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Heat Shock Factor-4 (HSF-4a) is a Repressor of HSF-1 Mediated Transcription

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Abstract Heat shock transcription factors (HSFs) regulate the expression of heat shock proteins and other molecular chaperones that are involved in cellular processes from higher order assembly to protein degradation and apoptosis. Among the human HSFs, HSF-4 is expressed as at least two splice variants. One isoform (HSF-4b) possesses a transcriptional activation domain, but this region is absent in the other isoform (HSF-4a). We have recently shown that the HSF-4a isoform represses basal transcription from heterologous promoters both in vitro and in vivo. Here we show that HSF-4a and HSF-4b have dramatically different effects on HSF-1-containing nuclear bodies, which form after heat shock. While the expression of HSF-4b colocalizes with nuclear granules, the expression of HSF-4a prevents their formation. In addition, there is a concurrent reduction of HSF-1 in the nucleus, and there is reduction in its DNA binding activity and in HSE-dependent transcription of a reporter gene. To better understand the mechanism by which HSF-4a represses transcription, we inducibly expressed HSF-4a in cells and found that HSF-4a binds to the heat shock element (HSE) during attenuation of the heat shock response. Thus HSF-4a is an active repressor of HSF-1-mediated transcription. This repressor function makes the HSF-4a isoform unique within the HSF family. J. Cell. Biochem. 82: 692–703, 2001.

Key words: heat shock factor-4; transcription

An understanding of the upstream regulators and signaling pathways that lead to the expression of heat shock proteins (HSPs) remains elusive. It is of special interest to determine the role of HSPs and other molecular chaperones in cellular transformation, since many human cancer cells express unusually high levels of inducible HSPs. A frequently overlooked group of regulatory molecules are the transcriptional repressors, which have important roles during development, differentiation, and cell growth. One of the several major classes of repressors consists of DNA binding proteins. The targets of such repressors can be activator proteins, coactivator proteins, corepressors, or other proteins that interact with basal transcription factors [Hanna-Rose and Hansen, 1996; Fisher and Caudy, 1998]. We have previously shown that a particular isoform of a heat shock transcription factor (HSF) inhibits basal transcription from a heterologous promoter by binding to basal transcription factor TFIIF [Frejtag et al., 2001]. The HSF-4a and 4b isoforms are generated by alternate splicing of exons 8 and 9, leading to different amino acid sequences in this region. In addition, HSF-4b contains 30 additional amino acids. Sequences in the *C*-terminal ends of the molecule are similar [Nakai et al., 1997; Tanabe et al., 1999]. While the HSF-4b isoform can activate transcription, the HSF-4a isoform has the ability to repress transcription and could potentially act as an active repressor of other HSFs through its ability to either directly bind to the HSE or to oligomerize with other members of the HSF family [Nakai et al., 1997; Tanabe et al., 1999; Frejtag et al., 2001]. In this study, we demonstrate that the differences in amino acid sequence that exist between the repressor and

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activator isoforms of HSF-4 have dramatic effects on the ability of HSF-1 to activate transcription. Expression of HSF-4a in cells in the first few hours after heat shock prevents the accumulation and formation of HSF-1 nuclear granules, which we have previously shown to colocalize with pre-nascent mRNA in the nucleus after heat shock [He et al., 1998; Dai et al., 2000]. This leads to a reduction in the heat-induced HSF-1-driven transcriptional activity of a reporter gene. In addition, we show that HSF-4a contains a repression motif that is capable of repressing a heterologous transcriptional activator. This is the same region that we have previously shown to repress basal transcription by binding to TFIIF transcription factor [Frejtag et al., 2001]. This is in contrast to the HSF-4b isoform, which itself colocalizes with HSF-1 nuclear granules. The effect of HSF-4 on HSF-1-mediated transcription, therefore, appears to occur at multiple levels.

MATERIALS AND METHODS

Cell Culture

H1299 is a human lung carcinoma cell line. H1299 cells, previously transfected with a tetracycline-responsive transcriptional activator (tTA), were stably transfected with a plasmid containing pTRE-hemaglutinin (HA) tagged-HSF-4a and pBabe, which contains the puromycin selectable marker. Transfected H1299 cells were routinely maintained in the presence of 3 μg/ml of tetracycline, but were cultured in the absence of tetracycline to induce HSF-4a expression for 48 h before the start of an experiment. Note that the clones of HSF-4a-expressing H1299 cells used in the experiments shown in Figures 2 to 4 contained ~20–30% cells that did not express HSF-4a.

Plasmids

All GAL4-HSF-4a chimeric fusion proteins were based on the parent vector pSG424, which contains the SV40 early promoter and origin of replication and the first 147 amino acid residues of the yeast protein GAL4 DNA binding domain followed by a multiple cloning site. The plasmid pSGVP16 contains the SV40 early promoter upstream of the first 147 amino acid residues of yeast protein GAL4 fused to amino acid residues 413–490 of the human herpes simplex virus type I transcriptional activator, VP-16 [Sadowski et al., 1988]. VP16 cDNA was amplified from pSGVP16 plasmids by PCR using primers that incorporated a Kpn I site for the amino-terminal primers and an Xba I site for the carboxyl-terminal primers. Primers to amplify VP-16 were:VP16-F413Kpn I: 5′-GGGG TACCCCGCCCCCCCGACCG ATGTC-3′; VP16-R490Xba I:5′-ATGCTCTAGACCCACCG-TACTCGTCAAT-3′. The PCR product was subcloned in-frame into pSG424 following the GAL4 DNA binding domain (1–147) and was called pSG424-VP16. The PCR products containing deleted fragments of HSF-4a were inserted in-frame into pSG424-VP16 following GAL4 (1–147) (at EcoR I site) and 5′ to VP16 (at Kpn I site). The numbers for each primer represent the location of the base pairs in the HSF-4a that were fused to the GAL4 DNA binding domain. For the Reverse primers, the numbers represent the base pairs at the carboxyl-terminal end of the protein. The HSF-4a Forward and Reverse primers were as follows: HSF-4-F373EcoR I 5′-GGAATTCGCGCTGC-GCGGCGACGACGGC-3′; HSF-4F582 EcoR I 5′-GGAATTCTTTGGGCCACTTCAGGCGGG-GCCG-3′; HSF4-F811EcoR I 5′-GGAATTCA-CATCCCAGAAGACTCTCCATCCC-3′; HSF-4-F1242EcoR I 5′-GGAATTCAAGGACCCC-ACGCTCGGGGCC-3′;HSF-4-R582-Kpn I 5′-GGGGTACCGAGACACTGGATCAGCTTGCC-3′; HSF-4-R811Kpn I 5′-GGGGTACCCAGA-GATGATGGGGCCCCT-3′; HSF-4-R1070Kpn I 5′-GGGGTACCAGG TT CCACACTTTCTTGA-GG-3′; HSF-4-R1373 Kpn I 5′-GGAATTCA-AGGACCCCACGCTCGGGGCC-3′.

The expression vectors, pcDNA3-HA (hemaglutinin) epitope tagged-HSF-4a, -4b, or -4c, or pTRE-HA-tagged HSF-4a, were constructed by PCR amplification of HSF-4 cDNA using primers that incorporated nucleotides coding for the HA tag [Dai et al., 2000]. HSF-4c was amplified from cDNA prepared from MCF-7 and LS174t, breast and colon carcinoma cell lines, respectively, as a 600 bp *N*-terminal fragment beginning at ATG. For this clone the missing *C*-terminal end was constructed similarly to HSF-4a. The HSF-4b isoform was amplified by PCR from the cDNA prepared from normal human skeletal muscle cells (BioWittekar). The appropriate restriction enzyme sites were included in the primers, and the resulting fragments were subcloned into expression vectors. The sequences of all PCR fragments were confirmed by sequencing.

Transient Transfection Assays and Reporter Plasmids

Transient transfections were performed by Lipofectamin (Gibco, BRL) as previously described [Dai et al., 2000]. pGAL4(×5)-E1b-CAT containing five GAL4 DNA binding sites upstream of a minimal promoter E1b TATA box drove the expression of the CAT reporter genes.

Indirect Immunofluorescence and Confocal Microscopy

Transiently transfected H1299 cells or H1299 cells inducibly expressing HSF-4a were plated in 8-chamber tissue culture slides and grown in the absence of tetracycline. After 48 h, cells were treated as described in the text, fixed with 4% paraformaldehyde, and stained and analyzed as previously reported [He et al., 1998; Dai et al., 2000].

Electrophoretic Mobility Shift Analysis

Electrophoretic mobility shift analysis (EMSA) using whole cell extracts has been described in detail previously [He et al., 1998; Dai et al., 2000]. The mutant double-stranded oligonucleotide used as nonspecific competitor was 5′-ATTCGATCGGGGCGGGGCAGC-3′. The double stranded oligonucleotide containing GAL4 binding site was as follows: 5′-GGGATCTCGGAGTACTGTCCTCCGA-3′ and 5′-GGTCGGAGGACAGTACTC CGAGAT-3′ [Newton et al., 1996].

RESULTS

The Effect of HSF-4 Activator and Repressor Isoforms on Accumulation of HSF-1 Nuclear Granules After Heat Shock

We wanted to determine whether, and how, the heat-induced dynamics of the different HSF-4 isoforms affected the ability of HSF-1 to form nuclear granules after heat shock. Upon heat shock, the latent form of HSF-1 is translocated into the nucleus, forms trimers, and appears as several intensely staining nuclear granules [Cotto et al., 1997; He et al., 1998]. Thus, we performed immunofluorescent and confocal microscopic analysis of transiently transfected HA-tagged HSF-4b, the isoform which is a transcriptional activator, and HSF-4a, which contains no activation domain and therefore, may act as a transcriptional repressor (Fig. 1A). We also transiently transfected another isoform of HSF-4, which we called HSF-4c, that we have recently isolated from MCF-7 and LS174t, human breast and colon carcinoma cells, respectively. This partial cDNA clone contains an in-frame 63 base pair deletion in the trimerization domain of the protein, which is required for its DNA binding activity (Fig. 1A) as determined by EMSA (data not shown). Thus, H1299 cells, which lack endogenous HSF-4 as detected by immunoblotting, were transfected with HA-tagged-HSF-4a, HSF-4b, or HSF-4c. Forty-eight hours after transfection, cells were left untreated or were heated at 45°C for 30 min and were incubated at 37°C for 4 h of recovery. Cells were then stained for the various HSF-4 isoforms and endogenous HSF-1, and were analyzed by confocal microscopy. Indirect immunofluorescence analysis of endogenous HSF-1 in all human cells tested so far shows HSF-1 appearing as multiple, intensely-staining granules after heat shock [He et al., 1998]. These granules colocalize with prenascent mRNA for as long as 4–8 h post heating. During recovery from heat shock, the granules disappear, which correlates with the loss of HSF-1 DNA binding activity [He et al., 1998; Dai et al., 2000]. As the data in Figure 1B indicate, the transcriptional activator HSF-4b isoform colocalizes with HSF-1 nuclear granules after heat shock, while the potential repressor HSF-4a isoform prevents HSF-1 from forming characteristic nuclear granules. HSF-4c, which lacks DNA binding activity, does not in anyway interfere with formation of HSF-1 nuclear bodies. In similar experiments, immunofluorescence analysis of unheated controls shows that HSF-1, and the HSF-4a, -4b, and -4c isoforms all display diffuse nuclear staining (data not shown).

To further analyze whether there could be an interaction between HSF-1 and HSF-4a, we used an in vitro transcription/translation coupled reaction using expression plasmids for either HSF-4a or HSF-1. Using the in vitro translated HSF-1 or HSF-4a, we performed electrophoretic mobility shift assays where a constant amount of HSF-1 was added to the increasing concentrations of HSF-4a. The fact that HSF-4a and HSF-1 have opposing DNA binding properties at different temperatures in vitro we chose a temperature (37°C) where both proteins are competent to bind to HSE containing oligonucleotides [Nakai et al., 1997 and data not shown]. In vitro translated lysates

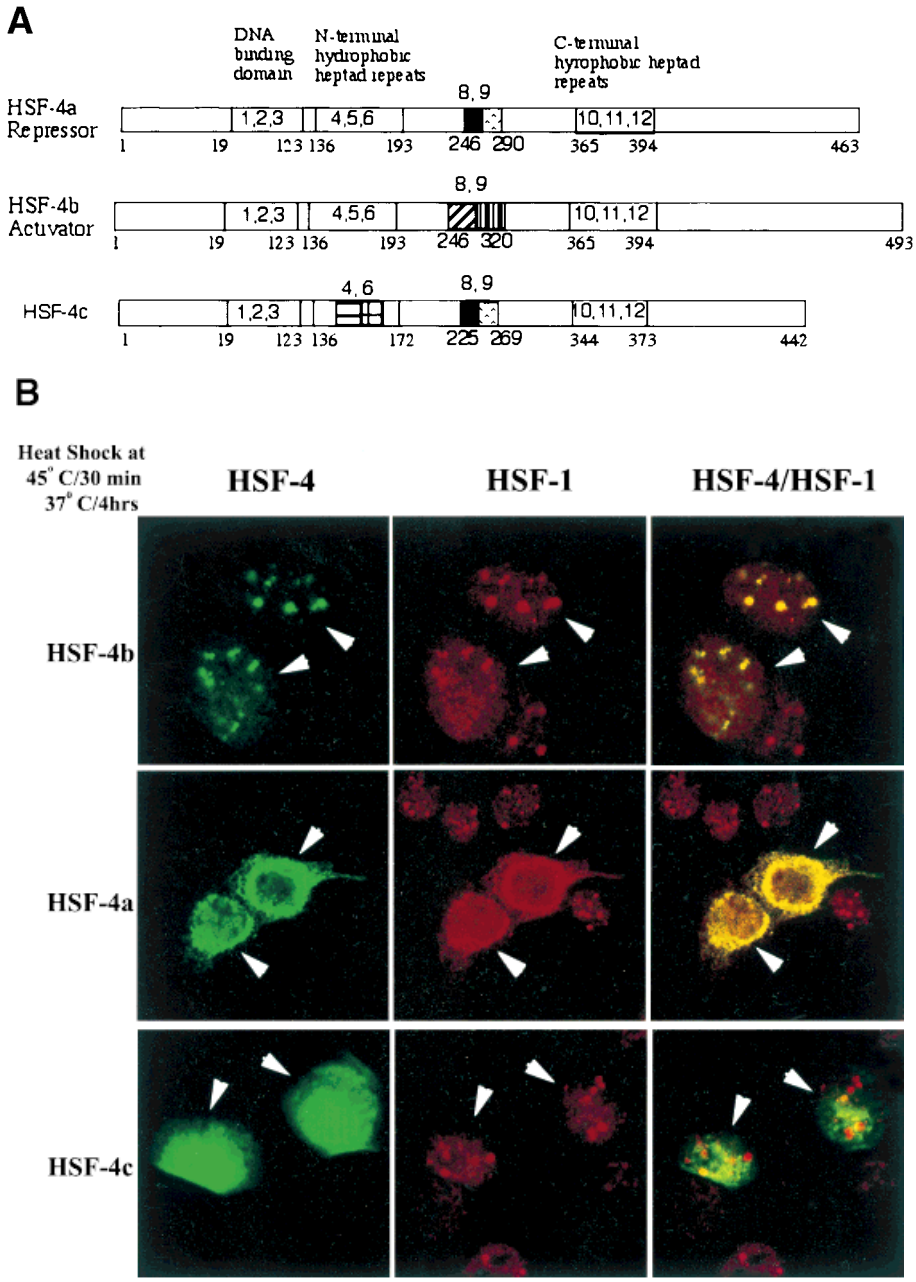

Fig. 1. HSF-4a, -4b, and -4c affect HSF-1 nuclear granules differently after heat shock. **A:** Structure of HSF-4a, HSF-4b, and HSF-4c cDNAs. Numbers above or inside the boxes represent segments encoded by different exons. Numbers below the boxes represent position of the amino acids relative to ATG. **B:** Representative immunofluorescence photographs (magnification, ×1000) of transiently transfected H1299 cells with HA-tagged HSF-4a, HSF-4b, or HSF-4c. Forty-eight hours after transfection, cells were left as untreated (control) (data not shown) or heated at 45°C for 30 min and allowed to recover at 37°C for 4 h. Expression of HSF-4 was detected with antibody to HA and FITC-conjugated secondary antibody. Endogenous HSF-1 was detected with antibody to HSF-1 and Texas Red-conjugated secondary antibody. The last column is when the first two panels were superimposed to show colocalization (as indicated by yellow staining) of the HSF-1 or HSF-4 proteins. Arrows in the panels indicate the cells with overexpressed HSF-4a, -4b, or -4c and the same cells stained for HSF-1. Note that HSF-1 in cells that do not express HSF-4a, -4b, or -4c show nuclear granules.

containing HSF-1 or HSF-4a were incubated at 37°C, in an experiment that contained either increasing amounts of HSF-4a in the absence of HSF-1, or the same increasing amounts of HSF-4a with an equal amount of HSF-1 added to each reaction. The quantitation of the EMSA indicate an increase (2-fold) in DNA binding ability of these two proteins as an equal amount of HSF-1

is added to increasing amounts of HSF-4a. The data indicated that the complexes formed appear to be heterogenous in nature and perhaps consist of a combination of the two proteins as trimers. Larger complexes than trimers could also conceivably be present (data not shown).

To further examine whether HSF-1 and HSF-4a could be interacting in vitro or in vivo, we performed in vitro pull-down experiments as well as immunoprecipitation followed by immunoblotting of HSF-1 and HSF-4a after cotransfection of expression constructs of these factors into H1299 cells. Immunoprecipitation experiments were performed with both untreated or heated cells that were incubated at 37°C for various lengths of recovery times. In either types of experiments, no interaction between HSF-1 and HSF-4a could be detected (data not shown). The results shown in Figure 1, panel B, therefore, could not be solely interpreted that there could be an interaction between HSF-1 and HSF-4a, especially because the addition of HSF-1 to increasing amounts of HSF-4a could conceivably stabilize homotrimerization of each protein. Similar experiments as above were not performed with HSF-4b isoform, which could potentially show a more stable interaction with HSF-1.

HSF-4a Represses the Formation of HSF-1 Nuclear Granules and Inhibits HSF-1 Mediated Transcription

To further analyze the mechanism of HSF-4a repression on HSF-1, we generated H1299 cells expressing HSF-4a under the control of a tetracycline-inducible promoter. In this cell line, ~70% of the cells can be induced to express HSF-4a. Forty-eight hours after removal of tetracycline, a time chosen so that sufficient amounts of HSF-4a could accumulate, as determined by immunoblotting (data not shown), cells were left untreated or were heated at 45°C for 5 or 30 min, or were heated at 45°C for 30 min and incubated at 37°C for 4, 8, and 24 h for recovery. These recovery times were selected for several reasons such as: 1) the DNA binding activity of HSF-1 is detectable for as long as 12–16 h post-heating and disappears entirely by 24 h post-heating [He et al., 1998; Dai et al., 2000], 2) the HSP-70 mRNA and protein continue to accumulate for at least 8–12 h [Mivechi et al., 1991; He et al., 1998; Dai et al., 2000], 3) the appearance of HSF-1 granules becomes more distinct at 4–8 h and the number of cells containing such granules peaks at 4–8 h post-heating (at this time over 90% of cells contain HSF-1 granules), whereas granules are no longer present at 24 h post-heating [He et al., 1998; Dai et al., 2000].

After heat shock or after specific periods of recovery, cells were fixed, stained, and examined by indirect immunofluorescence microscopy. The results show that in unheated cells the overexpressed HSF-4a accumulates in the nucleus. Endogenous HSF-1, however, is found in the cytoplasm, with some found in the nucleus (Fig. 2, control). Almost immediately after the exposure of the cells to heat shock (Fig. 2, 45°C, 5 and 30 min), in those cells that express HSF-4a, HSF-1 nuclear granules do not appear (as indicated by an arrow), while HSF-1 nuclear granules appear in cells not expressing HSF-4a. As cells are kept at 37°C to recover from heat shock, cells expressing HSF-4a continue to show no nuclear granules while those cells not expressing HSF-4a show nuclear granules (Fig. 2, 4 and 8 h post-heating). At these recovery times after heat shock, HSF-4a appearance becomes further diffuse and some staining also appears in the cytoplasm (Fig. 2, 4 h). Interestingly, HSF-1 staining also becomes diffuse and is almost undetectable in the cells during the first 4–8 h of recovery from heat shock (Fig. 2, 4 h). After 8 h of recovery after heat shock, HSF-4a appears to begin to return into the nuclei and HSF-1 can be detected in the nuclei in cells that express HSF-4a (Fig. 2, 8 h). After 24 h of recovery time from heat shock, HSF-4a returns to the nucleus and HSF-1 again can be observed in a staining pattern similar to that observed in the untreated control cells (Fig. 2, 24 h).

The absence of HSF-1 nuclear granules and reduction in HSF-1 staining after heat shock in cells expressing HSF-4a (Fig. 2) prompted us to determine at the biochemical level whether there was also a reduction in HSF-1 DNA binding activity. For this, cytoplasmic or nuclear fractions of control or heated H1299 cells cultured in the presence or absence of tetracycline were prepared and DNA binding ability of HSF-1 was analyzed using EMSA (Fig. 3A). Quantitation of the data indicates a 34 and 46% reduction in HSF-1 DNA binding activity of the nuclear extracts of heat-treated cells that expressed HSF-4a compared to cells that did not express HSF-4a following recovery at 37°C for 1 or 4 h, respectively. No DNA

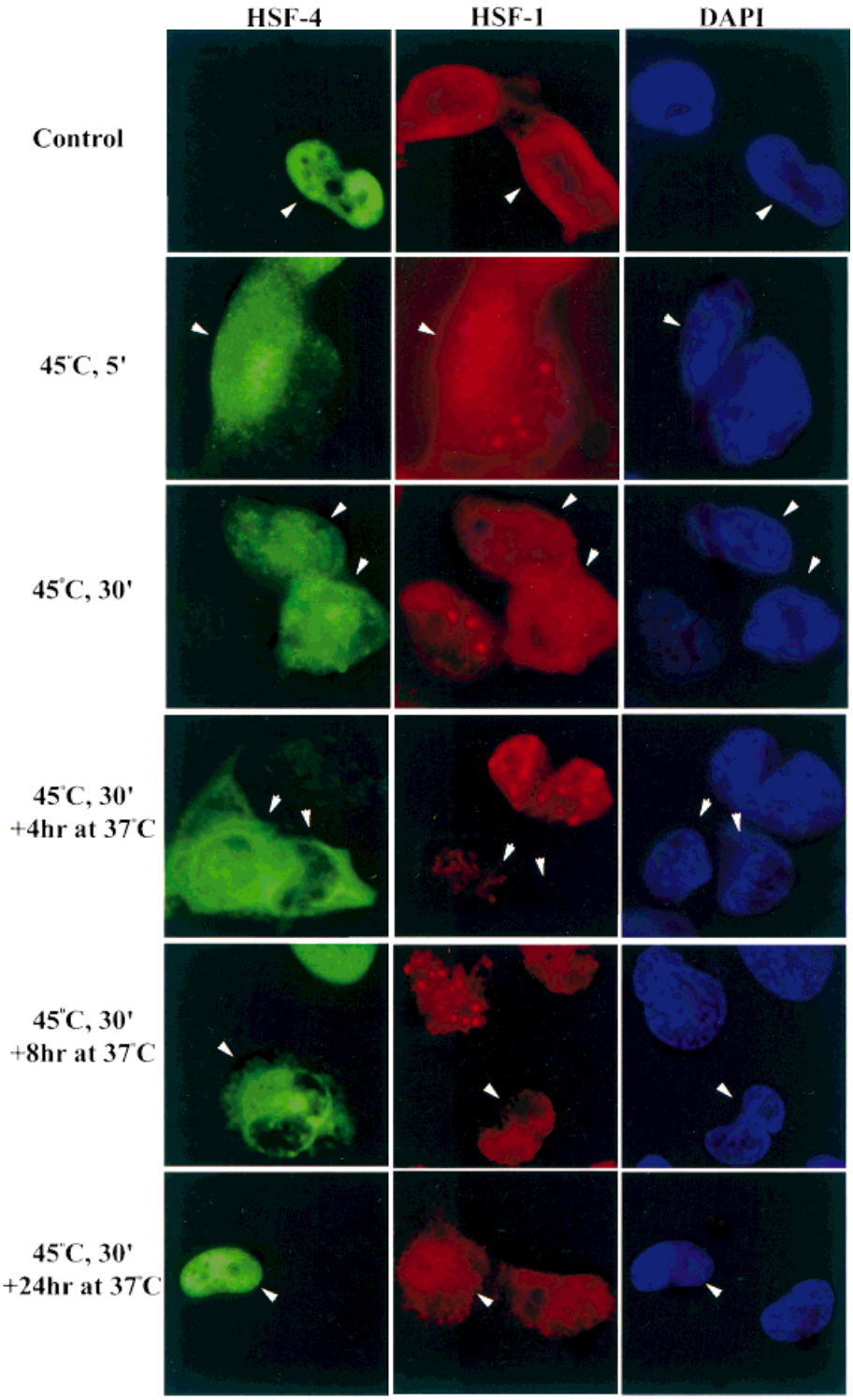

Fig. 2. Indirect immunofluorescence analysis of endogenous HSF-1 and HA-HSF-4a. Representative immunofluorescence photographs (magnification, ×1000) of cells stably transfected with plasmids containing pTRE-HA-HSF-4a. H1299 cells were grown in the absence of tetracycline for 48 h to increase HSF-4 expression. Cells were left as untreated (control), or heated at 45°C for 5 or 30 min, or were heated at 45°C for 30 min and allowed to recover at 37°C for 4, 8, or 24 h. HSF-4a (first column) was detected with antibody to HA and FITC-conjugated secondary antibody. The endogenous HSF-1 (middle column) was detected with antibody to HSF-1 and Texas Red-conjugated secondary antibody. Nuclei were detected by DAPI (last column). Arrows in the panels of first column indicate the cells expressing HSF-4a and arrows in the middle and third columns indicate the same cells stained for HSF-1 or DAPI.

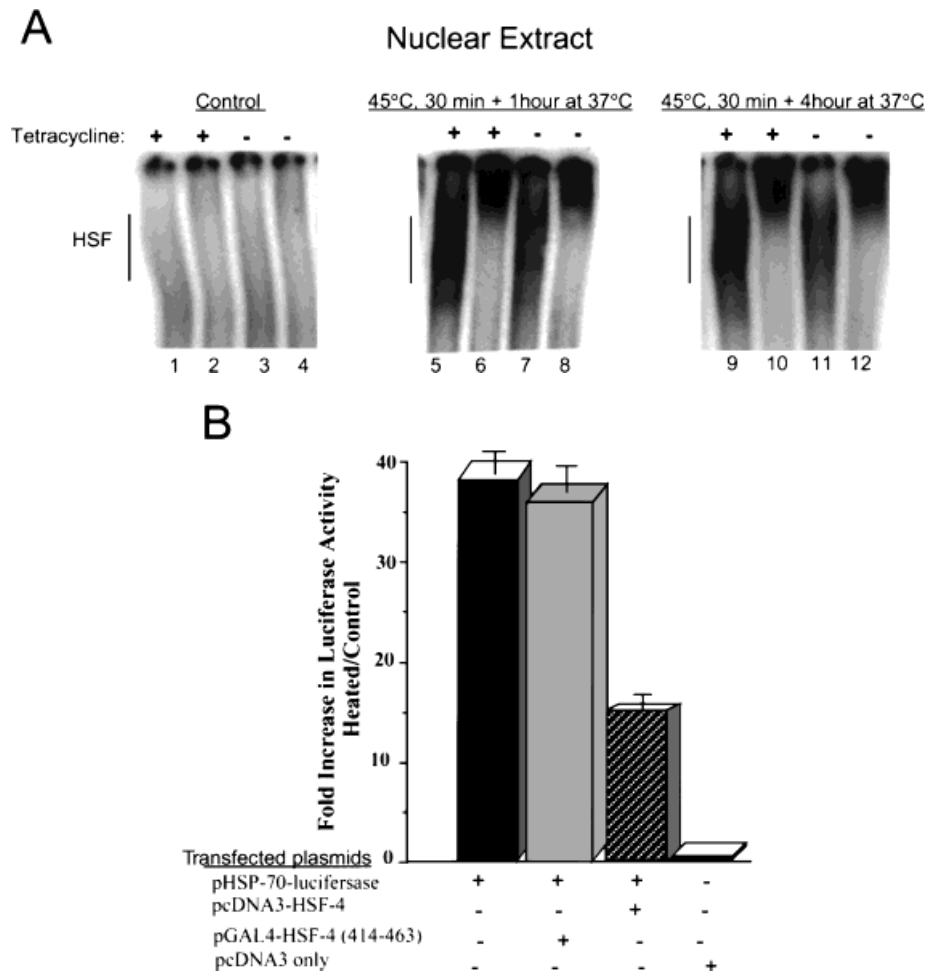

Fig. 3. HSF-4a reduces HSF-1's DNA binding ability in the nuclear fraction and reduces HSF-1's mediated transcription. **A:** H1299 cells grown in the presence (+) or absence (−) of tetracycline for 48 h were untreated or heated at 45°C for 30 min and were left to recover at 37°C for 1 or 4 h. Nuclear and cytoplasmic fractions were prepared according to manufacturer's instruction (Pierce, Rockford, IL), and equal amounts of nuclear extracts were analyzed by EMSA. Lanes 1–4, 5–8, and 9–12 are nuclear extracts of control or heated cells with 1 or 4 h of recovery after heat shock. Lanes 2, 4, 6, 8, 10, and 12 are the same extracts as in lanes 1, 3, 5, 7, 9, and 11, respectively, but the extracts were incubated in the presence of antibody to HSF-1. Note that antibody to HSF-1 supershifts all binding activity, suggesting that all binding is due to HSF-1 at these time points. **B:** H1299 cells were transiently cotransfected with plasmids containing HSP70-luciferase, Renilla luciferase with or without expression constructs pcDNA3-HA-HSF-4 (amino acid residues 1–463, wild-type), or pGAL4-HSF-4 (414–463), or pcDNA3 only. Twenty-four hours after transfection, cells were serum-starved with 0.5% FCS for 24 h to reduce background activity. Cells remained untreated or were heated at 45°C for 30 min and allowed to recover at 37°C for 6 h for expression of HSP-70-luciferase. Luciferase activity was determined in 20 μg of cell lysate for each sample. Data is presented as fold increase of heated/control and adjusted for the transfection frequency using Renilla luciferase. Cells transfected with plasmid construct pcDNA3 was used as a negative control in one group as indicated.

binding activity was observed in the cytoplasmic extracts of any of the groups (data not shown). This suggests that the disappearance of HSF-1 from the nuclei of heat-treated cells that express HSF-4a coincides with a reduction in DNA binding activity of HSF-1, when compared to cells that do not express HSF-4a.

To determine if the disappearance of HSF-1 nuclear granules and the reduction in heat-induced HSF-1 DNA binding activity observed in the nuclei of cells expressing HSF-4a inhibits HSF-1-driven transcription, transient cotransfection experiments in H1299 cells were performed using expression plasmids containing full length HSF-4a cDNA or GAL4-HSF-4a (residues 414–463) (used as a negative control) and reporter plasmids containing a human HSP-70 promoter fused to a luciferase gene.

Cells were left untreated or were heated and luciferase activity was determined. Results indicate that the activity of HSP-70-luciferase is increased after heat shock and 6 h recovery; however, its activity is dramatically reduced in cells that expressed full length HSF-4a (Fig. 3B). There was no effect on HSP-70 luciferase activity when cells were transfected with plasmids containing truncated GAL4-HSF-4a (residues 414–463) fusion protein (Fig. 3B) or cells transfected with empty vector. These results indicate that full length HSF-4a inhibits the heat-induced HSF-1-driven transcriptional activity.

HSF-4a Binds HSE During the Attenuation of the Heat Shock Response

The EMSA analysis of in vitro translated HSF-1 suggests that the major fraction of the protein does not bind DNA constitutively, but HSF-1 DNA binding activity is increased after lysate is incubated at 37 or 42°C for 1 h. In contrast, in vitro translated HSF-4a binds DNA constitutively and its DNA binding activity is sharply reduced when the lysate is incubated at 37 or 42°C for 1 h [data not shown, Nakai et al., 1997]. Both HSF-1 and HSF-4a, however, exhibit constitutive DNA binding activity when expressed at high levels in cells, perhaps due to the lack of sufficient negative regulators [Mivechi and Giaccia, 1995; Nakai et al., 1997]. Since the DNA binding properties of HSF-4a, in relation to HSF-1, in cells under control or heat shock conditions is not understood, we used H1299 cells that inducibly express HSF-4a to examine the kinetics of HSF-1 and HSF-4a DNA binding activity after heat shock. Forty-eight hours after removal of tetracycline, so that moderate and sufficient amounts of HSF-4a could accumulate, cells were left untreated or were heated and incubated at 37°C for up to 24 h. This period of recovery was selected because the heat-induced DNA binding activity of HSF-1 is retained for several hours [He et al., 1998]. The DNA binding activity of both HSF-1 and HSF-4a were examined by EMSA (Fig. 4A). The results show that HSF-1, but not HSF-4a, binds oligonucleotides containing an HSE immediately after heat shock and during the recovery phase for up to 16 h (lane 5, 8, 11, and 14). The DNA binding activity of HSF-1 begins to decline by 16–24 h of recovery after heat shock (lanes 14 and 17), at the same time that HSF-4a DNA binding activity begins to appear (lanes 15 and 18). This phenomena is only observed in cells expressing HSF-4a and not in cells that are

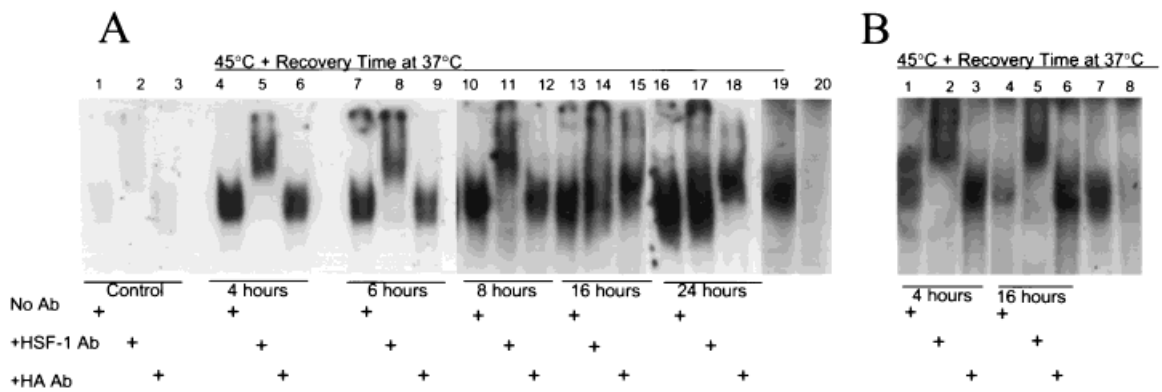

Fig. 4. HSF-4 binds HSE during the attenuation of the heat shock response. **A:** H1299 cells expressing pTRE-HA-HSF-4 were grown in the absence of tetracycline for 48 h. Cells were then left untreated or were heated at 45°C for 30 min and incubated at 37°C for recovery. At times indicated, cells were lysed and extracts prepared. Equal amounts of protein (10 μg) were incubated at 4°C with appropriate antibody for 20 min and then analyzed by EMSA using a ^{32}P-labeled HSE-containing DNA fragment. **Lanes 1–18:** Extracts from untreated cells or from cells heated that had recovered at 37°C for 4, 6, 8, 16, or 24 h were incubated without any antibody (lanes 1, 4, 7, 10, 13, and 16), with antibody to HSF-1 (lanes 2, 5, 8, 11, 14, and 17), or with antibody to HA to detect HSF-4 (lanes 3, 6, 9, 12, 15, and 18). **Lanes 19** and **20** are extracts from cells heated that had recovered at 37°C for 4 h and were incubated with nonspecific serum or with 200× excess unlabeled HSE as competitor respectively. **B:** H1299 cells that were cultured in the presence of tetracycline and therefore do not express HSF-4a are presented as a control for panel (A). Cells were treated as in (A). **Lanes 1, 2,** and **3:** Extracts from cells heated that had recovered at 37°C for 4 or 16 h were incubated without any antibody (lanes 1 and **4**), with antibody to HSF-1 (lanes 2 and **5**), or with antibody to HA to detect HSF-4 (lanes 3 and **6**). Lanes **7** and **8** are the same extracts from cells heated that had recovered at 37°C for 4 h and incubated with nonspecific serum (lane 7) or with 200× excess unlabeled HSE (lane 8) as competitor.

cultured in the presence of tetracycline and, therefore, do not express HSF-4a (Fig. 4B). H1299 cells cultured in the presence of tetracycline were heated and allowed to recover at 37°C for 4 or 16 h and the HSF-1 and HSF-4a DNA binding abilities were analyzed by EMSA. The results indicate that while antibody to HSF-1 supershifts HSF-1 at 4 and 16 h post-heating (lanes 2 and 5), antibody to HSF-4a has no effect (lanes 3 and 6).

HSF-4a Contains a Repression Domain That Inhibits the Activity of a Heterologous Chimeric Transcriptional Activator, VP16

Most active repressors, in addition to containing site-specific DNA binding ability, have a small repressor domain that is often rich in alanine, glutamine, proline, or charged amino acids [Hanna-Rose and Hansen, 1996]. While the *N*-terminal heptad repeat in the HSF-4a protein is hydrophobic and charged, the *C*-terminal fragment contains many alanine, proline, and glutamine residues, suggesting that both termini could have repressor function. To investigate this, we constructed a series of mutants where different fragments of HSF-4a were fused between the yeast GAL-4 DNA binding domain and the VP16 transcriptional activation domain [Sadowski et al., 1988] (Fig. 5A). Immunoblot and EMSA analyses using antibodies to VP16 or GAL4 show that all expression constructs expressed fusion proteins of the predicted molecular weight (Fig. 5B) and exhibited GAL4 DNA binding ability (Fig. 5C). These constructs were transiently transfected into H1299 cells cotransfected with plasmids containing the GAL4(×5)-E1b-CAT reporter construct and plasmids containing firefly luciferase as an indicator of transfection frequency. Forty-eight hours after transfection, CAT expression was determined in cells heated and left to recover at 37°C for 6 h, or in cells that were incubated under normal physiological growth conditions. Constructs containing amino acids 124–193, 124–269, and 124–361 conferred the most inhibitory effects on the VP16 activation domain (Fig. 5D and E). This portion of HSF-4a encodes the *N*-terminal hydrophobic heptad repeats, which extend from amino acid residues 137–194. Although constructs containing residues close to the *C*-terminal end are also inhibitory, for example residues 124–269 and 124–361, the inhibition is lost when residues 124–193 are deleted. The control plasmids pSG424, containing GAL4 DNA binding domain cotransfected with reporter plasmids GAL4(5×)-E1b-CAT, showed almost no CAT expression in untreated or heated cells. Interestingly, HSF-4a repressor activity remained essentially the same in control or heated cells.

DISCUSSION

The mechanisms underlying HSF-1 transcriptional activation and deactivation are poorly understood. Several recent reports indicate that phosphorylation of specific serine residues in the regulatory domain of HSF-1 represses HSF-1 activity under normal physiological growth conditions [Chu et al., 1996; Kline and Morimoto, 1997; He et al., 1998]. Interactions with other molecular chaperones, such as HSP-90, HSP-70, and HSF binding protein (HSBP1), also inhibit HSF-1 transcriptional activity [Morimoto, 1998]. One interesting aspect of HSF-1 regulation is the presence of multiple mechanisms that have evolved to repress an activated HSF-1 molecule. In this report, we describe a potential new mechanism of HSF-1 repression that occurs via the recently discovered heat shock factor, HSF-4. HSF-4 expresses as multiple isoforms [Tanabe et al., 1999] and often appears as aberrant splice forms in different tumor cell lines that we have analyzed (e.g., HSF-4c reported here). In order to investigate the function of the repressor isoform of HSF-4, we expressed HSF-4a under the control of an inducible system. Our results indicate that cells inducibly expressing HSF-4a do not show HSF-1 nuclear granules after heat shock. This is unique to HSF-4a, since the expression of HSF-4b, which possesses an activation domain and is able to activate HSE-driven transcription, causes the formation of characteristic nuclear granules after heat shock. HSF-4b-induced nuclear granules colocalize with HSF-1 granules and the kinetics of recovery of both types of granules after heat shock coincide (data not shown). The composition of these nuclear granules has not been well established. However, we previously showed that the granules are HSF-1 molecules at the sites of transcription of pre-nascent mRNA after heat shock [He et al., 1998]. We note, however, that other investigators have not been able to identify HSP-70-specific transcripts in the granules [Jolly et al., 1997].

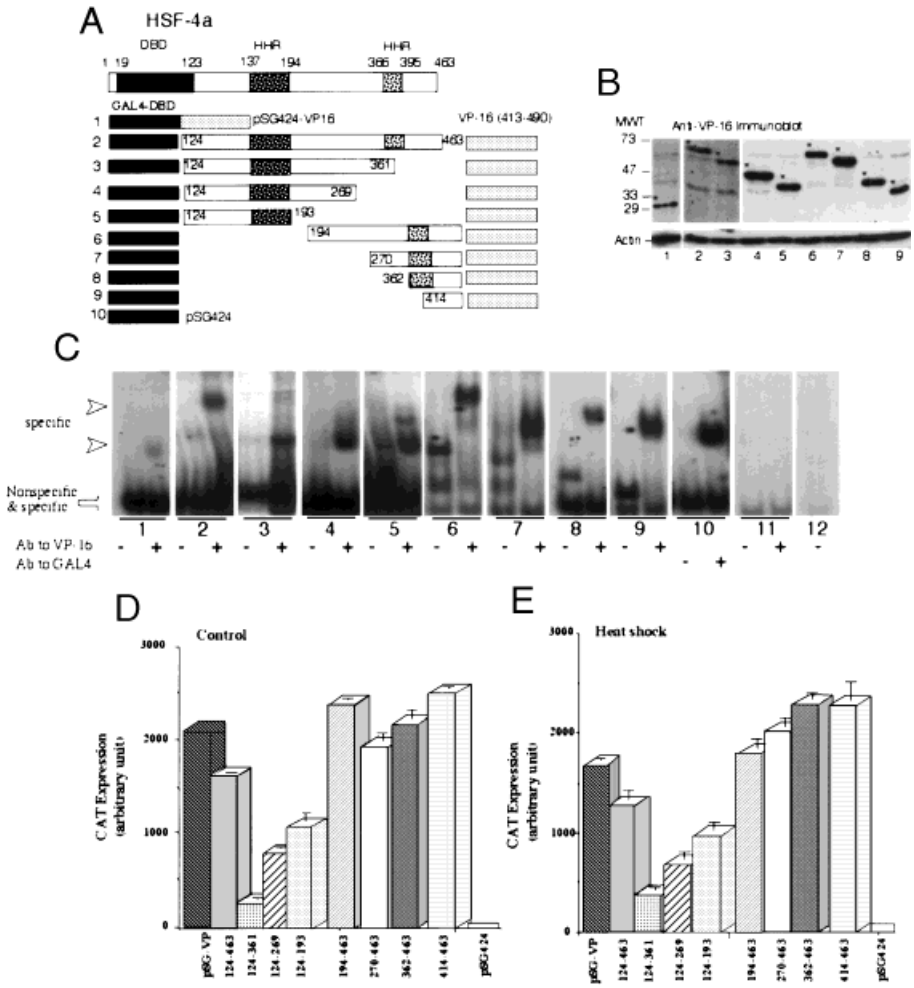

Fig. 5. HSF-4 contains a domain that represses the VP16 transcriptional activator. **A:** Constructs showing various deletion mutants of HSF-4 were fused between the DNA binding domain of yeast transcription factor GAL4 (1–147 amino acids) and the VP16 transcriptional activator. Constructs are named according to the amino acids present. DBD, DNA binding domain; HHR, hydrophobic heptad repeats. **B:** Immunoblot analysis of expression constructs (1–9) shown in (A). Forty-eight hours after transfection, 50 μg of cell lysates were analyzed by immunoblotting using antibody to VP16. Anti-actin antibody was used to indicate loading. * indicates appropriate molecular weight band for each fusion construct. Lanes represent constructs are shown in panel A. **C:** EMSA of constructs shown in (A). Forty-eight hours after transfection, 20 μg of cleared cell lysates were analyzed by EMSA. Plus samples are lysates incubated in the presence of antibody (1:20 dilution) for 20 min at 25°C. **Lanes 1** through **10** are constructs shown in panel A. **Lane 11** is untransfected cell lysate. **Lane 12** is the cell lysate as in **lane 9** but with 200× excess cold oligonucleotide added. ''Nonspecific and specific'' indicate the presence of a nonspecific band that is commonly present in mammalian cell lysates and binds oligonucleotide containing the GAL4 binding site (32). However, GAL4 protein in plasmid pSG424 as well as other small fragments in the same plasmid also run with the same mobility. Note that larger fragments, such as those show in lanes 5, 6, 7, 8, and 9 appear above the nonspecific band in − lanes. All fusion protein are supershifted by antibody to VP-16 or GAL4. The presence of multiple bands in − lanes most likely represent multimers. (D&E) Constructs shown in (A) were cotransfected into H1299 cells with the reporter construct GAL4(5×)E1b-CAT and firefly luciferase as an indicator of transfection frequency. Forty-eight hours after transfection, H1299 cells were left untreated (control) (**D**) or were heated at 45°C for 30 min (Heat Shock) (**E**) and incubated at 37°C for 6 h. Cells were lysed and CAT expression and luciferase activity determined from 120 μg and 20 μg of protein, respectively. Data are presented as CAT reporter expression relative to internal control.

Interestingly, HSF-1 granules also disappear when other HSF-1 repressors (such as GSK-3 or JNK) are transfected into cells [He et al., 1998; Dai et al., 2000]. In GSK3 expressing cells, HSF-1 granules appear after heat shock and then disappear very quickly once cells are incubated at 37°C [He et al., 1998]; however, in HSF-4a expressing cells, HSF-1 granules are unable to form. These results suggest a different mechanism of repression for GSK3, which phosphor-

ylates serine 303 in human HSF-1 protein and represses active HSF-1 protein, vs. HSF-4a, which affects the location of HSF-1 protein through an unknown mechanism.

HSF-4a inhibits HSF-1 transcriptional activity as determined by transient cotransfection of expression plasmids containing HSF-4a cDNA and the human HSP-70 promoter driving the luciferase reporter gene. These results support a view that HSF-4a inhibition of HSF-1 transcriptional activity could be in the context of binding to the HSE. HSF-4a may also reduce HSF-1 DNA binding activity in the first few hours after heat shock. This possibility is supported by the immunofluorescence experiments where HSF-1 is unable to form granules after heat shock as well as the decrease in HSF-1 DNA binding activity in the nuclear extracts of heated cells shown in Figure 3A. Our results are not sufficient to suggest that HSF-4a and HSF-1 interact directly and form heterocomplexes in vivo, but HSF-4a and HSF-1 appear to colocalize during first few hours of recovery after heat shock with a tentative reduction in HSF-1 protein in the nucleus. It is conceivable that HSF-1 and HSF-4a do not form heterocomplexes, but HSF-4a prevents HSF-1 from entering the complexes that constitute the HSF-1 granules that form after heat shock. HSF-4b, on the other hand, appears to stabilize and colocalize with HSF-1 nuclear granules after heat shock (Fig. 1B). Similar colocalization and stabilization of nuclear bodies have been observed for ARF, MDM2, and p53 protein. This association blocks the nuclear export and degradation of p53 and MDM2. Tumor-associated mutations in ARF exon 2, however, dissociate ARFs colocalization with the p53 and MDM2 proteins [Zhang and Xiong, 1999]. The possibility that the reduction in number of HSF-1 nuclear granules and the lower DNA binding activity observed in the nuclear extract of HSF-4a-expressing cells together with the lower reporter activity in cells expressing HSF-4a, could be associated with the destabilization and, perhaps, even the degradation of HSF-1 in the presence of HSF-4a after heat shock. We are now pursuing the possibility of ubiquitination and degradation of HSF-1 as it leaves the nuclei upon heat shock.

HSF-4a is also capable of repressing both herpes simplex virus thymidine kinase and adenovirus major late promoters, when targeted to these promoters via fusion of HSF-4a to the GAL4 DNA binding domain [Frejtag et al., 2001]. This silencing occurs by HSF-4a interacting with TFIIF, a component of the basal transcription machinery via the same region that also is capable of repressing a heterologous transcriptional activator (Fig. 5). The reason why the construct 124–463 is less repressive to VP16 than the construct 124–363 is not clear at the present time. A number of other repressors have been shown to repress basal transcription, including unliganded thyroid hormone receptor (TR), which binds the *N*-terminus of TFIIH, and the conserved region of the TATA binding protein, TBP [Fondell et al., 1993]. Other repressors such as the *Drosophila* gene *eve* and *kr* also interact with basal factors such as TBP and TFIIEγ [Um et al., 1995]. In the case of basal transcriptional repression by Rb, it has been shown that Rb also prevents the recruitment of preinitiation complexes by E2F [Ross et al., 1999]. A more detailed mechanism of repression mediated by Rb involves the recruitment of histone deacetylases (HDACs), which repress transcription by remodeling chromatin structure and assisting in the formation of nucleosomes.

In conclusion, HSF-4a appears to utilize several modes of repression that have been proposed for site-specific transcriptional repressors. Our hypothetical model for HSF-4a repression of HSF-1 is that first, as HSF-4a encodes a DNA binding domain that recognizes an HSE, it appears to bind the HSE alone, or less likely perhaps, it binds as a heterocomplex with HSF-1. This could prevent HSF-1 from making proper contact with the DNA, which is required for its activator function. Second, HSF-4a may also repress general transcription by interacting with TFIIF [Frejtag et al., 2001], which is consistent with its ability to interfere directly with the formation, or activity, of the basal transcription complex. Finally, although we show evidence that HSF-4a directly represses HSF-1 transcriptional activity in transient transfection assays, the question remains whether endogenous HSF-4a can function independently of HSF-1 as a repressor or silencer for specific HSE-containing genes. Previous studies have shown the reduction of HSP-90 and HSP-27 mRNA and to a certain extent, HSP-70 mRNA, in heated cells constitutively overexpressing high levels of HSF-4a [Nakai et al., 1997]. More studies will be required before we definitely know how HSF-4a carries out its repressor function.

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