

HSF4, a New Member of the Human Heat Shock Factor Family Which Lacks Properties of a Transcriptional Activator

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Heat shock transcription factors (HSFs) mediate the inducible transcriptional response of genes that encode heat shock proteins and molecular chaperones. In vertebrates, three related *HSF* genes (*HSF1* to -3) and the respective gene products (HSFs) have been characterized. We report the cloning and characterization of human HSF4 (hHSF4), a novel member of the hHSF family that shares properties with other members of the HSF family yet appears to be functionally distinct. hHSF4 lacks the carboxyl-terminal hydrophobic repeat which is shared among all vertebrate HSFs and has been suggested to be involved in the negative regulation of DNA binding activity. hHSF4 is preferentially expressed in the human heart, brain, skeletal muscle, and pancreas. Transient transfection of hHSF4 in HeLa cells, which do not express hHSF4, results in a constitutively active DNA binding trimer which, unlike other members of the HSF family, lacks the properties of a transcriptional activator. Constitutive overexpression of hHSF4 in HeLa cells results in reduced expression of the endogenous *hsp70*, *hsp90*, and *hsp27* genes. hHSF4 represents a novel HSF that exhibits tissue-specific expression and functions to repress the expression of genes encoding heat shock proteins and molecular chaperones.

The inducible transcription of heat shock genes is mediated by members of a family of heat shock transcription factors (HSFs) which share common properties. The vertebrate HSFs are negatively regulated for DNA binding and transactivation and become activated in response to heat shock and other forms of environmental and chemical stress to form transcriptionally active trimers.

An unexpected finding during the cloning of the *HSF* genes from plants and vertebrates was the identification of a *HSF* multigene family. At least three *HSF* genes have been isolated from the human, mouse, chicken, and tomato genomes, whereas only a single functional *HSF* gene has been characterized to date for yeasts and *Drosophila melanogaster* (3, 12, 17, 22, 24, 27, 28, 34, 40). Comparison of the sequences of the vertebrate HSFs reveals that within a single species the HSFs are ≈40% related in amino acid sequences (e.g., mouse HSF1 [mHSF1] and mHSF2 or chicken HSF1 [cHSF1], cHSF2, and cHSF3) (17, 22, 24, 27, 28). Interspecies comparisons, e.g., between hHSF1, mHSF1, and cHSF1, indicates 73 to 89% conservation in the amino acid sequence. Comparison of the three cHSFs with other cloned HSFs suggested that there was a common ancestor from which they diverged (17). By comparison, the tomato HSFs (HSF8, HSF24, and HSF30), although similar in structure, were isolated independently through binding-site screening; however, the relationships among the plant HSFs and between plant and animal HSFs are less certain (27).

All members of the HSF family share common structural features, including a conserved DNA binding domain which exhibits a winged helix-turn-helix motif (9, 38), an extended

hydrophobic repeat (HR-A/B) involved in trimerization, and a transactivation domain (15, 20, 35, 41). With the exception of those from budding yeasts, HSFs also have a carboxyl-terminal hydrophobic repeat (HR-C) which has been suggested to function in suppression of trimer formation by interaction with the amino-terminal hydrophobic repeats (3, 6, 16, 17, 22–24, 28, 43).

What is the role of multiple HSFs? One possibility is that larger, more complex organisms require multiple HSFs to respond to a diverse array of developmental and environmental cues. *Saccharomyces cerevisiae* HSF, which is encoded by an essential gene, has at least two transcriptional activation domains that respond to sustained or transient heat shock (2, 19, 33). Perhaps the duplication of *HSF* genes in larger eukaryotes was a response to evolutionary pressure to provide some specificity to the inducible regulation of heat shock gene expression. For example, HSF1 corresponds to the rapidly activated stress-responsive factor whereas the coexpressed HSF2 is activated in response to distinct developmental cues (1, 25, 31) or during hemin treatment of K562 erythroleukemia cells (32, 36). During mouse development, mHSF2 mRNA accumulated to high levels during early embryogenesis and in spermatocytes (5, 26). Studies on cHSF3 provide additional evidence for diversity in the pathways for activation of HSFs (16, 17). cHSF3 is expressed ubiquitously, and its activity is induced upon heat shock in chicken erythroblastic cells (HD6 cells). These data suggest that cHSF1 and cHSF3 could enhance the ability of the cell to tightly regulate the heat shock response (16).

Despite these differences in expression, HSF1, -2, and -3 all function as positive activators of transcription. In this paper, we describe the cloning and characterization of a novel human HSF (hHSF), hHSF4, which exhibits many of the properties of the previously cloned vertebrate HSFs yet is functionally distinct in that it lacks any activity as a positive transactivator.

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M V Q E A P A A L P T E R G P S P V P A F L G K L W A L V 29
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G D P G T D H L I R W S P S G T S F L V S D Q S R F A K E V 59
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L P Q Y F K H S N M A S F V R Q L N M Y G F R K V V S I E Q 89
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G G L L R P E R D H V E F Q H P S F V R G R E Q L L E R V R 119
GGCGGCTGCTTAGGCCGGAGCGCGACCACGTCGAGTTCAGCACCAGCTTCGTGCGCGGCCGCGAGCAGCTACTGGAGCGCGTGGC 360

R K V P A L R G D D G R W R P E D L G R L L G E V Q A L R G 149
GCGCAAGGTGCCCGCGCTGCGGGCGACGACGGCCGCTGGCGCCCGAGGACTGGGTCGACTACTGGGCGAGGTGCAGGCTTTGCGGGG 450

V Q E S T E A R L R E L R Q Q N E I L W R E V V T L R Q S H 179
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G Q Q H R V I G K L I Q C L F G P L Q A G P S N A G G K R K 209
CGGTGAGCAGCACCAGGTCATTGGCAAGCTGATCCAGTGTCTCTTTGGGCCACTTCAGGCGGGGCCAGCAATGCAGGAGGCAAGAGAAA 630

L S L M L D E G S S C P T P A K F N T C P L P G A L L Q D P 239
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T S Q K T L H P L R G P G F L P P V M A G A P P P L P V A V 299
GACATCCAGAAGACTTCCATCCCTGAGGGGACCAGGCTTCTCCCTCCAGTGATGGCAGGAGCCCCCGCCACTGCCTGTGGTGT 900

V Q A I L E G K G S F S P E G P R N A Q Q P E P G D P R E I 329
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P D R G P L G L E S G D R S P E S L L P P M L L Q P P Q E S 359
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ATAAACTCCGTTTTTTTTTTCACC 1555

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FIG. 1. Nucleotide and predicted amino acid sequences of hHSF4. Numbers indicate the nucleotide (lower numbers) and amino acid (upper numbers) positions starting at the first nucleotide and at a predicted initiator, ATG, respectively. Lines under the nucleotide sequences ATG and TAA indicate the predicted translation start site and the termination site, respectively. The underlined amino acids (nucleotides 19 to 123) indicate the predicted DNA binding domain (17). The open and closed rectangles show the heptad repeats of hydrophobic amino acids. The dotted line (nucleotides 1172 to 1241) indicates the sequences which are alternatively spliced out (data not shown).

Consensus	M-----P...G.S.VPA FL.KLW.LV. DP.TD..I.W S..G.SF.V.	50
hHSF4	MVQAPAAPL TEPGSPVPA FLGKLWALVG DPGTDHLIRW SPSTGSLVLS	50
hHSF1	M--DLPVG-P GAAGPSNVA FLTKLWTLVS DPDTDALICW SPSCNSFHVV	47
hHSF2	M----- --KQSSNVA FLSKLWTLVE EHTHNEFITW SQNGQSFLVL	39
cHSF3	M--REGSALP GAPGAAPVPG FLAKLWALVE DPQSDDVICW SRNGENFCIL	48
Consensus	D..RFAKE.L PKYFKHNNMA SFVRQLNMYG FRKV.V.IE.C	100
hHSF4	DQSRFAKEVL EQYFKHSNMA SFVRQLNMYG FRKVVSIEQG GLLRPERDHV	100
hHSF1	DQGGFAKEVL PKYFKHNNMA SFVRQLNMYG FRKVVHIEQG GLVKPERDDT	97
hHSF2	DEQRFAKEIL PKYFKHNNMA SFVRQLNMYG FRKVVHIDSG IVKQERDGPV	89
cHSF3	DEQRFAKELL EKYPKHNNIS SFIRQLNMYG FRKVVALENG MITAKNSVI	98
Consensus	EFQHP.F..G...LLENIKR KV..... ED.K.R.EDL .K.L..VQ..	150
hHSF4	EFQHPFVVRG REQLLEKVRK KVPAL--RG DGRWRPEDL GRLLGEVQAL	147
hHSF1	EFQHPFCPLRG QEQLLENIKR KVISVSTLKS EDKIRQDSV TKLLTDVQLM	147
hHSF2	EFQHPYFKQG QDDLLENIKR KVSIS--KP EENKIRQEDL TKIISSAQKV	136
cHSF3	EFQHPFFKQG NAHLENIKR KVSIV--RT EDLKVCAEDL HKVLSEVQEM	145
Consensus	...QE...R L...K.ENE. LW.EV..LRQ KH.QQQ.V.. K..QF...L.	200
hHSF4	RGVQSTEAR LRRLRQNEI LWREVVLTRQ SHGQQRHVIG KLIQCLFGLP	197
hHSF1	KGKQECMDSK LLAMKHENEA LWREVASLRQ KHAQQQKVVN KLIQFLISLV	197
hHSF2	QIKQETIESR LSELKSENS LWKRVSELRA KHAQQQVIR KIVQFVITLV	186
cHSF3	REQQNMDIR LANMKREKA LWKEVAVLRQ KHSQQQKLLS KILQFILLSM	195
Consensus	Q.N...-G.K RK..L.L... .S....K..	250
hHSF4	QAGPSNAGGK KKLRLMLDEG SSCPTPAKFN TCIPLGALLQ DPYF-----	241
hHSF1	QSNRIL-GVK RKIPLMLNDS GSAHSMFKYS RQFSLHVVHG SGPYSAPSPA	246
hHSF2	QNNQLV-SLK RKRPLLLNTN GAQKKNLPQH IVKREPTDNHH HKVPHSRTEG	235
cHSF3	RGNIV-GVK RKRBLTDAAG ASPSS--KYS RQYVRIPVES GQAMAFSEHN	241
Consensusa.....b.....c.....d.....e.....f.....	300
hHSF4	-----II. D.T.....S	267
hHSF1	-----IQS PSTYLSQRQ IVALALTGPG A-----PS	291
hHSF2	YSSSSLYAPD AVASSGPIIS DITELAPASP MAS--PGGS I--DERPLSS	279
cHSF3	LKPREISDD I-----IY DVTEQNADEE NIFVIPETNE DVISDPSNCS	285
hHSF4	SDEDEGNRTG L-----IIR DITDTLENAT NGLLAVAHTS GRDRETQTAL	285
ConsensusA.....	350
hHSF4	SLT----SQK TL--HPLR-----GPGF LPPVMAGAPP PLFVAVVDAI	303
hHSF1	SPLVVRKREP PSPQSPRVE EASPRPSSV D----TLLS-- --PTALLDRI	334
hHSF2	QYPDIVIVRD DNEDEYAPVI QSGEQNEPAR ESLSSGSDGS SPLMSSAVQL	329
cHSF3	DFGLPICQVS QPNELSCAEP IPPVHINDVS KPNEMGNVAV RLHTAQANAP	335
ConsensusD.....P.....L	400
hHSF4	LECKGSFSPF GPRNAQQPEP GDPREIPDRG P-----L	335
hHSF1	LRSEFPAPAS VTALTDARGH TITRGRFPSP PPTSTPEKCL SVACLDKNEL	384
hHSF2	NGSSSLTSEB FVTHMDRILM DMINLLKQVE -----L	360
cHSF3	-----RD FVSVIDSILN EN-NSGNQND PLLDREE-----I	364
Consensus	.D.L..D.. L...Q.MLS. .Q.S..... .D.F.PS... ..L.....	450
hHSF4	GLESQDRSPE SLLPPLMLQF PQESVPEPAGP LDVLGPSLQC REWTLMDLDM	385
hHSF1	SDHLDAMDSN LDNLQTMSS HGFSVDTLAL LDLPSPSVTV PDMSLPDLDS	434
hHSF2	LDYLDSDICS LEDFQAMLSG RQFSIDPDL VDLFTSSVQM NPTDYINMTR	410
cHSF3	QDFLNCIDAS LEELQAMLSG KQYSFSSRAP SDVFNPELPA LDMNLMETSP	414
ConsensusE...S...G... ..L.....G....	500
hHSF4	ELSLMQPLVP ERDEFELAVK GLNSPSPOKD PTLGAPLLD VQAAALGGPAL	435
hHSF1	SLASIOELLS PQSPRPPEA ENSPDSC-K QLVHYTAGEL FLLDFGSDVT	483
hHSF2	SENKGLETTK NNVVQPVSEE GRKSKSKPKR QIIQYTAGEL IAPLDGNPAS	460
cHSF3	GMENIANMED STEDLGASER ETAGSKGGQE GTESCDSEVL PQMVCVLRWNF	464
Consensus	550
hHSF4	GLPGALTIYS TPESTASYL GPEASPSP--	463
hHSF1	GSDNLPVLFQ LGEOSYFSEG DGFADPTIS LLTGSEPPKA KDPTVS----	529
hHSF2	SVEQASTTAS SEVLSSVDKP IEVDELLDSS LDPEPTQSKL VRLEPLTRAE	510
cHSF3	SSL-----	467
Consensus	600
hHSF4	-----	463
hHSF1	-----	529
hHSF2	ASEATLPYLC ELAPAPLDS MPLLDS-----	536
cHSF3	-----	467

FIG. 2. Comparison of predicted amino acid sequences. The sequence of hHSF4 is aligned with sequences of hHSF1 (22), hHSF2 (28), and cHSF3 (17). The four regions of identity are indicated as region I, corresponding to the DNA binding domain; region II, corresponding to the amino-terminal hydrophobic repeat (HR-A/B); region III, corresponding to the carboxyl-terminal hydrophobic repeat (HR-C); and region IV, corresponding to a DHR (downstream of hydrophobic repeat). The shaded regions indicated as sites a to f correspond to additional regions of identity among the four factors (17). Site f in hHSF4 and hHSF1 is a new region highly related in the two proteins. The numbers on the right indicate the amino acid positions of each protein. Site a is found in all four HSFs and is a part of two nuclear localization signals (29).

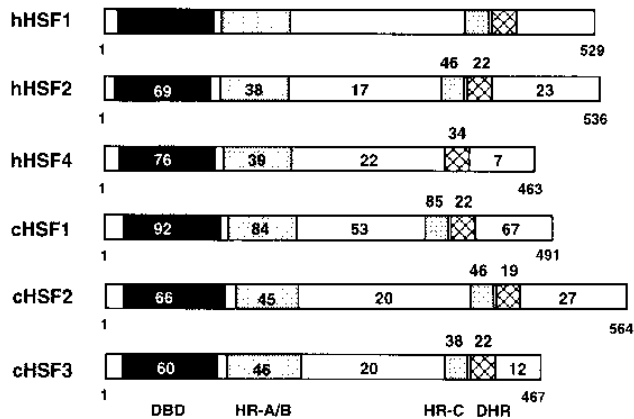


FIG. 3. Diagrammatic representation of all of the hHSFs (22, 28) and cHSFs (17). The percent identities between hHSF1 and each HSF were established by using the computer program GeneWorks. The numbers of amino acids of each HSF are shown at the amino-terminal end of each HSF. DBD, DNA binding domain; HR, hydrophobic heptad repeat; DHR, downstream of HR-C. Remarkably, hHSF4 does not have a region corresponding to HR-C.

MATERIALS AND METHODS

Screening of human cDNA libraries and nucleotide sequence analysis. A λ ZAPII library (Stratagene) of human HeLa cDNA (a gift from G. Dreyfuss) was screened by low-stringency hybridization with a 2.7-kb fragment of cHSF3 cDNA as a probe as described previously (17). The filters were rinsed four times with $2\times$ SSC ($1\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate (SDS) at room temperature for 5 min per rinse, washed twice in $2\times$ SSC–0.1% SDS at 50°C for 30 min, dried, and exposed to X-ray film. Forty-six positive clones were identified, of which two partially overlapping novel clones, AKI-25 and AKI-30, encoded a consensus HSF DNA binding domain. To isolate a full-length cDNA clone, we further screened a human heart cDNA library (Clontech) by high-stringency hybridization with AKI-25 cDNA as a probe. Of 15 clones isolated, the longest clone, pHHSF4-7a, was subcloned into the pGEM1 vector (Promega). The other clones corresponded to partial cDNA clones. Sequencing reactions were performed by using an AutoRead sequencing kit (Pharmacia) with synthetic oligonucleotides and Fluore-dATP labeling mix (Pharmacia). Sequences were analyzed by using an A.L.F. DNA sequencer (Pharmacia) and assembled with GeneWorks (IntelliGenetics, Inc., Mountain View, Calif.).

FISH. Fluorescence in situ hybridization (FISH) was performed as previously reported (11). Metaphase chromosomes were prepared by the thymidine synchronization bromodeoxyuridine release technique for chromosome banding. The AKI-25 cDNA was labeled with biotin-16-dUTP (Boehringer) by nick translation and hybridized to metaphase chromosomes. The hybridization signals

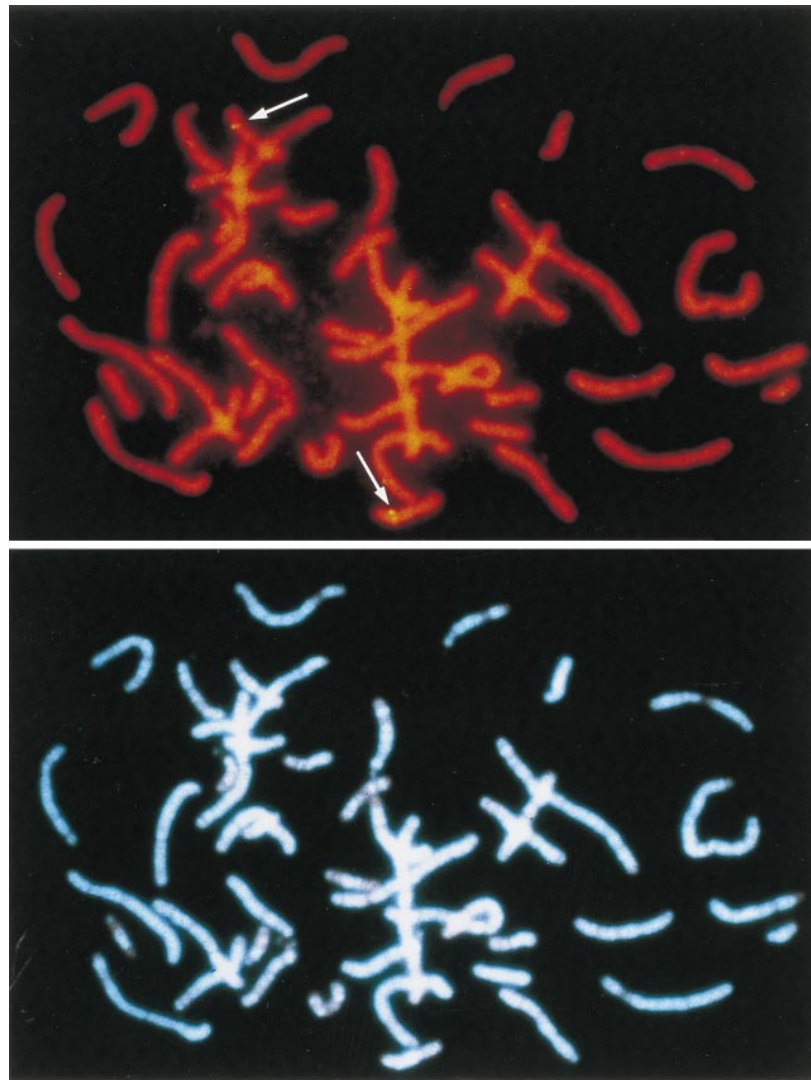


FIG. 4. Chromosomal assignment of hHSF4 cDNA by FISH. (Top) Metaphase chromosomes stained with propidium iodide, showing the twin-spot signals on the long arm of chromosome 16 (arrows). (Bottom) The G-banding pattern of the same chromosomes was delineated through a UV-2A filter (Nikon), indicating that hHSF4 cDNA is localized on chromosome 16q21.

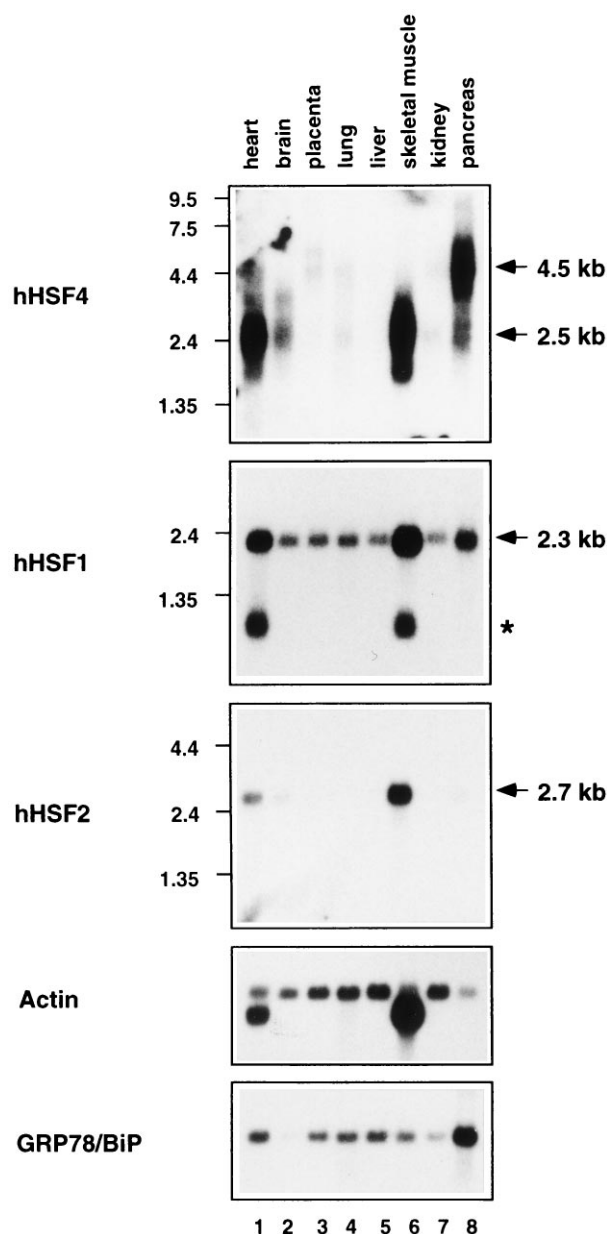


FIG. 5. Northern blot analysis of adult human tissues. RNA blots containing 2 μ g each of poly(A)⁺ RNA from various human tissues (MTN blot purchased from Clontech) were hybridized with hHSF4, hHSF1, and hHSF2 cDNA fragments; 2.5- and 4.5-kb mRNAs of hHSF4 were detected, as indicated by arrows in the top panel. A smaller mRNA for hHSF1, indicated by an asterisk in the second panel, was detected in the heart and skeletal muscle, in addition to the 2.3-kb mRNA. hHSF2 mRNA was detected at 2.7 kb (third column). Hybridizations to a human β -actin probe and GRP78/BiP are shown as a control for the intensity of the samples (bottom two columns). A smaller β -actin isoform exists in the heart and skeletal muscle.

were detected with fluorescein isothiocyanate (FITC)-avidin (Boehringer) and biotinylated antiavidin (Vector) as described previously (21).

Northern blot analysis. RNA blots containing 2 μ g (each) of poly(A)⁺ RNAs from various human tissues (MTN blot purchased from Clontech) were hybridized with a 1.6-kb *Eco*RI fragment of pHHSF4-7a; a 2.1-kb *Eco*RI fragment of an hHSF1 cDNA, pGEM-AKI5, which was isolated from the HeLa cDNA library described above (15a); a 2.4-kb *Eco*RI/*Xho*I fragment of pHSF2-1 cDNA (kindly provided by Robert E. Kingston) (28); a human β -actin cDNA fragment (Clontech); and a 2.0-kb *Bam*HI/*Eco*RI fragment of pHG2 (39). The filters were rinsed four times with 2 \times SSC-0.1% SDS at room temperature for 5 min per rinse, washed twice in 0.2 \times SSC-0.1% SDS at 65°C for 30 min, dried, and exposed to

X-ray film. To examine the expression of heat shock genes, human *hsp70* cDNA (a 2.3-kb *Hind*III/*Bam*HI fragment of pH2.3), human *hsp90* cDNA (a 0.7-kb *Sac*I fragment of pC1-11R) (42), and human *hsp27* cDNA (a 0.4-kb *Pst*I fragment of pHS208) (10) were used as probes.

Expression of hHSF4 in *Escherichia coli* and generation of antiserum. The full-length hHSF4 cDNA was modified by using PCR mutagenesis to introduce *Eco*RI sites and ligated into the pGEX-2T vector (Pharmacia) to create pGEX2T-hHSF4 (16). The two primers used were hHSF4-24 (5'-GAAGCCAGTCCCTCCCCTAAGAATTCGC-3') (underlined nucleotides indicate matched sequences, and boldface nucleotides are *Eco*RI recognition sites) and hHSF4-25 (5'-GCGAATTCCTGGTGCAGGAAGCGCCAG). A 50-ml culture of *E. coli* (strain DH1) transformed with pGEX2T-hHSF4 was treated with 0.4 mM IPTG (isopropyl- β -D-thiogalactopyranoside) for 3 h, suspended in 2 ml of HEMGN buffer (25 mM HEPES [pH 7.9], 300 mM KCl, 12.5 mM MgCl₂, 0.1 mM EDTA, 10% glycerol, 0.1% Nonidet P-40, and 0.1 mM phenylmethylsulfonyl fluoride) (22), and then sonicated. Soluble fractions were collected and stored at -20°C until used for gel shift assay and DNase I footprint analysis. Some degraded products could be observed in this preparation (see Fig. 6A).

For creating antibody to hHSF4, pGEX2T-hHSF4 Δ C was constructed, which encoded a product composed of the C-terminal side of hHSF4 (amino acids 300 to 463) fused to glutathione S-transferase (GST) by using two primers, hHSF4-24 and hHSF4-23 (5'-GCGAATTCCTGGTGCAGGCCATCCTGG-3'). A 500-ml culture of *E. coli* cells transformed with pGEX2T-hHSF4 Δ C was treated with 0.4 mM IPTG for 3 h. These cells were suspended in 30 ml of a buffer containing 50 mM Tris-Cl (pH 8.0), 2 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride and sonicated. After centrifugation, the pellet fraction was suspended in 10 ml of Laemmli's sample buffer, sonicated, and analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). GST-HSF4 Δ C was purified by electroelution from the gel. An water-in-oil emulsion of TiterMax adjuvant (Vaxel Inc., Norcross, Ga.) containing GST-hHSF4 Δ C was used as the rabbit immunogen to obtain an antiserum specific for hHSF4.

Gel mobility shift assay, DNase I footprint analysis, and in vitro transcription and translation. The gel mobility shift assay was performed as described previously (16). The antibody supershift experiments were performed with 2.0 μ l of antiserum diluted 1:10 in phosphate-buffered saline (PBS) and 2.0 μ l of cell lysates in a total volume of 10 μ l. After incubation on ice for 20 min, an oligonucleotide probe mixture containing ³²P-labeled self-complementary ideal heat shock element (HSE) oligonucleotides was mixed and incubated on ice for 20 min and then analyzed on 4% native polyacrylamide gels. To analyze the binding activity to the GAL4 site, gel shift assays were performed as previously described (30) except that 0.1 μ g of GAL4 antibody (RK5C1; Santa Cruz) was added to separate specific binding from nonspecific binding (18).

DNase I footprint analysis was performed as described previously (16).

HSF proteins were translated in vitro in a rabbit reticulocyte lysate as described previously (17).

Transfection, CAT assay, and luciferase assay. hHSF4 cDNA was subcloned into plasmid pCMV-GAL4 (37) to generate the hHSF4 expression plasmid pCMV-HSF4. Transient overexpression of HSFs in COS7 cells was obtained by the calcium phosphate transfection method (16). The chloramphenicol acetyltransferase (CAT) assay was performed with extracts of cells transfected with pCMV-HSF4 (1 to 8 μ g) or each HSF expression vector, pH β -HSF (16), 5 μ g of LSNWT as a reporter gene, and 0.5 μ g of the plasmid containing the human β -actin promoter upstream of the luciferase gene as an internal control. Cell lysates were prepared by standard methods with 0.25 M Tris-Cl as a lysis buffer, and the CAT activity and luciferase activity were assayed.

The expression plasmids pGAL4-hHSF4, pGAL4-cHSF1, and pGAL4-cHSF3, encoding a fusion protein with the GAL4 DNA binding domain (147 amino acids) and the carboxyl-terminal residues of hHSF4 (amino acids 200 to 463), cHSF1, or cHSF3, respectively, were constructed (16). COS7 cells were transfected by using calcium phosphate containing 20 μ g of each pGAL4-HSF expression plasmid, 5 μ g of reporter plasmid ptk-galp3-luc, and 1 μ g of pSVCAT as an internal control. The luciferase assay was performed as described previously (16).

To isolate a stable HeLa cell line expressing hHSF4, cells were transfected by calcium phosphate with the pH β APr-1-neo vector and an hHSF4 expression vector, pCMV-hHSF4. Stable transformants were isolated by selecting cells in the presence of 1.5 mg of Geneticin disulfate (Wako Pure Chemical, Osaka, Japan) per ml. These established lines were maintained in medium containing 0.75 mg of Geneticin disulfate per ml.

Immunofluorescence assay. HeLa cells were cultured on glass coverslips for 16 h, fixed with 50% methanol-50% acetone at -20°C for 1 min, dried, and then washed with PBS. After blocking with 10% normal goat serum-PBS, the cells were incubated with anti-HSF4 serum diluted 1:200 in 10% normal goat serum-PBS and then with FITC-conjugated goat anti-rabbit immunoglobulin G (IgG) antibody (1:200 dilution) (Cappel). The coverslips were washed and mounted in 80% glycerol in 10 mM Tris-HCl (pH 7.5) on a coverglass. Immunolabeled cells were visualized by fluorescence microscopy (Nikon, Tokyo, Japan).

Gel filtration. Whole-cell extracts (200 μ l containing 500 μ g of protein) were applied on a Superdex 200 HR column with fast protein liquid chromatography (Pharmacia) (16). The samples were eluted at 0.3 ml/min with a buffer containing 1% glycerol, 20 mM Tris-HCl (pH 7.9), 200 mM KCl, and 1.5 mM MgCl₂. The fractions (0.5 ml) were precipitated with trichloroacetic acid (10% final concen-

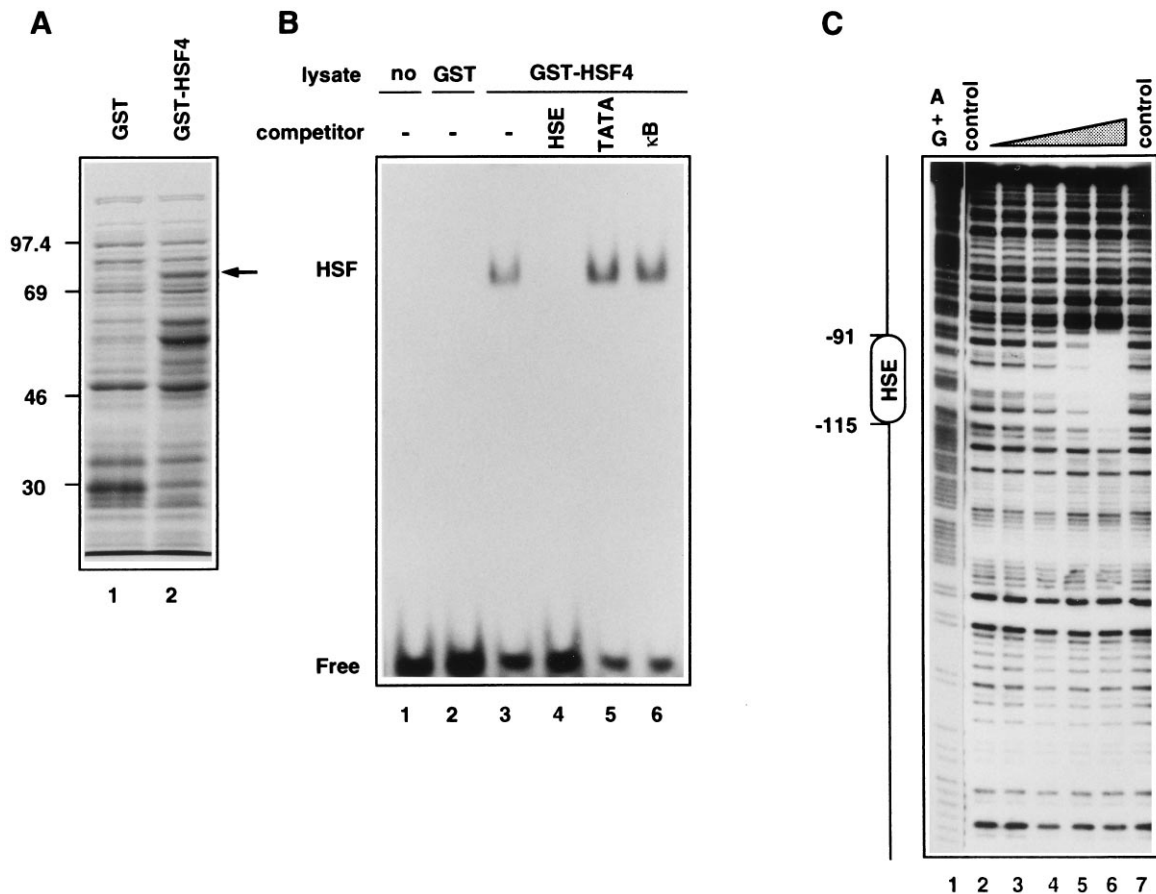


FIG. 6. Specific binding of hHSF4 to HSE. (A) Expression of hHSF4 fused to GST in *E. coli*. GST and a full-length hHSF4 fused to GST were expressed by treating *E. coli* transformed with pGEX2T or pGEX2T-hHSF4 with 0.4 mM IPTG for 3 h. Cell pellets were suspended in HEMGN buffer and sonicated. Soluble fractions were collected, and the lysates were subjected to SDS-10% PAGE and then stained with Coomassie brilliant blue. The arrow indicates the position of GST-hHSF4. Molecular mass standards are as follows: 200 kDa, myosin; 97.4 kDa, phosphorylase *b*; 69 kDa, bovine serum albumin; 46 kDa, ovalbumin; 30 kDa, carbonic anhydrase. (B) Specificity of hHSF4 binding to HSE. Bacterial lysates (0.5 μ l) without GST (lane 1), with GST (lane 2), or with GST-hHSF4 (lanes 3 to 6) were incubated with 32 P-labeled HSE oligonucleotides and loaded on a 4% native gel. In some case, 100-fold molar excesses of unlabeled HSE oligonucleotides (lane 4), TATA oligonucleotides (lane 5), or oligonucleotides containing a κ B site (lane 6) were added to the reaction mixtures (Promega). HSF, HSF-HSE complex; Free, free probe. (C) DNase I footprinting analysis of GST-hHSF4 binding to the promoter on the human *hsp70* gene. Increasing amounts of lysates containing GST-hHSF4 (0, 0.5, 1, 2, and 4 μ l of cell lysates) were assayed (lanes 2 to 6). A + G, A and G ladders. GST-hHSF4 can protect all five nGAAn units, as seen for mHSF1 and cHSF3 (16).

tration), washed with acetone, dried, suspended in gel sample buffer, and analyzed by SDS-PAGE and Western blotting. The peak positions of HSFs were estimated by quantifying the signals of each fraction. The protein standards were as follows: thyroglobulin, 669 kDa; ferritin, 440 kDa; aldolase, 158 kDa; and albumin, 67 kDa.

Western blot analysis. Western blot analysis was performed as described previously (16) by using rabbit anti-Hsp90 serum (a gift of Ichiro Yahara, Tokyo Metropolitan Institute for Medical Research), mouse anti-Hsp70 monoclonal IgG (SPA-810; StressGen), mouse anti-Hsp27 monoclonal IgG (SPP-715; StressGen), or mouse anti- β -actin monoclonal IgG (Chemicon International Inc.) as a first antibody and horseradish peroxidase-conjugated goat anti-rabbit IgG or anti-mouse IgG (Cappel) as a second antibody.

Nucleotide sequence accession number. The DDBJ accession number for the hHSF4 gene is D87673.

RESULTS

Cloning and general features of the hHSF4 gene. We have isolated a novel member of the hHSF gene family by screening a HeLa cDNA library with a full-length cHSF3 cDNA insert under conditions of low-stringency nucleic acid hybridization. From 46 positive clones, two overlapping partial cDNA clones isolated in the initial screen were used as hybridization probes to obtain a full-length cDNA from a human heart cDNA library. Of the 15 clones obtained, a single cDNA clone (re-

ferred to as phHSF4-7a) contained an insert of 1,555 nucleotides with a single open reading frame encoding 463 amino acids (Fig. 1). Comparison of this sequence to other previously characterized members of the HSF family (Fig. 2) revealed that phHSF4-7a encodes a novel member of the HSF family, which we refer to as hHSF4.

Our criterion that hHSF4 is a new member of the HSF family, and not the human homolog of cHSF3, is based on comparisons of the nucleotide and derived amino acid sequences with those of other members of the vertebrate HSF gene family. hHSF4 exhibits only limited sequence homology with the previously cloned HSF1, -2, and -3 genes (Fig. 2; summarized in Fig. 3). Overall, hHSF4 is 37% identical to hHSF1, 27% identical to hHSF2, and 27% identical to cHSF3. The regions of amino acid sequence identity among the HSFs are shown in Fig. 3 and correspond primarily to the DNA binding domain (amino acid residues 19 to 123) and the amino-terminal hydrophobic repeats (amino acid residues 130 to 203). Comparison of the amino acid sequences of the DNA binding domains of hHSF1 and cHSF1 reveals 92% identity; likewise, the corresponding regions of hHSF2 and cHSF2 are 86% identical (17). In contrast, the DNA binding domain of

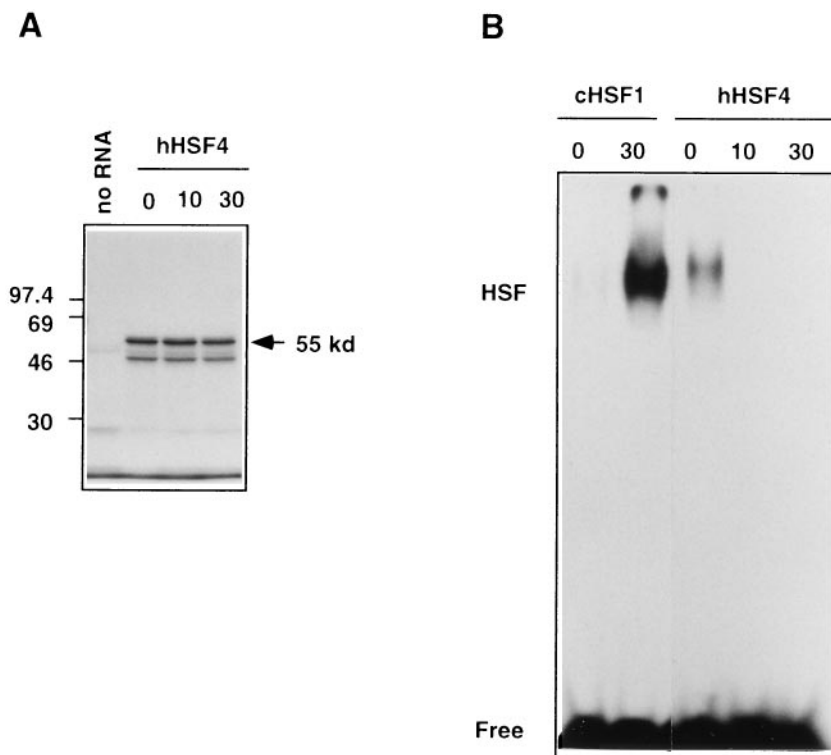


FIG. 7. DNA binding properties of hHSF4 synthesized in vitro. (A) Translation reaction mixtures with hHSF4 in the presence of [³⁵S]methionine were incubated on ice (0 min) or at 45°C for 10 or 30 min and were subjected to SDS-10% PAGE. The gel was fixed, soaked in sodium salicylate, dried, and then exposed on X-ray film. The position of hHSF4 is indicated by an arrow at 55 kDa. (B) hHSF4 and cHSF1 were translated in rabbit reticulocyte lysates. Five microliters of each translation reaction mixture was incubated on ice (0 min) or at 45°C for the 10 or 30 min and analyzed for HSE binding activity by gel shift assay.

HSF4 is 76, 61, and 56% identical to the corresponding region in hHSF1, hHSF2, and cHSF3, respectively. The low level (56%) of identity shared between the DNA binding domains of cHSF3 and hHSF4 provides evidence that the genes for these two proteins are no more closely related to each other than for other pairwise analyses of *HSF* genes. Therefore, based on these sequence relationships, we propose that HSF4 corresponds to a new member of the vertebrate HSF family.

Other conserved motifs found in hHSF4 include the hydrophobic repeat (HR-A/B) which functions as the oligomerization motif and is 39% identical to the corresponding region of hHSF1 (Fig. 3). In contrast, the carboxyl-terminal heptad repeat (HR-C), which has been proposed to have a role in intramolecular negative regulation of DNA binding activity in HSF1 and HSF3, is absent in hHSF4. We note that hHSF4 has a variant of the carboxyl-terminal hydrophobic repeat (amino acid residues 366 to 397) (Fig. 1 and 2) in which two internally positioned proline residues located at residues 371 and 392 distinguish this region from the HR-C regions of HSF1, -2, and -3. Similar alternative heptad repeats are also present in the corresponding location downstream of HR-C of HSF1, HSF2, and HSF3. We suggest that this heptad repeat is distinct from HR-C and refer to this motif as DHR (downstream of heptad repeat).

The analysis of copy number and gene mapping of hHSF4 reveal that the hHSF4 gene is present as a single copy in the human genome as determined by Southern blot analysis (data not shown) of genomic human DNA. The chromosomal location of hHSF4 was established by using fluorescence in situ hybridization with metaphase cells. Of the 47 sets of chromosomes examined, twin-spot signals were detected on both ho-

mologous chromosomes 16q21 (Fig. 4) of 6 chromosomes, whereas the other 21 chromosomes had twin-spot signals on one chromosome 16q21 and a single spot on another 16q21.

Tissue-specific expression of HSF4. The expression of the hHSF4 gene in human tissues was examined by Northern blot analysis (Fig. 5). The hHSF4 gene is selectively expressed as a 2.5-kb RNA species and is detected only in the heart, in the skeletal muscle, and, to a lesser extent, in the brain (Fig. 5, top panel). A distinct abundant 4.5-kb RNA homologous to the hHSF4 gene was detected in the pancreas (Fig. 5). Upon longer exposure of the autoradiogram, the 2.5-kb hHSF4 transcript is detected in other tissues (data not shown).

In contrast to the pattern of tissue-specific expression observed for hHSF4, the mRNA for hHSF1 is ubiquitous (Fig. 5, second panel) and expressed at higher levels in the heart, skeletal muscle, and pancreas. Similar results have been obtained for mHSF1 and cHSF1 (5, 17, 24). A novel hHSF1 mRNA of approximately 1 kb is detected in the heart and skeletal muscle (Fig. 5, second panel). The pattern of expression of hHSF2 mRNA reveals higher levels in the skeletal muscle and heart (Fig. 5, third panel). To ensure equal loading of the RNAs from each tissue, the expression patterns for actin and *grp78/BiP* mRNAs were also obtained (Fig. 5, fourth and fifth panels).

HSF4 exhibits DNA binding with the HSE. The DNA binding properties of hHSF4 were established by using a gel mobility shift assay with recombinant hHSF4 protein and the HSE as the sequence-specific DNA probe (Fig. 6A and B). The specificity of the DNA-protein complex was demonstrated by incubation of the hHSF4-HSE complex with excess unlabeled HSE or with oligonucleotides corresponding to the heterolo-

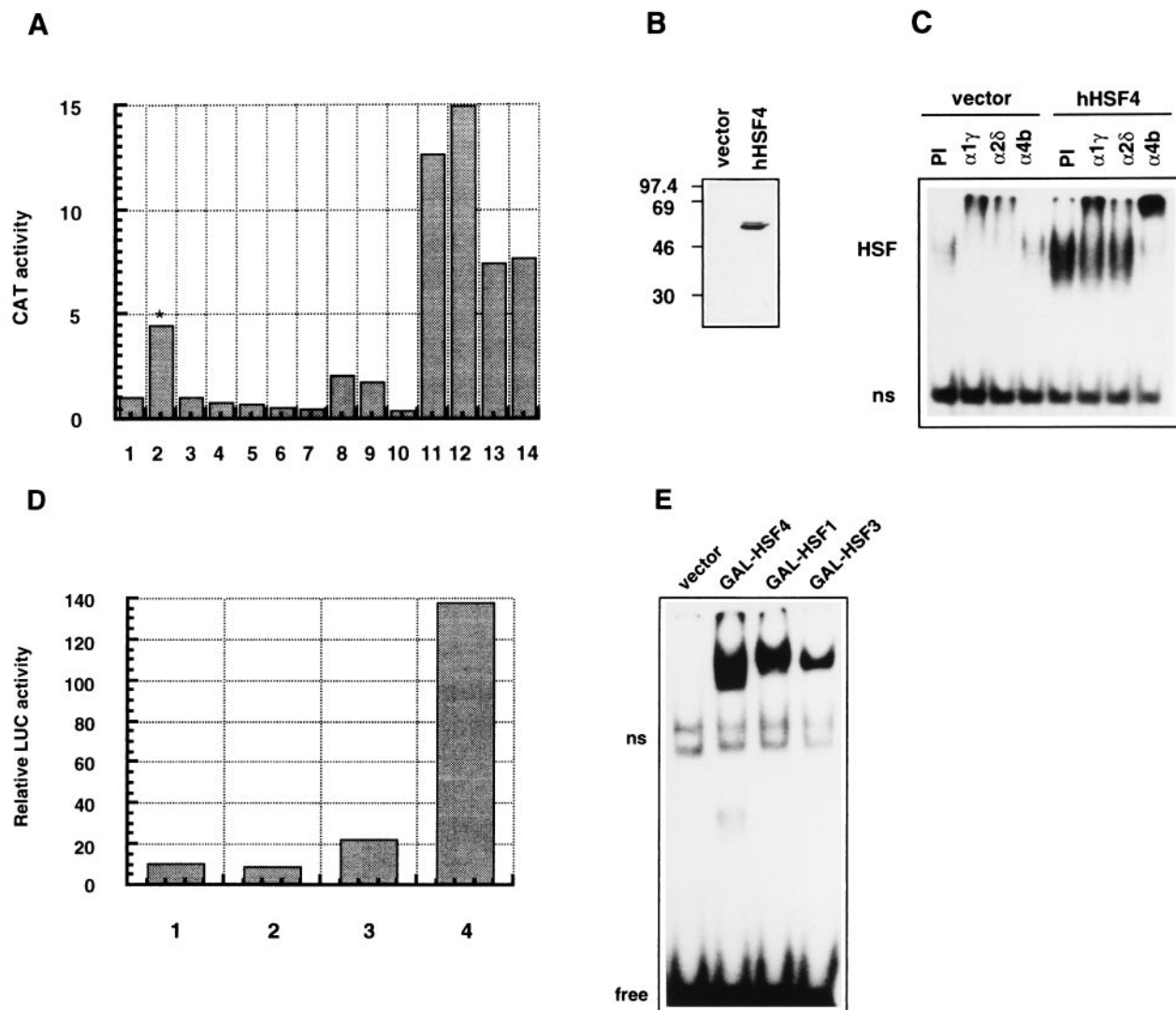


FIG. 8. hHSF4 does not have the ability to activate transcription. (A) Increasing amounts of hHSF4 (1, 2, 4, and 8 μ g for bars 3 to 6, respectively), cHSF1 (bars 7 to 10), or cHSF3 (bars 11 to 14) expression vector were cotransfected into COS7 cells with HSE-CAT vector LSNWT (5 μ g) as a reporter and a construct containing a human β -actin promoter upstream of a luciferase gene (0.5 μ g) as an internal control. At 12 h after transfection, the cells were washed with PBS and incubated for another 24 h. The CAT activities of these cell lysates were analyzed by thin-layer chromatography. The amount of acetylated chloramphenicol was quantitated with a Molecular Dynamics PhosphorImager (Bio-Rad). In some cells, only LSNWT and an internal control vector were transfected (bars 1 and 2); in one of these cases, the cells were heat shocked at 43°C for 30 min and then allowed to recover for 2 h (bar 2) (asterisk). (B) Cell extracts were prepared from cells transfected with vector or pCMV-hHSF4. Western blot analysis was performed with anti-HSF4 serum. Numbers on the left are molecular masses in kilodaltons. (C) HSE binding activity of hHSF4 shown by a supershift experiment. Cell extracts were incubated with preimmune serum (PI) or antiserum specific for HSF1 (α 1 γ), HSF2 (α 2 δ), or HSF4 (α 4b). After being mixed with a 32 P-labeled HSE probe, these extracts were loaded on a 4% native gel. ns, nonspecific binding. (D) The expression vector for the GAL4 DNA binding domain (147 amino acids) (bar 1), GAL4-hHSF4 (bar 2), GAL4-cHSF1 (bar 3), or GAL4-cHSF3 (bar 4) (20 μ g each) was cotransfected into COS7 cells together with 5 μ g of the reporter plasmid ptk-galp3-luc and 1 μ g of pSVCAT as an internal control. After 36 h of transfection, cell lysates were prepared and luciferase (LUC) activities were assayed. The relative levels of the luciferase activities (in arbitrary units) were 10.2, 8.7, 22.4, and 138.0, respectively. (E) Whole-cell extracts prepared from cells transfected as described for panel D were subjected to gel shift assay with a GAL4 site oligonucleotide as a probe (30). To separate specific binding from nonspecific binding, 0.1 μ g of GAL4 antibody was added to the binding mixture (18).

gous TATA box or NF- κ B binding sites (Fig. 6B). The DNA binding properties of hHSF4 were corroborated by using the 55-kDa in vitro-translated hHSF4 (Fig. 7A), which exhibits constitutive DNA binding activity when expressed in the reticulocyte lysate (Fig. 7B). Incubation of lysates expressing hHSF4 at 45°C results in a loss of DNA binding activity (Fig. 7B) without any effect on the levels of hHSF4 (Fig. 7A). These features are similar to the in vitro properties of HSF2, but not those of HSF1 or HSF3, which require exposure to heat shock or other stressful conditions for activation of DNA binding activity (Fig. 7B) (17, 24).

The specificity of hHSF4-HSE interactions was further established by DNase I footprint analysis with the well-characterized human *hsp70* promoter. In the presence of recombinant hHSF4, a region of DNA corresponding to nucleotides -120 to -85 on the coding strand and -115 to -88 on the noncoding strand (data not shown) of the human *hsp70* promoter was protected from digestion with DNase I (Fig. 6C). The boundaries established for hHSF4 binding on the *hsp70* promoter correspond to a region which is identical to that previously observed with mHSF1 or cHSF3 and distinct from a smaller region of the promoter which is footprinted by mHSF2

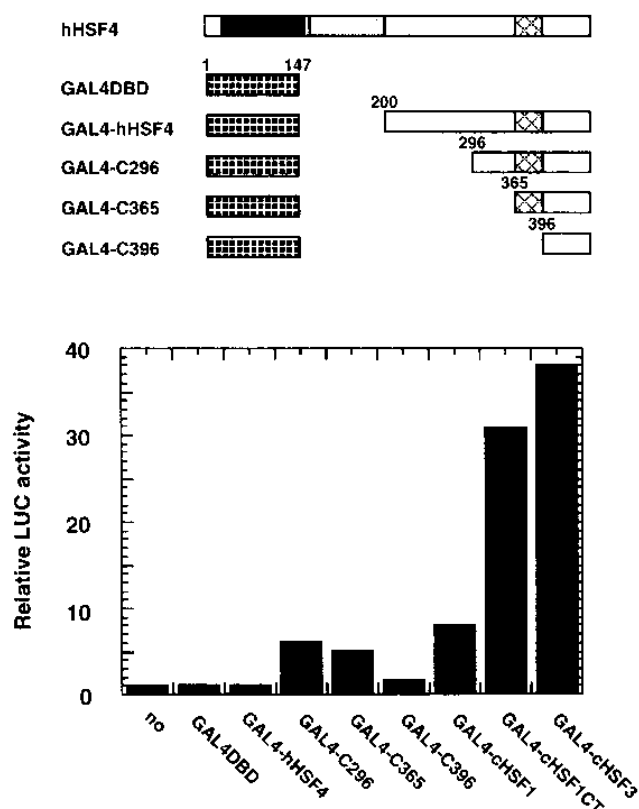


FIG. 9. Deletion analysis of the carboxyl terminus of hHSF4. Each mutant of hHSF4 is schematically shown at the top. At the bottom is the relative luciferase (LUC) activity after normalization by CAT activity. Assays were carried out as described for Fig. 8D. To compare the transcriptional activation potentials, the activities of GAL4-cHSF1 (amino acids 209 to 491 of cHSF1), GAL4-cHSF1CT (amino acids 313 to 491), and GAL4-cHSF3 (amino acids 202 to 467 of cHSF3) are shown.

(16). Taken together, these results demonstrate that HSF4 exhibits sequence-specific interaction with the HSE indistinguishable from that of previously characterized HSFs.

HSF4 lacks the properties of a positive transcription activator. We next examined whether hHSF4 had properties of a transcriptional activator by cotransfection into COS7 cells of an expression plasmid encoding hHSF4 together with the corresponding reporter construct, HSE-CAT. Overexpression of hHSF4 (Fig. 8B) does not stimulate transcription of the CAT reporter construct (Fig. 8A, bars 3 to 6) despite the presence of constitutive DNA binding activity (Fig. 8C). In contrast, cotransfection with expression plasmids for cHSF1 and cHSF3 resulted in 2.1- and 14.9-fold-increased levels of CAT activity, respectively (Fig. 8A, bars 8 and 12) (16). The level of activation of the reporter genes by exogenous cHSF1 or cHSF3 was affected by high levels of the HSF constructs. By comparison, transfection of COS7 cells with the reporter gene alone, which contains the minimal heat shock-responsive human *hsp70* promoter upstream of the CAT gene (LSNWT), resulted in a 4.5-fold increase of CAT activity following heat shock (Fig. 8A, bar 2).

The DNA binding properties of HSF1, -2, and -3 are negatively regulated; therefore, we analyzed the properties of chimeric GAL4-hHSF4 proteins in which the endogenous hHSF4 DNA binding domain was replaced with the constitutive GAL4 DNA binding domain. As shown in Fig. 8D, expression of GAL4-hHSF4 did not stimulate transcription of the cotrans-

ected luciferase reporter construct, despite the high levels of GAL4-hHSF4 DNA binding activity (Fig. 8E). In parallel experiments the activities of GAL4-cHSF1 and GAL4-cHSF3 constructs stimulated reporter activity by 2.2- and 13.5-fold, respectively (Fig. 8D and E).

A possible explanation for the lack of hHSF4 activity is that the transcription activation domain is negatively regulated, as has been previously shown for HSF1. Similar analyses of GAL4-hHSF1 constructs have shown that the transactivation domain is repressed at control temperatures and derepressed upon heat shock or upon deletion of sequences within hHSF1 which negatively regulate the activation domain (7, 30). To address the possibility that the lack of transcriptional activity of hHSF4 was due to intramolecular negative regulation, we generated a collection of hHSF4 deletion mutants fused to the GAL4 DNA binding domain. As shown in Fig. 9, the GAL4-hHSF4 protein did not stimulate the reporter. Deletion of sequences between the hydrophobic repeats of hHSF4, extending to residue 396, resulted in two- to fivefold activation; however, even this level of transactivation was below the basal transcription activity of GAL4-cHSF1 in its repressed state. Therefore, we conclude that hHSF4 has very little activity of a positive activator and is functionally distinct from HSF1 or HSF3.

Properties of HSF4 in HeLa cells stably overexpressing HSF4. In order to pursue additional questions about hHSF4 function, it was necessary to create cell lines expressing hHSF4. Therefore, we stably transfected the hHSF4 gene into HeLa cells, which lack expression of hHSF4 (Fig. 10A). Five HeLa cell lines which stably expressed variable levels of hHSF4 (designated HEF4a to HEF4e) (Fig. 10A) and a single mock-transfected cell line (HEF4x) were obtained. Overexpression of hHSF4 affected cell growth; for example, cell lines HEF4x and HEF4a to HEF4c had an average doubling time of approximately 20 h, whereas cell lines HEF4d and -e exhibited doubling times of 30 and 38 h, respectively. Constitutive expression of hHSF4 in lines HEF4a, -b, and -d resulted in constitutive HSE DNA binding activity (Fig. 10B). There does not appear to be any correlation between the level of expression of hHSF4 and either the constitutive hHSF4 DNA binding activity or effects on cell growth. To establish whether the constitutive DNA binding activity corresponded to hHSF4, antibody supershift experiments with specific polyclonal antibodies to HSF1, HSF2, and HSF4 were performed (Fig. 10C). The specificity of the anti-HSF4 antibody was demonstrated by complementary experiments in which hHSF1, activated in HeLa cells upon exposure to heat shock, did not form a complex with the anti-HSF4 antisera (Fig. 10C, lanes 5 to 11).

The availability of these HeLa-derived cell lines that expressed hHSF4 allowed us to examine the native size of the DNA binding form of hHSF4. Extracts of HEF4d cells were subjected to gel filtration, and the native sizes of hHSF4 and hHSF1 were established by Western blot analysis with anti-HSF4 and anti-HSF1 antibodies. The DNA binding form of hHSF4 has a molecular size similar to that of the trimeric form of hHSF1 (Fig. 10D, lanes 16 to 18) (16) and distinct from that of the monomeric form of hHSF1 (lanes 21 and 22) (16, 32). We infer from these studies that the constitutive DNA binding form of hHSF4 is a trimer. The subcellular localization of hHSF4 in HEF4d cells was determined by indirect immunofluorescence, which shows that the constitutively expressed DNA binding trimer is localized to the nucleus and observed as nucleus-localized speckles (Fig. 10E).

Taken together, constitutive expression of hHSF4 results in a nucleus-localized form of the protein that exhibits constitu-

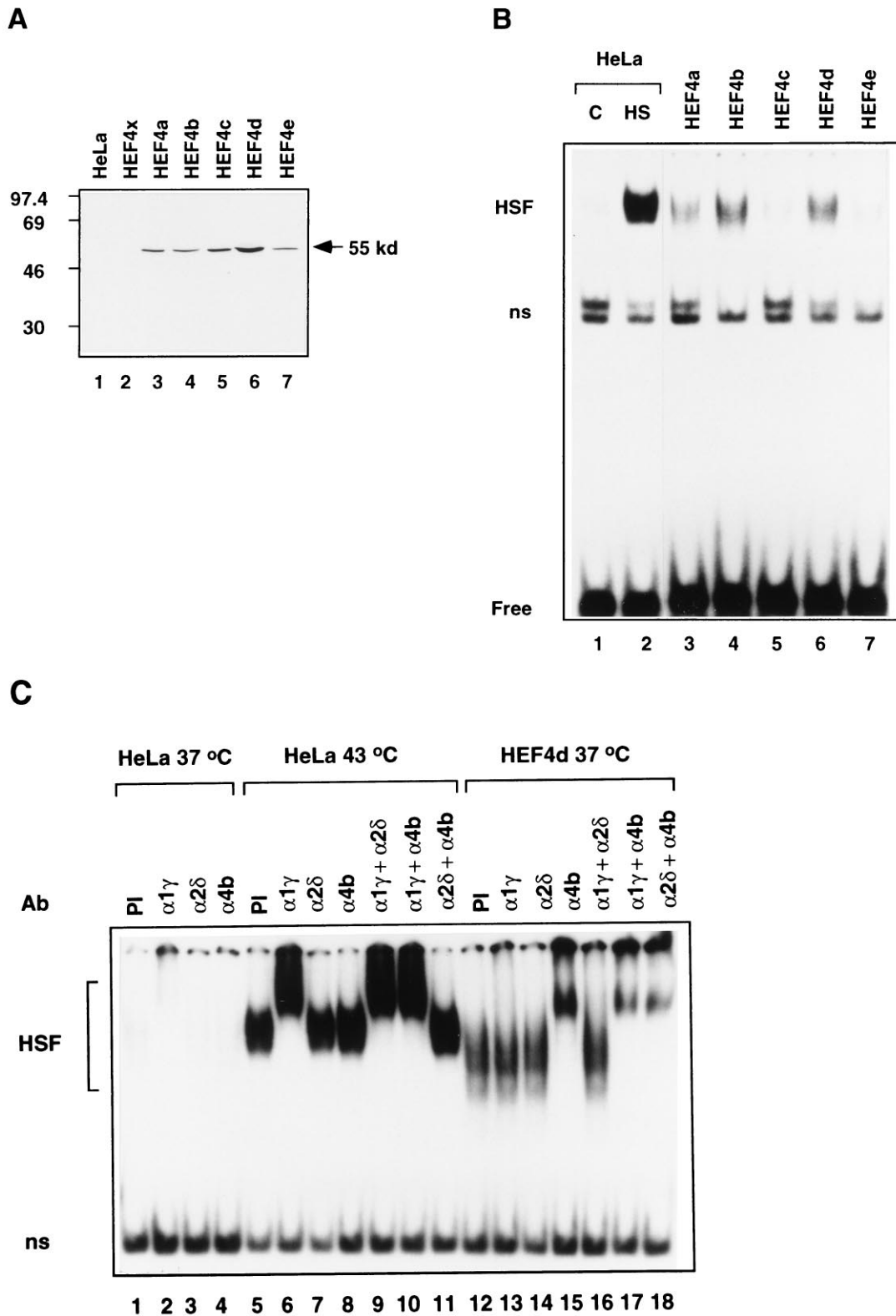


FIG. 10.

tive DNA binding with a native size consistent with a trimer. These features are also common to HSF1, -2, and -3, and the overexpression of these HSFs overrides a critical threshold component of negative regulation.

Constitutive expression of hHSF4 does not interfere with the heat shock response. One consequence of constitutive expression of hHSF4 is that the inducible expression of heat shock genes could be blunted due to competition between the

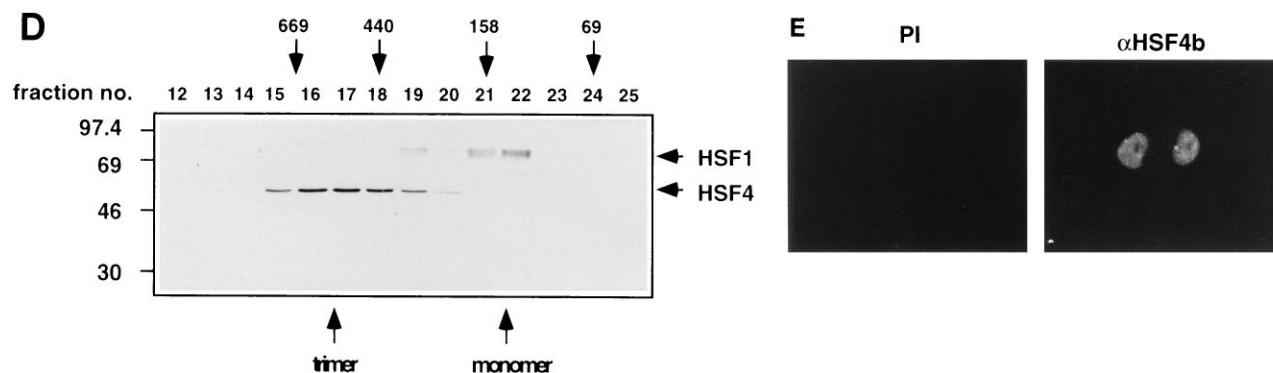


FIG. 10. Characterization of hHSF4 overexpressed in HeLa cells. (A) HeLa cells were cotransfected with a p β APr-1-neo vector and pCMV-hHSF4. Stable transformants (HEF4a to HEF4e) were isolated by selection in the presence of 1.5 mg of Geneticin disulfate per ml. HEF4x is a drug-resistant line which does not express hHSF4. Whole-cell extracts of each line were subjected to SDS-10% PAGE, and Western blot analysis was performed with anti-HSF4b. Signals were detected with the ECL system (Amersham). The position of hHSF4 is indicated by an arrow at 55 kDa. (B) Whole-cell extracts (6 μ g) of control (C) and heat-shocked (HS) HeLa cells (lanes 1 and 2, respectively), and cells from lines HEF4a to HEF4e (lanes 3 to 7, respectively) were subjected to gel shift assay. ns, nonspecific binding. (C) Whole-cell extracts (6 μ g) of control HeLa cells (lanes 1 to 4), heat-shocked (43°C for 30 min) HeLa cells (lanes 5 to 11), and HEF4d cells (lanes 12 to 18) were incubated with a preimmune serum (PI) or antiserum (Ab) against HSF1 β (α 1 γ), HSF2 δ (α 2 δ), or HSF4b (α 4b) (2 μ l of 1:10-diluted serum in a final volume of 10 μ l in PBS) on ice for 20 min, and the 32 P-labeled probe was added. These mixtures were loaded on a native 4% polyacrylamide gel. (D) A whole-cell extract of HEF4d incubated at 37°C was fractionated by gel filtration with a Superdex 200 HR column (Pharmacia). Proteins of each fraction were precipitated with trichloroacetic acid and analyzed by SDS-PAGE and Western blotting with antisera against HSF4b and HSF1 β . Numbers on the left are molecular masses in kilodaltons. (E) Localization of overexpressed hHSF4 in HEF4d cells. HEF4d cells were fixed with 50% methanol-50% acetone and stained with preimmune serum or anti-HSF4b as a first antibody and then with FITC-conjugated goat anti-rabbit IgG antibody (Cappel). Cells were visualized by fluorescence microscopy. A diffuse nuclear staining, except in the nucleolus, was observed, with some specks.

constitutively expressed hHSF4 and inducible hHSF1. To address this possibility, we examined the levels of *hsp70* mRNA in the HEF4d cell line compared with those in HeLa cells. As shown in Fig. 11, the relative levels of *hsp70* mRNA induced upon heat shock are indistinguishable; therefore, we conclude that despite the constitutive expression of hHSF4, which lacks transactivator activity, there is no obvious negative effect on hHSF1-mediated activation of heat shock gene transcription after heat shock.

Constitutive expression of HSF4 reduces the basal expression of the *hsp90*, *hsp70*, and *hsp27* genes. Another possible outcome of constitutive hHSF4 DNA binding activity is an effect on target genes under the regulation of an HSE. For example, one possibility is that constitutive expression of hHSF4 could interfere with the basal expression of heat shock genes. To address this possibility, we compared the levels of expression of three prominent heat shock genes which are known to be regulated by hHSF1 through direct inducible binding at the respective HSEs for the *hsp90*, *hsp70*, and *hsp27* genes. As shown in Fig. 12A, the steady-state levels of Hsp90 and Hsp27 in the hHSF4 cell lines constitutively overexpress-

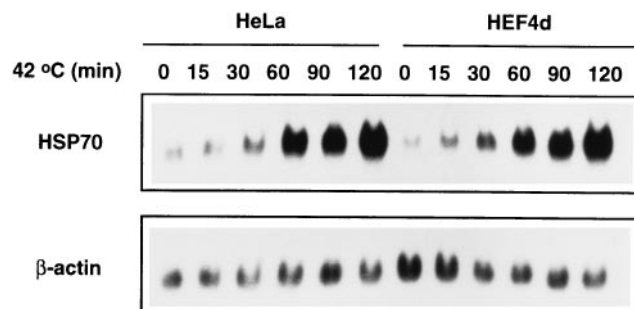


FIG. 11. hHSF4 does not suppress the heat shock response. HeLa and HEF4d cells were incubated at 42°C for the indicated times. Total RNA was isolated and subjected to Northern blot hybridization with *hsp70* or human β -actin cDNA (Clontech) as a probe.

ing hHSF4 (HEF4d and -e) are reduced relative to those in control HeLa cells and mock-transfected HEF4x cells as measured by Western blot analysis. Similar effects on *hsp90* and *hsp27* mRNA levels were also observed by Northern blot analysis (Fig. 12B). The constitutive expression of hHSF4 re-

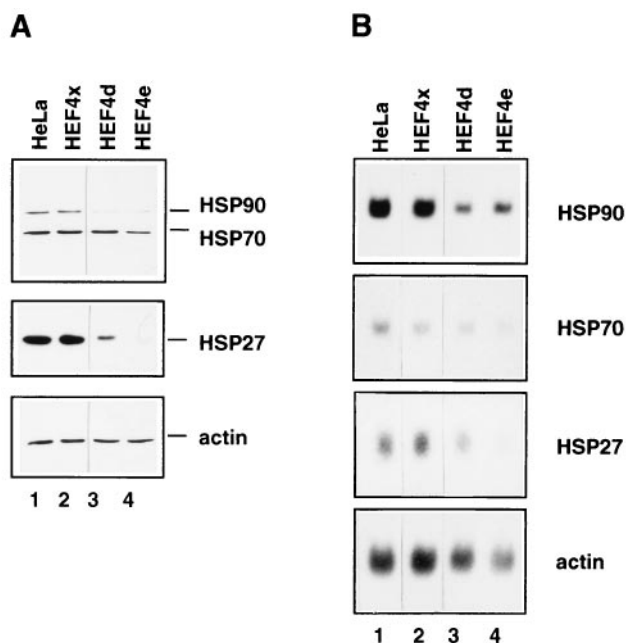


FIG. 12. Basal expression of heat shock proteins in cells expressing hHSF4. (A) Whole-cell extracts of HeLa cells and cells expressing hHSF4 were subjected to SDS-PAGE, and Western blot analysis was performed with rabbit anti-Hsp90 serum, mouse anti-Hsp70 monoclonal IgG, mouse anti-Hsp27 monoclonal IgG, or mouse anti- β -actin monoclonal IgG as a first antibody. The signals were detected with the ECL system (Amersham). (B) Total RNA was isolated from cells as described for panel A, and Northern blot hybridization analysis was performed with each specific cDNA probe.

pressed the levels of *hsp70* mRNA (Fig. 12B), with less striking effects on Hsp70 protein levels. These results demonstrate that hHSF4 can repress the expression of heat shock genes, presumably through the constitutive DNA binding at the HSEs resulting in down-regulation of basal transcription.

DISCUSSION

hHSF4 is a new member of the hHSF family which has the unusual feature of lacking the properties of a transcriptional activator despite having in common many of the biochemical features of other previously cloned HSFs. By use of both transient-transfection studies and stably transfected cell lines, we come to the same conclusion, that hHSF4 or GAL4-hHSF4 proteins bind to the expected DNA sequences yet have little or no positive stimulatory effect on transcription. Overexpression of hHSF4 leads to the repression of target genes that are regulated through HSE promoter sequences. This unusual feature may be related to the fact that hHSF4 lacks the carboxyl-terminal hydrophobic repeat which is shared among all previously characterized members of the HSF gene family which are negatively regulated and exhibits a highly restricted expression pattern in human tissues.

Our *in vivo* analysis of hHSF4 is based on transient-transfection assays and on stably transfected cell lines; therefore, we can only infer the properties of native hHSF4. The DNA binding specificity of hHSF4 to the HSE has been clearly demonstrated; however, our data do not establish whether endogenous hHSF4 normally exists in a repressed or activated state with regard to DNA binding. We would predict from the absence of the hydrophobic repeat HR-C, which is essential in HSF1 and HSF3 for negative regulation of DNA binding, that hHSF4 would exhibit constitutive DNA binding (16, 17, 23, 43). Therefore, it remains to be established whether hHSF4 is a novel negative regulator of heat shock gene expression which exerts its activity in a highly tissue-restricted manner. An example of a tissue-specific regulator is RelB, which is highly expressed in lymphoid tissues and results in constitutive DNA binding activity. RelB is recognized as a cell-type-specific transcriptional activator in the inducible NF- κ B system, which regulates the basal expression of target genes (13, 14).

Comparison of the vertebrate HSFs to the single yeast HSF provides some additional useful insights. The yeast HSF is essential for normal growth and is an essential component of heat shock-induced expression of stress genes. In higher eukaryotes, heat shock induction is regulated primarily by HSF1 and HSF3, with HSF2 providing a developmental profile for heat shock gene expression. A potentially interesting role for hHSF4 is as a repressor whose activity is to balance HSE-targeted transcriptional activity.

Among the cloned HSFs identified in diverse species, only hHSF4 to date has been shown to lack activity as a positive transactivator. The evidence that we have presented for hHSF4 clearly demonstrates biochemical regulatory properties distinct from those of HSF1, -2, and -3, all of which function as positive transcriptional regulators. An alternative, however unlikely, explanation is that hHSF4 requires a cell-type-specific coactivator to function in COS7 or HeLa cells, a feature which would also be unique to hHSF4. The previously cloned mammalian HSFs do not exhibit any cell type specificity. The repression of basal transcription of cellular heat shock genes regulated by the HSE (i.e., *hsp90*, *hsp70*, and *hsp27*) could result from direct binding of HSF4 to the HSEs and subsequent inhibitory effects either on the initiation complex or during transcriptional elongation. It is tempting to speculate that the function of hHSF4 as a negative regulator of basal transcription of heat shock

genes provides a novel means to modulate heat shock gene transcription. Overexpression of heat shock proteins is detrimental to growth at normal temperatures, which provides indirect support for a regulatory mechanism to carefully control the expression of heat shock genes (4, 8).

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