Heat Shock Factor-4 (HSF-4a) Represses Basal Transcription through Interaction with TFIIF*

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The heat shock transcription factors (HSFs) regulate the expression of heat shock proteins (hspS), which are critical for normal cellular proliferation and differentiation. One of the HSFs, HSF-4, contains two alternative splice variants, one of which possesses transcriptional repressor properties in vivo. This repressor isoform inhibits basal transcription of hspS 27 and 90 in tissue culture cells. The molecular mechanisms of HSF-4a isoform-mediated transcriptional repression is unknown. Here, we present evidence that HSF-4a inhibits basal transcription in vivo when it is artificially targeted to basal promoters via the DNA-binding domain of the yeast transcription factor, GAL4. By using a highly purified, reconstituted in vitro transcription system, we show that HSF-4a represses basal transcription at an early step during preinitiation complex assembly, as pre-assembled preinitiation complexes are refractory to the inhibitory effect on transcription. This repression occurs by the HSF-4a isoform, but not by the HSF-4b isoform, which we show is capable of activating transcription from a heat shock element-driven promoter in vitro. The repression of basal transcription by HSF-4a occurs through interaction with the basal transcription factor TFIIF. TFIIF interacts with a segment of HSF-4a that is required for the trimerization of HSF-4a, and deletion of this segment no longer inhibits basal transcription. These studies suggest that HSF-4a inhibits basal transcription both in vivo and in vitro. Furthermore, this is the first report identifying an interaction between a transcriptional repressor with the basal transcription factor TFIIF.

In mammalian cells, three heat shock transcription factors, HSF-1, -2, and -4, have been isolated. These factors share high levels of sequence homology in their DNA-binding domains and hydrophobic heptad repeats (1–5). Various HSF1 family members are expressed as at least two isoforms, and the precise function of the different HSF isoforms is unclear; however, they may play a regulatory role in heat shock element (HSE)-driven transcription (1, 6). The recently cloned human HSF-4 is structurally different from other members of the HSF family. Alternative splicing between the two isoforms, namely HSF-4a and -b introduces a frameshift leading to a smaller HSF-4a protein (463 versus 493 amino acid residues) (7). The HSF-4a contains a DNA-binding domain and the N-terminal hydrophobic heptad repeats, but it lacks a transcriptional activation domain (2, 7). This suggests that this HSF-4 isoform may act as a repressor of other HSFs through its ability to bind either directly to the HSE or to oligomerize with other members of the family. Cells expressing exogenous HSF-4a protein exhibit lower levels of basal and inducible heat shock protein expression (2). The HSF-4b protein appears to be a relatively weak transcriptional activator after heat shock, when compared with other members of the HSF family, such as HSF-1 (7). Interestingly, HSF-4b contains putative mitogen-activated protein kinase phosphorylation sites, which are absent from the HSF-4a isoform. The HSF-4 is expressed in many tissues in human and mouse, but as with other HSF family members, the molecular function, as well as the ratio of expression of the different isoforms in different tissues and at different times, has not been clearly determined.

Transcriptional repressors play an important role in regulation of gene expression, particularly during development, differentiation, and cell growth (8, 9). Of several major classes of repressors, one class consists of DNA-binding proteins that repress transcription via active repression. The targets of such repressors can be activator or co-activator proteins, co-repressors, or proteins that interact with basal transcription factors or GTFs. The repressors that repress activators or co-activators can only inhibit a limited number of promoters (9). The YY1 protein is an example of one such repressor; it represses the fos promoter and requires a DNA-binding site for YY1 as well as a proximal cyclic AMP response element (10). Those repressors that interact with co-repressors exert their effect via interaction of the co-repressor with the transcriptional machinery. Examples of such co-repressors are mSin3A and mSin3B, which interact with Mad and MxiI, which in turn are transcriptional repressors when they dimerize with other members of the e-Myc family (11, 12). The Mad proteins contain a motif that interacts with a paired amphipathic helix 3 present in Sin3 proteins. Recruitment of Sin3, the transcriptional corepressor N-Cor, and a histone deacetylase is how Mad proteins are thought to suppress transcription (13, 14). Finally, the repressors that interact with basal transcription factors repress a minimal RNA polymerase II promoter containing an initiator element or a TATA box (9, 15–17). The Drosophila homeodomain protein Eve and the DNA-binding protein Krup-
pel (KR) are capable of repressing basal transcription by binding to general transcription factors TBP and TFIIE, respectively (18, 19). Other repressors such as the Drosophila Dorsal Switch Protein (DSP1) act as a transcriptional repressor for several activator families in vitro. DSP1 binds directly to the TATA-binding protein (TBP) complexes containing TFIIA and displaces TFIIA from binding to TBP (20). Another repressor that has been shown to interact with basal factors is the retinoblastoma tumor suppressor protein, which represses the activation of transcription mediated by E2F by preventing TFIIB/TFIID from entering the preinitiation complex (21).

Transcriptional initiation by RNA polymerase II in an in vitro transcription system occurs in the presence of five general transcription factors, namely TFIIA, TFIIB, TFIIE, TFIIF, and TFIH. The initiation of transcription occurs following the assembly of the preinitiation complex (PIC), which consists of RNA polymerase II and general transcription factors binding to a promoter (22–25). For those genes that contain a consensus TATA element, the assembly of factors begins with the binding of TBP, which is the TATA box-binding protein and is a subunit of TFIID. Complex formation is completed by the assembly of other general transcription factors and RNA polymerase II (22–26).

The basal transcription factor TFIIF is a heterotrimer of RNA polymerase II-associating protein RAP30 and RAP30 subunits (27). TFIIF is involved in both the initiation and elongation stages of transcription and has been shown to be essential for transcription of all RNA polymerase II promoters that have been examined (28). The RAP30 subunit of TFIIF is involved in the recruitment of RNA polymerase II to promoter-bound TBP and TFIIB. TFIIF has been shown to bind directly to other basal factors, TFIID, TFIIE, and TFIIF as well as RNA polymerase II. TFIIE and RNA polymerase II binding occurs at conserved region III of RAP74. The binding of TAF250 and TFIIE to RAP30 occurs at conserved region I (29). Transcriptional activators, such as serum response factor (SRF), have been shown to bind RAP74 in the middle of the molecule, which encodes conserved region II (30). On promoters such as adenovirus major late promoter, the RAP74 subunit helps to wrap the DNA approximately one turn around the general transcription factors and RNA polymerase II. The TFIIF transcription factor may have a role in isomerization of the preinitiation complex, resulting in helix untwisting before the open complex is formed (31). TFIIF is proposed to play a role in recruitment, isomerization, initiation, and stimulation of elongation by RNA polymerase II. The RAP30 component of TFIIF can enhance the assembly of RNA polymerase II into the initiation complex, and RAP74 binding to the initiation complex will allow RNA polymerase II to make promoter contact (29, 31, 32).

We report here studies on the molecular mechanisms of transcriptional repression by the HSF-4a isoform, using both in vivo and cell-free transcription assays. Our findings indicate that the target of HSF-4a repression is the basal transcription machinery, and the repression occurs through inhibition of the early step in PIC assembly. We further show that the basal transcription factor TFIIF is the specific target of HSF-4a-mediated repression. This repression occurs through the interaction of TFIIF with HSF-4a, and deletion of amino acid residues 124–194 from HSF-4a renders the protein incapable of inhibiting basal transcription.

MATERIALS AND METHODS

Cell Culture—H1299 and HeLa cells are derived from human lung and cervical carcinomas, respectively, and were purchased from American Type Culture Collection (ATCC). These cells were maintained in Dulbecco’s minimal essential medium, supplemented with 10% fetal calf serum.

Plasmids—For in vitro transcription assays, three different G-less cassette constructs were used as follows: one promoter was 2x HSEs upstream of the core heat shock promoter, another had the HSEs removed, and the other contained the complete adenovirus major late promoter (MLP). These plasmids were prepared as follows. For the G-less cassette containing two HSEs, the previously reported plasmids pHSP50HSE2, which contained one or two tandem heat shock elements (HSEs) followed by a TATA element, was used (33). The G-less cassette with no HSE was similar to pHSP50HSE2 but with no HSE. Both G-less cassettes generated 190-bp sized transcripts. The MLP-containing G-less cassette generated a 170-bp sized transcript (gift of Dr. D. Peterson, Texas A & M University, Houston).

H1299-HSF-4a deletion mutants were constructed as described: using the HSF-4a cDNA as a template, different fragments were amplified using primers that incorporated an EcoRI site at the 5′-terminal end and a HindIII site at the 3′-terminal end of the HSF-4a cDNA. The fragments were subcloned in-frame into pSG424 downstream of the GAL4 (1–147) DNA-binding domain (34, 35). The sequences of the primers are as follows: HSF-4 R1373HindIII EcoRI, 5′-GGAATTCCCACGTTAGGGGAGGAGGCAGTGGCTTTCCGG-3′; HSF4F82EcoRI, 5′-G-GAATCTTTGGCCACTCGGAGGCGGCGG-3′; HSF4F811 EcoRI, 5′-GGAATTCACATCCAGAGACATCTCATCCATCC-3′; HSF4F1110 EcoRI, 5′-GGAATTCCTAGTGTG-TGGCCTCCAGACG-3′; HSF4F714 EcoRI, 5′-GGAATTCACAGGGCCACCCACGCCTGGGGCCG-3′.

The N-terminal His6-tagged HSF-4a deletion mutants were constructed as described above, except that the restriction enzyme recognition sites incorporated were NheI and HindIII at the 5′- and 3′-ends of HSF-4a cDNA, respectively. The fragments were inserted into pcET28b vector (Novagen, Madison, WI), and their nucleotide sequences were confirmed by automated DNA sequencing. The human HSF-4a cDNA was the gift of Dr. A. Nakai (Kyoto University, Japan). The human HSF-4b isoform was amplified by polymerase chain reaction using cDNA obtained from normal human skeletal muscle cells (BioWhittaker, Inc.). The reporter plasmids, GAL4-TK-CAT and GAL4-MLP-CAT, were obtained from Dr. D. Dean (Washington University, St. Louis) (36).

Transient Transfection Assays—Transient transfections were performed by calcium phosphate precipitation technique or by LipofectAMINE (Life Technologies, Inc.). Transfected DNA mixes included 2 μg of expression plasmid DNA and, where indicated, 1.5 μg of GAL4-TK-CAT or GAL4-MLP-CAT DNA, and 0.1 μg of firefly luciferase DNA with pBlueScript carrier DNA added to a total of 4 μg. The DNA mixes were added to 0.5 to 1 × 10⁶ cells. Forty eight hours after transfection, cells were lysed, and firefly luciferase activity was determined according to the manufacturer’s instruction (Promega, Madison, WI). CAT activity was determined by chromatography or enzyme-linked immunosorbent assay (Roche Molecular Biochemicals). The activity of firefly luciferase in which a cytomegalovirus promoter drives its constitutive expression was used as an indicator of transfection frequency (37, 38). All transient transfection experiments were performed at least 3 times with both H1299 and HeLa cells, and results were consistent.

Electrophoretic Mobility Shift Assays—Electrophoretic mobility shift assays have been described in detail previously (38–40). Briefly, after each treatment, cells were rinsed with phosphate-buffered saline and lysed in 100 μl of extraction buffer (10 mM HEPES (pH 7.9), 0.4 mM NaCl, 0.1 mM EDTA, 0.5 mM dithiothreitol, 5% glycerol, 0.5 mM phenylmethylsulfonyl fluoride). The protein concentration of samples was estimated by the bicinchoninic acid method. Equal amounts of protein (10 μg) in extraction buffer (volume not exceeding 15 μl) were added to the reaction mixture, which contained 4 μl of binding buffer (37.5 mM NaCl, 15 mM Tris-HCl (pH 7.4), 0.1 mM EDTA, 0.5 mM dithiothreitol, 5% glycerol), 10 μg of poly(dI-dC), and 1 ng of [32P]-labeled GAL4 oligonucleotide (see below)). The mixture was incubated for 15 min at 25 °C and resolved on a 4.5% nondenaturing gel. After depurination, gels were fixed in 7% (v/v) acetic acid for 5 min, rinsed once in distilled water, dried under vacuum, and exposed to x-ray film. The double-stranded oligonucleotide containing the GAL4-binding site was as follows: 5′-GGATCTTCCGGA GTACTGTCCTTCCGA-3′ and 5′-GGTGCGGAGGACAGTACTCCGAG-3′ (41). The oligonucleotide was labeled using Klenow fragment of DNA polymerase I, deoxyxynucleotide triphosphates, and [α-32P]dCTP. Antibody to GAL4 DNA-binding domain used in supershift experiments (10 μg of cell extract incubated with 0.1 μg of antibody for 20 min at 25 °C prior to further analysis) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).
Fig. 1. HSF-4a inhibits transcription via basal promoters. A, constructs showing deletion mutants of HSF-4a that were fused to the DNA-binding domain of yeast transcription factor GAL4 (1-147). Constructs are presented according to the amino acid residues of HSF-4a present in the construct. DBD, DNA-binding domain; MLP, HSF, hydrophobic leucine repeat; leucine zipper 1–3. B, electrophoretic mobility shift assays of constructs shown in A. 48 h after transfection, equal amounts of cleared cell lysates were analyzed by gel mobility shift assays. +Ab samples are lysates incubated in the presence of antibody to GAL4 (1:20 dilution) for 20 min at 25 °C before analysis. Panels 1–6 are constructs shown in A. Panel 7 is untransfected cell lysate. Panel 8 is the cell lysate as in panel 3 but with 200× excess cold oligonucleotide added. Nonspecific and specific indicate the presence of a nonspecific band that is commonly seen with some mammalian cell lysates using oligonucleotides containing GAL4-binding sites (41). However, the GAL4 DNA-binding domain fragment encoded by plasmid PSG424 as well as the other small mutant proteins also run with the same mobility (see −Ab in lanes 5 and 6). Note that larger fragments such as those shown in lanes 2-4 appear above the nonspecific band in the −Ab lanes. Antibody to GAL4 in +Ab lanes are able to supershift all fusion proteins. The presence of multiple bands in −Ab lanes most likely represent multimers. C, CAT assays. Constructs 1-6 shown in A were co-transfected into H1299 cells with the reporter construct GAL4-MLP-CAT or GAL4-TK-CAT and firefly luciferase as an indicator of transcriptional frequency. 48 h post-transfection, cells were lysed, and CAT and luciferase activity was determined from 80 or 20 μg of protein, respectively. D and E, quantitation of the data shown in C as well as data from other experiments. Data are presented as % conversion of [14C]chloramphenicol acetyltransferase to major acetylated forms relative to internal control.

RESULTS

HSF-4a Inhibits Transcription via Basal Promoter Elements in Vivo—The HSF-4a isoform has been shown to inhibit basal expression of heat shock protein 90 and 27 in tissue culture cell lines (7). This isoform of HSF-4 also inhibits the heat inducibility of reporter plasmids containing an HSE. These results and the fact that this HSF-4 isoform has a DNA-binding domain, but lacks a transcriptional activation domain, suggests that it could be an active, transcriptional repressor (9). To determine whether HSF-4a inhibits transcription from basal promoters as other active repressors do, we constructed a series of chimeric proteins in which the HSF-4a DNA-binding domain was replaced by a yeast GAL-4 transcription factor. In addition, the remainder of the HSF-4a protein was truncated as shown in Fig. 1A. These constructs included the remaining regions of HSF-4a, which included amino acid residues...
124–463 (construct 2 in Fig. 1A); residues 194–463 (construct 3 in Fig. 1A); residues 270–463 (construct 4 in Fig. 1A); 366–463, and residues 414–463 (constructs 5 and 6, respectively, in Fig. 1A). The ability of each construct to bind to the GAL4-binding site was determined using electrophoretic mobility shift assays following the transient transfection of each construct into H1299 cells. All constructs were capable of binding to an oligonucleotide containing a GAL-4 binding site (Fig. 1B).

The expression constructs shown in Fig. 1A were co-transfected with reporter plasmids GAL4-MLP-CAT or GAL4-TK-CAT into H1299 cells and 48 h following transfection, and CAT activity for each construct was determined. Results indicate that the GAL4-HSF-4a construct containing amino acid residues 124–463 repressed the expression of both thymidine kinase and MLP basal promoters by more than 5-fold when targeted to these promoters (Fig. 1, C–E). HSF-4a repression of transcription of these basal promoters was less pronounced in the more severe GAL4-HSF-4a deletion mutants. There was no reduction in CAT expression when cells were co-transfected with plasmid construct pSG424, which contained the GAL4-(1–147) DNA-binding domain (construct 1), when compared with transcription of cells with reporter genes alone.

The results of these experiments suggest that sequences encoding the amino acid residues 124–194 of HSF-4a protein are required for repression of basal transcription. This region encodes the N-terminal hydrophobic heptad repeats (HHR in Fig. 1A).

To ensure that the inhibition of basal transcription that was observed in the presence of GAL4-HSF-4a (Fig. 1) was not due to nonspecific squelching, the following experiment was performed. Reporter plasmids GAL4-TK-CAT or GAL4-MLP-CAT containing GAL4-binding sites were co-transfected with or without an expression construct containing full-length (hemagglutinin)-tagged HSF-4a. This HSF-4a protein contains a DNA-binding domain that requires heat shock element for binding and, therefore, is unable to bind to the GAL4-binding sites present in the reporter constructs. However, HSF-4a would be able to bind to other factors present in the cell and cause nonspecific squelching of transcription of these reporter constructs. 48 h after transfection, CAT expression was determined. The results indicate that there was no inhibition of basal transcription driving GAL4-MLP-CAT or GAL4-TK-CAT by HSF-4a if the protein was not targeted specifically to these promoters (Fig. 2).

**HSF-4a Inhibits Basal Transcription in an in Vitro Model System Prior to the Formation of the Preinitiation Complex**—Both the thymidine kinase and adenovirus major late promoters used in the previous experiments are minimal basal promoters containing a consensus TATA element. For transcription, these promoters have been shown to require RNA polymerase II and the so-called "basal" transcription factors (also known as general transcription factors, or GTFs), which include the TBP (TATA-binding polypeptide) subunit of TFIIID and TFIIA, TFIIH, TFIIIE, TFIIF, and TFIIH (24). To explore the mechanisms of HSF-4a repression of basal transcription, these promoters have been shown to require RNA polymerase II, recombinant TFIIA, TFIIB, and other factors such as TFIIA, TFIIH, TFIIE, TFIIF, and TFIIH purified from HeLa cell nuclear fractions. GTFs and a G-less cassette containing an hsp 70 promoter with two HSEs were incubated for 30 min at 30 °C and then terminated. As shown in Fig. 3B, additions of 30, 60, or 120 ng of purified recombinant HSF-4a protein to the transcription reaction inhibited basal transcription in a concentration-dependent manner. The addition of 120 ng of puri-
fied HSF-4a protein inhibited transcription by more than 5-fold.

We then asked whether HSF-4a could inhibit basal transcription if it was added after the assembly of the preinitiation complex on the DNA. For this, GTFs were allowed to incubate with the DNA template for 30 min at 30 °C in the absence of nucleotides. Following this incubation period, HSF-4a and nucleotide triphosphates were added; transcription was allowed to proceed for 45 min at 30 °C, and samples were analyzed. No inhibition of basal transcription by HSF-4a was detected under these conditions (Fig. 3C). The results of these experiments suggest that HSF-4a inhibits basal transcription at an early step during the PIC assembly.

**HSF-4a Inhibits in Vitro Transcription from Promoters with No HSE**—We and others (Ref. 2 and data not shown) have shown that recombinant or in vitro translated HSF-4a protein can bind constitutively to the HSE. To test whether HSF-4a binding to the HSE was required for its inhibition of basal transcription, two additional constructs lacking HSEs were tested. One was identical to the HSE construct used in Fig. 3 except the HSEs were removed, and the other was a construct with the adenovirus major late core promoter. For these experiments, GTFs were allowed to incubate with DNA, with or without 120 ng of purified HSF-4a protein, for 30 min at 30 °C before the transcription reaction was allowed to proceed at 30 °C for 45 min (see Fig. 4A for schematic). The results indicate that HSF-4a inhibited transcription from all promoters used (compare lanes 1 and 2, Fig. 4B–D) that lack DNA-binding domain, respectively, to show the position of the two proteins. Lanes 1 and 2, input full-length HSF-4a (residues 1–463) that lacks DNA-binding domain, respectively.

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**FIG. 4.** HSF-4a inhibits basal transcription with several promoters. A, schematic of transcription reaction. Indicated GTFs and template DNA with or without HSF-4a were incubated at 30 °C for 30 min. Nucleotides and HSF-4a were then added, and the reaction was allowed to proceed for 45 min. Reactions were then stopped, and samples were processed as described under “Materials and Methods,” analyzed by gel electrophoresis, and quantitated by PhosphorImager. B, template DNA contained 2 HSEs. C, template DNA contained no HSEs. D, template DNA was MLP. Lanes 1–3 are as follows: 1, GTFs and nucleotides, without HSF-4a; 2, GTFs and nucleotides with the addition of 120 ng of HSF-4a; 3, HSF-4a added to the reaction after the formation of the preinitiation complex assembly. E, DNA beads were incubated with HSF-4a protein in 70 μl reaction. After 20 min at 25 °C, beads were washed 4 times with 200 μl of 0.5 × HE buffer. Transcription was then carried out in duplicate by the addition of various components of in vitro transcription. Products were analyzed as above. Lanes 1 and 2 indicate DNA beads only; lanes 3 and 4, DNA beads plus HSF-4a (residues 124–463); lanes 5 and 6, DNA beads plus HSF-4a (residues 1–463). F, quantitation of the data shown in E as well as in other experiments. G, immunoblot analysis showing the fraction of HSF-4a that bound to HSE beads after HSF-4a was incubated with HSE containing DNA beads. Lanes 1 and 2, input full-length HSF-4a (residues 1–453) and truncated HSF-4a (residues 124–463) that lacks DNA-binding domain, respectively, to show the position of the two proteins. Lanes 3 and 4, fraction of full-length and truncated HSF-4a bound to DNA, respectively. Lanes 5 and 6, fraction of full-length and truncated HSF-4a that were not bound to DNA and were present in the first wash, respectively.
Promoter-bound HSF-4a Inhibits Transcription—The fact that HSF-4a could potentially bind to a component of the basal transcription machinery and inhibit in vitro transcription would be reasonable if we assumed that the factors are more easily accessible to each other while in a test tube, but that in vivo the HSE is critical for targeting HSF-4a to the promoter. To explore if the fraction of HSF-4a that is bound to the HSE is indeed inhibitory to in vitro basal transcription, we performed the following experiment. A biotinylated DNA fragment (1.2 μg or 0.7 pmol final concentration) containing 2 HSEs bound to streptavidin-coated beads was incubated with full-length HSF-4a (400 ng or 2.6 pmol final concentration) or with the same amounts of truncated HSF-4a lacking the DNA-binding domain at 25 °C for 20 min. The mixture was then rinsed to remove excess HSF-4a, and basal transcription was performed as above. The results show that only the full-length HSF-4a bound to the HSE is able to inhibit in vitro basal transcription (Fig. 4E, lanes 5 and 6 and 4F), whereas the construct HSF-4a (residues 124-463), which does not bind to the HSE, does not inhibit basal transcription (Fig. 4E, lanes 3 and 4, and F). The fraction of the full-length and truncated HSF-4a that bound to the DNA beads is shown in the immunoblot analysis in Fig. 4G. Our previous experiments using gel mobility shift analysis to measure HSF-4a DNA binding ability to HSE indicate that purified HSF-4a binds specifically to HSE maximally when it is preincubated at 4 or 25 °C, and its DNA-binding ability is reduced when it is preincubated at elevated temperatures.2

HSF-4a Interacts with the TFIIH Transcription Factor, Leading to Inhibition of Basal Transcription—Two observations suggested to us that HSF-4a might be interacting with one of the GTFs and thus inhibit transcription: 1) HSF-4a exerted its inhibitory effect independent of the HSE, and 2) HSF-4a lost this inhibitory effect if added after the PIC had already been formed. To address this question, we performed in vitro transcription assays as outlined in Fig. 5A. These assays were designed to determine the minimal set of GTFs that were needed to make the HSF-4a repression-resistant complex seen in Figs. 3 and 4. In this experiment, selected GTFs were left out of the initial preincubation step, allowing formation of the partial PICs. The “deleted” GTFs were then added to the reaction, in the presence or absence of HSF-4a, along with nucleotides, and transcription was allowed to proceed. TFIIA, -B, -D and -E can form a stable complex on DNA (24). Preinitiation complexes containing these factors were still susceptible to repression by HSF-4a (Fig. 5A and B). Addition of RNA polymerase II and TFIIA, -B, -D did not significantly alter these results (Fig. 5C). Significantly, when TFIIH was included (TFIIA, -B, and -D), RNA polymerase II, and TFIIH (Fig. 5D) or TFIIA, -B, -D, -E, and -F and RNA polymerase II (Fig. 5E), transcription could no longer be repressed.

To test further TFIIH in HSF-4a-mediated repression, we determined whether the presence of TFIIH was critical for the promoter that was used in this in vitro transcription model. For this experiment, the preincubation reaction included RNA polymerase II and all the GTFs except for TFIIH. A 5-fold reduction in basal transcription occurred when recombinant TFIIH was left out (Fig. 6, A–C, compare lane 2 to 1). We also asked whether addition of TFIIH to the transcription reaction after a 30-min preincubation period would have allowed transcription to take place, because the order of PIC assembly requires TFIIIE and TFIIH to enter the complex after TFIIF. The results show that the level of transcription was not affected by the late addition of TFIIH to the reaction (Fig. 6, B and C, compare lanes 3 to 1), and HSF-4a was inhibitory to the transcription reaction (compare lanes 4 to 3).

From the data shown in Fig. 6, it therefore appeared that HSF-4a inhibited basal transcription by interfering with TFIIH function. One possible mechanism for the interference of TFIIH function is through a direct interaction of HSF-4a with TFIIH. We therefore tested for an interaction in pull-down experiments. Purified HSF-4a and TFIIH were incubated at 30 °C for 30 min followed by immunoprecipitation using antibody to RAP74, a component of TFIIH. Complexes were immunoblotted with antibody to the His6 to detect the co-immunoprecipitated His6-tagged HSF-4a protein. As shown in Fig. 6D, HSF-4a specifically co-immunoprecipitated with TFIIH (Fig. 6D, lane 3), suggesting that HSF-4a interacts with a component of the TFIIH complex.

Another transcriptional activator that has been shown to
FIG. 6. TFIIF is the target of HSF-4a-mediated transcriptional repression. A, schematic of transcription reaction. Indicated GTFs and template DNA were incubated at 30 °C for 30 min. Nucleotides with or without HSF-4a were then added to the reaction, which was allowed to proceed for 45 min. TFIIF was added with or without HSF-4a during PIC assembly or after 30 min of preincubation as indicated. Reactions were then stopped, and samples were processed as in Fig. 4. B, transcription reactions. Lane 1, GTFs with TFIIF added during PIC assembly. Lane 2, GTFs without TFIIF during PIC assembly. Lane 3, GTFs were incubated for 30 min to assemble PICs without TFIIF, which was added at 30 min at the onset of transcription reaction. Lane 4, GTFs were incubated for 30 min to assemble PICs without TFIIF which was then added at 30 min with HSF-4a at the onset of transcription. C, quantitation of the data shown in B and other experiments using PhosphorImager. D, pull-down experiments showing HSF-4a interaction with TFIIF. TFIIF was preincubated with purified histidine-tagged HSF-4a protein (30 min at 30 °C). Anti-RAP74 (large subunit of TFIIF) was then added to the mixture, and following further incubation, protein A beads were added. The co-immunoprecipitated materials were analyzed by SDS-polyacrylamide gel electrophoresis and detected using antibody to His6 to detect presence of recombinant HSF-4a. Lane 1, purified HSF-4a protein in the input reaction. Lane 2, HSF-4a unbound to protein A beads. Lane 3, HSF-4a/TFIIF co-immunoprecipitates. Lane 4, immunoprecipitation (IP) of HSF-4a without the addition of TFIIF to the reaction. Lane 5, control reaction mixture containing HSF-4a, TFIIF, and protein A but not using antibody to RAP74 for immunoprecipitation. E, HSF-4a bound to TFIIF added prior to the assembly of the transcription components does not inhibit basal transcription. HSF-4a was incubated for 20 min with TFIIF, and the mixture was added to the components of the transcription that contained TFIIF. Lane 1, transcription reaction containing all the components. Lane 2, HSF-4a added to the transcription reaction. Lane 3, same as lane 2 but preincubated HSF-4a/TFIIF was added before the onset of the transcription. F, quantitation of the data shown in E. Lane numbers correspond to that for E.

FIG. 7. TFIIF targets HSF-4a at a region encoding the N-terminal hydrophobic heptad repeats. A, constructs showing various deletion mutants of HSF-4a that were fused to the His6 tag. Constructs are indicated according to the amino acids of HSF-4a present in the construct. DBD, DNA-binding domain; HHR, hydrophobic heptad repeats (N-terminal leucine zippers). B, Coomassie Blue staining of HSF-4a deletion mutants (constructs 1–5 shown in A). Molecular weight markers are shown on the right. C, schematic of transcription reaction. D, transcription reaction contained no HSF-4a (lane indicated as −) or with HSF-4a wild type (lane 1), HSF-4a with amino acid residues 124–463 (lane 2), HSF-4a with amino acid residues 194–463 (lane 3), HSF-4a with amino acid residues 270–463 (lane 4), HSF-4a with amino acid residues 366–463 (lane 5). E, quantitation of the results in D as well as other experiments using PhosphorImager. Interact with TFIIF is SRF. Addition of SRF to the in vitro basal transcription system enhances transcription. However, increasing amounts of SRF leads to inhibition, possibly through a mechanism called squelching (30). "Squelching" by SRF could be overcome by the addition of TFIIF. To investigate whether adding TFIIF back to the transcription reaction would rescue the inhibitory effect observed following addition of HSF-4a to the basal transcription reaction, experiments were performed where 0.26 pmol (40 ng) of HSF-4a trimers was incubated with 0.36 pmol (75 ng) of TFIIF tetramers for 20 min at 25 °C. This mixture was then added to the standard GTF mixture which also included TFIIF, and these factors were allowed to assemble on DNA for 30 min before the onset of transcription reaction. The results indicate that if sufficient amounts of TFIIF are prebound to HSF-4a, HSF-4a no longer inhibits transcription (Fig. 6, E and F).

The Domain of HSF-4a That Inhibits TFIIF Function Is Encoded by Amino Acid Residues 124–194—To determine the region of HSF-4a that is responsible for interacting with TFIIF during transcription, a series of His6-tagged HSF-4a deletion mutants were constructed (Fig. 7A) and were purified using Ni2+–chelate nitriotriacetic acid chromatography (Fig. 7B). These mutant proteins were then added to the preincubation...
stage of the transcription reaction together with GTFs (Fig. 7C). Only the full-length HSF-4a protein or the HSF-4a deletion mutant containing amino acid residues 124–463 was able to inhibit basal transcription 3–4-fold (Fig. 7, D and E, constructs 1 and 2). Construct 3, containing amino acid residues 194–463, was slightly inhibitory (reduced transcription by 20–30%), but constructs containing amino acid residues 270–463 or 366–463 (constructs 4 and 5) did not inhibit the in vitro transcription reaction (Fig. 7, D and E). These results suggest that the region of HSF-4a that interferes with transcription is encoded by amino acid residues 124 to a few residues beyond amino acid residue 194, which includes the N-terminal hydrophobic heptad repeat or leucine zippers 1–3 (N-terminal HHR in Fig. 7A). These findings are consistent with the regions of HSF-4a required for repression of basal transcription in vitro (Fig. 1).

**The Transcriptional Activator HSF-4b Isosform Can Activate HSE-driven Transcription In Vitro**—To determine if HSF-4a and HSF-4b, which differ in part of their amino acid sequence, have the opposite effect on HSE-driven transcription, we titrated HSF-4a or HSF-4b to the in vitro transcription reactions (Fig. 8A). We observed that HSF-4a was not able to activate transcription at any of the concentrations tested (5–120 ng). Rather transcription was inhibited in the 30–120-ng range (Fig. 8B). In sharp contrast, HSF-4b stimulated transcription at all the concentrations tested except at the highest (120 ng per reaction). Under the same conditions of in vitro transcription, addition of another HSF family member, HSF-1, activates an HSE-driven transcription (Fig. 8C). These results are consistent with our conclusion that HSF-4a is a transcriptional inhibitor, and HSF-4b is a transcriptional activator.

**DISCUSSION**

HSF-4 has been detected in two isoforms. One isoform (HSF-4a) appears to be a transcriptional repressor, because it contains a conserved DNA-binding domain but lacks an activation domain. This suggests that this isoform can bind an HSE without being able to activate transcription. The second isoform, HSF-4b, which is predicted to have differences in residues in the central domain of the protein due to alternative splicing, can activate transcription (7). The HSF-4 transcription factor is expressed in several tissues in both mouse and human as determined by Northern blots as well as polymerase chain reaction analysis of cDNA obtained from human tissues (7). HSF-4 binds an HSE constitutively but loses its DNA binding activity upon a mild heat shock when it is synthesized in an in vitro transcription/translation-coupled reaction (7). However, the exact role of HSF-4 in transcriptional regulation of heat shock proteins in vivo is not known.

In these studies, we analyzed the molecular mechanisms underlying HSF-4a-mediated transcriptional repression. We found that HSF-4a represses basal transcription when it is targeted artificially, through fusion with a GAL4 DNA-binding domain, to basal promoters containing GAL4-binding sites fused to the thymidine kinase or the adenovirus major late promoters in vivo. Interestingly, other transcriptional repressors, such as Rb, inhibit specific basal promoters when targeted to them (36). Rb repression of basal promoters occurs via two different mechanisms. One mechanism is dependent on histone deacetylase activity. This is used for Rb repression of the adenovirus MLP (36). The second mechanism, which is used with the SV-40 enhancer, is independent of histone deacetylase activity and occurs by direct inhibition of transcription factors at the promoter (36). Further studies using in vitro transcription models to understand the molecular mechanism of Rb-mediated repression indicate that Rb prevents the TFIID-TFIIA complex from contacting the consensus TATA element (21). To understand in more detail how HSF-4a-mediated repression of basal transcription occurs, we performed experiments using purified HSF-4a and a purified in vitro transcription system. We demonstrate here that HSF-4a can inhibit basal transcription in this model system. HSF-4a inhibited transcription from core promoters with a consensus TATA element without HSE, although greater inhibitory activity was seen for promoters that contained HSE. This suggests that HSF-4a repression of basal transcription occurs through its interference with one of the factors involved in basal transcription. By using order-of-addition experiments, we demonstrate binding of HSF-4a to TFIIF. TFIIF is a complex of 74- and 30-kDa subunits, and our preliminary results suggest that the larger RAP74 subunit directly interacts with HSF-4a (data not shown). The RAP74 subunit of TFIIF has been shown to interact with transcriptional activators such as SRF and GTFs such as TAFII250, RAP30, TFIIB, and RNA polymerase II (24, 30, 43). The activation domain of SRF, for example, associates with amino acid residues 172–357 of RAP74 (30). It has therefore been suggested that the SRF interaction with TFIIF is required for SRF-activated transcription. Furthermore, since TFIIF binds RNA polymerase II through the RAP30 subunit, SRF could facilitate the recruitment of RNA polymerase II to the promoter or alter the conformation of the initiation complex (30). By using amino acid deletion studies, we were able to identify the region of interaction of TFIIF with HSF-4a. Deletion of N-terminal amino acid residues on the C-terminal side of residue 194 exerts weak to no inhibition of transcription. This suggests that the region that spans leucine zippers 1–3 of HSF-4a is the
The HSF-4a interaction with TFIIF could inhibit the ability of TFIIF to interact with other factors such as RNA polymerase II. HSF4a could function in preventing the proper assembly of preinitiation complexes as indicated by its inability to inhibit transcription from PICs that already contain TFIIF. Since the HSF-4b isoform contains the leucine zipper 1–3 region but also contains amino acid residues that differ from that of the HSF-4a isoform, HSF-4b could potentially generate a weaker interaction with TFIIF. Conversely, these differences in amino acid residues between HSF-4a and HSF-4b may be critical for the function of HSF-4b in its ability to activate transcription. Interestingly, addition of HSF-4b to an in vitro transcription reaction is capable of activating transcription in the range of concentration for HSF-4a that was found to be repressive to basal transcription. Since other members of the HSF family, namely HSF-1 and HSF-2, also contain leucine zippers 1–3 and have high homology to HSF-4b, it is conceivable that all HSF family members potentially interact with TFIIF to exert their positive effect on transcription. HSF-1 and HSF-2 also have other isoforms as well, but their isoforms lack a whole exon, and in all cases the amino acid sequence encoding the activation domain remains intact. More studies are needed to investigate the role of these various HSF isoforms in the regulation of heat shock protein gene expression in vivo.

Extensive studies indicate that the PIC assembly pathway differs from promoter to promoter and is dependent on both the specific elements involved as well as the promoter context. The TFIIF transcription factor, however, has been shown to be required for all transcriptional activators so far tested (24). Nevertheless, the requirements for the general transcription factors and the dependence of the HSE promoter used was tested to ensure the requirement for TFIIF of such promoters. We found that TFIIF was required for transcription from basal promoters, since elimination of recombinant TFIIF from PIC assembly significantly decreased basal transcription. From this and other results presented, a hypothetical model is presented in Fig. 9 to describe our findings. Under the conditions whereby HSF-4a binds an HSE, proper assembly of the preinitiation complex is hindered or prevented, perhaps by interfering with the recruitment of RNA polymerase II and other factors to the complex through its association with TFIIF. HSF-4b, on the other hand, could also potentially interact with TFIIF, but this interaction has a positive, rather than a negative, regulatory role on transcription.

In conclusion, we have presented evidence of an interaction of the transcriptional repressor HSF-4a with TFIIF, leading to inhibition of transcription. Future experiments will help to determine better the details of the HSF-4a-TFIIF interaction and the mechanism by which HSF-4a functions as transcriptional repressor.

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