MOLECULAR AND CELLULAR BIOLOGY, Aug. 1995, p. 4309–4318 Vol. 15, No. 8
0270-7306/95/$04.00+0

The Carboxyl-Terminal Transactivation Domain of Heat Shock Factor 1 Is Negatively Regulated and Stress Responsive

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Received 10 February 1995/Returned for modification 10 April 1995/Accepted 8 May 1995

We have characterized a stress-responsive transcriptional activation domain of mouse heat shock factor 1 (HSF1) by using chimeric GAL4-HSF1 fusion proteins. Fusion of the GAL4 DNA-binding domain to residues 124 to 503 of HSF1 results in a chimeric factor that binds DNA yet lacks any transcriptional activity. Transactivation is acquired upon exposure to heat shock or by deletion of a negative regulatory domain including part of the DNA-binding-domain-proximal leucine zippers. Analysis of a collection of GAL4-HSF1 deletion mutants revealed the minimal region for the constitutive transcriptional activator to map within the extreme carboxyl-terminal 108 amino acids, corresponding to a region rich in acidic and hydrophobic residues. Loss of residues 395 to 425 or 451 to 503, which are located at either end of this activation domain, severely diminished activity, indicating that the entire domain is required for transactivation. The minimal activation domain of HSF1 also confers enhanced transcriptional response to heat shock or cadmium treatment. These results demonstrate that the transcriptional activation domain of HSF1 is negatively regulated and that the signal for stress induction is mediated by interactions between the amino-terminal negative regulator and the carboxyl-terminal transcriptional activation domain.

The induction of HSP70 transcription during heat shock is a well-studied example of inducible gene expression because of the magnitude of transcriptional induction (20- to 50-fold) and the rapid kinetics of the response (29, 35). Heat shock genes are also induced in response to a wide range of environmental and physiological conditions including exposure to heavy metals, amino acids analogs, oxidative stress, fatty acids, and various therapeutic drugs (5, 9, 10, 20, 25, 27, 28, 37, 38, 43, 46, 50, 51, 58, 66). The stress-mediated induction of HSP70 gene transcription is dependent on the inducible activity of heat shock transcription factors (6, 40, 46, 51). Transcription of the HSP70 gene in vertebrate cells is also regulated in the absence of stress during the cell cycle and in response to growth factors, oncogenes, and developmental and differentiation cues through DNA-protein interactions with ubiquitous transcription factors such as CCAAT-binding factor (CBF), CCAAT transcription factor (CTF), simian protein 1 (Sp1), and activating transcription factor (ATF) that bind to the human HSP70 promoter (19, 22, 23, 30, 33, 34, 63, 64, 65). In addition, the interplay between basal transcription factors and heat shock factor (HSF) may be important for interactions with chromatin and for modulation of the inducible response (4, 8, 32, 57, 62).

Analysis of HSF genes from various organisms has identified a single HSF gene in yeasts (*Saccharomyces cerevisiae*, *Kluyveromyces lactis*, *Schizosaccharomyces pombe*) and fruitflies (*Drosophila melanogaster*), whereas multiple HSFs have been cloned from the tomato, chicken, mouse, and human genomes (16, 39, 44, 47–49, 61). In vertebrate cells, the HSFs are constitutively expressed; however, the DNA-binding and transcriptional activities are negatively regulated and induced in response to stress or certain developmental cues (39, 46, 50, 51). The activation of HSF1, the predominant stress-responsive factor, involves multiple steps, including translocation to the nuclear compartment, oligomerization from the latent monomer to a trimer, acquisition of DNA-binding activity, inducible serine phosphorylation, and subsequent transcriptional activity (6, 17, 46, 50, 51). The magnitude and kinetics of HSF1 activation depend on the severity of the stress, as exemplified by the temperature dependence of the heat shock response (3). Other inducers which activate HSF1 DNA-binding and transcriptional activity include transition heavy metals, arachidonic acid, antiinflammatory drugs, oxidative stress, and amino acid analogs; however, these treatments result in a delayed response compared with the rapid induction following heat shock (3, 25, 26, 36, 38, 63).

The nature of the cellular events that mediate HSF1 regulation remains obscure. However, a commonly held hypothesis links the appearance of malfolded nascent proteins with the activation of HSF1. Heat shock or stress-induced changes in the conformation of nascent or preexisting proteins would lead to the formation of stable chaperone-substrate complexes. Consequently, less HSP70 and other molecular chaperones would be available to participate in the events of protein synthesis, folding, and translocation and to prevent the aggregation of malfolded proteins. Therefore, the association of HSP70 with protein substrates can result in the release of HSF1 from negative regulation during heat shock. Notably, HSP70 has been implicated as a component in the negative regulation of its own activator, HSF1 (2, 7, 12, 13, 18, 35, 56). This hypothesis is consistent with studies that have demonstrated that overexpression of HSF1 in mouse NIH 3T3 cells results in constitutive DNA binding and trimerization (46).

How does HSF1 activate transcription? Previous studies of *S. cerevisiae* HSF (ScHSF) demonstrated that, unlike its higher eukaryotic counterparts, ScHSF exhibits constitutive DNA binding and yet is negatively regulated as a heat shock-responsive transcriptional activator (41, 53). During heat shock, ScHSF was shown to undergo a significant change in apparent molecular weight because of phosphorylation (55). Studies from several laboratories have confirmed this observation for

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other HSFs and have suggested that the conformational change that occurs during trimerization may include the unmasking and modification of a hidden activator sequence (46, 50, 60). Since the amino terminus of HSF1 contains the DNA-binding domain and trimerization domain, it was likely that the transcriptional activation domain would map to the carboxyl terminus. Recently, the activation domains of the yeast HSFs (*S. cerevisiae* and *K. lactis*) and the tomato HSFs have been localized to the carboxyl terminus, although there is no evidence for a conserved regulatory motif (14, 59). In *K. lactis*, the activator corresponds to a 32-amino-acid sequence (residues 592 to 623) with predicted α-helical character and acidic quality. In contrast, the *S. cerevisiae* carboxyl-terminal activation domain is approximately 180 amino acids long and appears to be bipartite, with one domain responsible for activation (residues 595 to 713) and the adjacent sequences (residues 713 to 780) having a supporting function for the activator (14). The tomato HSFs apparently have a another type of activation motif located in the carboxyl terminus which is composed of acidic sequences with a central tryptophan residue (59).

In order to understand the functional properties of vertebrate HSFs, specifically the features required for the activation of transcription and stress responsiveness by HSF1, we constructed chimeric transcription factors that replaced the endogenous HSF1 DNA-binding domain with the yeast GAL4 DNA-binding domain. In the course of this analysis, we mapped the activation domain of HSF1 to the extreme carboxyl terminus of the protein. We can conclude that this activation domain is negatively regulated by intramolecular interactions and induced by exposure to heat shock and cadmium in a manner analogous to that of the endogenous HSF1 protein.

MATERIALS AND METHODS

Plasmids and transfection constructs. The GAL4 (residues 1 to 147)-HSF1 fusion proteins were made in the vector pSG424, originally constructed in Mark Ptashne's laboratory (gift of N. Jones). pSG424 contains the simian virus 40 early promoter and origin of replication and the first 147 amino acid residues of the yeast GAL4 protein. The GAL4 region (residues 1 to 147) is followed by a polylinker that contains several unique restriction sites for the in-frame insertion of DNA sequences of interest. All constructs were subjected to dideoxy nucleotide sequencing across the junction between the GAL4 and HSF1 sequences to ensure that the HSF1 reading frame was maintained and to confirm the boundaries of the deletion mutants. All GAL4-HSF1 fusion constructs used in this study are labeled numerically as shown in Fig. 1 and 2. The activities of the GAL4-HSF1 fusion proteins were measured by cotransfection with a reporter plasmid, G_5BCAT, that contains the chloramphenicol acetyl transferase (CAT) gene expressed from a minimal promoter by utilizing an E1b TATA box with five GAL4-binding sites 5′ to the TATA box. As an internal control for transfection efficiency, RSVβgal was cotransfected into cells and the levels of β-galactosidase were measured.

Transfection of NIH 3T3 cells. NIH 3T3 cells were cultured in Dulbecco's modified Eagle's medium (GIBCO-BRL) with 5% calf serum at 37°C and 5% CO_2. Cells at a density of 40 to 50% confluence were transfected with 20 μg of total DNA per 10-cm plate. In general, 5 μg of activator, 5 μg of CAT reporter, 2 μg of RSVβgal, and 8 μg of sheared salmon sperm DNA were combined with 250 mM $CaCl_2$ in a 500-μl final volume. After chilling on ice, the DNA-Ca^{2+} solution was added dropwise to 500 μl of 2× HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid)-buffered saline (pH 7.06; 2× HEBES = 0.28 M NaCl, 0.0015 M Na_2HPO_4, 0.05 M HEPES). After 20 min at room temperature, the precipitate was added to the cells dropwise and allowed to settle on the cells for 6 to 8 h. The plates were then removed from the incubator and washed twice with 10 ml of 1× phosphate-buffered saline (PBS)–1 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) and replaced with fresh medium. Cells were harvested 42 to 48 h posttransfection.

For heat shock treatment of control and transfected cells, the plates were wrapped with Parafilm and submerged in a water bath at the indicated temperatures. Following heat shock, cells were harvested immediately in order to prepare whole-cell extract or total RNA. Treatment with cadmium sulfate was performed at 37°C with the concentrations indicated in the text.

Preparation of whole-cell extracts and RNA from transfected cells. Plates of cells were placed on ice and washed with PBS, and the cells were pelleted and frozen on dry ice. The RNA was prepared as described elsewhere and stored at −70°C (15). Whole-cell extracts for CAT assays or gel shift analysis were prepared as described, with the addition of 4 packed cell volumes of buffer C with the protease inhibitors phenylmethylsulfonyl fluoride, leupeptin, and pepstatin (37). Whole-cell extracts were also stored at −70°C, and protein levels were quantified with a Bio-Rad kit (Bio-Rad Laboratories, Hercules, Calif.).

CAT assays were performed with 12.5 μg of whole-cell extract from mock or transfected NIH 3T3 cells as described previously (21). The acetylated products were developed by chromatography in 19:1 chloroform-methanol and were visualized by autoradiography. The amount of CAT activity was quantified by direct imaging with the Molecular Dynamics PhosphorImager. The data are presented as relative units (RU) of CAT activity. Each experiment was performed at least twice and in triplicate.

Primer extension analysis of RNA from mock-transfected and transfected cells. Ten micrograms of the RNA was mixed with 1 μl of end-labeled CAT primer (50 fmol) and 1 μl of mouse HSP70-specific primer (1 fmol) in a final volume of 50 μl of 1× hybridization buffer (5× hybridization buffer = 1.25 M KCl, 10 mM Tris-HCl [pH 7.9], and 1 mM EDTA), denatured at 85°C for 5 min, and then hybridized for 3 to 4 h at 56°C. The CAT primer was complementary to a 24-nucleotide region of the CAT coding sequence (from nucleotides +26 to +49 of the CAT coding sequence), and the mHSP70 primer was complementary to a 24-nucleotide region of the mHSP70 sequence (24) (nucleotides +40 to +63 from its transcription start site) with the same GC ratio. The hybridization reaction mixtures were placed on ice and diluted with 115 μl of primer extension buffer (1× primer extension buffer = 20 mM Tris-HCl [pH 8.0; at 23°C], 10 mM $MgCl_2$, 5 mM dithiothreitol, 10 μg of actinomycin D per ml, and 0.5 mM each deoxynucleoside triphosphate). The reaction mixtures were warmed to 42°C, and 200 U of mouse mammary leukemia virus reverse transcriptase (BRL; Bethesda, Md.) was added. After 1 h at 42°C, the reaction mixtures were precipitated with ethanol, centrifuged, resuspended in denaturing sample buffer, and analyzed on an 8% denaturing acrylamide gel. *Msp*I-digested pBR322 that was end labeled with the Klenow fragment of DNA polymerase I and [α-^{32}P]dCTP was used as a marker for size estimations of the extended products. Gels were dried and visualized by autoradiography and quantified with the Molecular Dynamics PhosphorImager.

Gel mobility shift assay. Ten micrograms of whole-cell extract from mock or transfected cells was combined with 0.1 ng of the labeled GAL4 probe

5′-GATCTCGGAGTACTGTCCTCCGA-3′
5′-GATCTCGGAGGACAGTACTCCGA-3′

in 1× binding buffer (20 mM HEPES [pH 7.5], 50 mM KCl, 5 mM $MgCl_2$, 10 μM $ZnCl_2$, 6% glycerol), and the mixture was incubated at 23°C for 20 min. Competitor oligonucleotides were added to the reaction mixture prior to the addition of proteins and as indicated in the figure legends. The samples were loaded onto a 4% 40:1 native acrylamide gel in 0.25× Tris-borate buffer. After electrophoresis at 180 V for 2 h, the gels were dried and exposed to X-ray film at −70°C.

RESULTS

The HSF1 transcription activation domain is negatively regulated and heat shock responsive. While there has been considerable effort to examine the kinetics of HSF1 trimerization and the subsequent effects of HSF1 binding on HSP70 transcription, less is known about the characteristics of the transcriptional activation domain of HSF1 and how these sequences are regulated. To identify the sequences of HSF1 required for transcriptional activation, we prepared a series of protein fusions based on the DNA-binding properties of the amino-terminal 147 amino acids of the yeast GAL4 protein (31), to which we fused a collection of HSF1 fragments lacking the corresponding HSF1 DNA-binding domain (Fig. 1). These constructs were cotransfected into NIH 3T3 cells with the CAT reporter gene G_5BCAT and the internal control gene RSVβgal. We included pSG424 vector (GAL4 DNA-binding domain only) as the negative control (0.35 RU of CAT activity) and GAL4-VP16 as the positive control (100 RU of CAT activity), respectively (Fig. 1).

The GAL4-HSF1 fusion constructs GAL4-HSF1-I (residues 124 to 503) and -II (residues 124 to 471) both contain the region of HSF1 distal to the HSF1 DNA-binding domain (Fig. 1). Neither chimera exhibits transcriptional activity (0.2 RU of CAT activity) when expressed transiently in NIH 3T3 cells (Fig. 1 and 2A). The lack of transcriptional activity is not due to the inability of the GAL4-HSF1 construct to bind DNA, as demonstrated by using a gel shift analysis with the GAL4 DNA-binding site (Fig. 2B). These data reveal that the tran-

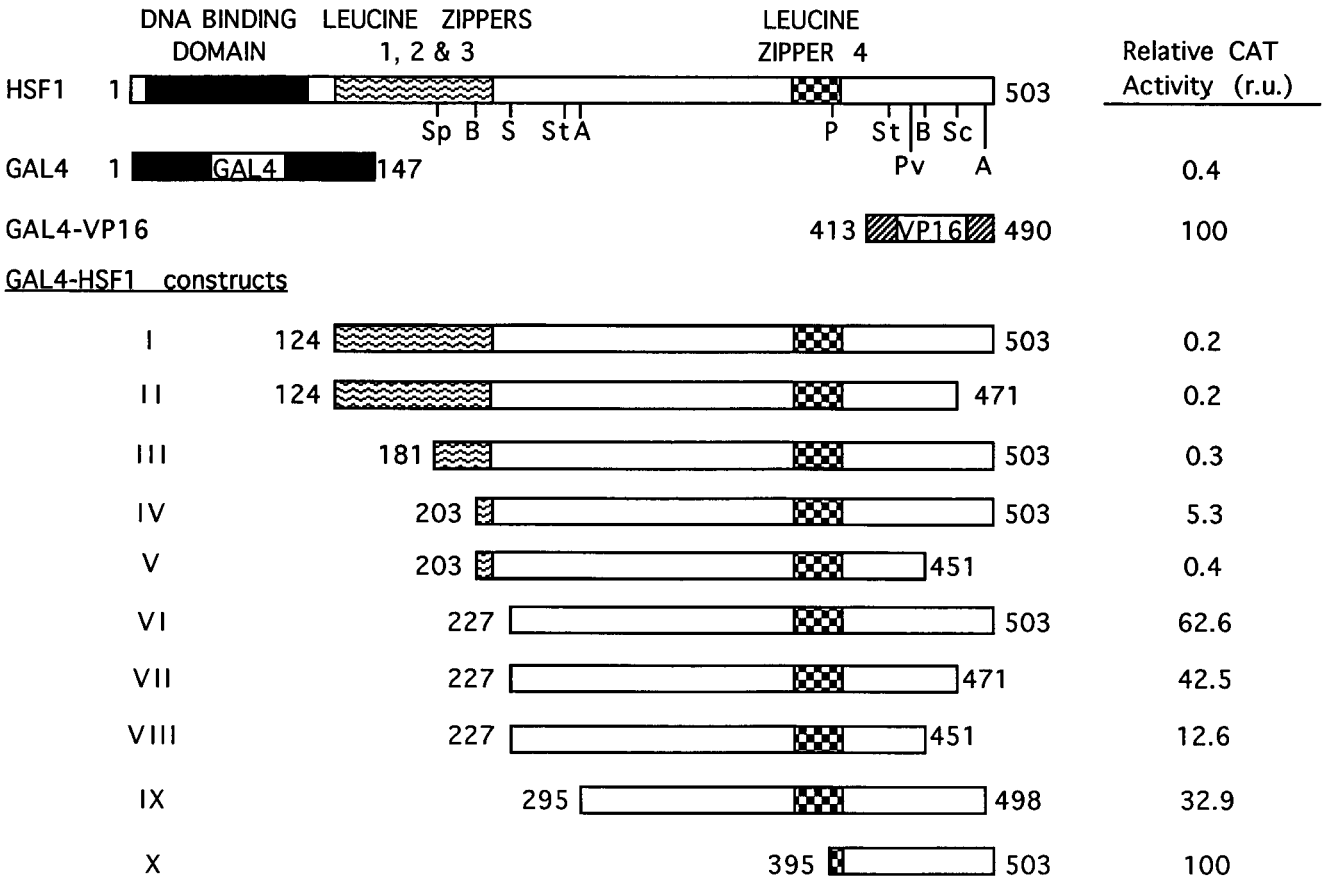

FIG. 1. Analysis of GAL4-HSF1 deletion mutants. A schematic of mouse HSF1 (residues 1 to 503) is shown at the top. Filled box, the DNA-binding domain; wave-lined box, the amino-terminal leucine zippers (1 to 3); checkered box, the carboxyl-terminal leucine zipper 4. Shown below the schematic of HSF1 are the positions of various restriction enzyme sites used to create the HSF1 mutants (Sp, *Sph*I; B, *Bam*HI; S, *Sal*I; St, *Stu*I, A, *Ava*II; P, *Pst*I; Pv, *Pvu*II; Sc, *Sac*I). Black box, the GAL4 DNA-binding domain (residues 1 to 147); dashed box, GAL4-VP16 (residues 413 to 490). The levels of CAT enzyme activity are given in relative units, with the maximal level of GAL4-HSF1 (construct X) as 100 RU. Constructs I to XI are indicated as the regions of HSF1 fused to the GAL4 DNA-binding domain. The boundaries of each deletion mutation and the levels of CAT activity induced in transfected NIH 3T3 cells are indicated.

scriptional activation of HSF1 is negatively regulated. The lack of HSF1 transcription activity was also detected in construct III (residues 181 to 503) despite exhibiting DNA-binding properties (Fig. 1 and 2A and B). Further deletion to residue 203 (construct IV [residues 203 to 503]) uncovered a low but significant level of CAT activity (5 RU), whereas deletion of an additional 24 amino acids to residue 227 (construct VI [residues 227 to 503]) unveiled a strong constitutive activation domain (62.6 RU of CAT activity). These data reveal that sequences in HSF1 encompassing residues 181 to 227 have a potent negative effect (200-fold inhibition) on its transcriptional activity, with the minimum negative regulatory element located between residues 203 and 227 (12-fold inhibition). These sequences include a component of the leucine zippers (zippers 1 to 3); furthermore, these leucine zippers have been proposed to be involved in the intramolecular negative regulation of HSF1 DNA-binding activity. The transcriptional activities of these constructs are independent of their DNA-binding properties, as demonstrated by the constitutive GAL4 DNA-binding activity (for example compare the CAT and gel shift activities for constructs I and VI [Fig. 2]). These data demonstrate that the use of a heterologous DNA-binding domain allows us to establish that the transcription activation domain is negatively regulated.

We then examined whether construct I, which binds to DNA but does not exhibit transactivation activity, is responsive to heat shock. We transfected construct I or VI together with the reporter G_5BCAT and the internal control RSV-CAT into NIH 3T3 cells and measured the level of expression in control and heat-shocked cells by primer extension analysis. This allowed us to directly compare the activities of the transfected GAL4-HSF1 fusion constructs with that of the cotransfected RSV-CAT internal control. Furthermore, primer extension analysis of RNA allowed for a more-sensitive assay of heat shock responsiveness. As shown in Fig. 2C, the level of G_5BCAT RNA expressed in cells expressing construct I was undetectable in control cells and was strongly induced (greater than 40-fold) upon heat shock. By comparison, construct VI exhibits substantial basal expression which was further enhanced 3-fold by heat shock. These data reveal that the transactivation domain in construct I is negatively regulated and that heat shock relieves this repression.

The boundaries of the HSF1 activation domain were determined by maintaining the amino-terminal boundary at residue 227 and creating a set of carboxyl-terminal deletion mutants (constructs VII [residues 227 to 471] and VIII [residues 227 to 451] [Fig. 1]). Whereas construct VI is a strong activator (62.6 RU of CAT activity), loss of 32 amino acids (construct VII) resulted in a reduction in CAT activity to 42.5 RU, while deletion to residue 451 (construct VIII) led to a fivefold loss of

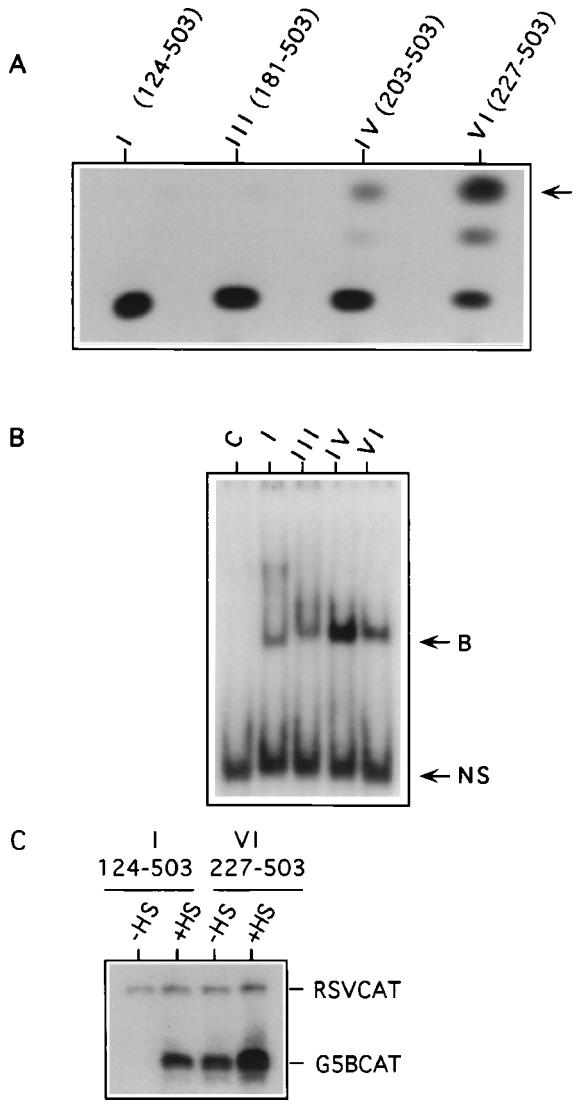

FIG. 2. Analysis of the HSF1 amino-terminal leucine zipper deletion mutants under control and heat shock conditions. (A) CAT activities of constructs I (residues 124 to 503), III (residues 181 to 503), IV (residues 203 to 503), and VI (residues 227 to 503) in transfected cells. (B) GAL4 DNA binding of the constructs shown in panel A and the nontransfected control (C). Only the top part of the gel, indicating the GAL4-HSF1 complex (B) and a nonspecific complex (NS), is shown. (C) Transcriptional activities of constructs I and VI in control and heat-shocked cells. NIH 3T3 cells were transfected with construct I or VI, the reporter gene G_5BCAT, and the internal control (RSV-CAT). The transfected cells were either maintained at control temperature or exposed to heat shock for 2 h at 43°C, RNA was isolated, and reverse transcription primer extension was performed to measure the levels of G_5BCAT RNA and the internal control RSV-CAT RNA.

CAT activity (12.6 RU). These data suggest that the activation domain is located in the extreme carboxyl terminus of HSF1. Additional support for this suggestion follows from construct V (residues 203 to 451), which exhibits a 10-fold reduction in activity compared with construct IV.

The importance of the extreme carboxyl terminus of HSF1 is revealed by the high level of transcriptional activity (100 RU) of construct X (residues 395 to 503), which is nearly two-fold more potent than the level of construct VI and equal to the activity of VP16 (Fig. 1). These data demonstrate that HSF1 contains a potent carboxyl-terminal activation domain whose activity is completely sequestered by interactions mediated by residues 181 to 227, with additional negative effects mediated through sequences between residues 295 to 395.

Our deletion analyses of a transcriptional activator that exhibits constitutive activity when appended to GAL4 lends support to the evidence that HSF1 activity is negatively regulated even in the presence of a heterologous DNA-binding domain. The negative regulatory effects influence transcription primarily through the residues that lie between residues 181 and 227. In addition, we detect a second level of negative regulation, in that deletion of residues 295 to 395 is necessary to reveal the potent activator domain of HSF1.

Dissection of the carboxyl-terminal activation domain. The sequences responsible for activation of transcription by HSF1 were further analyzed by using a series of amino and carboxyl-terminal deletion mutants of the GAL4-HSF1 activation domain (residues 395 to 503) (Fig. 3). Deletion into the amino-terminal boundary (residue 395) revealed that the loss of 16 residues (construct XI [residues 411 to 503]) led to a 1.4-fold reduction in activity, and the deletion of an additional 14 residues (construct XII [residues 425 to 503]) resulted in a 6-fold reduction. Further deletion into residues 439 (construct XIII [residues 439 to 503]) and 451 (construct XIV [residues 451 to 503]) resulted in nearly complete loss of activity. On the basis of these data, we conclude that residues 395 to 425 are important for HSF1 transcriptional activity.

To corroborate the results for the amino-terminal deletion mutants, we analyzed the activities of a set of deletion mutants in which the amino boundary was held at residue 395 and residues were removed from the carboxyl terminus (residue 503). Deletion into the carboxyl terminus from 503 to 471 (construct XV [residues 395 to 471]) resulted in a 2.4-fold reduction, and the loss of an additional 20 residues (construct XVI [residues 395 to 451]) resulted in a 30-fold reduction. Constructs XVII (residues 395 to 439) and XVIII (residues 395 to 411) revealed very low levels of transcriptional activity. These data establish that loss of residues 451 to 503 impairs the transcriptional activity of HSF1. One interpretation of the data from both the amino- and carboxyl-terminal mutants is that the HSF1 transcription activation domain does not correspond to a short linear array of residues. That is, neither region functions independently. The suggestion of dependent carboxyl-terminally localized regions within the activation domain is further supported by the lack of activity of two internal deletion mutants retaining residues 425 to 471 or 451 to 471 (data not shown).

To ascertain that the transcriptional activities of the various GAL4-HSF1 constructs were not due to differences in DNA-binding activity, we performed both Western blot (immunoblot) analyses using anti-GAL4 antiserum (data not shown) and gel mobility shift assays using a GAL4-binding site. As shown in Fig. 4, the levels of DNA-binding activities exhibited by the GAL4-HSF1 constructs IX, X, XI, and XV were nearly equivalent. Higher levels of GAL4-HSF1 DNA-binding activity were consistently detected for constructs XII, XIV, and XIX (residues 451 to 471). However, for this latter collection of mutants, all were transcriptionally inert. Since the binding activities of all constructs tested here were similar to or above the level exhibited by the best activator, construct X, we can conclude that the absolute amount of DNA binding is not indicative of the potential for transcriptional activation. Therefore, we could assume that the observed differences in transcriptional activation levels reflect the ability of the HSF1 sequences to stimulate transcription from the G_5BCAT promoter when tethered to GAL4 (residues 1 to 147).

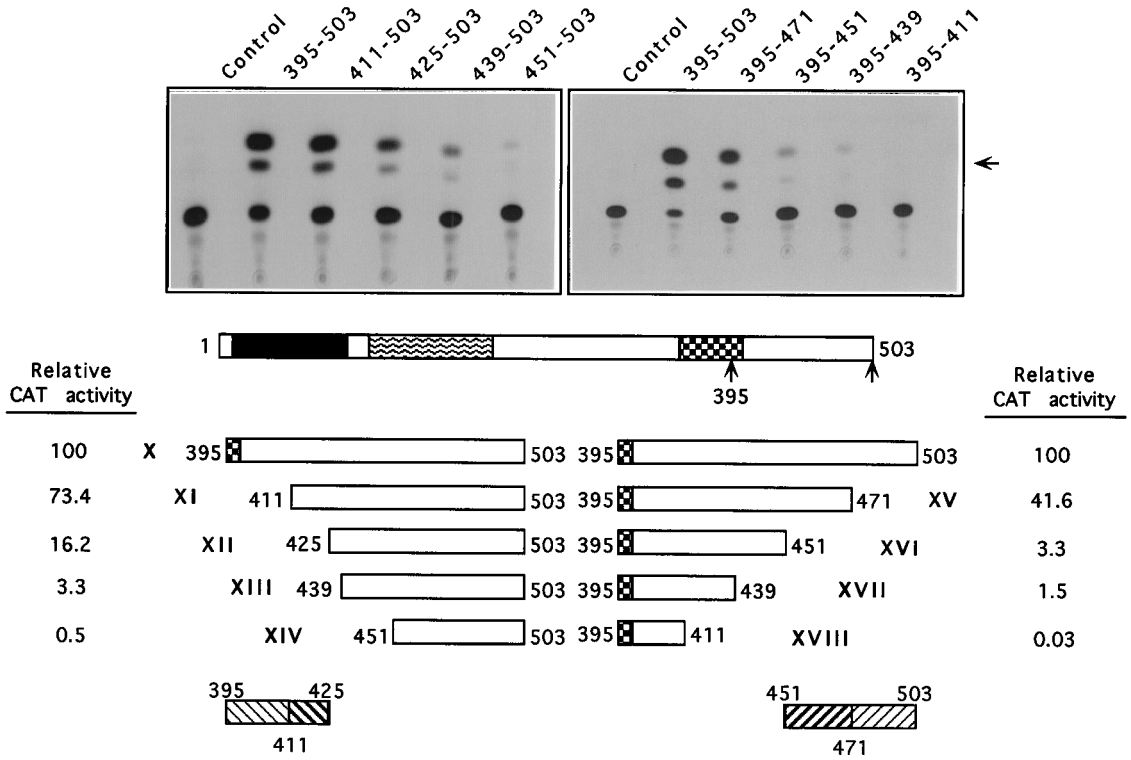

FIG. 3. Deletion analysis of the carboxyl-terminal activation domain of HSF1. CAT activities for constructs X to XVIII are shown at the top. Below is a schematic of the entire HSF1 protein indicating the carboxyl-terminal 108 residues (arrows) which were analyzed by creation of an additional set of deletion mutants. Each mutant is schematically shown below and indicated by a roman numeral (X to XVIII), along with the boundaries of each mutant and the relative level of CAT activity.

The transcriptional activity of GAL4-HSF1 is heat shock and cadmium inducible. Another well-characterized activity of HSF1 is stress responsiveness. We examined the GAL4-HSF1 constructs for stress responsiveness by transfection of NIH 3T3 cells with the GAL4-HSF1 construct IX (residues 295 to 498) and subsequent exposure to heat shock or the heavy metal cadmium sulfate. The response to GAL4-HSF1 in control or stressed cells was measured by primer extension analysis of CAT mRNA transcribed from the G_5BCAT reporter. The cells were cotransfected with RSV-CAT as an internal control, and the kinetics of endogenous HSP70 message levels were measured with an HSP70-specific primer. Finally, we performed gel shift analyses of GAL4-HSF1 from control or stressed cells. Thus, we were able to directly compare the activities of the transfected GAL4-HSF1 fusion constructs with the endogenous HSF1 activity.

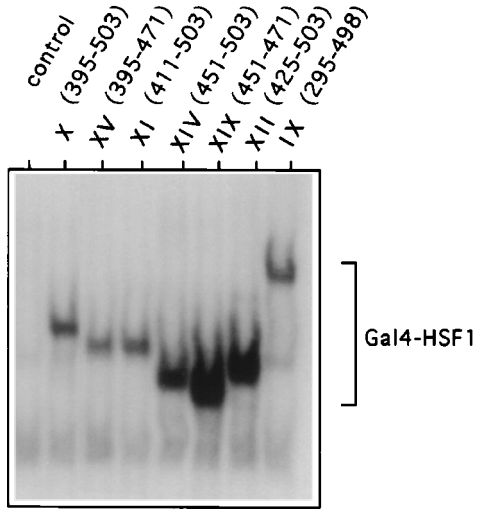

FIG. 4. Carboxyl-terminal deletion mutants of HSF1 exhibit constitutive DNA-binding activity. Extracts were prepared from NIH 3T3 cells transfected with the indicated constructs. Only the top part of the gel corresponding to the GAL4-HSF1 DNA-binding complex is shown.

We initially analyzed the level of G_5BCAT mRNA as a function of heat shock temperature (Fig. 5A). Exposure of the transfected cells to elevated temperatures (42 to 43°C) for 2 h resulted in increased levels of the G_5BCAT mRNA. After 2 h at 43°C, there was a 4-fold increase in the level of G_5BCAT mRNA and a 37-fold increase in the level of endogenous HSP70 mRNA. Since maximal activation of transcription occurred at 43°C, we measured the kinetics of activation of transcription by both the GAL4-HSF1 fusion and the endogenous mHSF1 protein during a continuous 2-h heat shock at 43°C. The activation of G_5BCAT transcription was detected even at 30 min of heat shock as a 1.3-fold induction, while the endogenous HSP70 message levels were induced by 1.7-fold. Comparison with the induction of the endogenous HSP70 gene demonstrated that both genes were activated with similar kinetics, although not to the same absolute level. This difference is due to the high basal transcription activity of the GAL4-HSF1 protein (37°C control [Fig. 6]) above which transcription is induced in response to heat shock treatment. In contrast, the endogenous HSF1 protein is negatively regulated at 37°C, and thus the magnitude of the fold increase of HSP70 transcription is greater during the continuous 43°C heat shock.

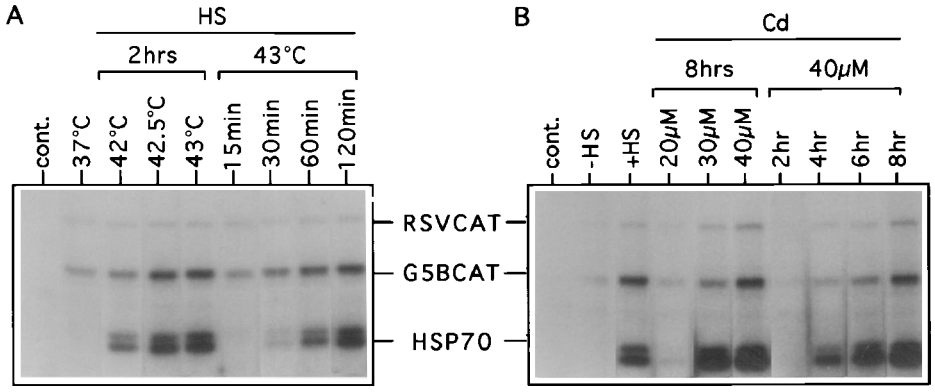

FIG. 5. Effects of heat shock and cadmium on the transcriptional activity of GAL4-HSF1. NIH 3T3 cells were transfected with construct IX (residues 295 to 498), the reporter gene G_5BCAT, and the internal control RSV-CAT. The transfected cells were either exposed to heat shock for 2 h at 42, 42.5, and 43°C and for periods of from 15 min to 2 h at 43°C (A) or were exposed to various concentrations of cadmium sulfate (20 to 40 μM) for 8 h or for various times (2 to 8 h) at 40 μM (B). The treated, untreated transfected cells (37°C or HS) or nontransfected control cells (cont.) were lysed, RNA was isolated, and reverse transcription primer extension was performed to measure the levels of G_5BCAT RNA, endogenous HSP70 RNA, and internal control RSV-CAT RNA.

Since HSF1 is also activated in response to heavy metals, we tested the GAL4-HSF1 fusion (construct IX) for sensitivity to various concentrations and exposure times to cadmium. In contrast to the rapid kinetics of HSP70 induction by heat shock, the induction of transcription by cadmium is a gradual process requiring hours of exposure. NIH 3T3 cells transfected with GAL4-HSF1 (construct IX) were exposed to 20 to 40 μM cadmium for 8 h. Examination of G_5BCAT mRNA demonstrated that the highest mRNA levels occurred after exposure to 40 μM cadmium, and this result correlated with the induction of the endogenous HSP70 mRNA. During exposure to 40 μM cadmium, G_5BCAT and HSP70 transcription increased 2.3- and 8.3-fold, respectively, after 6 h of cadmium treatment and 3.9- and 40-fold, respectively, after 8 h (Fig. 5B). We

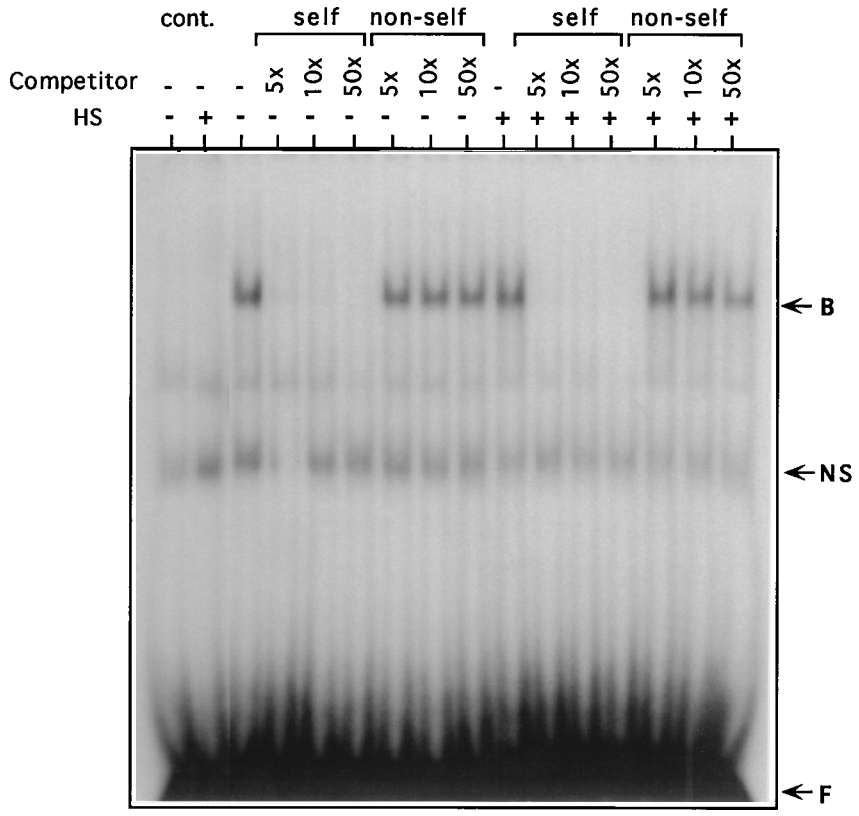

FIG. 6. Heat shock does not affect GAL4-HSF1 DNA-binding activity. Replicate samples were analyzed for gel mobility shift assays as for Fig. 5. Transfected cell were either maintained at control temperatures (−) or heat shocked (HS) at 43°C for 2 h (+). Nontransfected cells (cont.) with (+) or without (−) heat shock treatment were included as controls. The GAL4-HSF1 DNA-binding complex (B), nonspecific complex (NS), and free GAL4 oligonucleotide (F) are indicated. Competition was performed with unlabeled GAL4 oligonucleotide (self) or a non-self oligonucleotide (non-self) at 5-, 10-, or 50-fold molar concentration of labeled GAL4 oligonucleotide.

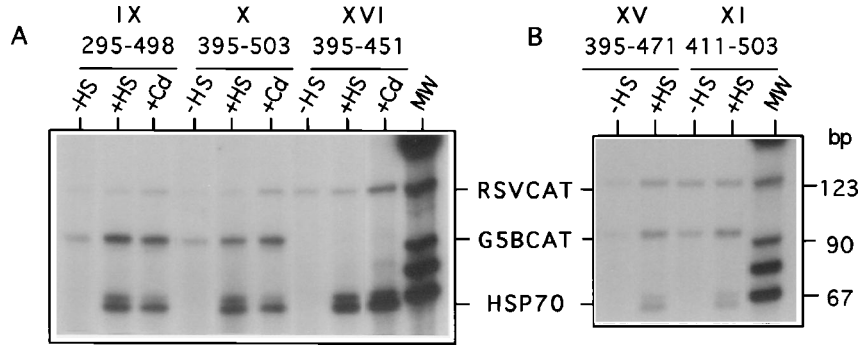

FIG. 7. Effects of heat shock (HS) and cadmium (Cd) on carboxyl-terminal deletion mutants. Constructs IX, X, or XVI (A) or XV or XI (B) were cotransfected with the reporter gene G_5BCAT and the internal control RSV-CAT. The transfected cells were either exposed to heat shock (2 h at 43°C) or cadmium sulfate (8 h at 40 μM). RNA was isolated from the transfected cells and analyzed as described in the legend to Fig. 5. Transfected cells without treatment (−HS) were included as controls. *Msp*I-digested pBR322 which was labeled with the Klenow fragment of DNA polymerase I and [α-^{32}P]dCIP was used as a marker. The sizes of the extended products are indicated in base pairs. MW, molecular weight.

observed some variation in RSV-CAT mRNA levels in the cadmium treatment experiment, in part because of the effects of cadmium on the expression of the RSV-CAT. Overall, these experiments demonstrate that despite the unveiling of the constitutive activator domain, the GAL4-HSF1 construct is induced an additional 4-fold by heat shock and heavy-metal exposure.

Heat shock does not influence the DNA-binding activity of the GAL4-HSF1 protein. Heat shock activates the endogenous HSF1 protein by uncovering both its DNA-binding activity and its transcriptional activity. Consequently, one concern was to ascertain whether exposure to heat shock influenced the DNA-binding properties of GAL4-HSF1. To examine this, we compared the GAL4-HSF1 DNA-binding properties in extracts from control or heat shocked cells (Fig. 6). Untransfected cells did not contain any GAL4 DNA-binding activity, whereas cells transfected with GAL4-HSF1 exhibited a GAL4 DNA-protein complex which was specifically inhibited by the GAL4 oligonucleotide but not by an unrelated oligonucleotide. In addition, exposure of transfected cells to heat shock had no effect on the amount or mobility of the GAL4-HSF1 complex (Fig. 6). These results demonstrate that the GAL4-HSF1 fusion protein binds constitutively to DNA and that heat shock does not noticeably influence the binding activity of the fusion protein. We conclude that the GAL4-HSF1 constructs that have transcriptional activities are heat shock and cadmium responsive.

The carboxyl-terminal transcription activation domain is heat shock and cadmium responsive. We compared the transactivation activities of some of the carboxyl-terminal activators in control and heat-shocked cells by directly measuring G_5BCAT message levels. As shown in Fig. 7, constructs IX, X, XI, and XV were constitutive activators and also heat shock responsive, whereas construct XVI was inactive in both control and heat-shocked cells. Our interpretation of these data is that the transcriptional activation domain is also stress responsive.

DISCUSSION

The analysis of DNA-binding and transcriptional activation properties of HSF1 has identified a stress-responsive carboxyl-terminal activation domain that is negatively regulated. Previous studies of *Drosophila* and mammalian cells have established that HSF1 does not exhibit DNA-binding activity in control cells. Consequently, the negative regulation of DNA binding intrinsic to the HSFs precludes the ability to measure transcription activity of this factor. However, by replacement of the HSF1 DNA-binding domain with the heterologous GAL4 DNA-binding domain, we demonstrate that the chimeric GAL4-HSF1 binds constitutively to DNA but remains transcriptionally inert. The transcriptional activity of HSF1 is uncovered either by heat shock or upon deletion of residues 181 to 227 which lie within and adjacent to the extended leucine zipper motif (45). Thus, removal of these negative regulatory sequences revealed a potent constitutive transcriptional activator which is heat shock and heavy-metal responsive.

One of the prominent features of HSF1 activity is the role of negative regulation. *Drosophila* and vertebrate HSFs contain two arrays of leucine zippers. It is known that the DNA-binding-domain-proximal zippers (zippers 1 to 3) form coiled-coil structures that are also important for trimer formation (42, 54). It has been previously suggested that intramolecular zipper interactions suppressed trimer formation to maintain HSF in its negatively regulated state in that mutation or deletion of zipper 4 resulted in a loss of negative regulation and constitutive trimer formation (39, 45). Our analysis with chimeric GAL4-HSF1 proteins demonstrates that the amino acid residues between 181 and 227 have a potent negative effect on the transcriptional activity of HSF1. Constructs that retain the sequences between 181 and 203 exhibit no transcriptional activity, whereas the construct with the boundary at 203 reveals a low level of transcriptional activity and deletion to residue 227 unveils a potent transcriptional activity. Alternatively, exposure of cells expressing a construct (I) that retains this amino-terminal region to heat shock also unveils this transcription activation domain. The observations presented here reveal that the sequences in the amino terminus of HSF1 (residues 181 to 227), including the leucine zippers, have a profound negative regulatory effect on the carboxyl-terminal transcription activation domain and that these events are affected by heat shock.

The transcription activation sequences of HSF1 are localized to the carboxyl-terminal 108 amino acid residues. The sequences within the HSF1 transcription activation domain are highly conserved among the mouse, chicken, and human HSF1 proteins; however, relative to chicken and human HSF1, the mouse HSF1 transcription activation domain lacks 22 residues (amino acids 415 to 437) within this region (Fig. 8). Residues 395 to 425 contain scattered hydrophobic and acidic residues, whereas residues 451 to 503 contain clusters of hydrophobic residues and acidic residues (Fig. 8). In contrast to the high degree of sequence conservation of HSF1 in vertebrates, a

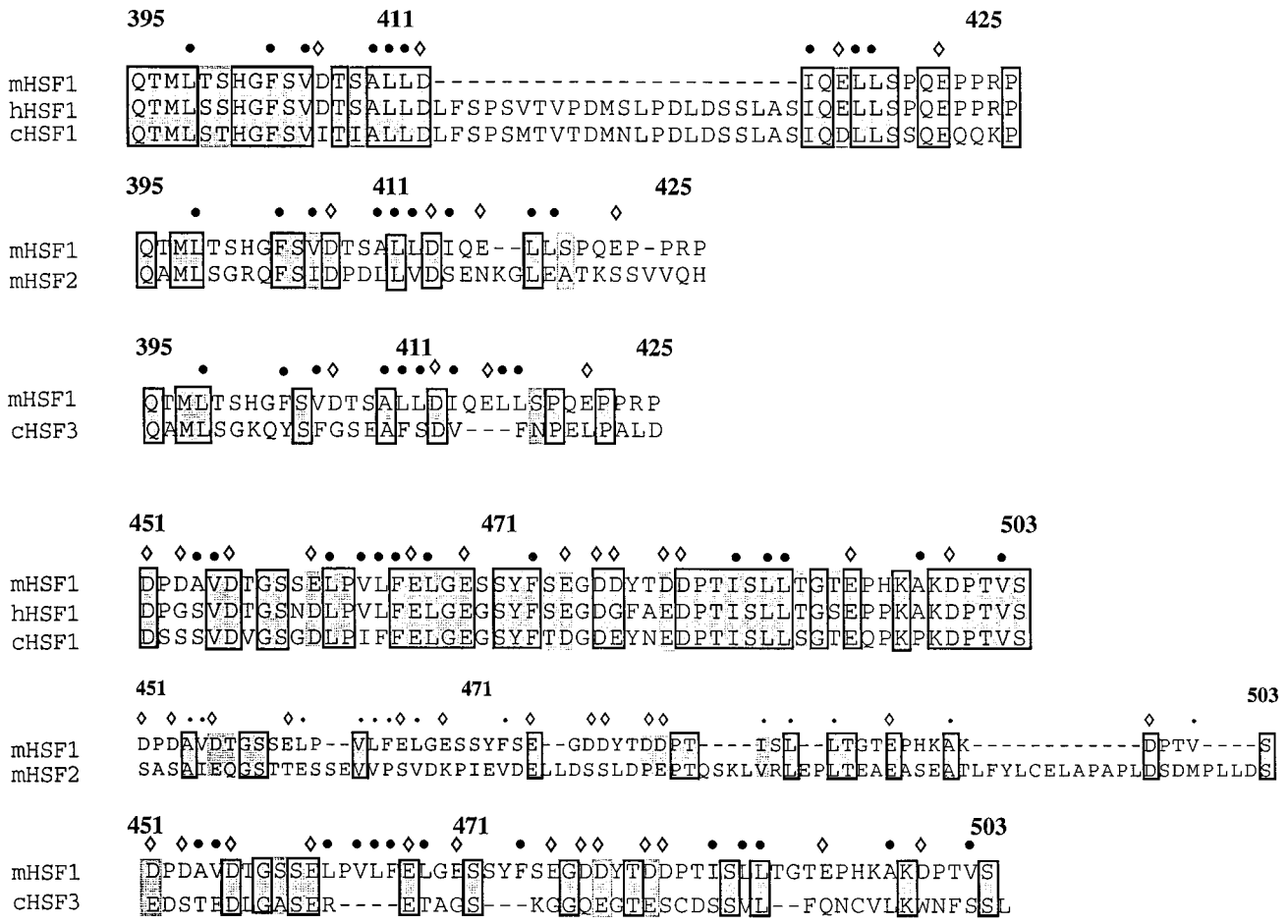

FIG. 8. Amino acid residues in the mouse HSF1 activation domain and comparison with other vertebrate HSF1, HSF2, and HSF3. The amino acid sequences of the mouse HSF1 (mHSF1) activation domain (residues 395 to 425 and 451 to 503) were compared with human (h) and chicken (c) HSF1 and mouse HSF2 or chicken HSF3. Identical residues are shaded and boxed, similar but unidentical residues are shaded, hydrophobic residues are indicated filled circles, and acidic residues are indicated by open diamonds.

comparison of the HSF1 activation domain with the corresponding residues in vertebrate HSF2 or HSF3 reveals limited sequence identity in this region. The corresponding region of HSF2 has weak activation properties, as measured with a chimeric GAL4-HSF2 (27a); however, GAL4-HSF3 exhibits potent transcription activation properties (40). The transcription activation domain of yeast HSF has also been localized to the carboxyl terminus (14, 41, 52). The *K. lactis* HSF corresponds to a domain of 32 residues that is slightly acidic and is required for both the heat shock response and growth at high temperatures (14). In *S. cerevisiae*, two distinct transcriptional activation regions have been identified, corresponding to an amino-terminal domain (AR1) containing the trimerization motifs which are required for the transient response to heat shock and a carboxyl-terminal activation region (AR2) implicated in the sustained response (50). Studies of the *S. cerevisiae* HSF fused to the VP16 transcriptional activation domain have shown that the low level of transcriptional activity of this chimeric factor detected at normal growth temperatures is strongly induced upon heat shock (11). The transient response in yeasts corresponds to the classical heat shock response in other eukaryotes. Despite the lack of convincing sequence homologies, a comparison of the properties of yeast HSF with those of mouse HSF1 reveals a parallel requirement in the transient heat shock response for two regions composed of an amino-terminal negative regulatory domain and a separate activation domain.

The mouse HSF1 activation domain belongs to a class of transcription activators in which the requirements for activity extend throughout the entire extended domain. Comparison of the residues in the mouse HSF1 transcription activation domain with other cloned transcription factors in the database reveals that the mouse HSF1 activation domain shares certain conserved features with the transcription activation domains of mouse Fos and Jun. Alignment of the transcription activation domain of HSF1 with those of Fos and Jun reveals extensive similarities in the number and spacing of hydrophobic residues (data not shown). The activation domain of Jun corresponds to a single large domain of 96 residues, which is similar to the activation domain of HSF1, whereas the Fos activator is composed of two regions (1). As has been shown for HSF1, the Fos and Jun activation domains are negatively regulated.

The minimum domain required for transcription activation of HSF1 also retains responsiveness to both heat shock and cadmium treatment. These data suggest that HSF1 transcriptional activity is under two forms of negative regulation in which the constitutive activity is detected upon deletion of sequences including components of zippers 1 to 3 and is enhanced by stress. The relative rates of transcription following heat shock and cadmium induction of GAL4-HSF1 are similar

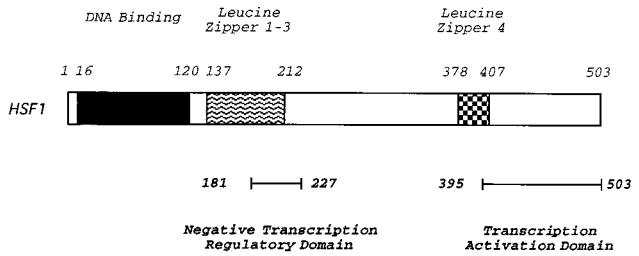

FIG. 9. Localization of functional domains of HSF1. The boundaries for the domains for DNA binding, leucine zippers 1 to 3, and leucine zipper 4 are based on previous analyses. At the bottom are shown the locations of the negative transcriptional regulatory domain and the transcription activation domain.

to those observed for the endogenous HSF1, suggesting that the transcription activation domain is also stress responsive. The ability of heat shock to induce the activity of the carboxyl-terminal transcription activation domain of HSF1 is not solely due to the effects of elevated temperature on the conformation of this domain, since the transcription stimulatory effect was also observed in cells treated with cadmium sulfate at 37°C. The general features of the domains of HSF1 are summarized in Fig. 9 which indicates the positions of the known domains for DNA binding and the proximal and distal leucine zippers. We have placed onto this linear map the locations of the negative transcriptional regulatory domain and the transactivation domain identified in this study. Our observations leave open the questions of whether heat shock or cadmium acts directly or indirectly on the carboxyl-terminal activation domain or whether these stress conditions modulate the interactions between HSF1 and components of the transcriptional apparatus, thus leading to elevated transcription levels. Additional studies will be required to identify the critical residues in the transcription activation domain of HSF1 and to describe how these residues interact with components of the transcriptional machinery and how these interactions are stimulated by exposure of the cell to physiological stress.

ACKNOWLEDGMENTS

We thank Sue Fox for her excellent technical assistance, Jose Cotto for his expertise in photography and image analysis, and Anna-Lisa Paul and Diana Montgomery for comments on the manuscript.

P.K. was supported by a postdoctoral fellowship (NRSA) from NIH. These studies were supported by grant GM38109 from NIH and by a grant from the March of Dimes.

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