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# Function of the C-terminal transactivation domain of human heat shock factor 2 is modulated by the adjacent negative regulatory segment

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## ABSTRACT

**DNA binding of heat shock factor 2 (HSF2) is induced during hemin-induced differentiation of human erythroleukemia cell line K562. To identify the transcriptional activation and the regulatory domains of HSF2, we constructed a series of deletion derivatives fused to the yeast GAL4 DNA binding domain and analyzed their transactivation activity. A minimal transactivation domain of HSF2 was localized to the C-terminus (residues 472–536), as in HSF1, although amino acid sequence similarity for these regions was rather limited and the potential transactivation ability was about 25% that of HSF1. The transactivation mediated by this region of HSF2 was found to be negatively regulated by the adjacent 18 amino acid segment (residues 428–445) under normal conditions. Furthermore, the latter segment, when fused to the GAL4 activation domain, markedly inhibited GAL4 activity. Extract containing most derivatives of HSF2 retaining this segment exhibited doublet or triplet bands in gel mobility shift assays with heat shock element-containing DNA, suggesting possible involvement of some factors interacting with that segment in the negative regulation. Another putative transactivation domain and two negative regulatory regions were also localized within the internal region.**

## INTRODUCTION

To respond to heat shock and various other environmental stresses, both prokaryotic and eukaryotic cells rapidly induce transcription of heat shock genes (1). In eukaryotes, transcriptional induction of heat shock genes is mediated by heat shock factors (HSFs) (2). Whereas only a single HSF has been identified from yeasts and *Drosophila* (3–7), multiple HSFs have been reported from higher eukaryotes (chicken, HSF1, HSF2 and HSF3; mouse, HSF1 and HSF2; human, HSF1, HSF2 and HSF4) (8–12).

Several common functional motifs, whose amino acid sequences are highly conserved among most HSFs, have been identified. A DNA binding domain essential for binding to the heat shock element (HSE) consisting of inverted repeat modules (NGAAN) is located in the N-terminus of all HSFs (13,14). Hydrophobic repeats (HR-A/B) located adjacent to the DNA binding domain are essential for the formation of active trimers (15), which confer on all HSFs the ability to specifically bind to DNA. Another short hydrophobic repeat (HR-C) is located towards the C-terminal region in most HSFs except for yeast HSF (*Saccharomyces cerevisiae* and *Kluyveromyces lactis*) and human HSF4. HR-C is thought to be necessary for suppression of trimerization by means of coiled coil intramolecular interaction with HR-A/B (16,17). Indeed, the yeast HSF and human HSF4 lacking the HR-C motif are constitutively trimerized and bound to HSE (12,15,18).

In higher eukaryotes, HSF1 is rapidly activated by heat and other stresses and induces transcription of *hsp* genes (19,20). The transactivation domain of human HSF1 is located in the C-terminal region, whose activity is manifested under stress conditions (17,21,22): this activity is negatively modulated by the central region located between HR-A/B and HR-C (21). The central region appears to be essential and sufficient to sense heat stress, because replacement of the DNA binding domain and the transactivation domain by those of other transcription factors does not affect the characteristic heat shock response (23).

HSF2 was previously reported to exhibit DNA binding ability on hemin treatment of K562 cells (24,25). On the other hand, constitutive binding of HSF2 to HSE has been found with spermatogenic cells (26), post-implantation embryos (27) and embryonal carcinoma (EC) cells in mouse (28). These observations suggested roles for HSF2 in differentiation and development.

The primary sequences that correspond to the DNA binding, HR-A/B and HR-C domains are highly conserved among all HSFs, including HSF2 (9,12), suggesting functional similarity of these regions between HSF2 and HSF1. However, functions of the central and C-terminal regions of HSF2 with much less sequence similarity remained to be characterized. In this work, a series of human HSF2 deletion derivatives fused to the GAL4 DNA binding domain (GAL4-BD) were analyzed to identify regions involved in the control of HSF2 transactivation and clarify the nature of activation mechanisms. We have now identified the transactivation domains and the separate regions involved in negative regulation, which is basic to understanding HSF2-mediated transcription.

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## MATERIALS AND METHODS

### Plasmid constructs

*HSF2* and *HSF1* deletion mutants on pAS2 (Clontech) were constructed as described elsewhere (29). DNA fragments containing these deletions fused with GAL4-BD or GAL4-BD alone were isolated from the pAS2 derivatives and inserted into the mammalian expression vector pCAGGS that carries a cytomegalovirus (CMV) enhancer and chicken β-actin promoter (30). The numbers following 'N' or 'C' for the deletion mutants obtained refer to those of N- or C-terminal residues deleted respectively.

The DNA fragment containing the GAL4 transactivation domain (AD) was excised from pGBT9 (Clontech) and fused to GAL4-BD on pAS2 and the GAL4-BD–AD fusion was inserted into pCAGGS. The firefly luciferase reporter plasmid pGLG4E5 was described previously (29). *Renilla* luciferase expression plasmid pRLSV40 (Promega) was used as a reference plasmid to normalize for transfection efficiency.

### Transient transfection and luciferase assay

K562 cells (ATCC CCL243) were cultured in RPMI 1640 medium containing 10% fetal bovine serum in 5% $CO_2$ at 37°C. Transfections were done by electroporation. Cells ($5 \times 10^6$) were washed twice with phosphate-buffered saline (PBS) and suspended in 0.4 ml PBS containing 30 µg plasmid. After incubation for 10 min at room temperature, the cells were exposed to an electric pulse (300 V/cm, 500 µF) with a Gene Pulser (BioRad). The cells were left at room temperature for 10 min and then incubated in RPMI 1640 medium containing 10% fetal bovine serum in 5% $CO_2$ at 37°C for 48 h. Harvested cells were washed twice with PBS, divided into two aliquots for luciferase assay and for preparation of whole cell extracts and stored at –80°C. The luciferase assay was performed using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions.

### Preparation of whole cell extracts

Frozen cells were suspended in 20 mM HEPES (pH 7.5), 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol, 25% glycerol, 1 mM phenylmethylsulfonyl fluoride, 1 µg/ml leupeptin, 1 µg/ml pepstatin A and 10 µg/ml aprotinin. After addition of NaCl at a final concentration of 0.42 M, the lysates were sat on ice for 5 min and centrifuged at 100 000 *g* for 15 min at 4°C. The supernatants were used as whole cell extracts. The protein concentrations were determined with a BioRad protein assay kit (BioRad) using bovine serum albumin as the standard.

### Gel mobility shift assay

Whole cell extracts (5 µg protein) were mixed with 5 fmol $^{32}$P-labeled DNA probe (5′-GATCCGGTCGGACTGTCCTCC-GACAGATC-3′) containing a single GAL4 binding site in 10 µl reaction buffer (20 mM HEPES, pH 7.7, 100 mM NaCl, 5 mM $MgCl_2$, 10 µM $ZnCl_2$, 6% glycerol) containing 0.4 µg poly(dI·dC) (Pharmacia) and 2 µg salmon sperm DNA. The mixture was incubated for 20 min at 25°C and loaded onto a 4% polyacrylamide gel in 0.25× TBE. After electrophoresis at 180 V for 2 h at 4°C, the gels were dried on DE81 paper and exposed to X-ray film.

## RESULTS

### The C-terminal region of HSF2 acts as a transactivation domain

A series of deletion derivatives of the *HSF2* gene was first constructed to identify the transcriptional activation domain of HSF2. To distinguish the activity of these mutant constructs from that of endogenous HSF2, the DNA binding domain of HSF2 was replaced by that of yeast GAL4 protein (residues 1–147, GAL4-BD). A construct 'GAL4' that consisted of yeast GAL4-BD and the GAL4 transactivation domain (amino acids 761–881) served as a positive control. Each of the GAL4-BD–*HSF2* fusion constructs was co-introduced into K562 cells with a luciferase reporter plasmid pGLG4E5 and a reference plasmid pRLSV40.

N96 containing most of HSF2 but lacking the DNA binding domain (Fig. 1A, lane 4) was the basic construct used to obtain the deletion derivatives examined in this study. N199 further lacked the trimerization domains (lane 5), whereas N328 contained HR-C and the C-terminal region (lane 6) and N446 retained only 91 C-terminal residues (446–536) (lane 7). Three C-terminal deletion derivatives, N96C444, N96C368 and N96C198, contained approximately the regions deleted in N446, N328 and N199 respectively (lanes 8–10). Among these eight constructs tested, the only construct that significantly activated the luciferase reporter was N446, which contained a short C-terminal region, indicating that the C-terminal region exhibits transactivation, though the activity was much lower than that observed with 'GAL4'. The fact that N96, N199 and N328 failed to activate the reporter suggested that they contained an internal region that inhibited transactivation mediated by the C-terminal activation region.

DNA binding activity of these fusion proteins expressed in K562 cells was then examined by gel mobility shift assays using the respective cell extracts and a $^{32}$P-labeled oligodeoxynucleotide containing the GAL4 binding site as probe (Fig. 2, lanes 1–6, 12, 16, 18 and 19). The DNA binding activity of each fusion protein was confirmed by specific bands observed at appropriate positions, although the amounts of protein–DNA complex detected varied appreciably among the constructs. The significance of differential band patterns obtained, singlet versus doublet/triplet bands, will be considered in the Discussion. These data taken together indicated that a transactivation domain of HSF2 is located in the C-terminal region (residues 446–536) and that the activation is negatively modulated by the internal region spanning residues 328–445.

### The C-terminal transactivation domain is negatively regulated

To further define the regions responsible for transactivation and for its negative control, fine deletion analysis starting with construct N328 was performed. The six constructs deleted up to residue 428 (constructs N328–N428) hardly exhibited activity, whereas deletion of 18 additional residues (N446) gave rise to marked activation (Fig. 1B, lanes 2–8). Further deletions up to residue 472 (N451 and N472) retained the activity, whereas deletion to residue 500 (N500) completely abolished transactivation (lanes 9–11). All of these constructs showed constitutive binding to the GAL4 binding site (Fig. 2, lanes 6–15), though the binding was much reduced with some of the constructs. These results demonstrate that the C-terminal region of HSF2 (residues 472–536)

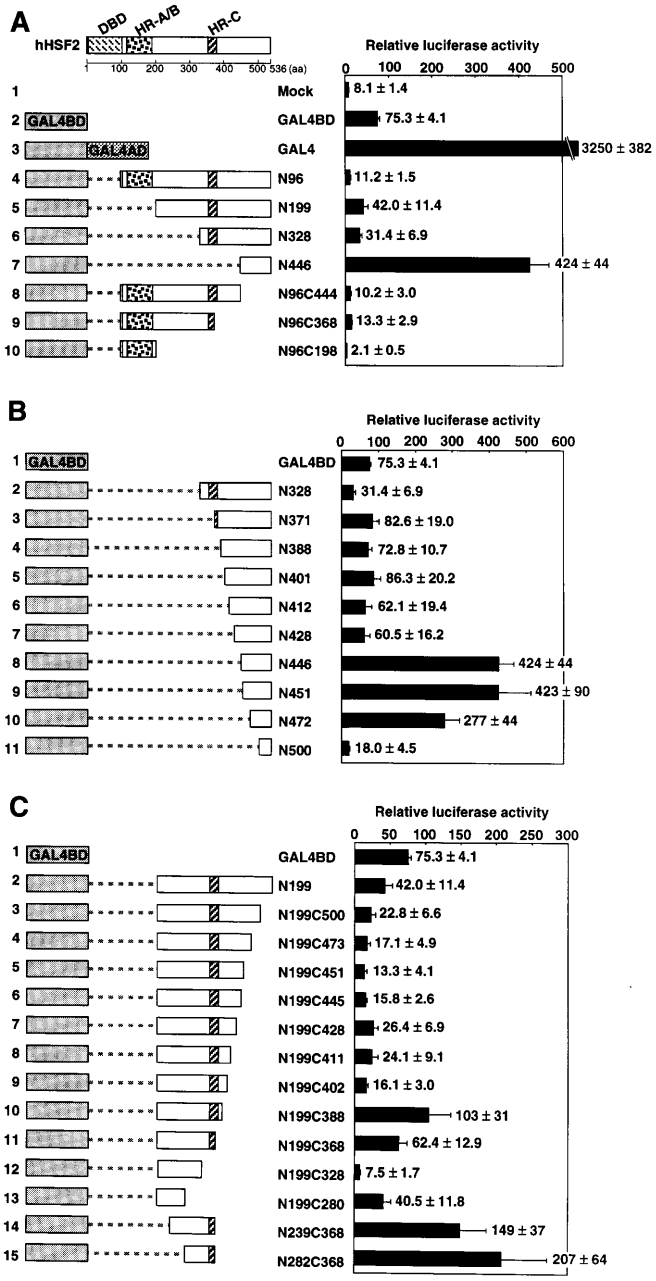


**Figure 1.** Transactivation activities of deletion derivatives of HSF2 in K562 cells. HSF2 deletion mutants fused to the GAL4 DNA binding domain constructed on the expression plasmid pCAGGS are shown schematically on the left. K562 cells were transfected with 5 µg each expression plasmid, 2 µg reporter plasmid pGLG4E5, 2 µg reference plasmid pRLSV40 and 21 µg pBluescriptII as a carrier. Cells were harvested after 48 h and subjected to dual luciferase assays. Activities of firefly luciferase were normalized to those of *Renilla* luciferase and presented as the mean ± SD (bars) from four independent experiments. The results are presented in three sections (A–C) for convenience. (**A**) An initial set of HSF2 deletion mutants roughly deleted from the N- or C-terminus. Schematic representation of human HSF2 is shown at the top. DBD, DNA binding domain; HR-A/B, hydrophobic repeats; HR-C, C-terminal hydrophobic repeat; Mock, transfection with pCAGGS. (**B**) Fine analysis of the N-terminal deletions. (**C**) Deletion analysis of C-terminal and internal regions.

is functional as a transactivation domain. Furthermore, it was suggested that the 18 amino acid segment (residues 428–445) is

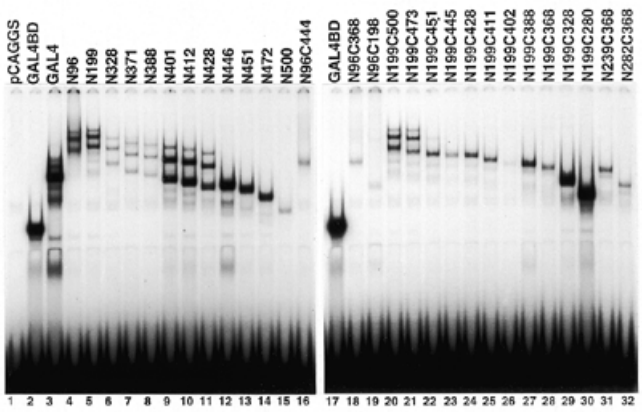


**Figure 2.** Gel mobility shift analysis of products of GAL4-BD–HSF2 deletion constructs. Whole cell extracts were prepared from K562 cells transiently transfected with each of the plasmid constructs indicated in Figure 1. Each extract (5 µg protein) was incubated with a $^{32}$P-labeled DNA probe containing a GAL4 binding site and the mixture was analyzed by native PAGE, as described in Materials and Methods.

responsible for repressing transactivation mediated by the C-terminal activation domain.

## Functional analysis of the central region of HSF2

To examine the potential regulatory function for the central region of HSF2, a series of C-terminal deletions were constructed, starting with the construct N199, which lacks the DNA binding and trimerization domains (Fig. 1C, lanes 3–11). These deletion mutants (N199–N199C368) exhibited very low transactivation activities, except N199C388 and N199C368, which showed significant activities. All these proteins were expressed and bound to the GAL4 binding site at comparable efficiencies (Fig. 2, lanes 5 and 20–28), except for N199C402, which showed a barely visible band (lane 26). For this reason, we excluded N199C402 from further interpretation of the results. It thus seemed likely that an additional minor transactivation domain exists in the internal region spanning residues 199–368, besides the C-terminal activation domain. Because N199C411, containing the whole region of N199C388 but not the C-terminal negative regulatory motif (residues 428–445), exhibited very low activity, another negative regulatory segment appeared to exist in the region flanked by residues 389 and 411.

The above finding that the central region (residues 199–368), which corresponds to the heat shock-responsive domain of HSF1 (21,23), contains a separate transactivation domain was substantiated by examining four additional constructs further deleted from either direction (Fig. 1C, lanes 12–15). Deletion from the C-terminal side (N199C328 and N199C280) showed lower activities than N199C368 (Fig. 1C, lanes 11–13), though their DNA binding activities were apparently much higher than that of N199C368 (Fig. 2, lanes 28–30). On the other hand, deletion from the N-terminal side (N239C368 and N282C368) activated transcription more efficiently than did N199C368, suggesting that the region flanked by residues 199 and 238 might act as another negative segment in the present experimental system. These observations also agreed with the lack of transactivation found with the C-terminal deletion constructs N96C444 and N96C368 (Fig. 1A, lanes 8 and 9). We conclude

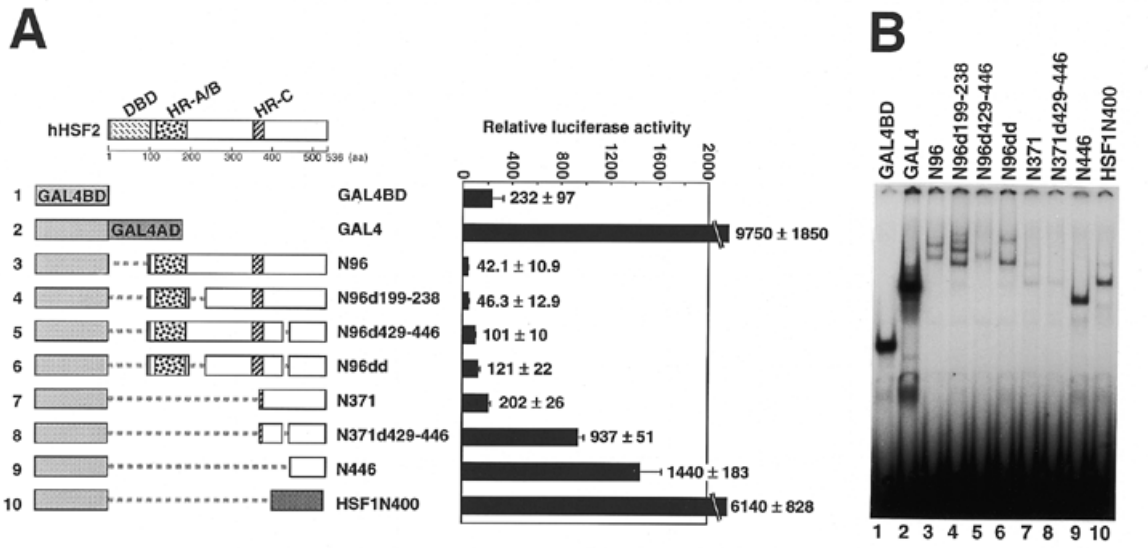


**Figure 3.** Deletion analysis of negative regulatory domains of HSF2. (**A**) Deletion constructs are shown on the left, transient transfection and luciferase assay were done as in Figure 1 and relative luciferase activities observed are shown on the right. (**B**) Gel mobility shift assay for the HSF2 deletion mutants shown in (A), carried out as in Figure 2.

that the internal region flanked by residues 282 and 368 contains a second transcriptional activation domain and that the adjacent segment flanked by residues 199 and 238 acts as a third negative regulatory motif.

### The C-terminal negative regulatory segment can act on the GAL4 activation domain

To further define the apparently negative regulatory roles of the C-terminal (residues 428–445) and the internal (residues 199–238) segments in HSF2 activation, we tested the set of constructs specifically lacking these regions. As shown in Figure 3A, deletion of residues 429–446 resulted in a slight increase in activity (2.4-fold) as compared with the parental construct N96, whereas deletion of residues 199–238 failed to show any significant effects (lanes 3–6). The small increase observed with the N96d429–446 construct is presumably due to the limited trimerization caused by intramolecular interaction between HR-A/B and HR-C (see Discussion). In the absence of intact HR-A/B and HR-C, the activity of N371d429–446 was 4.5-fold higher than that of N371 (lanes 7 and 8). These observations taken together suggest that residues 428–445 but not 199–238 can repress the transactivation function of the C-terminal domain. The levels of DNA binding of these fusion proteins were confirmed by gel shift assay (Fig. 3B, lanes 1–9).

To examine a possible negative effect of residues 428–445 on heterologous transcription factors, appropriate C-terminal regions of HSF2 were fused to the positive control ‘GAL4’ construct and tested for their activities. Indeed, the segment containing residues 428–445 reduced the GAL4 activity by ~50%: both GAL4N428 and GAL4N428C446 were approximately half as active as GAL4 (Fig. 4A, lanes 1, 2 and 4). In contrast, construct GAL4N446, lacking the segment, activated the reporter gene beyond that obtained with the GAL4 activation domain (lanes 1 and 3). These results demonstrate that residues 428–445 of HSF2 by itself can exert at least part of the negative regulatory function on a heterologous transcription factor such as GAL4. The locations of the functional domains of HSF2 so far identified are summarized in Figure 5.

### Relative strengths of the C-terminal and internal transactivation domains

We compared the activities of two transcriptional activation domains of HSF2 with that of HSF1, known to have an activity comparable with that of a strong viral transcription factor VP16 (23). Since HSF1 has two transactivation domains, AD1 and AD2, both located in the C-terminal region beyond residue 400 (23), activity of this region fused to GAL4-BD (construct HSF1N400) was compared with that of HSF2 N446. In the present reporter assay, the C-terminal transactivation domain of HSF2 was found to be 4.3-fold weaker than that of HSF1 (Fig. 3A, lanes 9 and 10). Thus the potential strength of the C-terminal activation domain of HSF2 appeared to be appreciable but modest. The internal transactivation domain of HSF2 (residues 282–368) was about half as active as the C-terminal activation domain (compare Fig. 1C, lane 15, and A, lane 7).

## DISCUSSION

By using a series of plasmid constructs containing various portions of HSF2 fused to the DNA binding domain of yeast GAL4, the segments of HSF2 responsible for transcriptional activation of the reporter luciferase gene were determined in K562 cells (Figs 1 and 3). The results revealed that the C-terminal region (residues 472–536) represents a major transactivation domain, whereas the adjacent segment (residues 428–445) represses functioning of the C-terminal activation domain under non-stress conditions. The activities of these regions were further substantiated by analysis of chimeric proteins with the active

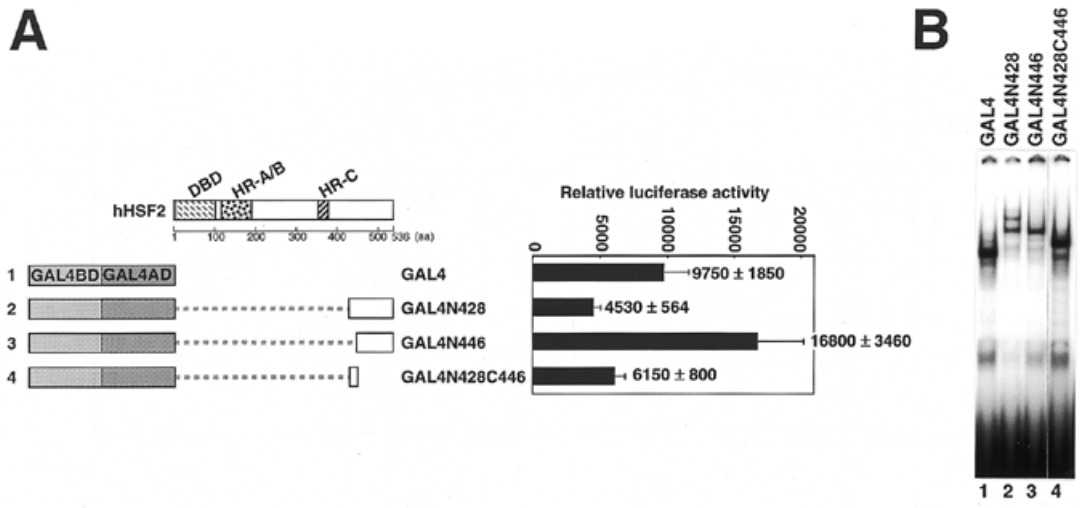


**Figure 4.** Effect of the 18 amino acid negative regulatory segment of HSF2 on transactivation of GAL4. (**A**) The negative regulatory segment of HSF2 (residues 428–445) and/or the neighboring region were fused to the GAL4-BD and GAL4 activation domain and the activities were determined as in Figure 1. (**B**) Gel shift assay with the constructs shown in (A) was done as in Figure 2.

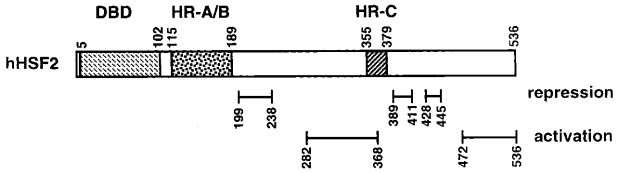


**Figure 5.** Localization of functional domains of HSF2. The localization of the DBD, HR-A/B and HR-C with the amino acid boundaries was taken from a previous report (38). The three negative regulatory domains and two transactivation domains are presented at the bottom.

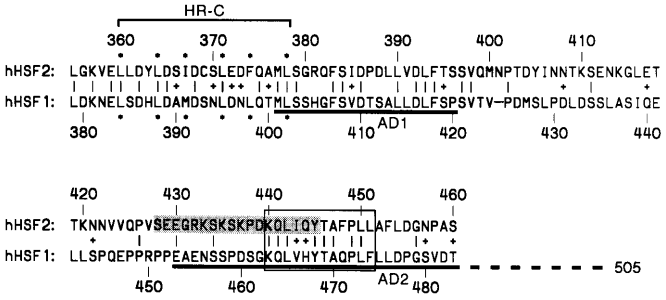


**Figure 6.** Alignment of the C-terminal regions of human HSF2 and HSF1. Residues 355–460 of HSF2 and 379–483 of HSF1 were compared. Bar and + indicate identical and similar amino acids, respectively. Asterisks indicate hydrophobic residues expected to be involved in hydrophobic interaction in the HR-C region and the open box shows the conserved 12 amino acids between HSF1 and HSF2 (16). Thick bars indicate the HSF1 transcriptional activation domains AD1 and AD2 (23). The shadow box represents the 18 amino acid negative regulatory segment of HSF2.

GAL4 construct (Fig. 4). The second transcriptional activation domain detected in the internal region, residues 282–368, was also shown to be negatively modulated under normal conditions, apparently mediated by the segments spanning residues 199–238 and residues 389–411 (Fig. 1C). In parallel experiments reported elsewhere (29), a second activation domain near the HR-C region of human HSF2 was implicated by expression analysis in yeast cells. Further work is required, however, to understand their modes of involvement in transactivation.

Analysis of the products of HSF2 deletion constructs by gel mobility shift assays revealed that most constructs containing the negative regulatory segment (residue 428–445) exhibit doublet or triplet bands (Fig. 2, lanes 4–11 and 20–22, Fig. 3B, lanes 3 and 4, and Fig. 4B, lane 2), whereas those lacking this segment form single bands. Apparent exceptions were N96C444, N199C445 and GAL4N428C446, which formed single bands despite the presence of such segments (Fig. 2, lanes 16 and 23, and Fig. 4B, lane 4); this may be explained by the fact that they lack the region immediately C-terminal of the negative regulatory domain, unlike all the other constructs. These results strongly suggest that a cellular factor(s) interacts with this segment plus at least several adjacent residues (up to residue 451) participating in negative regulation. Interestingly, Rabindran *et al.* reported that HR-C and a 12 residue segment (463–474) of human HSF1, conserved among vertebrate HSFs, are essential to maintain the monomeric form under non-stress conditions (16). The negative regulatory region we have identified (residues 428–445) partially overlaps with the latter segment and the putative binding site for hypothetical cellular factors (residues 428–451) completely includes this segment (see Fig. 6).

On the other hand, the above results and interpretation of the negative regulatory region of HSF2 differ strikingly from those previously reported for HSF1 by Newton *et al.* (23). They showed that a segment spanning residues 453–505 of human HSF1 including the 12 conserved residues acts as a transcriptional activation domain, called AD2. Furthermore, another strong activation domain (AD1) was identified at residues 401–420; the corresponding region of HSF2 (residues 377–396) partially overlaps with one of the negative regulatory segments (residues 389–411)

(Fig. 6). Specifically, the phenylalanine residue, most critical for activation, at position 418 in AD1 of HSF1 (23) is conserved in HSF2. Nevertheless, this region of HSF2 can hardly activate transcription (cf. Fig. 1B, lanes 3–5). It thus seems evident that the C-terminus side of HR-C is functionally different in HSF1 and HSF2, despite the moderate conservation in amino acid sequence.

Although the 18 amino acid negative regulatory segment (residues 428–445) of HSF2 can markedly repress transcription mediated by the C-terminal activation domain (Fig. 1B, lanes 7 and 8), elimination of this segment from 'parental' N96 enhanced transcription only slightly (Fig. 3A, lanes 3 and 5), presumably because the coiled coil intramolecular interaction between the HR-A/B and HR-C regions is sufficient to maintain an inactive conformation. Thus, the above negative segment appears to counteract the C-terminal activation domain, in addition to the coiled coil intramolecular interaction, under normal conditions. Since this segment can function by itself (Fig. 4), HSF2 seems to be activated in at least two steps, as has been demonstrated for HSF1 (31,32). However, post-translational modification, such as stress-induced phosphorylation, known to be involved in activation and deactivation of HSF1 (33–37), has not yet been reported.

The mechanism of activation of HSF1 by heat and other stresses has been well characterized (17,21–23). When K562 cells are exposed to hemin, the DNA binding activity of HSF2 is induced and expression of the *hsp70* gene is increased (24). If HSF2 is indeed responsible for the induction of HSP70, a hemin-responsive domain would be expected to be present in HSF2, just like the stress-responsive domain found in HSF1. However, all constructs of the HSF2 deletion derivatives examined in this study failed to respond significantly to hemin treatment in K562 cells (Yoshima, unpublished results), thus precluding identification of such domains. Involvement of other HSFs or transcription factors might explain hemin-induced activation of *hsp70* transcription.

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