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The Regulatory Domain of Human Heat Shock Factor 1 Is Sufficient To Sense Heat Stress

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Heat shock factor (HSF) activates transcription in response to cellular stress. Human HSF1 has a central regulatory domain which can repress the activity of its activation domains at the control temperature and render them heat shock inducible. To determine whether the regulatory domain works in tandem with specific features of the HSF1 transcriptional activation domains, we first used deletion and point mutagenesis to define these activation domains. One of the activation domains can be reduced to just 20 amino acids. A GAL4 fusion protein containing the HSF1 regulatory domain and this 20-amino-acid activation domain is repressed at the control temperature but potently activates transcription in response to heat shock. No specific amino acids in this activation domain are required for response to the regulatory domain; in particular, none of the potentially phosphorylated serine and threonine residues are required for heat induction, implying that heat-induced phosphorylation of the transcriptional activation domains is not required for induction. The regulatory domain is able to confer heat responsiveness to an otherwise completely heterologous chimeric activator that contains a portion of the VP16 activation domain, suggesting that the regulatory domain can sense heat in the absence of other portions of HSF1.

Eukaryotic cells respond to cellular stress and heat shock by inducing the transcription of genes encoding heat shock proteins (reviewed in references 9 and 21). This large increase in transcription, upwards of 50-fold, is due to the activation of a preexisting transcription factor termed heat shock factor (HSF) (17, 18, 33, 34). In mammalian and *Drosophila* cells, HSF is in a monomeric form in nonstress conditions and is unable to bind to DNA (5, 17, 32, 33). Upon heat shock, HSF trimerizes and is then able to bind specifically to the heat shock element which is located upstream of all heat shock protein genes. However, there are several instances in which induction of DNA binding is insufficient to activate the transcription of heat shock protein genes, indicating an additional regulatory step at the level of transcriptional activation (3, 8, 14, 23).

It has recently been shown that the transcriptional activation domains of human HSF1, located in the C-terminal one-third of the protein, can be heat regulated (7, 27, 35). This regulation of transcriptional activation is dependent on a central region of the protein located between the trimerization domain and the activation domains that we refer to as the regulatory domain (7). The regulatory domain is capable of repressing the transactivation potential of the activation domains at the control temperature and rendering them heat shock inducible, as demonstrated by using GAL4 fusion constructs in mammalian cells (7, 27) or by microinjection of human HSF1 deletion mutants into *Xenopus* oocytes (35). The heat shock induction of these chimeric constructs was not caused by increased DNA binding, and thus this regulation appears to function at the level of transcriptional activation.

How does the regulatory domain regulate the activation potential of the HSF1 activation domains? In the yeast *Saccharomyces cerevisiae*, HSF can bind to strong heat shock elements at the control temperature; however, the factor is capable of efficiently activating transcription only after heat activation (6, 12, 29). This increase in transcriptional activation was correlated with an increase in phosphorylation of the factor, and this hyperphosphorylation has been proposed to be responsible for the increase in this factor's activation potential, although no causal link was established (28, 30). Human HSF1 is also hyperphosphorylated in response to heat shock, but this phosphorylation is not necessary for either the monomer-to-trimer transition or the acquisition of DNA binding ability (18, 19) and therefore has also been proposed to be involved in transcriptional activation (8, 18, 25). By defining features of transcriptional activation domains that are necessary for induction by the regulatory domain of human HSF1, we hoped to determine whether phosphorylation plays a necessary role in heat induction and whether any other features of the HSF1 activation domains play a key role in response to heat.

We created an extensive series of GAL4 fusion constructs to determine what portions of HSF1 are necessary to respond to heat shock. We found that one of the two activation domains present in HSF1, AD1, can be reduced to just 20 amino acids, resulting in an activation domain that still potently activates transcription and is heat shock responsive. All of the potential phosphorylation sites can be removed from this activation domain without affecting the heat shock-inducible regulation. In fact, there appears to be no intrinsic property of the HSF1 activation domains that allow them to respond to heat, as activation domains from the viral protein VP16 can also be rendered heat inducible when fused with the central regulatory domain of HSF1. These results demonstrate that the regulatory domain of HSF1 plays a key role in sensing heat stress in humans.

MATERIALS AND METHODS

Plasmid constructions and mutagenesis. (i) Deletions mutants. All GAL4 expression plasmids were based on the parent vector pBXG1 (kindly provided by M. Ptashne), which expresses the first 147 amino acids of the yeast protein GAL4 [GAL4(1-147)] followed by a multiple cloning site. Deletion mutants were made by using PCR with primers that incorporated the appropriate restriction site

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(*Eco*RI for the forward primer; a stop codon followed by *Bam*HI for the reverse primer). PCR was performed by using *Taq* polymerase (Boehringer Mannheim) according to manufacturer's instructions. PCR products were digested with the appropriate enzymes and subcloned into pBXG1. The GAL4-VP16 construct in Fig. 1 and 2 is BXGALVP, also a gift of M. Ptashne, which contains amino acids 413 to 490 of VP16.

All GAL4 (G4 in construct names) constructs containing the regulatory domain (RD in construct names) were made from a parent plasmid in which amino acids 201 to 370 of HSF1 were PCR amplified by using a forward primer that included a single point mutation that removes the naturally occurring *Bam*HI site in HSF1 without changing the amino acid sequence, and a reverse primer with an *Eco*RI site, so that different activation domains could be subcloned into this site. This PCR product was subcloned into pBXG1 in a blunt-end–*Eco*RI ligation.

(ii) Point mutations. The domain to be mutagenized was subcloned into pBluescript SK+ (Stratagene) and transformed into the *dut ung Escherichia coli* strain R382 to make single-stranded uracil-substituted DNA, using M13K07 helper phage (Pharmacia). Point mutations were introduced by using Kunkel mutagenesis as previously described (26), with the following modifications: the elongation and ligation step was done by incubation for 5 min on ice and 5 min at room temperature followed by 2 h at 37°C, and the plasmids were transformed into *E. coli* XL1Blue cells (Stratagene). Point mutations were confirmed by sequencing and then subcloned back into the appropriate GAL4 expression vector.

(iii) Reporter plasmids. The reporter construct used in these experiments was G540CAT, which contains five GAL4 DNA binding sites at position −40 of the *hsp70* basal promoter driving expression of the chloramphenicol acetyltransferase (CAT) gene (31). The internal reference plasmid was pIR17-84, which contains *hsp70* promoter sequences to −84 (thus deleting the heat shock elements), with a deletion in the 5′ untranslated region driving expression of the CAT gene (16). This produces an S1 reference signal that is 130 nucleotides, compared to 230 nucleotides for the reporter.

Transient transfections. Transfections were done with HeLa cells grown in Dulbecco modified Eagle medium containing 10% calf serum at 37°C in 5% CO_2. Plates at approximately 90% confluency were split 1:10 the day before transfection. Transfections were by the $CaPO_4$ method (1) with 5 μg of GAL4 expression plasmid, 5 μg of G540CAT reporter plasmid, and 5 μg of pIR17-84 internal reference plasmid. Chloroquine was added to the medium at a final concentration of 100 μM immediately before precipitate was added. Precipitate was left on cells for 4 h, and then cells were washed with phosphate-buffered saline (PBS) and refed with Dulbecco modified Eagle medium containing 10% calf serum. RNA was harvested approximately 48 h posttransfection. For heat shock experiments, cells were incubated at 43°C for 4 h before harvest.

RNA harvest and S1 analysis. Total cellular RNA was isolated from transfected cells by using RNeasy prep columns (Qiagen) according to the manufacturer's instructions. Single-stranded 5′-end-labeled S1 probe was prepared as previously described (16) and contains sequences from +229 to −133 of the *hsp70*-CAT fusion construct. Total cellular RNA (20 to 30 μg) was mixed with S1 probe (5×10^4 dpm), ethanol precipitated, air dried, and resuspended in hybridization buffer [40 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES; pH 6.4), 1 mM EDTA, 0.4 M NaCl, 80% deionized formamide]. Hybridization was at 30°C for 12 to 16 h. S1 nuclease digestion and analysis on 8% denaturing polyacrylamide gels were done by standard procedures (1). Gels were quantitated with a Molecular Dynamics PhosphorImager.

Electrophoretic mobility shift assay. Transfected HeLa cells were washed with ice-cold PBS, duplicate plates were pooled, and nuclear extracts were prepared as previously described (20). Total protein concentrations were determined by the Bradford assay (Bio-Rad). Ten micrograms of protein, except where noted, was brought up to 10 μl in buffer D (20 mM *N*-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid [HEPES; pH 7.9], 20% glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol), and 11 μl of a salt mix (20 mM HEPES [pH 7.5], 16.67 mM KCl, 8.83 mM $MgCl_2$, 16.67 μM $ZnCl_2$, 6.63 mM spermidine) was added; 0.1 μg of GAL4 antibody (RK5C1; Santa Cruz Biotechnology Inc.) was added, and the mixture was incubated on ice for 30 min. Then 5 μg of poly(dI-dC) (Pharmacia) and 2.5 ng of double-stranded radiolabeled probe containing a single GAL4 site (5′CGGAGTACTGTCCTCCG) were added, and the mixture was incubated at 30°C for 15 min and resolved on a native 4.5% acrylamide gel in 0.5× Tris-borate-EDTA.

In vivo labeling and immunoprecipitation. HeLa cells were seeded into 60-mm-diameter dishes and transfected with the appropriate GAL4 expression plasmids as described above. The same DNA-$CaPO_4$ precipitate was used for parallel samples (control and heat shock-treated cells). In addition, the transfection efficiency was monitored by using a human growth hormone-based transient assay (Nichols Institute). At 36 h posttransfection, cells were washed in phosphate-free minimal essential medium (Gibco BRL) and labeled in 1 ml of medium supplemented with 2% dialyzed fetal bovine serum and 1 mCi of $^{32}P_i$ (DuPont/New England Nuclear) for 90 min at 37 or 43°C.

Immunoprecipitations were carried out essentially as described elsewhere (1). Briefly, cells were washed with ice-cold PBS, and extracts were prepared from cells lysed directly in radioimmunoprecipitation assay buffer (10 mM Tris [pH 8.0], 0.1 M NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS]) supplemented with 50 mM β-glycerophosphate, 1 mM Na_3VO_4, 40 mM NaF, 2 μM leupeptin, 400 μM phenylmethylsulfonyl fluoride, and 10 kallekrein-inhibiting units of Trasylol per ml. Lysates were centrifuged at 4°C for 30 min at 12,000 × *g* and preincubated with protein A-Sepharose (Pharmacia) to reduce the background. Equal counts from all samples were incubated with a rabbit antiserum against human HSF1 (kindly provided by R. Morimoto) or GAL4 (kindly provided by M. Ptashne) for 2 h at 4°C, protein A-Sepharose was added, and incubation was continued for another hour. Immune complexes were washed four times with radioimmunoprecipitation assay buffer, which was supplemented with 1 M NaCl in the first washing step. Pellets were boiled in SDS sample buffer and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) (10% acrylamide) and autoradiography.

RESULTS

It has previously been shown that human HSF1 has a central regulatory domain that is able to repress function of the HSF1 activation domains and render them heat inducible (7, 27, 35). We have analyzed the HSF1 activation domains in order to determine whether specific amino acids in the activation domains are required for heat-induced regulation. There are two potent and separable activation domains in the C-terminal one-third of HSF1 (7); the proximal activation domain, AD1, is located within amino acids 371 to 430, and the distal domain, AD2, is located within amino acids 431 to 529 (Fig. 1A). AD1 is predicted to be helical in structure and is centered around a region that is conserved between HSF1 and HSF2, while AD2 is very glycine and proline rich and therefore predicted to be nonhelical in structure.

AD1 of HSF1 is only 20 amino acids. We performed deletion analysis on both HSF1 activation domains in order to further define amino acids necessary for transcriptional activation. Activation domains were fused to the GAL4 DNA binding domain, and their function was evaluated by cotransfection with a reporter containing five GAL4 sites and by using S1 analysis to measure resultant transcript levels (Fig. 1B). To normalize for transfection efficiency, an internal reference plasmid that contains the basal *hsp70* promoter with a deletion in the 5′ untranslated region that results in a shorter S1 product was included. Intensities of the resultant S1 products were quantified by a PhosphorImager, and the ability of each GAL4 fusion protein to activate transcription was determined by dividing the signal from the reporter plasmid by that from the internal reference. Transcriptional activation values in the figures are for the gel shown; repeat experiments gave values within 15% of those reported below.

Deletion analysis of the 60-amino-acid region from amino acids 371 to 430 defined 20 amino acids (401 to 420) as the primary determinant of AD1 activity (Fig. 1B). These 20 amino acids had activity comparable to that of the VP16 activation domain (compare lanes 2 and 8). Deletion into this 20-amino-acid region from either side almost completely abolished transactivation (Fig. 1B, lanes 5 and 9).

Deletion analysis of AD2 did not reveal boundaries as defined as those of AD1 (Fig. 1C). Amino acids 453 to 505 of this domain activated transcription approximately 30% as well as VP16 (Fig. 1C; compare lanes 1 and 10); however, inspection of the data reveals that amino acids 431 to 441 can also play important roles in activating transcription in certain deletion mutations (e.g., compare lanes 6 and 9). Therefore, functional regions of AD2 are spread out over approximately 75 amino acids.

Hydrophobic and acidic residues are critical for activation by AD1. The small size and high activity of AD1 made it a good candidate for point mutagenesis. We scanned across the important 20 amino acids by point mutating 2 amino acids at a time to alanine. An alanine (position 413) found in the wild-type sequence was not mutated, and aspartic acid residues were mutated to lysine in order to reverse the negative charge. Single-point mutations were made across a critical segment of

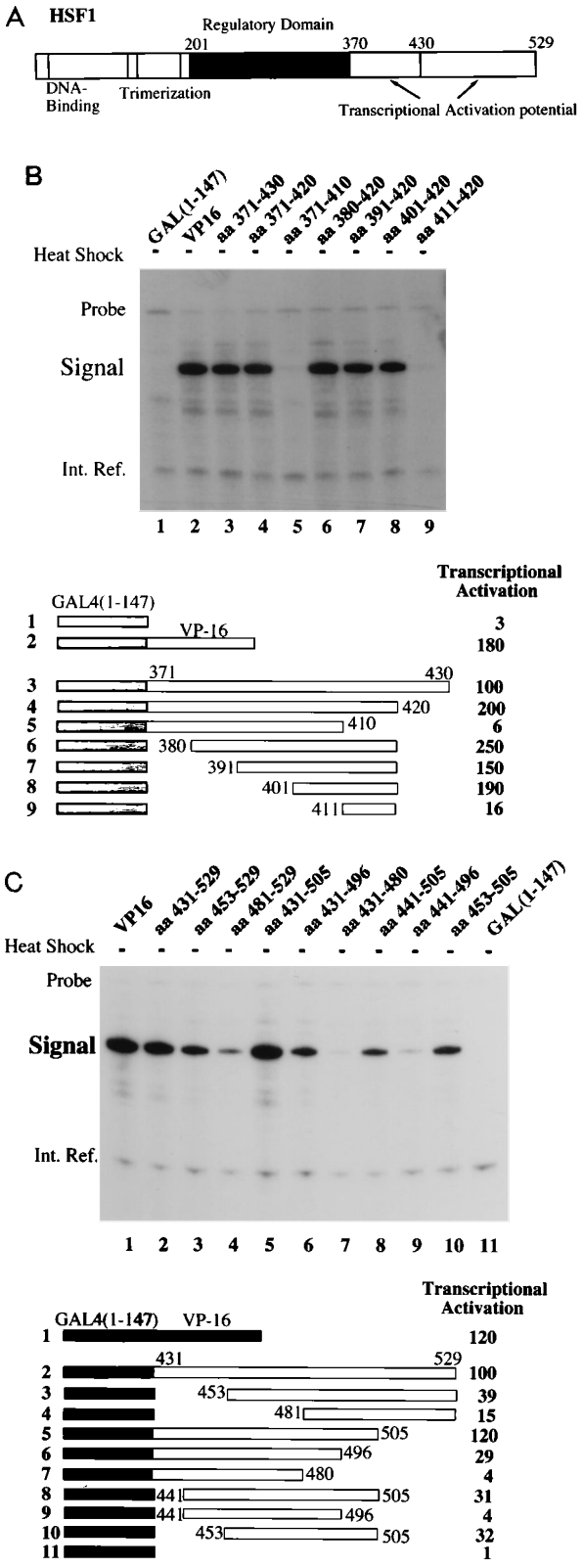

FIG. 1. Deletion mutagenesis of HSF1 activation domains. (A) Schematic representation of human HSF1. The DNA binding domain and trimerization domain are defined by homology with other characterized HSFs. The regulatory domain and transcriptional activation domains are as defined by Green et al. (7), with numbering referring to the amino acid at the border of the domain. (B) Deletion mutagenesis of the proximal activation domain. S1 analysis was done on 20 μg of total RNA harvested from normally growing HeLa cells with the indicated activator construct. The resultant signal was quantified by a PhosphorImager, and transcriptional activation was calculated by dividing the signal band by the internal reference (Int. Ref.) band. Schematics of deletion constructs are shown below. GAL4 VP-16 and GAL4(1-147) are included as positive and negative controls, respectively. There was no heat shock in this experiment. aa, amino acids. (C) S1 analysis of 20 μg of RNA from HeLa cells transfected with the indicated deletion mutants of the distal activation domain of HSF1. Quantitation was done as described for panel B.

the activation domain (Fig. 2A). We analyzed the point mutations within the context of a 60-amino-acid segment that is conserved between mammalian HSF1 and HSF2; this was done to ensure that there is no functional redundancy in the region immediately surrounding AD1. This analysis demonstrated that several hydrophobic amino acids are critical for activation (Fig. 2A, lanes 1, 5, and 7 to 13) as well as the two acidic residues (lanes 5 and 11). These amino acids include a phenylalanine residue at position 418, where mutation to alanine causes a 10-fold decrease in activation, reminiscent of the mutational analysis of VP16 (4). Point mutagenesis of serine and threonine residues had little effect on the activation potential of this domain (lanes 2 and 6; see below).

To ensure that the effect on transcriptional activation was not due to an effect on stability or binding of the GAL4 fusion protein, we performed a gel shift analysis using extracts made from transfected cells and a single GAL4 binding site. The shifted band produced by GAL4 fusion proteins in this experiment is obscured by a band caused by nonspecific binding by a protein(s) found in untransfected cells (Fig. 2B, lanes 27 and 28). Therefore, the amount of GAL4 DNA binding activity was determined by supershifting the complex with an antibody specific for the GAL4 DNA binding domain. This antibody has no effect on the mock-transfected cells (Fig. 2B, lane 28). All of the mutants that have decreased transcriptional activation have DNA binding activity equal to or greater than the wild-type level (Fig. 2B). The only mutant that appears to have lower than wild-type expression is the histidine-glycine mutant, which has wild-type levels of transcriptional activation, indicating that amounts of protein that are barely detectable in an in vitro gel shift assay are likely saturating in vivo.

The regulatory domain of HSF1 is capable of regulating the 20-amino-acid AD1. To use mutations in AD1 to determine whether any specific amino acids were required for response to the regulatory domain, we first had to verify that the wild-type 20-amino-acid AD1 responded appropriately to regulation. The potent activation potential of this 20-amino-acid segment is significantly repressed by fusion to the regulatory domain (Fig. 3A, lanes 7 and 3). Heat shocking the cells induced this fusion protein to activate transcription (compare lanes 7 and 8), while the AD1 segment without the regulatory domain was not heat inducible (compare lanes 3 and 4). The 20-amino-acid AD1 responds similarly in these assays to the 60-amino-acid conserved portion of HSF1 that contains AD1 (compare lanes 3, 4, 7, and 8 with 1, 2, 5, and 6). Gel shift analysis demonstrated that the DNA binding activity of the fusion proteins that contain the regulatory domain was not heat inducible (Fig. 3B). Fusion proteins containing the regulatory domain were expressed at a much higher level than the activation domains alone, a fact also noted previously (7). Perhaps this is due to a toxic effect of this potent activation domain on the cell. Thus, fusion of the regulatory domain to AD1 creates a highly defined factor whose activation capability is heat responsive, allowing us to determine if there are any specific amino acids of the activation domain required for the response to heat shock.

Phosphorylation of AD1 is not necessary for heat shock-induced transactivation. Endogenous HSF1 has been shown to

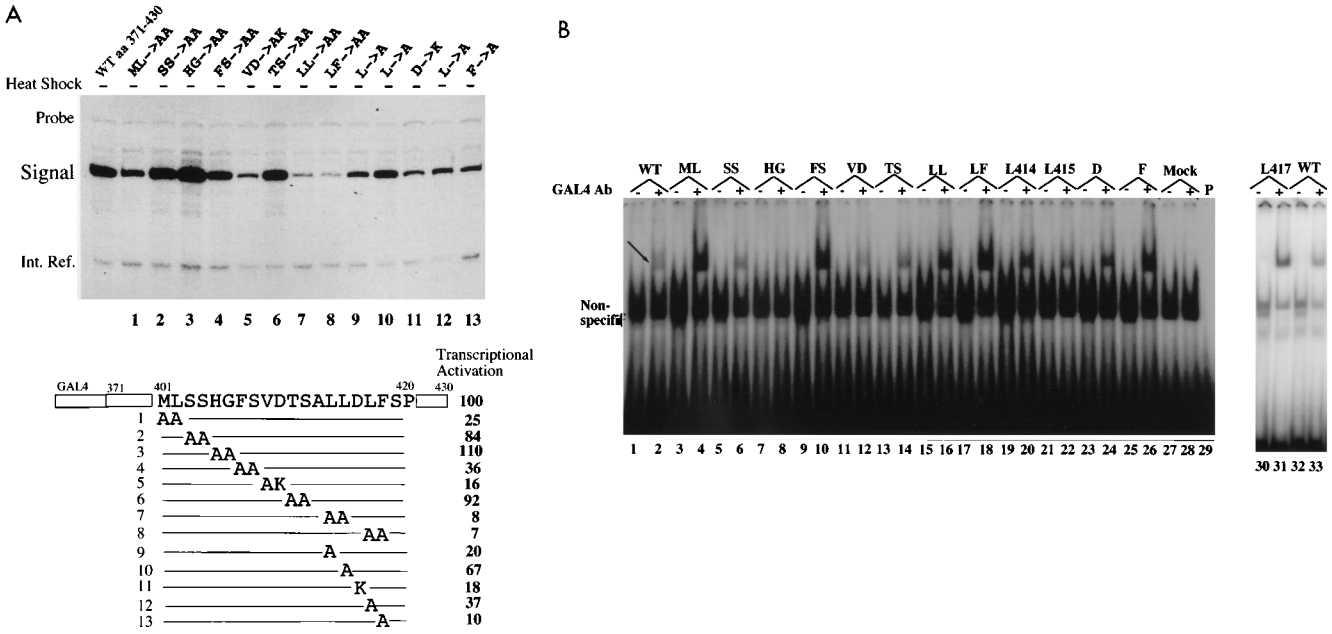

FIG. 2. Point mutagenesis of AD1 shows the importance of hydrophobic and acidic amino acids. (A) S1 analysis of 20 μg of RNA from cells transfected with the indicated point mutations in AD1. Amino acids (aa) were mutated two at a time to alanine to scan across the 20-amino-acid region of AD1 in the context of amino acids 371 to 430. Acidic residues were mutated to lysine to determine the effect of reversing the charge. Constructs 7 and 8 had such reduced activity that the single amino acid changes were made across this region. WT, wild type; Int. Ref., internal reference. (B) Gel shift analysis of the expression of mutant GAL4 fusion proteins relative to wild-type expression. Extracts were prepared from HeLa cells transfected exactly as for panel A with the indicated constructs (letters above the lanes refer to the mutated amino acids). Ten micrograms of protein was used in each lane. The GAL4 DNA binding domain-containing species were supershifted with a GAL4-specific antibody (Ab) in order to separate the GAL4 signal from an obscuring band of cellular origin (lanes 28 and 29). The supershifted species is indicated with an arrow. Lanes 30 to 33 were from a different experiment using a different batch of poly(dI-dC) competitor which resulted in less prominent nonspecific bands.

be hyperphosphorylated upon heat shock (18). This phosphorylation is not necessary for the acquisition of DNA binding activity and has been proposed to be involved in transcriptional activation (8, 18, 25). We used regulatory domain-AD1 fusion proteins to determine whether induced phosphorylation was required for increased transcriptional activation following heat shock. There are six possible phosphorylation sites in AD1 that might play a role in regulation by heat. We mutated all five serines and one threonine residue in AD1 and fused this mutated activation domain to the regulatory domain. This mutant construct, through both deletion and point mutagenesis, has all of the possible phosphorylation sites following amino acid 370 removed and therefore has none of the phosphorylation sites normally found within 30 amino acids of the transcriptional activation domains.

Mutation of these potential phosphorylation sites did not disrupt the heat shock regulation of the factor (Fig. 4A, lanes 7 and 8). The mutant construct is repressed at the control temperature and strongly heat shock inducible. This activation domain alone is less capable of transcriptional activation than the wild type (Fig. 4A; compare lanes 1 and 2 with lanes 5 and 6), a result that is not surprising considering that 6 amino acids out of 20 have been mutated to alanine. Note that following heat shock, the mutated AD1-regulatory domain construct is significantly more active than the mutated AD1 construct that lacks the regulatory domain (compare lane 8 with lanes 5 and 6). This observation emphasizes the conclusion that phosphorylation of the activation domain is not required for induction of transcriptional activation following heat shock.

To ensure that we have in fact removed all of the heat-inducible phosphorylation sites of HSF1 in our mutant construct, we performed in vivo labeling with $^{32}P_i$ followed by immunoprecipitation with either an HSF- or a GAL4-specific antiserum. Endogenous HSF from mock-transfected cells is partly phosphorylated at the control temperature and highly phosphorylated after heat shock (Fig. 4B, lanes 7 and 8) (18, 25). Both G4-RD-AD1 and G4-RD-AD1:SallA (SallA is defined in the legend to Fig. 4) are phosphorylated at the control temperature, but the level of phosphorylation is not increased after heat shock (lanes 3 to 6). GAL4 alone is not labeled to detectable levels, suggesting that the constitutive phosphorylation that we observe is in the regulatory domain (lanes 1 and 2). To ensure that these proteins are expressed at similar levels, we performed gel shift analysis of extracts from cells transfected with G4-RD-AD1 and G4-RD-AD1:SallA and found equal levels of DNA binding activity in cells with similar transfection efficiencies as determine by human growth hormone assays (Fig. 4C). These data indicate that heat-induced phosphorylation is not necessary for heat induction of AD1. (We have not addressed the role of phosphorylation in regulation of AD2 activity.)

We determined next whether any of the other point mutations in AD1 had an effect on regulation. When all of the point mutants discussed above (Fig. 2) were fused with the regulatory domain and examined for heat induction, they all exhibited appropriate regulation; that is, they were all repressed at the control temperature and induced by heat shock (data not shown). Thus, mutation of any individual amino acid does not eliminate response to the regulatory domain.

The regulatory domain is able to render VP16 heat shock inducible. The data presented above led to a testable hypothesis concerning the function of the regulatory domain: if no specific amino acids in AD1 are required for response to the regulatory domain, then heterologous activation domains might also respond to the regulatory domain. We created fusion proteins to test this hypothesis. As a control in this exper-

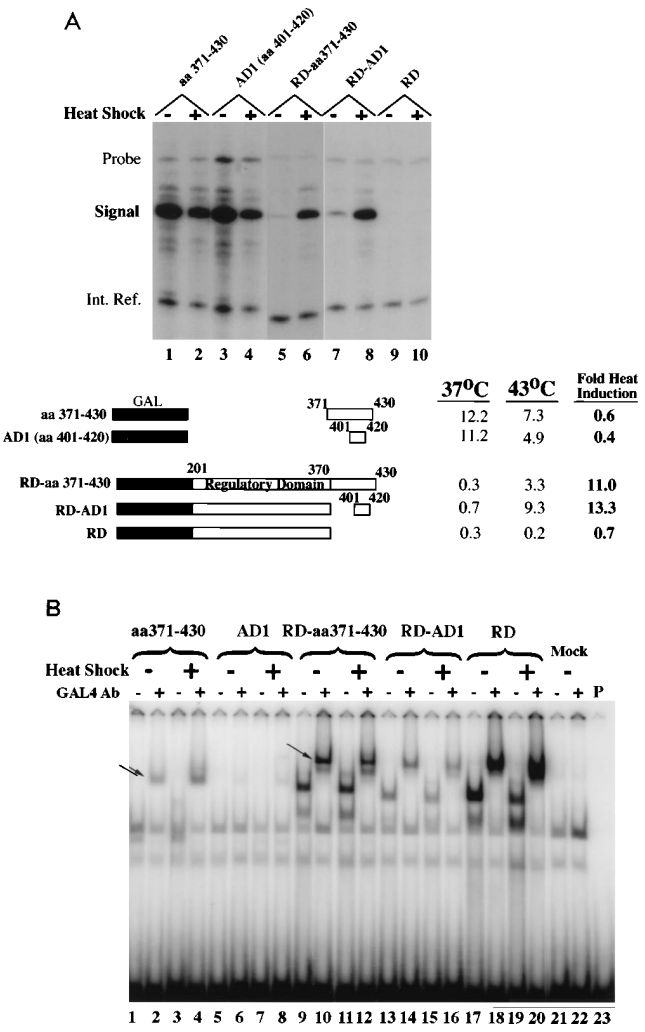

FIG. 3. AD1 is heat regulated when fused with the regulatory domain. (A) S1 analysis of 25 μg of total RNA harvested from cells transfected with the constructs diagrammed at the bottom. Heat shock lanes represent RNA harvested immediately after a 4-h heat shock at 43°C. Quantification is shown below. Fold heat induction is the level at 43°C divided by the level at 37°C. Lanes 5 and 6 are from a different gel. aa, amino acids; Int. Ref., internal reference. (B) Gel shift analysis demonstrating the ability of the fusion proteins to bind DNA both before and after heat shock. Supershifted species are indicated with arrows. Ten micrograms of protein was used in lanes 1 to 12, 21, and 22, while 5 μg of protein was used in lanes 13 to 20, as these constructs express at a very high level. Ab, antibody.

iment, we used a point mutation that inactivates the ability of the regulatory domain to modulate activity of the HSF activation domains and verified that this mutation would also interfere with regulatory domain function when the domain was not fused to the HSF activation domains. The point mutation used below (Lys-298 to Ala [K298A]) was identified during a general mutagenesis of the regulatory domain (21a). This mutation essentially eliminates the ability of the regulatory domain to repress AD1 function (Fig. 5A; compare lanes 1 and 3) and the ability to confer heat induction (compare lanes 1 and 2 with lanes 3 and 4).

To determine whether the regulatory domain could function with a heterologous activation domain, we fused the regulatory domain to both subdomains of the transcriptional activator, VP16 (amino acids 413 to 456 and 452 to 490). We used these subdomains of VP16 because they are both well characterized (4, 24). Both of these domains are able to potently activate transcription when fused to the GAL4 DNA binding domain, and neither is heat inducible (Fig. 5B, lanes 1, 2, 5, and 6). However, both of these activation domains were repressed at the control temperature and heat shock inducible when fused with the regulatory domain of HSF1 (Fig. 5B, lanes 3, 4, 7, and 8). The K298A point mutation in the regulatory domain eliminated repression and heat induction of the VP16 activation domains (Fig. 5B, lanes 9 to 12), as seen with the HSF AD1 (Fig. 5A). We conclude that the regulatory domain can function with heterologous activation domains.

DISCUSSION

The activity of HSF1 is highly regulated, both at the level of DNA binding and at the level of transcriptional activation. It had previously been shown that HSF1 has two separable activation domains that can both be regulated by heat when fused with the central regulatory domain (7). It was not clear from these previous studies whether the nature of the HSF1 activation domains contributed significantly to the ability of HSF1 to respond to heat. The central conclusion of this study is that the regulatory domain is capable of playing a primary role in sensing heat stress; no specific feature of AD1 is required for response to heat shock. Three major lines of evidence support this conclusion. First, point mutagenesis across the entire sequence of AD1 revealed no specific amino acids necessary for heat shock regulation of this domain. Second, when the HSF1 activation domains were functionally replaced by activation domains from VP16, the factor remained heat regulated. Third, in contrast to mutations in the activation domain, a mutation in the regulatory domain that abolished regulation of both homologous and heterologous activation domains was found. These data demonstrate that the regulatory domain of HSF1 is functional and has the ability to receive a heat signal when it is removed from its natural context.

It has long been known that HSFs from *S. cerevisiae* to humans are hyperphosphorylated in response to heat shock (18, 30). HSF is also hyperphosphorylated when treated with other inducers of transcriptional activation such as cadmium and arachidonic acid (15, 25). Close examination reveals a strong correlation between heat induction of phosphorylation and heat induction of activation for both mouse HSF1 and yeast HSF (25, 28). We have shown that heat-inducible phosphorylation is not necessary for regulation of transcriptional activation of AD1, as removal of all of the heat-inducible phosphorylation sites does not affect heat-inducible transcriptional activation (Fig. 4). It is possible that heat-induced phosphorylation plays a role in regulation of AD2 and AD1 in the context of intact HSF1; however, our results concerning regulation of AD1 make this possibility less likely. These data are consistent with results found upon treating cells with the amino acid analog azetidine, which induces heat shock gene transcription but does not cause hyperphosphorylation of murine HSF1 (25). Studies of HSF from the budding yeast *Kluyveromyces lactis* also showed that heat-induced phosphorylation of serines responsible for the mobility shift is not necessary for activation and may in fact be involved in deactivating the factor (10, 13). However, in this case, the serines are located outside the activation domains of this protein and are not conserved between *K. lactis* and mammalian HSFs. Thus, the function of heat-induced phosphorylation of HSF1 remains unknown.

In the course of addressing the role of the activation domains in heat regulation, we further characterized these portions of the factor. AD1 shares characteristics with the activation domains of VP16 with respect to both potency and

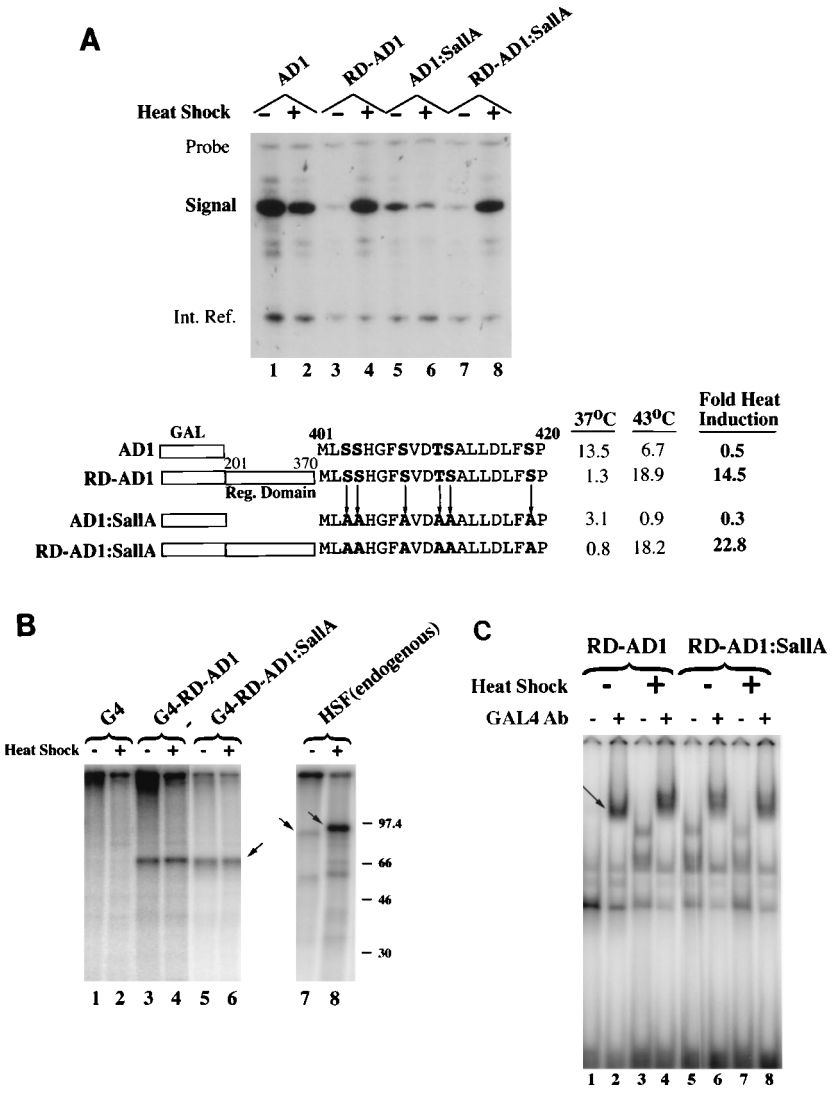

FIG. 4. Phosphorylation sites in AD1 are unnecessary for heat regulation. (A) S1 analysis of 30 μg of RNA harvested from transfected HeLa cells containing the regulatory (Reg.) domain with either wild-type AD1 or AD1 with all potential phosphorylation sites mutated to alanine (AD1:SallA) as in the schematic shown below. Transfected cells were heat shocked as indicated. Int. Ref., internal reference. (B) In vivo phosphorylation of transfected GAL4 fusion constructs compared with that of endogenous HSF. Cells were labeled with $^{32}P_i$ at 37 or 43°C for 90 min. Cell extracts were immunoprecipitated with an anti-GAL4 (lanes 1 to 6) or anti-HSF1 (lanes 7 and 8) antibody (Ab) and analyzed by SDS-PAGE and autoradiography. The exposure time for each pair of lanes was adjusted for transfection efficiency as determined by human growth hormone assays. (C) Gel shift analysis demonstrating equal amounts of protein being expressed by RD-AD1 and RD-AD1:SallA, both at the control temperature and after heat shock. Ten micrograms of protein was used in each lane, as the two constructs had similar transfection efficiencies in this experiment. The GAL4 DNA binding domain-containing species were supershifted by GAL4 antibodies as indicated, and the supershifted species are indicated with arrows.

richness in bulky hydrophobic and acidic amino acids. Both AD1 and the VP16 N-terminal activation domain also contain a critical phenylalanine residue. AD2 (amino acids 431 to 505) is similarly rich in hydrophobic and acidic amino acids but, in contrast to AD1 and VP16, has a high proline and glycine content (21%). In addition, the transcriptional activation potential of AD2 is spread out over a large area with ill-defined borders, similar to findings of studies on other activators such as GCN4 (11). Despite the differences between AD1 and AD2, it remains possible that AD1 and AD2 of HSF1 activate transcription through mechanisms similar to each other and to that of the VP16 activation domains, as all have similar hydrophobic and acidic amino acid contents. The regulatory domain might function most efficiently when paired with activation domains of this class. The possibility that certain classes of activation domains are optimal for heat-regulated transcription has been suggested by studies of HSFs from different budding yeasts. The VP16 activation domain fused with portions of *S. cerevisiae* HSF also created a heterologous HSF, while the activation domain of GCN4 was not heat inducible in a fusion protein with *K. lactis* HSF sequences (2, 13). It will be necessary to elucidate the mechanisms of action both of these activation domains and of the regulatory domain to determine whether certain classes of activation domain work best in heat induction.

The HSF1 regulatory domain acts as a repressor of activated transcription at the control temperature. While it is not clear how the ability to repress relates to heat induction, our experiments indicate that heat induction may not be simply derepression. There are cases when the heat-induced level of tran-

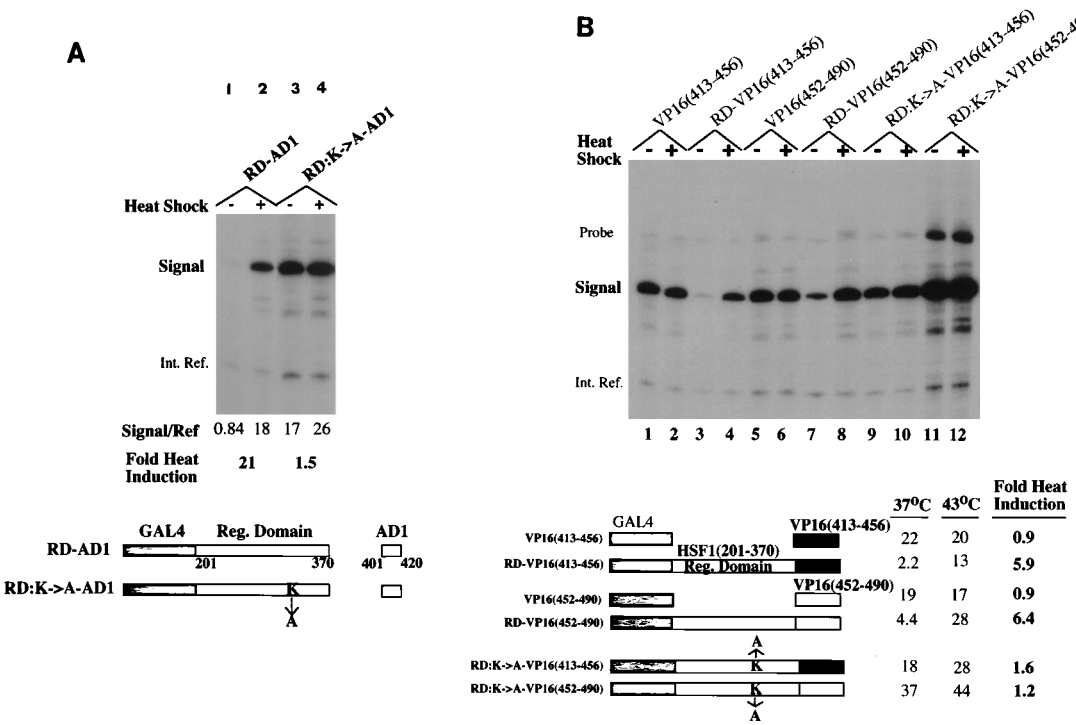

FIG. 5. The regulatory domain can confer heat regulation on the VP16 activation domains. (A) Effect of a single point mutation in the regulatory (Reg.) domain on heat regulation, determined by S1 analysis of 20 μg of RNA isolated from HeLa cells transfected with the indicated activator constructs. RD:K→A-AD1 contains a mutation of lysine 298 to alanine. Int. Ref., internal reference. (B) S1 analysis of 20 μg of RNA from HeLa cells transfected with regulatory domain-VP16 fusions, constructed as shown in the schematic at the bottom. Quantification of these data was performed as described in the legend to Fig. 3.

scriptional activation with the regulatory domain is above the intrinsic potential of the activation domain itself, such as with AD1 when all five serines and one threonine are mutated to alanine (Fig. 4A). This could be an indication that heat induction is more than derepression, or it could be the result of having the activation domain further displaced from the DNA binding domain in this fusion protein.

We have demonstrated that the regulatory domain of HSF1 has both repression and heat induction activity when isolated from the rest of the HSF1 protein, confirming that this is a separable domain. This domain is responsible for significant repression and heat induction when paired with the heterologous VP16 activation domains and the GAL4 DNA binding domain (Fig. 5). Transcriptional activators frequently have separable modules that are capable of sequence specific DNA binding and of transcriptional activation. It is apparent that HSF1, like the steroid receptors (22), has a third module that is capable of playing a regulatory role.

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