Cloning and characterization of two mouse heat shock factors with distinct inducible and constitutive DNA-binding ability

Kevin D. Sarge,[1] Vincenzo Zimarino,[2] Karin Holm,[2] Carl Wu,[3] and Richard I. Morimoto[1,4]

[1]Department of Biochemistry, Molecular Biology, and Cell Biology, Northwestern University, Evanston, Illinois 60208 USA; [2]Institute of Microbiology, University of Copenhagen, Copenhagen, Denmark; [3]Laboratory of Biochemistry, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892 USA

We have cloned two distinct mouse heat shock transcription factor genes, mHSF1 and mHSF2. The mHSF1 and mHSF2 open reading frames are similar in size, containing 503 and 517 amino acids, respectively. Although mHSFl and mHSF2 are quite divergent overall (only 38% identity), they display extensive homology in the DNA-binding and oligomerization domains that are conserved in the heat shock factors of *Saccharomyces cerevisiae*, *Kluyveromyces lactis*, *Drosophila*, tomato, and human. The ability of these two mouse heat shock factors to bind to the heat shock element (HSE) is regulated by heat. mHSFl is expressed in an in vitro translation system in an inactive form that is activated to DNA binding by incubation at temperatures >41°C, the same temperatures that activate heat shock factor DNA binding and the stress response in mouse cells in vivo. mHSF2, on the other hand, is expressed in a form that binds DNA constitutively but loses DNA binding by incubation at >41°C. Both mHSFl and mHSF2 are encoded by single-copy genes, and neither is transcriptionally regulated by heat shock. However, there is a striking difference in the levels of mHSFl mRNA in different tissues of the mouse.

[*Key Words*: Mouse; heat shock factor; inducible]

Received June 3, 1991; revised version accepted July 19, 1991.

Cells respond to a rise in temperature above physiological levels by rapidly inducing the expression of heat shock proteins that are thought to protect the cell from the harmful effects of prolonged exposure to elevated temperatures. This induction is mediated by heat shock transcription factor (HSF), which binds to heat shock elements (HSEs) in the promoters of heat shock genes (Amin et al. 1988; Xiao and Lis 1988; Abravaya et al. 1991a). While the sequence motif to which HSFs in different organisms bind—inverted repeats of the sequence NGAAN—is highly conserved, there are at least two different mechanisms by which organisms control the transcriptional activity of HSF. In the yeast *Saccharomyces cerevisiae*, for example, HSF exists in nonstressed cells in a form that is already bound to DNA and then undergoes heat shock-dependent phosphorylation concomitant with transcriptional competence (Jakobsen and Pelham 1988; Sorger and Pelham 1988; Sorger 1990). In *Drosophila* and vertebrate cells, however, HSF exists in nonstressed cells in a form that is unable to bind DNA and is converted to a DNA-binding form by heat shock (Kingston et al. 1987; Sorger et al. 1987; Zimarino and Wu 1987; Mosser et al. 1988).

Genes encoding HSF have been isolated from the yeasts *S. cerevisiae* and *Kluyveromyces lactis*, *Drosophila*, tomato, and human (Sorger and Pelham 1988; Wiederrecht et al. 1988; Clos et al. 1990; Scharf et al. 1990; Jakobsen and Pelham 1991; Rabindran et al. 1991; Schuetz et al. 1991). In *S. cerevisiae* and *K. lactis* HSF exists as unique single-copy genes, and in *Drosophila* only one HSF gene has been isolated. In the tomato, however, at least three different HSF genes are expressed, and in human there are at least two distinct HSF genes. We report the cloning of two distinct HSF genes in mouse, mHSF1 and mHSF2. One has the property of constitutive DNA binding similar to *S. cerevisiae* and *K. lactis*, whereas the other shows inducible DNA binding like that present in *Drosophila* and vertebrate cells. The presence of two different HSFs in one species, one with inducible and the other with constitutive DNA-binding ability, raises interesting questions about the regulation of heat shock gene expression in eukaryotes.

[4]Corresponding author.

Results

Mouse has two distinct HSFs with conserved DNA-binding and oligomerization domains

We have been studying the mammalian heat shock response and in particular have been interested in the biochemical and functional properties as well as the mechanism of activation of HSF. To facilitate our ability to study the relationship between the structure of HSF and its functional properties, we sought to clone mouse HSF. To this end, a probe containing a portion of the human HSF1 gene containing the DNA-binding and oligomerization domains was used to screen a mouse liver and WEHI-3 cell line cDNA library. Ten independent clones were isolated and subjected to partial sequence analysis. Of these 10 clones (C1–C9 and C12), the 2 longest (C12 and C9) were chosen for further characterization. Sequencing of substantial portions of the other eight clones indicates that each represents a partial cDNA contained in either the C12 or C9 cDNAs. Clones C12 and C9 were completely sequenced by using an overlapping series of exonuclease III deletion mutants by the Sanger chain-termination method. Hereafter we will refer to C12 as the mHSF1 cDNA and C9 as the mHSF2 cDNA.

The nucleotide and predicted amino acid sequences of mHSFl and mHSF2 are shown in Figures 1 and 2. The mHSF1 and mHSF2 cDNAs are 1947 and 1972 nucleotides, respectively. Each of the cDNAs contain only one long open reading frame. The open reading frames shown for mHSF1 and mHSF2 code for proteins of 503 and 517 amino acids, with estimated molecular masses of 54,800 and 58,160 daltons, respectively. A comparison of the amino acid homologies between mHSF1 and mHSF2 reveals that although there are regions of extensive homology, the sequences of these two factors are quite divergent (Fig. 3A). Overall, the identity is only 38% (by the Wisconsin GCG GAP program with default parameters). mHSF1 and mHSF2 have nearly as much identity with *Drosophila* HSF (36% and 32%, respectively) as they do with each other. Most of the homology is located in the DNA-binding and oligomerization domains in the amino-terminal half of the proteins. In these domains there is extensive homology with HSFs from *S. cerevisiae*, *K. lactis*, *Drosophila*, tomato, and human. A comparison of the DNA-binding domains of mHSF1 and mHSF2 with the other cloned HSFs is shown in Figure 3B. The homology between mHSF1 and mHSF2 in this region is 79% (83% if conservative substitution is allowed). Analysis of the consensus sequence for this domain shows that although there are well-conserved amino acid positions throughout this region there is a core of extreme homology at the center. This 26-amino-acid sequence (corresponding to *S. cerevisiae* HSF 213–238) is (V/L/I)LPKYFKH (N/S)N(M/F)ASFVRQLNMYG-(F/W)(R/H)K(V/I). This block of amino acids also contains the sequence similarity noted previously between *Drosophila* HSF, *S. cerevisiae* HSF, and the bacterial σ-32 and σ-70 factors (Clos et al. 1990).

The only other sequences in mHSF1 and mHSF2 that exhibit significant homology with each other and with HSFs from other species are contained in the leucine zipper structural motifs implicated in oligomerization of

```
   1 CTGCGGCCATGCGAGGCCTTGCTGTGTGTGCGCAGCGGGCGGCGGAGCGGCCCGGACGGCAGGGGCAGCGACGACACTAGCTCAGCCTTC  90

   1                                          M  D  L  A  V  G  P  G  A  A  G  P  S  N  14
  91 AGCCACTCTTCTAAAAGGCCACCCCAGCCTCTGCCTGCTTTCGTCCGAGATGGATCTGGCCGTGGGCCCCGGTGCAGCGGGGCCCAGCAA 180

  15  V  P  A  F  L  T  K  L  W  T  L  V  S  D  P  D  T  D  A  L  I  C  W  S  P  S  G  N  S  F  44
 181 CGTCCCGGCCTTCCTAACCAAGCTGTGGACCCTCGTGAGCGACCCGGACACAGACGCGCTCATCTGCTGGAGCCCGAGTGGGAACAGCTT 270

  45  H  V  F  D  Q  G  Q  F  A  K  E  V  L  P  K  Y  F  K  H  N  N  M  A  S  F  V  R  Q  L  N  74
 271 CCACGTGTTTGACCAGGGCCAGTTTGCCAAGGAGGTGCTGCCCAAGTACTTCAAGCACAACAACATGGCTAGCTTCGTGCGGCAGCTCAA 360

  75  M  Y  G  F  R  K  V  V  H  I  E  Q  G  G  L  V  K  P  E  R  D  D  T  E  F  Q  H  P  C  F  104
 361 CATGTATGGCTTCCGAAAAGTAGTCCACATTGAGCAGGGTGGCCTGGTCAAGCCTGAGAGAGATGACACCGAGTTCCAGCATCCTTGTTT 450

 105  L  R  G  Q  E  Q  L  L  E  N  I  K  R  K  V  T  S  V  S  T  L  K  S  E  D  I  K  I  R  Q  134
 451 CTTGCGTGGACAGGAACAGCTCCTTGAGAACATCAAGAGGAAAGTGACCAGCGTGTCCACCCTGAAGAGTGAGGACATAAAAATACGCCA 540

 135  D  S  V  T  R  L  L  T  D  V  Q  L  M  K  G  K  Q  E  C  M  D  S  K  L  L  A  M  K  H  E  164
 541 GGACAGTGTCACCCGGCTGTTGACAGATGTGCAGCTGATGAAGGGGAAACAGGAGTGTATGGACTCCAAGCTCCTGGCCATGAAGCACGA 630

 165  N  E  A  L  W  R  E  V  A  S  L  R  Q  K  H  A  Q  Q  Q  K  V  V  N  K  L  I  Q  F  L  I  194
 631 GAACGAGGCCCTGTGGCGGGAGGTGGCCAGCCTTCGGCAGAAGCATGCCCAGCAGCAAAAAGTTGTCAACAAGCTCATTCAGTTCCTGAT 720

 195  S  L  V  Q  S  N  R  I  L  G  V  K  R  K  I  P  L  M  L  S  D  S  N  S  A  H  S  V  P  K  224
 721 CTCACTGGTGCAGTCGAACCGGATCCTGGGGGTGAAGAGAAAGATCCCTCTGATGTTGAGTGACAGCAACTCAGCACACTCTGTGCCCAA 810

 225  Y  G  R  Q  Y  S  L  E  H  V  H  G  P  G  P  Y  S  A  P  S  P  A  Y  S  S  S  S  L  Y  S  254
 811 GTATGGTCGACAGTACTCCCTGGAGCATGTCCATGGTCCTGGCCCATACTCAGCTCCATCTCCAGCCTACAGCAGCTCTAGCCTTTACTC 900

 255  S  D  A  V  T  S  S  G  P  I  I  S  D  I  T  E  L  A  P  T  S  P  L  A  S  P  G  R  S  I  284
 901 CTCTGATGCTGTCACCAGCTCTGGACCCATAATCTCCGATATCACTGAGCTGGCTCCCACCAGCCCTTTGGCCTCCCCAGGCAGGAGCAT 990

 285  D  E  R  P  L  S  S  S  T  L  V  R  V  K  Q  E  P  P  S  P  P  H  S  P  R  V  L  E  A  S  314
 991 AGATGAGAGGCCTCTGTCCAGCAGCACTCTGGTCCGTGTCAAGCAAGAGCCCCCCAGCCCACCTCACAGCCCTCGGGTACTGGAGGCGAG 1080

 315  P  G  R  P  S  S  M  D  T  P  L  S  P  T  A  F  I  D  S  I  L  R  E  S  E  P  T  P  A  A  344
1081 CCCTGGGCGCCCATCCTCCATGGATACCCCTTTGTCCCCAACTGCCTTCATTGACTCCATCCTTCGAGAGAGCGAGCCTACCCCTGCTGC 1170

 345  S  N  T  A  P  M  D  T  T  G  A  Q  A  P  A  L  P  T  P  S  T  P  E  K  C  L  S  V  A  C  374
1171 CTCAAACACAGCCCCTATGGACACAACCGGAGCCCAAGCCCCCGCACTCCCGACCCCCTCCACCCCTGAGAAGTGCCTCAGCGTAGCCTG 1260

 375  L  D  K  N  E  L  S  D  H  L  D  A  M  D  S  N  L  D  N  L  Q  T  M  L  T  S  H  G  F  S  404
1261 CCTAGACAAGAACGAGCTAAGTGATCACCTGGATGCCATGGACTCCAACCTGGACAACCTGCAGACCATGCTGACAAGCCACGGCTTCAG 1350

 405  V  D  T  S  A  L  L  D  I  Q  E  L  L  S  P  Q  E  P  P  R  P  I  E  A  E  N  S  N  P  D  434
1351 TGTGGACACCAGTGCCCTGCTGGACATTCAGGAGCTTCTGTCTCCACAAGAGCCTCCCAGGCCTATTGAGGCAGAGAACAGTAACCCCGA 1440

 435  S  G  K  Q  L  V  H  Y  T  A  Q  P  L  F  L  L  D  P  D  A  V  D  T  G  S  S  E  L  P  V  464
1441 CTCAGGAAAGCAGCTGGTGCACTACACGGCTCAGCCTCTGTTCCTGCTGGATCCTGATGCTGTGGACACAGGGAGCAGTGAGCTGCCTGT 1530

 465  L  F  E  L  G  E  S  S  Y  F  S  E  G  D  D  Y  T  D  D  P  T  I  S  L  L  T  G  T  E  P  494
1531 GCTCTTTGAGCTGGGGGAGAGCTCCTACTTCTCTGAGGGGGATGACTACACGGATGATCCCACCATCTCTCTTCTGACAGGCACTGAACC 1620

 495  H  K  A  K  D  P  T  V  S  *                                                          503
1621 CCATAAAGCCAAGGACCCCACTGTCTCCTAGAGCTCTCAGGAGTTGTCAGGCTGGCTTGTGCCTGGCCCCCAACCTATCCCTAGGACATG 1710

1711 GCTGGTCCTAGGGAGACAAAACAGTTGGGTAGTCCAGGGGACCCTAGGTCAAGCCACCACAACCCCAGTGGAGCACAGATGGAACTTGGT 1800

1801 CCTGGGCAGTACCTTGGATCAGGAGGAAGATCCTGAGGGCTGCATACCTGCTGCCTTTACCCCAGCCCCAGGTCTACTCTCTGGTCACAG 1890

1891 CTTCACAGCCACACTTGGACTGACCCTGCAGGTTGTTCATAAAATTGTATTTTGGCC                                  1947
```

Figure 1. Nucleotide and predicted amino acid sequence of mHSF1. The DNA sequence presented was obtained by sequencing an overlapping set of exonuclease III deletion mutants of cDNA clone C12 (mHSF1). Start and stop codons are indicated by underlining. Sequence data for mHSF1 have been submitted to EMBL/GenBank data libraries under accession number X61753.

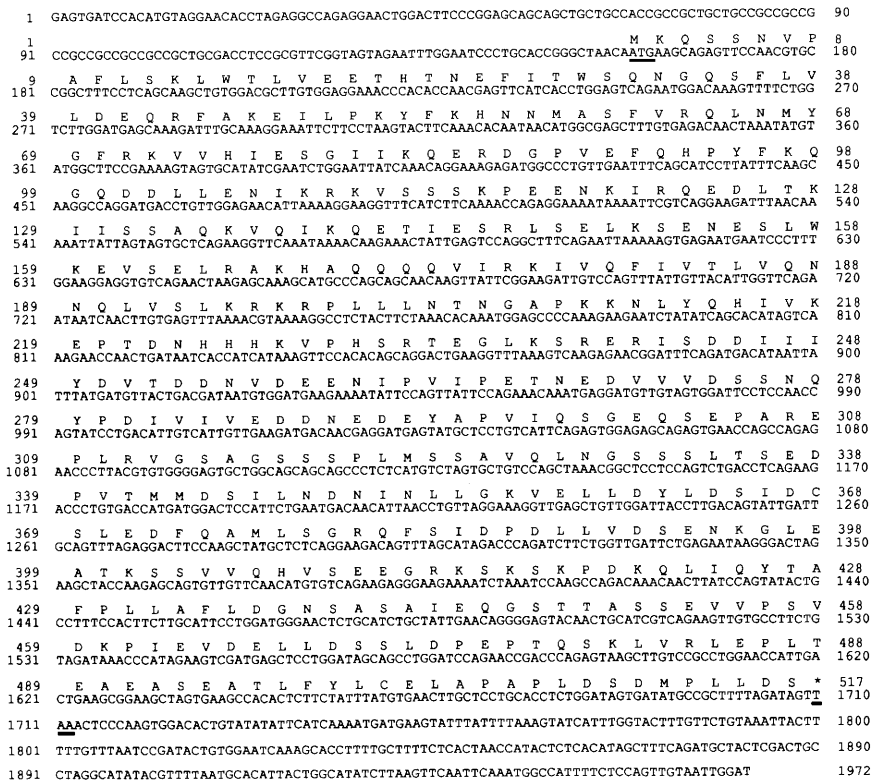

Figure 2. Nucleotide and predicted amino acid sequence of mHSF2 (for details, see legend to Fig. 1). Sequence data for mHSF2 have been submitted to EMBL/GenBank data libraries under accession number X61754.

Drosophila and *S. cerevisiae* HSF (Sorger and Nelson 1989; Clos et al. 1990). Figure 3C shows that the oligomerization domains of mHSFl and mHSF2 exhibit a nearly identical pattern of the three leucine zipper heptad repeats of hydrophobic amino acids with *Drosophila* HSF (Clos et al. 1990), as well as a fourth newly identified carboxy-terminal leucine zipper motif conserved in *Drosophila* HSF, human HSF1, and human HSF2 (Clos et al. 1990; Rabindran et al. 1991; Schuetz et al. 1991). In support of the functional importance of each of these leucine zipper motifs in *Drosophila* HSF, mHSFl, and mHSF2 are the numerous conservative mutations maintaining hydrophobic amino acid residues at required positions in each heptad repeat. The function of the fourth carboxy-terminal leucine zipper is unknown but has been speculated to be involved in formation of the inactive state of HSF under nonshock conditions or as part of a transcriptional activation motif (Rabindran et al. 1991; Schuetz et al. 1991).

Examination of the DNA-binding domains and leucine zipper motifs of mHSF1 and mHSF2, with respect to position in the proteins, shows that their organization is very similar (Fig. 3D). One interesting aspect of this organization relative to other HSFs is the localization of these two functional domains at the extreme amino terminus of each protein. This appears to be a trend in the evolution of HSF structure. In the HSFs of the yeasts *K. lactis* and *S. cerevisiae* there are 195 and 173 amino acids, respectively, from the amino terminus to the beginning of the DNA-binding domain. In *Drosophila* HSF, this distance has been reduced to 48 amino acids; and in mHSF1 and mHSF2, only 16 and 8 amino acids, respectively, separate the amino-terminal methionine from the DNA-binding domain. In the three tomato HSFs this distance is also quite short, with 30, 22, and 7 amino acids (Scharf et al. 1990).

Southern and Northern blot analyses indicate that mHSF1 and mHSF2 are encoded by single-copy genes whose expression is not induced by heat shock

Southern blot analysis of mouse liver genomic DNA cut with *Eco*RI, *Pst*I, or *Eco*RI and *Pst*I and probed with oligonucleotides complementary to 5′-untranslated sequences of the mHSF1 and mHSF2 cDNAs (nucleotides 58–97 of mHSF1 cDNA; 105–146 of mHSF2 cDNA) indicates that the genes encoding mHSF1 and mHSF2 are both present as single copies in the mouse genome (Fig. 4). However, this experiment does not rule out the possibility that the mouse genome encodes other members of an HSF gene family that contain coding sequence homologies to mHSFl or mHSF2.

Northern blot analysis of poly(A)$^+$ RNA from control and heat-shocked mouse NIH–3T3 cells indicates that the sizes of the mature mRNAs encoding mHSF1 and mHSF2 are ~2000 and 2100 nucleotides, respectively, which is in good agreement with cDNA sizes for mHSF1 and mHSF2 of 1947 and 1972 bp, respectively (Fig. 5A). Additionally, at least two minor smaller mRNA species appear to be in RNA lanes probed with mHSF2 cDNA, but it is not known whether these species correspond to other members of a related gene family or whether they

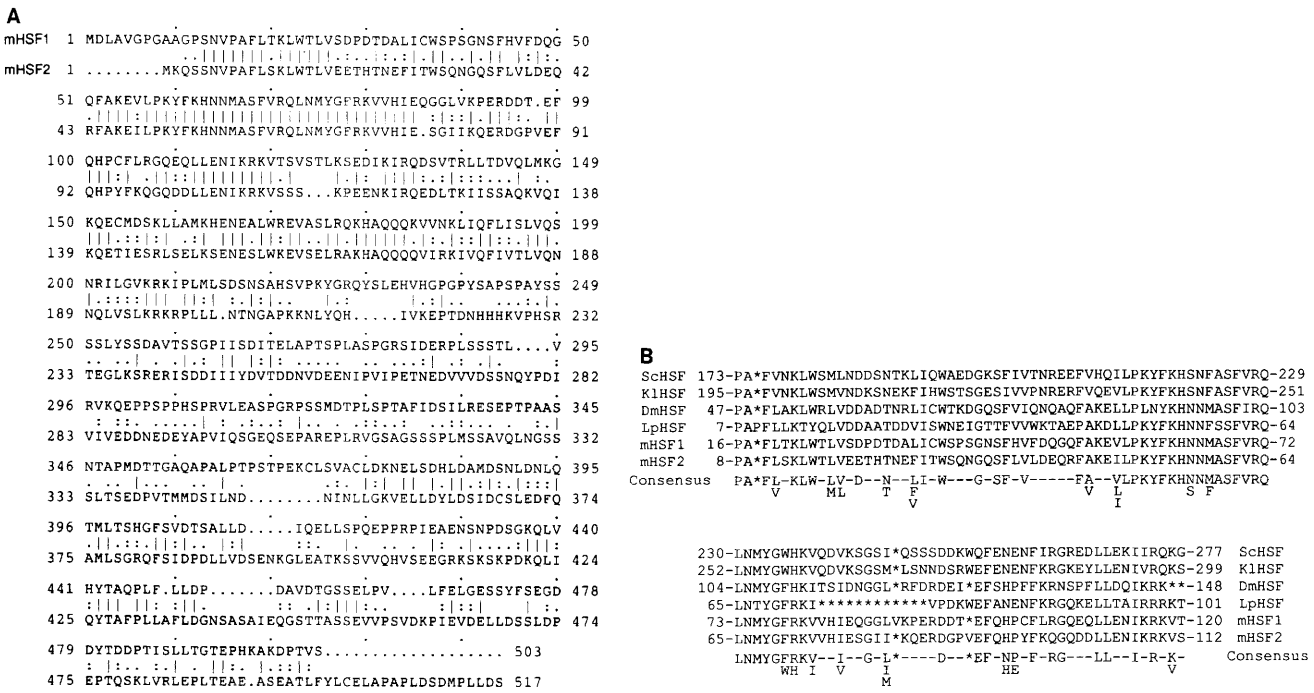

Figure 3. (*A*) Amino acid homologies between mHSF1 and mHSF2. Comparison was generated by the Wisconsin GCG GAP program by using default parameters. (*B*) Amino acid homologies between DNA-binding domains of *S. cerevisiae* HSF, *K. lactis* HSF, *Drosophila* HSF, tomato HSF24, mHSF1, and mHSF2. Gaps placed in individual sequences to allow lineup of highest homology are indicated by asterisks (*). A consensus amino acid sequence considering conservation of three or fewer amino acids at each position is indicated below the individual sequences. Matches of five of six at a position are listed only as the predominant amino acid. (*C*) Conserved heptad repeats in leucine zipper motifs of mHSF1, mHSF2, and *Drosophila* HSF. Heptad repeats of hydrophobic amino acids of leucine zippers 1, 2, and 3 of conserved oligomerization domain (*top*) and carboxy-terminal leucine zipper 4 (*bottom*) are indicated by ◊ and ◆. Heptad repeats of *Drosophila* HSF oligomerization domain (*top*) are taken from Clos et al. (1990). (*D*) Organization of DNA-binding domain and leucine zipper motifs of mHSF1 and mHSF2.

represent alternative splicing products of a single precursor mHSF2 mRNA, utilization of different start sites or 3′-processing sites, or possibly intermediates in the degradation pathway. The levels of mHSF1 and mHSF2 mRNA are not altered by heat shock, and run-on transcription analysis of nuclei isolated from control and heat-shocked 3T3 cells shows no change in expression of mHSF1 or mHSF2 mRNA (B. Phillips, unpubl.). Reprobing the blots for actin mRNA and *hsp70* mRNA confirmed that equal amounts of mRNA were present in each lane and that heat shock induction had occurred (Fig. 5B).

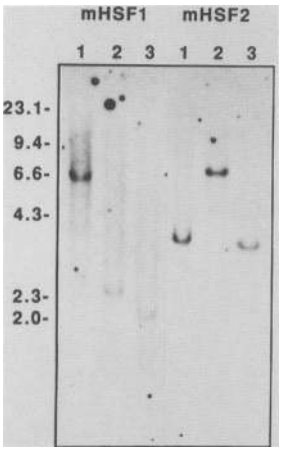

Figure 4. Southern blot analysis of mHSF1 and mHSF2 genes in mouse. Mouse liver genomic DNA digested with *Eco*RI, *Pst*I, or both *Eco*RI and *Pst*I (lanes *1*, *2*, and *3*, respectively) was separated by agarose gel electrophoresis, blotted onto nitrocellulose, and probed with oligonucleotides that are complementary to sequences in the 5′-untranslated region of mHSF1 or mHSF2 (for location of oligonucleotide binding sites, see Materials and methods). Migration of DNA size markers is indicated at *left*.

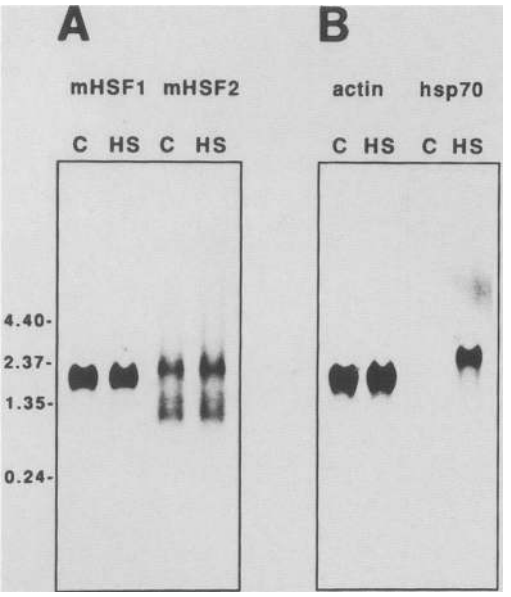

Figure 5. Northern blot analysis of mHSF1 and mHSF2 mRNA in control and heat-shocked cells. (*A*) Poly(A)$^+$ (3 μg) RNA from NIH–3T3 cells grown at 37°C (control) or heat-shocked at 43°C for 1 hr was separated on a 1% agarose gel containing formaldehyde, blotted onto nitrocellulose, and probed with primer-labeled cDNAs for mHSF1 (C12) or mHSF2 (C9). Migration of RNA size markers is indicated at *left*. (*B*) Northern blots reprobed with human β-actin cDNA and human *hsp70* cDNA.

mHSFl is expressed in an inactive form induced to DNA binding by heat treatment while mHSF2 binds DNA constitutively

To characterize the biochemical and functional properties of these two mHSFs, both mHSF genes were expressed in vitro by using T7 polymerase to make synthetic capped mRNAs, which were translated in a rabbit reticulocyte lysate in vitro translation system. The proteins expressed by in vitro translation of mHSF1 and mHSF2 in the presence of [^{35}S]methionine are 75 and 72 kD, respectively, on SDS–polyacrylamide gels (Fig. 6). These sizes are substantially larger than the derived molecular masses for mHSF1 and mHSF2 of 54,800 and 58,160 daltons, respectively. Such discrepancies between measured and estimated molecular masses appear to be characteristic of the several HSFs that have been cloned to date and, at least in our system, may result from either post-translational modification, which may occur in our in vitro translation system, or some not-yet-understood anomalous behavior of these proteins in SDS-PAGE gels.

The ability of mHSF1 and mHSF2 to bind the HSE was then examined by gel-shift assay of the in vitro-translated proteins. Figure 7A shows that the DNA-binding ability of these two factors is altered in very different ways by heat treatment. In the case of mHSF1, the protein produced by in vitro translation is unable to bind DNA when it is left on ice or incubated at 37°C for 1 hr but acquires DNA-binding ability when it is incubated at 43°C. In the case of mHSF2, however, the in vitro-translated protein binds DNA constitutively at 0°C or 37°C for 1 hr but completely loses DNA binding at 43°C. These changes in DNA-binding ability, which have been consistently reproducible for both mHSF1 and mHSF2, are not caused by any obvious changes in protein amounts or degradation of the mHSF1 and mHSF2 polypeptides induced by post-translation heat treatments as analyzed by SDS–polyacrylamide gels of the [^{35}S]methionine-incorporated proteins (Fig. 7B). Although we have not yet been able to measure the native size of the DNA-binding forms of mHSF1 and mHSF2, the nearly equal gel-shift mobility of these factors with HSF extracted from heat-shocked mouse cells suggests that they are also forming large multimers.

Both the activation of DNA binding of mHSF1 and the

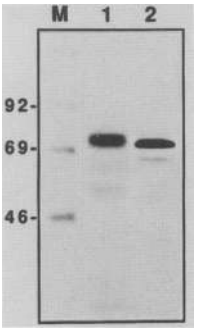

Figure 6. Denatured size of mHSF1 and mHSF2 protein expressed in vitro. Synthetic RNAs transcribed in vitro from the mHSF1 and mHSF2 cDNAs were translated in a rabbit reticulocyte lysate in the presence of [^{35}S]methionine and analyzed by SDS-PAGE. (Lane *M*) ^{14}C-Labeled protein size markers.

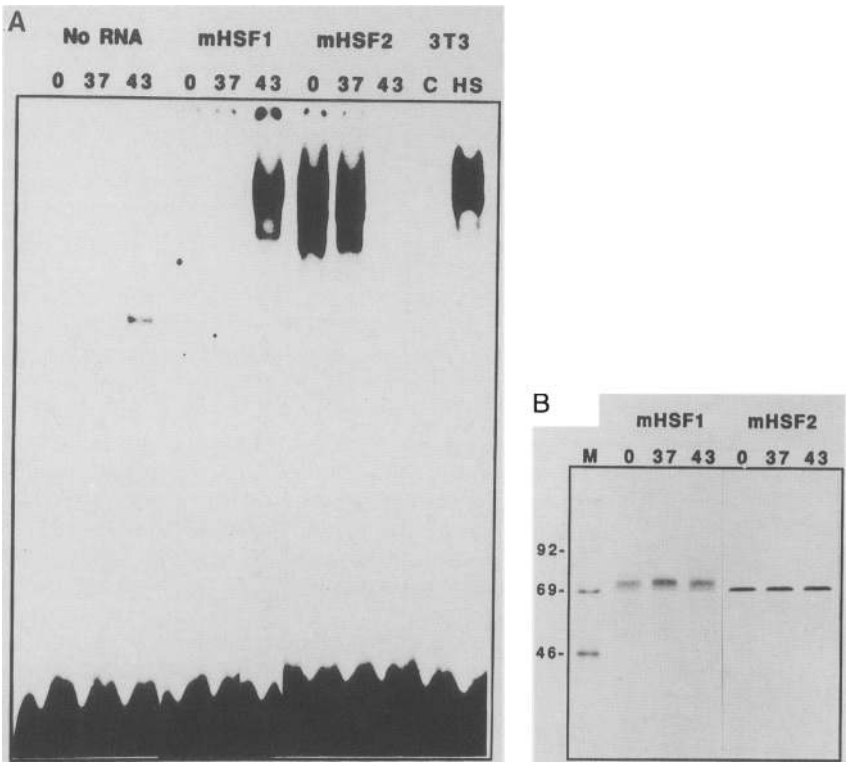

Figure 7. Regulation of DNA binding of mHSF1 and mHSF2 by heat. (*A*) Aliquots of mHSF1 and mHSF2 in vitro translation reactions were incubated either on ice (0°C), at 37°C, or at 43°C for 1 hr and analyzed by gel-shift assay using an oligonucleotide containing four inverted NGAAN repeats. In vitro translation reactions containing no added exogenous RNA are indicated, as are lanes containing shifts of whole-cell extracts of control (37°C) and heat-shocked (43°C, 1 hr) NIH–3T3 cells. (*B*) In vitro translation reactions and post-translation incubations were performed exactly as in *A* except that [^{35}S]methionine was substituted for cold methionine. Samples were analyzed by SDS-PAGE and fluorography. (Lane *M*) ^{14}C-Labeled protein size markers.

loss of DNA binding of mHSF2 by treatment at 43°C are very rapid and nearly complete after only 20 min of incubation (Fig. 8A). An examination of the temperature profile for activation of mHSF1 and loss of mHSF2 DNA binding shows an increase in mHSF1 binding with treatment at 39°C, an apparent peak at 41°C, and sustained binding activity up to 45°C, whereas the mHSF2 profile shows substantial loss of constitutive binding at 39°C, increasing to near total loss at 41°C, and no detectable binding at >43°C (Fig. 8B). Attempts to recover DNA-binding activity from heat-treated mHSF2 have been unsuccessful so far.

It is important to note that the constitutive DNA-binding form of mHSF2 has a very distinct mobility on native gels from that of a constitutive HSE-binding activity (CHBA), which we have observed in extracts of non-heat-shocked HeLa cells and mouse 3T3 cells (Mosser et al. 1988; K.D. Sarge, unpubl.). In addition, the mHSF2 constitutive activity binds equally well to all HSE oligonucleotides that we have tested, including a human *hsp70* HSE, mouse *hsp70* HSE, and the ideal HSE shown in Figure 7A; whereas the CHBA factor binds to the human and mouse *hsp70* HSEs but does not interact with the idealized HSE (K.D. Sarge, unpubl.). Therefore, at least in this system and under the conditions that we are using, neither mHSF1 nor mHSF2 gives rise to CHBA activity.

mHSF1 and mHSF2 bind the HSE specifically and contact the same G residues as HSF extracted from heat-shocked mouse cells

Specificity of mHSF1 and mHSF2 DNA binding for the HSE was demonstrated both by competition gel-shift as-

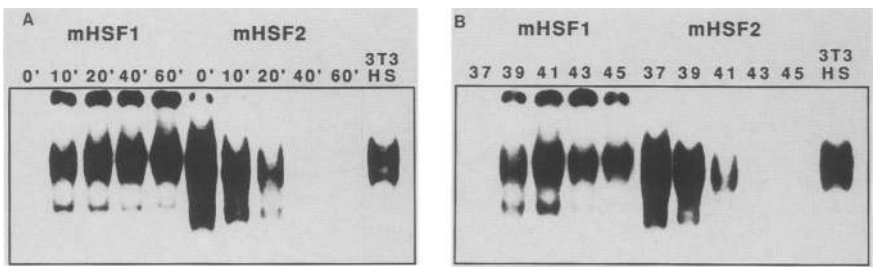

Figure 8. Temperature and time dependence of heat-regulated DNA binding of mHSF1 and mHSF2. Aliquots of in vitro translation reactions were incubated at 43°C for different times (*A*) or different temperatures (*B*) for 60 min. The last lane in each panel contains extract from heat-shocked mouse 3T3 cells (3T3 HS).

says and methylation interference analysis. Figure 9A shows that both the in vitro-activated DNA-binding form of mHSFl (43°C, 60 min) and the constitutively binding mHSF2 are specifically competed from binding to the labeled HSE by a 50-fold molar excess of cold HSE oligonucleotide but not by a 50-fold molar excess of an oligonucleotide containing a CCAAT-binding site. Methylation interference analysis shows that mHSF1, mHSF2, and HSF extracted from heat-shocked NIH–3T3 cells all contact the same G residues in the ideal HSE-binding site (Fig. 9B), which is depicted in Figure 9C. Identical results have been obtained from several different methylation interference experiments. We have also performed methylation interference analysis with an oligonucleotide containing an HSE from the mouse *hsp70* gene promoter (Hunt and Calderwood 1990); a summary of these results also shows indistinguishable patterns of contact G residues for binding of mHSF1, mHSF2, and HSF from heat-shocked mouse 3T3 cells (Fig. 9C). This is not surprising in view of the high degree of homology between mHSF1 and mHSF2 in the DNA-binding domain.

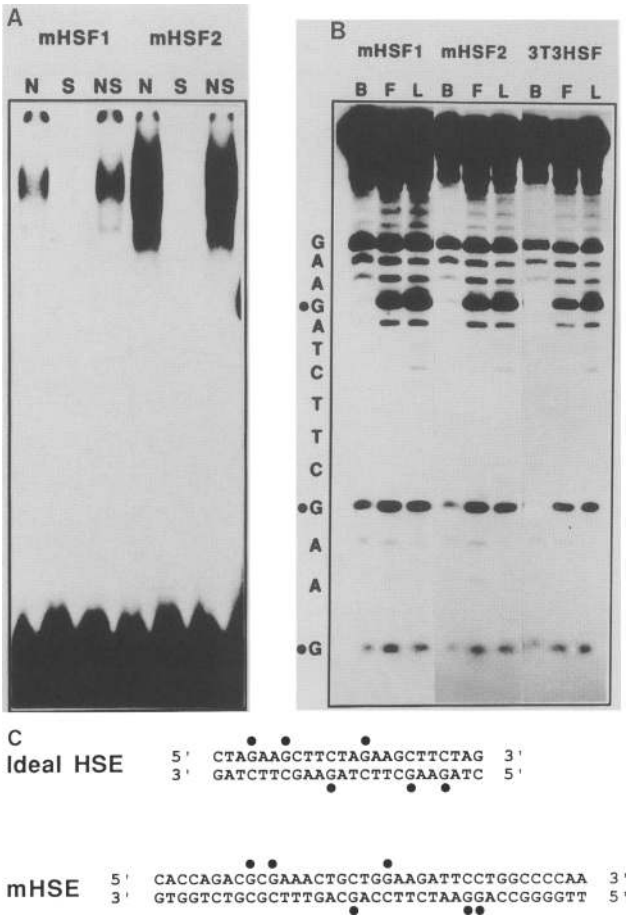

Figure 9. Specificity of DNA binding by competition and methylation interference. (*A*) Aliquots of in vitro-translated mHSF1 (activated at 43°C for 60 min) and mHSF2 were analyzed by gel-shift assay without added competitor (N), with a 50-fold molar excess of unlabeled HSE (S), or with a 50-fold molar excess of a CCAAT-binding site oligonucleotide (NS). (*B*) In vitro-translated mHSF1 (activated at 43°C, for 60 min) and mHSF2 and HSF extracted from heat-shocked mouse 3T3 cells were subjected to methylation interference analysis. The methylation patterns of the fraction of HSE DNA bound to protein (B) or free of protein (F) is shown next to the G residue ladder (L). (*C*) A summary of the methylation interference results for the ideal HSE and the mouse *hsp70* HSE is shown. (●) Guanine residues that interfered with factor binding.

Variation in amounts of mHSF1 mRNA in different mouse tissues

As a precursor for future studies on the role of mHSF1 and mHSF2 in mouse cells, we were interested to know whether there was any regulation of mHSFl mRNA levels in different mouse tissues. Northern blot analysis of total RNA extracted from mouse tissues probed with mHSF1 cDNA shows that there is a surprising variation in the levels of mHSF1 mRNA in various tissues (Fig. 10). Of the selected tissues shown, ovary has the highest amount of mHSF1 RNA, with slightly less in placenta and heart. Lower levels are observed in testis and fetal brain, and it is undetectable in the uterus and maternal brain. Probing of this blot with a β-actin cDNA verified that there were equal amounts of RNA in each lane (data not shown). These large variations are surprising in view of the supposed universal nature of the heat shock response but may reflect different requirements in different tissues for more or less heat shock response capability. We also attempted to examine mHSF2 mRNA levels in these same tissues, but the amounts were below detectable levels in total RNA samples.

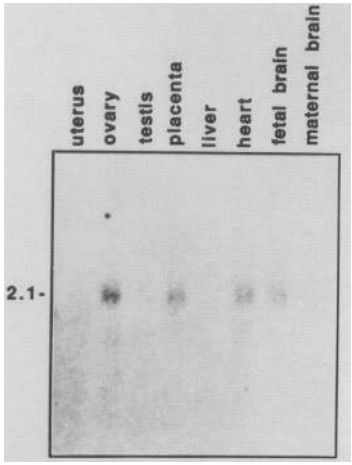

Figure 10. Variation of mHSF1 mRNA levels in different tissues. Total RNA from tissues of normal adult mice and fetal and maternal brain were subjected to Northern blot analysis by using the full-length mHSF1 cDNA as probe. The size of the mRNA (in kilonucleotides), as determined by comparison with RNA size markers, is indicated at *left*.

Discussion

Regulation of DNA-binding ability of mHSF1 and mHSF2

In the budding yeasts *S. cerevisiae* and *K. lactis*, HSF exists in a form that constitutively binds the heat shock element (Jakobsen and Pelham 1988, 1991). In most other species studied, including *Drosophila*, tomato, and vertebrates, HSF only binds to DNA after exposure to heat or other stresses (Kingston et al. 1987; Zimarino and Wu 1987; Scharf et al. 1990). This inducible DNA binding is accompanied by oligomerization and may be triggered by protein conformational changes caused by heat or other stress signals (Mosser et al. 1990; Abravaya et al. 1991b). Of the two mouse HSFs that we have cloned, one has the property of inducible DNA binding while the other binds DNA constitutively. Given the high degree of homology and similar organization of the DNA-binding and oligomerization domains of mHSF1 and mHSF2, we can postulate two possibilities for the different regulation of DNA-binding ability of these two factors. In the first scenario, the DNA-binding ability of mHSF1, presumably mediated by the ability to oligomerize, is somehow masked and requires some protein conformational changes in one or more domains to be unmasked. The impetus for this conformational change would be provided by heat or other stress signals. mHSF2, on the other hand, could be expressed in a form that is not masked and may immediately oligomerize and bind DNA.

In the second scenario, the DNA-binding ability of mHSF1 may be controlled by the binding of a repressor protein, and the stress signals would activate mHSF1 by disrupting this interaction. This repressor protein may not be able to bind to mHSF2; hence, it would be free to oligomerize and bind DNA. This model has already been proposed for cloned *Drosophila* HSF which, like mHSF2, also binds DNA constitutively (Clos et al. 1990). For this second model to be true there would have to be a protein or other molecule in a rabbit reticulocyte lysate that could bind to and repress mouse HSF. Others have already postulated that heat shock proteins, which are well-conserved and might be capable of this kind of cross-species functional interaction, would be logical candidates for HSF repressors; however, as yet, no direct evidence exists to support this hypothesis. Experiments are in progress to determine whether the regulation that we have observed for mHSF1 and mHSF2 DNA binding is specific to expression in reticulocyte lysates or whether it is also observed in other expression systems such as *Escherichia coli* or mouse cell lines.

The need for multiple HSFs in a single species

Despite the existence of multiple HSF proteins in organisms as widely separated evolutionarily as mammals and plants, it is not known how general this phenomenon may be. The reason for multiple HSFs in these species is unclear, but it raises the possibility that each factor has a different role or function in regulating transcription of heat shock genes. For example, each factor could respond to a different stress signal or set of signals. This could explain the seeming unrelatedness of many of the myriad known inducers of the stress response (Morimoto et al. 1990).

Another possibility is that one factor could be acting positively and the other acting negatively on heat shock protein expression. However, at least in HeLa cells no repressor-like HSE-binding activity has been observed on the *hsp70* promoter in vivo prior to or during attenuation of a heat shock response (Abravaya et al. 1991a).

Alternatively, it is possible that some species have divided HSF function into separate inducible and constitutively active factors. The inducible activity could respond to stress signals and activate the classical heat shock response, whereas the constitutive activity could be used to turn on heat shock genes in the absence of stress, for example, during specific developmental stages or perhaps in cells that require higher basal levels of heat shock proteins. This possibility is consistent with the observations of developmentally regulated expression of heat shock proteins during mouse embryogenesis and differentiation of the mouse male germ line and the high levels of constitutive HSE-binding activity in unstressed embryonal carcinoma cells (Barnier et al. 1987; Zakeri and Wolgemuth 1987; Mezger et al. 1989). Thus, the need for both an inducible and constitutive HSF may simply be a requirement of the need for heat shock proteins not only in the stress response but also in many aspects of normal cell development and function.

The presence of multiple HSFs in a single species raises one more very interesting question. Are HSF multimers composed only of identical HSF subunits (homomultimers) or can they contain a mixture of different HSF subunits (heteromultimers)? Given the high degree of homology between the oligomerization domains of mHSF1 and mHSF2 it seems likely that they could potentially co-oligomerize. However, although our Northern analysis shows that both mHSF1 and mHSF2 mRNAs are expressed (at least in mouse 3T3 cells), we do not know whether both proteins exist in the same cell. If heteromultimers do form, regulation of the relative stoichiometry of HSF subunits could modulate the function of the HSF complex. This might provide different cells with the ability to fine-tune the function of HSF to suit their individual needs.

Materials and methods

Library screening and sequencing

A mouse liver cDNA library purchased from Clontech [oligo(dT)- and random-primed] and a mouse WEHI-3 cell line cDNA library [oligo(dT)-primed] (a generous gift of Silvana Obici) were screened by hybridization with a 468-nucleotide sequence corresponding to a portion of hHSF1 sequence generated by PCR with degenerate oligonucleotide primers based on conserved *S. cerevisiae* and *Drosophila* HSF sequences and subcloned into pGEM1. Greater than 2×10^6 PFU was screened from the Clontech mouse liver cDNA library and 1×10^6 PFU from the WEHI-3 cDNA library. Nine independent cDNA clones (C1–C9) were obtained from the Clontech library, and

one was obtained from the WEHI-3 library (C12). Two of these, C12 and C9, appeared to be full-length and were chosen for further analysis. The cDNA inserts were subcloned into the *Eco*RI site of pGEM1.

A series of deletion mutants of Cl2 and C9 were prepared by exonuclease III digestion by standard protocols (Sambrook et al. 1989), and overlapping sets of mutants were sequenced by the Sanger chain-termination method.

Southern and Northern hybridization analysis

For Southern blot analysis, 40 μg of mouse liver genomic DNA was digested with *Eco*RI, *Pst*I, or both *Eco*RI and *Pst*I and electrophoresed on a 0.7% agarose gel in 0.5× TBE (45 mM Tris-borate, 1 mM EDTA) and blotted onto nitrocellulose by using standard protocols (Sambrook et al. 1989). The blots were hybridized with either a 40- or 42-mer oligonucleotide complementary to nucleotides 58–97 or 105–146 in the 5′-untranslated regions of the mHSF1 or mHSF2 cDNAs, respectively. The blots were washed to final conditions of 57°C, 1× SSC, 0.1% SDS.

For Northern blot analysis, 3 μg of poly(A)$^+$ RNA isolated from control (37°C) and heat-shocked (43°C, 1 hr) NIH–3T3 cells was separated on a 1% agarose gel containing formaldehyde and blotted onto nitrocellulose by using standard protocols (Sambrook et al. 1989). The blots were hybridized with primer-labeled C12 (mHSF1) or C9 (mHSF2) cDNAs and washed to final conditions of 68°C, 0.2× SSC. The blots were subsequently stripped and reprobed with a human β-actin probe to verify equal amounts of mRNA in each lane and also reprobed with a human *hsp70* probe to confirm heat shock induction. For Northern analysis of RNAs from tissues, 30 μg of total RNA was loaded in each lane and probed with mHSF1 cDNA as described above. The blot was probed for actin mRNA to verify equal loading of mRNA in each lane.

In vitro translation

Full-length capped synthetic RNA was made from the cDNAs for mHSF1 (C12) and mHSF2 (C9) by T7 RNA polymerase transcription of template linearized by *Sma*I digestion. Reactions contained 40 mM Tris-HCl (pH 7.5); 6 mM $MgCl_2$, 2 mM spermidine; 10 mM NaCl; 10 mM dithiothreitol; 0.5 mM nucleotides A, C, and U, and 0.05 mM nucleotide G; 0.5 mM ^{7}mGpppG cap analog; 40 units of RNasin; 2 μg of linearized template; and 50 units of T7 RNA polymerase. Reactions were incubated at 37°C for 2 hr and, after digestion, by 10 units of RNase-free DNase at 37°C for 20 min; the transcribed RNA was extracted with phenol–chloroform and precipitated with ethanol. The RNA was collected by centrifugation, washed with 70% ethanol, air-dried, and resuspended in water at a concentration of 1 μg/μl.

Synthetic capped mHSF1 and mHSF2 RNAs were translated in a rabbit reticulocyte lysate in vitro translation system (Promega). Each 20-μl reaction contained 15 μl of lysate, 25 μM amino acids, 10 units of RNasin, and 2 μg of RNA, at 30°C for 2 hr. Translations incorporating labeled methionine contained 20 μCi of [^{35}S]methionine (800 Ci/mmole) in place of the unlabeled amino acid. [^{35}S]Methionine-incorporated proteins were analyzed on SDS–polyacrylamide gels followed by fluorography.

Gel-shift assay and methylation interference analysis

In vitro-translated mHSF1 and mHSF2 and extracts from control and heat-shocked NIH–3T3 cells were assayed by gel shift as described previously (Mosser et al. 1990) with a self-complementary ideal HSE oligonucleotide (5′-CTAGAAGCTTCTAGAAGCTTCTAG-3′), which contains four perfect inverted NGAAN repeats when annealed. Competition gel-shift analysis was performed as described previously (Mosser et al. 1990).

Binding of in vitro-translated mHSF1 and mHSF2 to both the ideal HSE oligonucleotide described above and a mouse HSE oligonucleotide (top strand; 5′-CACCAGACGCGAAACTGCTGGAAGATTCCTGGCCCCAA-3′), based on the mouse *hsp70* gene promoter (Hunt and Calderwood 1990), was examined by methylation interference analysis as described previously (Mosser et al. 1988) either as translated (mHSF2) or after in vitro activation at 43°C for 1 hr (mHSF1).

Acknowledgments

We thank Professor Francesco Blasi for his support of this project at the Institute of Microbiology, University of Copenhagen. We also thank Silvana Obici for the kind gift of the mouse WEHI-3 cDNA library, Sridhar Rabindran for hHSF1 sequence information prior to publication, Robert Kingston for hHSF2 sequence information prior to publication, Doug Engel for the mouse tissue RNA Northern blot, and Ok-Kyong Park for helpful discussions during the library screening. These studies were supported by grants from the National Institutes of Health, March of Dimes, and an American Cancer Society Faculty Research Award to R.I.M., a grant from Kraeftens Bekaempelse (Danish Cancer Society) (grant 90–085) supporting V.Z. and K.H., and an Individual National Research Service Award to K.D.S.

The publication costs of this article were defrayed in part by payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 USC section 1734 solely to indicate this fact.

References

Abravaya, K., B. Phillips, and R.I. Morimoto. 1991a. Heat shock induced interactions of heat shock transcription factor and the human hsp70 promoter examined by in vivo footprinting. *Mol. Cell. Biol.* **11:** 586–592.

Abravaya, K., K.D. Sarge, B. Phillips, V. Zimarino, and R.I. Morimoto. 1991b. In vivo and in vitro studies on the activation and binding of human heat shock transcription factor. In *Heat shock* (ed. S. Lindquist and B. Maresca). Springer Verlag, New York (In press.).

Amin, J., J. Ananthan, and R. Voellmy. 1988. Key features of heat shock regulatory elements. *Mol. Cell. Biol.* **8:** 3761–3769.

Barnier, J.V., O. Bensaude, M. Morange, and C. Babinet. 1987. Mouse 89 kD heat shock protein—Two polypeptides with distinct developmental regulation. *Exp. Cell Res.* **170:** 186–194.

Clos, J., J.T. Westwood, P.B. Becker, S. Wilson, K. Lambert, and C. Wu. 1990. Molecular cloning and expression of a hexameric Drosophila heat shock factor subject to negative regulation. *Cell* **63:** 1085–1097.

Hunt, C. and S. Calderwood. 1990. Characterization and sequence of a mouse hsp70 gene and its expression in mouse cell lines. *Gene* **87:** 199–204.

Jakobsen, B.K. and H.R.B. Pelham. 1988. Constitutive binding of yeast heat shock factor to DNA in vivo. *Mol. Cell. Biol.* **8:** 5040–5042.

———. 1991. A conserved heptapeptide restrains the activity of the yeast heat shock transcription factor. *EMBO J.* **10:** 369–375.

Kingston, R.E., T.J. Schuetz, and Z. Larin. 1987. Heat-inducible human factor that binds to a human hsp70 promoter. *Mol. Cell. Biol.* **7:** 1530–1534.

Mezger, V., O. Bensaude, and M. Morange. 1989. Unusual levels of heat shock element-binding activity in embryonal carcinoma cells. *Mol. Cell. Biol.* **9:** 3888–3896.

Morimoto, R.I., A. Tissieres, and C. Georgopoulos. 1990. The stress response, functions of the proteins, and perspectives. In *Stress proteins in biology and medicine* (ed. R.I. Morimoto, A. Tissieres, and C. Georgopoulos), pp. 1–36. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Mosser, D.D., N.G. Theodorakis, and R.I. Morimoto. 1988. Coordinate changes in heat shock element-binding activity and hsp70 gene transcription rates in human cells. *Mol. Cell. Biol.* **8:** 4736–4744.

Mosser, D.D., P.T. Kotzbauer, K.D. Sarge, and R.I. Morimoto. 1990. In vitro activation of heat shock transcription factor DNA-binding by calcium and biochemical conditions that affect protein conformation. *Proc. Natl. Acad. Sci.* **87:** 3748–3752.

Rabindran, S.K., G. Giorgi, J. Clos, and C. Wu. 1991. Molecular cloning and expression of a human heat shock factor, HSF1. *Proc. Natl. Acad. Sci.* **88:** 6906–6910.

Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. *Molecular cloning: A laboratory manual,* 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Scharf, K.-D., S. Rose, W. Zott, F. Schoff, and L. Nover. 1990. Three tomato genes code for heat stress transcription factors with a remarkable degree of homology to the DNA-binding domain of the yeast HSF. *EMBO J.* **9:** 4495–4501.

Schuetz, T.J., G.J. Gallo, L. Sheldon, P. Tempst, and R.E. Kingston. 1991. Isolation of a cDNA for HSF2: Evidence for two heat shock factor genes in humans. *Proc. Natl. Acad. Sci.* **88:** 6911–6915.

Sorger, P.K. 1990. Yeast heat shock factor contains separable transient and sustained response transcriptional activators. *Cell* **62:** 793–805.

Sorger, P.K. and H.C.M. Nelson. 1989. Trimerization of a yeast transcriptional activator via a coiled-coil motif. *Cell* **59:** 807–813.

Sorger, P.K. and H.R.B. Pelham. 1988. Yeast heat shock factor is an essential DNA-binding protein that exhibits temperature-dependent phosphorylation. *Cell* **54:** 855–864.

Sorger, P.K., M.J. Lewis, and H.R.B. Pelham. 1987. Heat shock factor is regulated differently in yeast and HeLa cells. *Nature* **329:** 81–84.

Wiederrecht, G., D. Seto, and C.S. Parker. 1988. Isolation of the gene encoding the S. cerevisiae heat shock transcription factor. *Cell* **54:** 841–853.

Xiao, H. and J.T. Lis. 1988. Germline transformation used to define key features of the heat shock response element. *Science* **239:** 1139–1142.

Zakeri, Z.F. and D.J. Wolgemuth. 1987. Developmental-stage-specific expression of the hsp70 gene family during differentiation of the mammalian male germ line. *Mol. Cell. Biol.* **7:** 1791–1796.

Zimarino, V. and C. Wu. 1987. Induction of sequence-specific binding of Drosophila heat shock activator proteins without protein synthesis. *Nature* **327:** 727–730.

Cloning and characterization of two mouse heat shock factors with distinct inducible and constitutive DNA-binding ability.

K D Sarge, V Zimarino, K Holm, et al.

Genes Dev. 1991, **5:**
Access the most recent version at doi:10.1101/gad.5.10.1902