and 82 kDa to 95 kDa band(s) in heat-shocked cells (42°C, 15 minutes), corresponding to differentially phosphorylated forms of HsHSF1. The relative mobility of hypophosphorylated and hyperphosphorylated HsHSF1 reported here is in good agreement with the previously described relative mobility of HsHSF1 (Rabindran et al., 1991, 1994). Both affinity-purified PM95-1 antibodies and SR191 also recognized a 71 kDa protein, which could be a degradation product of HsHSF1 (Rabindran et al., 1994).

Localization of HSF1 by biochemical fractionation of cells

Two groups have judged HsHSF1 to be a cytoplasmic protein under non-shock conditions based on the biochemical fractionation of unshocked and heat-shocked cells (Baler et al., 1993; Sarge et al., 1993; Sistonen et al., 1994). In this study we used two different biochemical fractionation techniques to produce cytoplasmic and nuclear extracts; a Dignam-type fractionation scheme (Lee et al., 1994) and a fractionation scheme based on the nonionic detergent NP-40 (Baler et al., 1993). The nuclei of unshocked and heat shocked HeLa cells were biochemically fractionated from the cytosol and the presence of HSF1 was tested by western blot analysis (Fig. 2A,B) and electrophoretic mobility-shift assay (EMSA) (Fig. 3A,B). Purity of the fractions was assessed by examining each fraction for the presence or absence of IκBα, a cytoplasmic marker, and histone H1, a nuclear marker (Fig. 2A,B). As expected, IκBα was only detected in the cytoplasmic fractions. Histone H1 was only found in the nuclear fractions for the NP-40 fractionation scheme (Fig. 2A) and almost all of it was found in the insoluble nuclear fractions for the Dignam fractionation scheme (Fig. 2B). The HsHSF1 fractionation results are in agreement with previous studies, i.e. that HSF1 in unshocked cells fractionates to the cytosol. However, the proportion of HSF1 in the cytoplasmic fraction differs, depending on the fractionation scheme used. That is, with the NP-40 based fractionation procedure, almost all of the unshocked HSF1 was found in the cytoplasmic fraction (Fig. 2A). Using the modified Dignam procedure, a large portion of the unshocked HSF1 was found in the nuclear fraction and some in the insoluble fraction (IN) (Fig. 2B). In cells heat shocked for either 15 minutes (Fig. 2) or 180 minutes (not shown) at 42°C, most of the HSF1 fractionated to the nucleus but there was a small portion of HSF1 that was still found in the cytoplasm (Fig. 2A,B).

The protein extracts made using the two different extraction procedures were also examined for HSF1 binding
2769HSF1 is a nuclear protein in unstressed cells. Unshocked (37°C) and heat-shocked (42°C, 15 or 180 minutes) HeLa cells were fractionated either by (A) an NP-40 based, or (B) the modified Dignam fractionation procedure. Fractions were examined for the presence of active HSF1 using gel mobility-shift analysis (EMSAs).

Localization of HSF1 by indirect immunofluorescence

The distribution of HSF1 within cells was examined by indirect immunofluorescence in three different cell types: human HeLa cells (Fig. 4), human kidney A549 cells (Fig. 5) and green monkey kidney Vero cells (Fig. 6). HSF1 staining was examined in both unshocked (37°C) and heat-shocked (42°C, 15 minutes) cells using two different HSF1 antibodies: SR191, a polyclonal antisera made against bacterially produced human HSF1; and affinity-purified PM95-1 containing polyclonal antibodies made against a bacterially produced maltose binding protein-human HSF1 fusion protein. Both antisera gave essentially the same results in all three cell types. In both unshocked and heat-shocked cells, HSF1 is seen mainly in the nucleus but not in the nucleolus (Figs 4F, I, L, O; 5C, F, I, L; 6C, F, I, L). Some fluorescence is observed in the cytoplasm. However, a proportion of this fluorescence can be attributed to the fluorescence seen using the secondary antibody alone, which stains the entire cell faintly (Fig. 4C). The distribution of HSF1 we observed is entirely consistent with previous studies that used the SR191 antisera (Rabindran et al., 1991; Wu et al., 1994; Martinez-Balbas et al., 1995). They are also consistent with results obtained with a polyclonal anti-mouse HSF1 antisera used to stain HeLa and other mammalian cell lines (M. Vujanac, O. Bensaude and E. Zimarino, manuscript in preparation). We also examined the distribution of hsc/hsp 70 before and after heat shock (Fig. 4Q, S). Hsc/hsp70 was predominantly cytoplasmic prior to heat shock (Fig. 4Q) and predominantly nuclear after heat shock (Fig. 4S). These results are consistent with previous findings using this antibody (Welch and Feramisco, 1984) and confirm that the cells were

activity to HSEs in an EMSA. Unshocked cells showed virtually no HSF1 binding activity in either the cytoplasmic or nuclear fractions (Fig. 3). In heat-shocked extracts, virtually all of the HSF1 binding activity was found in the nuclear fractions for both fractionation schemes, although a small amount of activity is seen in the 15 minute heat-shocked NP-40 cytoplasmic fraction (Fig. 3B). These results are consistent with those obtained previously by others (Sarge et al., 1993; Baler et al., 1993), with the exception that we did not observe as much attenuation of HSF1 binding activity or reversion to cytoplasmic localization during prolonged heat shock (Sarge et al., 1993).
**Fig. 4.** Human HSF1 is a nuclear protein before and after stress in HeLa cells. Cells were either kept in non-shock conditions (37°C) or were heat shocked (42°C, 15 minutes), fixed, then were incubated with one of two different anti-human HSF1 antibodies: affinity-purified PM95-1 (F,I) or SR191 (L,O), followed by staining with a fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit secondary antibody. Fluorescence staining due to the secondary antibody alone is shown in (C). DNA was visualized by staining with Hoechst 33342 (B,E,H,K,N). Whole cells were visualized using Hoffman modulation contrast microscopy (A,D,G,J,M). Other cells were incubated with an anti-hsp70 antibody (SPA-10) followed by staining with a rhodamine-conjugated secondary antibody (Q,S).

**Fig. 5.** HSF1 is a nuclear protein before and after stress in human A549 cells. Human A549 cells were kept in non-shock conditions (37°C) or were heat shocked (42°C, 15 minutes), fixed, then stained with one of two different anti-human HSF1 antibodies, as described in the legend of Fig. 4.
HSF1 is a nuclear protein in unstressed cells and indeed heat shocked and that our fixation technique worked for both nuclear and cytoplasmically localized proteins.

We examined the distribution of HSF1 before and after heat shock using image analysis software (Northern Exposure) on digitally captured images. This analysis was performed on cells stained in three separate experiments, all giving essentially the same results. The results of one such analysis are shown in Table 1. Several different measurements and calculations were made to try and address three major points. First, what proportion of the fluorescence observed is due to HSF1? Second, what is the amount of HSF1 staining in the nucleus relative to the cytoplasm? Third, does either the total amount or the distribution of HSF1 staining change when comparing unshocked to heat shocked cells? To determine the amount of fluorescence that can be attributed to HSF1, the total amount of fluorescence for each nucleus and each cytoplasm was determined and the average of 40 to 50 cells was calculated. From this value, the fluorescence due to the FITC secondary antibody alone (no anti-HSF1 antibodies) was subtracted. Secondary antibody staining alone produced a uniform staining pattern over the entire cell in both unshocked (Fig. 4C) and heat-shocked cells (not shown). Approximately 29% of the total nuclear fluorescence could be attributed to the secondary antibody in both unshocked and heat-shocked cells (see Table 1). Approximately 63% of the total cytoplasmic fluorescence could be attributed to the secondary antibody in both unshocked and heat-shocked cells.

Unshocked cells appeared to have slightly more HSF1 staining than heat-shocked cells (186 versus 167 units), but this difference is well within the standard deviation of each value. The large standard deviations observed in the fluorescence in both the nucleus and cytoplasm appears to be mostly due to the large variation in nuclear and cytoplasmic size from cell to cell. The average amount of HSF1 staining per unit area of nucleus and cytoplasm was almost identical between control and heat-shocked cells.

The proportion of total HSF1 in the nucleus was essentially the same in unshocked and heat-shocked cells (78% and 79%, respectively). These results indicate that most (approximately 80%) of the HSF1 appears to be in the nucleus before and after heat shock. The remaining 20% of the staining attributed to HSF1 appears to be localized throughout the cytoplasm and does not appear to be translocated into the nucleus upon heat shock.

| Table 1. Subcellular distribution of HSF1 in unshocked and heat-shocked HeLa cells |
|------------------------------------------|-----------------|-----------------|
|                                         | Unshocked (37°C) | Heat shocked (42°C, 15 minutes) |
| Nuclear HSF1 staining (arbitrary units) |                 |                               |
| Total average fluorescence per nucleus¹ | 209±62           | 184±91                       |
| Fluorescence due to secondary antibody alone² | 64±2           | 51±4                        |
| Average HSF1 staining per nucleus (Total fluorescence – secondary antibody fluorescence) | 145 | 125 |
| Average nuclear area (arbitrary units) | 161±47 | 149±53 |
| Average HSF1 staining/unit nuclear area³ | 0.88 | 0.89 |
| Cytoplasmic HSF1 staining |                 |                               |
| Total average fluorescence per cytoplasm¹ | 114±35 | 90±50 |
| Fluorescence due to secondary antibody alone² | 73±25 | 64±4 |
| Average HSF1 staining per cytoplasm² | 41 | 34 |
| Average cytoplasmic area (arbitrary units) | 185±59 | 163±83 |
| Average HSF1 staining/unit area of cytoplasm³ | 0.22 | 0.21 |
| Percentage of total HSF1 in nucleus⁴ | 78% | 79% |

¹Total average fluorescence per nucleus (or cytoplasm) = sum of the fluorescence measured in nucleus/total number of cells measured. The fluorescence for each cell cytoplasm was measured by subtracting nuclear fluorescence from total fluorescence for that cell. Values are ± s.d. (unshocked, n=42 cells; heat shocked, n=50 cells).
²Fluorescence due to secondary antibody staining = average staining of secondary antibody alone/unit cell area × average size of nucleus (or cytoplasm). Values are ± s.d. (unshocked, n=7; heat shocked, n=5).
³Average HSF1 staining/unit area of nucleus (or cytoplasm) = average HSF1 staining per nucleus (or cytoplasm) / average size of nucleus (or cytoplasm).
⁴Percentage of total HSF1 in nucleus = average HSF1 staining per nucleus × 100 / (average HSF1 staining per nucleus + average HSF1 staining per cytoplasm).
DISCUSSION

Regulation of mammalian hs genes by HSF1 has been postulated to involve translocation of HSF1 from the cytoplasm to the nucleus (Sarge et al., 1993; Baler et al., 1993; Sistonen et al., 1994; Morimoto, 1998). Some of the early studies determining the subcellular localization of HSF1 involved biochemical fractionation of mammalian cells followed by western blot analysis (Baler et al., 1993; Sistonen et al., 1994). These studies suggested that HSF1 was a cytoplasmic protein that translocated from the cytoplasm to the nucleus with heat stress. Using a similar western blot analysis of biochemically fractionated mammalian cells, we obtained essentially the same results. That is, after biochemical fractionation, HsHSF1 from unshocked cells was found in the cytoplasmic fraction, while after heat shock, HsHSF1 was found predominantly in the nuclear fraction. However, the difference in the amount of HsHSF1 found in the cytoplasm of unshocked cells differs depending on which fractionation technique is used. Using a modified Dignam extraction procedure, a large proportion of the HSF1 was found in the nuclear fraction prior to heat shock. Using an NP-40 based extraction procedure, there was almost no detectable HSF1 in the nuclear fraction prior to heat shock. Therefore, the distribution of HSF1 prior to heat shock is highly dependent on the fractionation procedure used and raises questions as to whether biochemical fractionation can accurately determine the location of HSF1.

A few studies have examined the localization of mammalian HSF1 by immunofluorescence microscopy. One study, using an anti-mouse HSF1 polyclonal antisera, has shown that in mouse 3T3 and in human HeLa cells, HSF1 is predominantly cytoplasmic before heat shock and predominantly nuclear after heat shock (Sarge et al., 1993). Other studies, which have used an anti-human HSF1 antibody (SR191), have shown that in HeLa cells, HSF1 is found almost exclusively in the nucleus before and after heat shock (Wu et al., 1994; Martinez-Balbas et al., 1995). An exception to this localization pattern was observed in mitotic cells where HSF1 was found to be distributed throughout the entire cell and excluded from condensed chromosomes (Martinez-Balbas et al., 1995). One of the cell types examined in all the studies was human HeLa cells, indicating that the observed differences are likely due to differences in the two antibodies used and not to different cells being examined. Using a different affinity-purified antibodies against HsHSF1, we obtained results that were virtually identical to those obtained by the latter studies. That is, HSF1 was found to be predominantly a nuclear protein in unshocked and heat-shocked interphase cells.

A factor that might contribute to the different results obtained with the different anti-HSF1 antibodies is the preparation of the HSF1 protein used as antigen. Some studies (e.g. Sarge et al., 1993) used bacterially expressed mouse HSF1 extracted from a denaturing SDS gel as antigen, while others (Wu et al., 1994; Martinez-Balbas et al., 1995) used human HSF1 that was purified by chromatography and was not denatured prior to introduction into rabbits. The antigen produced in our study was not denatured prior to injection. This raises the possibility that antibodies raised against nondenatured HSF1 may not recognize all forms, and, in particular, the inactive monomeric form of HSF1. We do not believe this to be the case because the amount of nuclear HSF1 staining, as measured by fluorescence quantification of immunostained cells, was the same before and after heat shock, indicating that the anti-HSF1 antiserum recognized unshocked HSF1 with the same efficiency as shocked HSF1.

Studies by Cotto et al. (1997) and Jolly et al. (1997) have examined the subnuclear localization of mammalian HSF1 by fluorescence microscopy before and after heat and other stresses. Using a green fluorescent protein-HSF1 fusion protein as well as monoclonal antibodies that recognize either HSF1 directly or an HSF1-Flag tagged fusion protein, it was found that HSF1 stained the entire nucleus during heat shock (except for the nucleolus) (Cotto et al., 1997). In addition, HSF1 formed bright irregularly shaped granules whose appearance and disappearance roughly correlated with HSF1 transcriptional activity. In agreement with our current findings, both natural HSF1 and the transfected HSF1 fusion proteins appeared to be predominantly nuclear in unstressed cells (Cotto et al., 1997; Jolly et al., 1997).

The behaviour we observe for human HSF1 in this study is identical to what has been previously observed for Drosophila HSF (Westwood et al., 1991; Wu et al., 1994; Wisniewski et al., 1996; Orosz et al., 1996). That is, biochemical fractionation of Drosophila SL2 tissue culture cells showed that HSF appeared to be a cytoplasmic protein before heat shock and a nuclear protein after heat shock (Wu et al., 1994). Immunofluorescence microscopy revealed that Drosophila HSF was a nuclear protein before and after heat shock (Westwood et al., 1991). This conclusion was based on experiments using three different polyclonal antisera made against Drosophila HSF1 and two types of Drosophila cells: Schneider line 2 (SL2) and the salivary gland cells of Drosophila melanogaster third instar larvae. Two of the polyclonal antisera were made against bacterially produced and purified Drosophila HSF (Westwood et al., 1991; Wu et al., 1994; Wisniewski et al., 1996; J. T. Westwood and C. Wu, unpublished) while the third was made against a peptide sequence found within HSF (Orosz et al., 1996). A fourth polyclonal antisera against Drosophila HSF immunostained polytene chromosomes of salivary glands in a similar fashion (Shopland et al., 1995; Shopland and Lis, 1996). In Drosophila embryos, it has been found that HSF is excluded from the nucleus in both unshocked and heat-shocked embryos until stage 13, at which time it becomes a nuclear protein in both unshocked and heat-shocked embryos (Wang and Lindquist, 1998). The redistribution of HSF correlates with the embryo's ability to induce heat-shock gene transcription, i.e. hsp70 transcription only occurs when HSF is a nuclear protein and when embryos are heat shocked.

When biochemically fractionated nuclei from heat-shocked Drosophila SL2 cells were examined by immunofluorescence microscopy, they stained brightly with anti-HSF1 antibody, whereas nuclei from unshocked cells had virtually no HSF (Wu et al., 1994). Consistent with these results, it has previously been shown, using immunofluorescence microscopy, that unshocked HeLa cells extracted with a buffer containing NP-40 no longer showed HSF1 staining in the nucleus (or any other location), whereas HSF1 in heat-shocked cells was resistant to NP-40 extraction and was still present in the nucleus (Martinez-Balbas et al., 1995).

A study by Orosz et al. (1996) has identified the amino acid sequence responsible for HSF nuclear localization in
HSF1 is a nuclear protein in unstrained cells

Drosophila, and this sequence conformed to a nuclear localization motif found in other nuclear proteins. Mutant HSF1 molecules lacking this sequence localize to the cytoplasm rather than the nucleus in both unshocked and heat-shocked cells when examined by immunofluorescence microscopy, while wild-type HSF1 was nuclear before and after heat shock (Orosz et al., 1996).

Not all studies examining the localization of Drosophila HSF have given the same results. Using immunofluorescence microscopy, Zandi et al. (1997) concluded that Drosophila HSF is a cytoplasmic protein prior to heat shock and a nuclear protein after heat shock. It is not clear why there is such a discrepancy in the localization results but it is most likely due to the different antibodies being used, since the cell line that was examined (SL2) was apparently the same in both studies. Further experimentation with more cell types and quantification of the staining should help clarify the issue.

It is intriguing that one of the heat shock proteins, hsp27, has localization and biochemical fractionation properties similar to HSF. Immunofluorescence microscopy suggested that, like HSF, hsp27 was predominantly a nuclear protein in Drosophila S3 cells that were untreated, heat shocked, heat shocked and allowed to recover at control temperatures, or treated with edcsyone (Beaulieu et al., 1989). When cells were biochemically fractionated using an extraction buffer containing NP-40, hsp27 was found in the detergent insoluble fraction of heat-shocked cells whereas in recovering and edcsyone-treated cells, hsp27 was found in the detergent-soluble fraction. Other proteins have also been shown to have different solubilities after heat shock. Michels et al. (1995) demonstrated that both cytoplasmic and nuclear localized luciferase expressed in mammalian cells were detergent (Triton X-100)-soluble in unstressed cells but were detergent-insoluble after heat shock.

Apparent discrepancies between biochemical fractionation and immunomicroscopy results have also been observed for other transcription factors. For example, it was believed for many years that unliganded (non-DNA binding) steroid receptors were cytoplasmic while the liganded DNA binding form of the receptor was nuclear. These conclusions were based primarily on the location of the receptor after standard biochemical fractionation of cells (Perrot-Applanat et al., 1992). The availability of specific monoclonal antibodies as well as different fractionation procedures has shown that the estrogen receptor, and in fact all steroid receptors except the glucocorticoid receptor, are predominantly nuclear proteins at all times (King and Greene, 1984; Welschons et al., 1984; Perrot-Applanat et al., 1992).

We have demonstrated using immunofluorescence microscopy that for several cell types, mammalian HSF1 is predominantly a nuclear protein in unshocked and heat-shocked cells. This observation differs from results obtained using biochemical fractionation procedures where HSF1 is found mostly in the cytoplasmic fraction of unshocked cells. However, because the amount of HSF1 fractionating to the ‘cytoplasm’ is dependent on the make-up of the buffer being used, we argue that most, if not all, of the HSF1 seen in the cytoplasmic fraction is probably an artifact of the fractionation procedure. Thus, monomeric HSF1 ‘leaches out’ or is extracted from the nucleus during fractionation, whereas trimeric HSF1 remains tightly associated with the nucleus (DNA) during extraction. We have previously demonstrated that Xenopus HSF1 is a nuclear protein in unshocked (and heat-shocked) mature oocytes (Mercier et al., 1997). In that study, nuclei were physically dissected from the oocyte under oil which prevented any possible ‘leaching’ of HSF1. The results of this and other studies (Westwood et al., 1991; Wu et al., 1994; Martinez-Balbas et al., 1995; Orosz et al., 1996; Mercier et al., 1997) demonstrate that Drosophila, Xenopus, and mammalian HSF1 appear to be predominantly nuclear proteins before and after heat shock. This observation implies that the evolutionarily conserved mechanism designed to detect heat and other environmental responses may be a nuclear response with HSF1 responding to stress-induced changes occurring within the nucleus.

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distinct inducible and constitutive DNA-binding ability. Cloning and characterization of two mouse heat shock factors with expression of a human heat shock factor, HSF1. Immunolocalization of steroid hormone receptors in normal and tumour tissues. Heat stressed neuronal and glial cell types in postnatal rat


Note added in proof

Brown and Rush (Brain Res. 821, 333-340 (1999)) have recently demonstrated using immunocytochemistry that HSF1 is predominantly localized in the nucleus of unstressed and heat stressed neuronal and glial cell types in postnatal rat tissues.