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Repression of the Heat Shock Factor 1 Transcriptional Activation Domain Is Modulated by Constitutive Phosphorylation

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Heat shock transcription factor 1 (HSF1) is constitutively expressed in mammalian cells and negatively regulated for DNA binding and transcriptional activity. Upon exposure to heat shock and other forms of chemical and physiological stress, these activities of HSF1 are rapidly induced. In this report, we demonstrate that constitutive phosphorylation of HSF1 at serine residues distal to the transcriptional activation domain functions to repress transactivation. Tryptic phosphopeptide analysis of a collection of chimeric GAL4-HSF1 deletion and point mutants identified a region of constitutive phosphorylation encompassing serine residues 303 and 307. The significance of phosphorylation at serines 303 and 307 in the regulation of HSF1 transcriptional activity was demonstrated by transient transfection and assay of a chloramphenicol acetyltransferase reporter construct. Whereas the transfected wild-type GAL4-HSF1 chimera is repressed for transcriptional activity and derepressed by heat shock, mutation of serines 303 and 307 to alanine results in derepression to a high level of constitutive activity. Similar results were obtained with mutation of these serine residues in the context of full-length HSF1. These data reveal that constitutive phosphorylation of serines 303 and 307 has an important role in the negative regulation of HSF1 transcriptional activity at control temperatures.

A hallmark of the heat shock response is the rapid, inducible transcription of genes that encode heat shock proteins and molecular chaperones. These events are mediated through heat shock transcription factor 1 (HSF1), a ubiquitous and constitutively expressed protein which is activated upon exposure to heat shock and other chemical, environmental, and physiological stresses (29, 31, 32, 46). Under normal growth conditions, HSF1 exists in a repressed state for both DNA binding and transcriptional activities. Activation is associated with transitions in the oligomeric state of HSF1 from a repressed monomer to a nucleus-localized trimer which possesses DNA binding activity (4, 34, 38, 39, 42, 44, 47). Treatment of cells with salicylate and other nonsteroidal anti-inflammatory drugs induces formation of the HSF1 DNA binding trimer in the absence of transcriptional activity (10, 18–20), demonstrating that the DNA binding and transcriptional activities of HSF1 can be uncoupled in mammalian cells and that the two activities may be silenced via distinct mechanisms.

The mechanism by which HSF1 is negatively regulated is complex. For example, the analysis of chimeric HSF1 proteins, in which a heterologous DNA binding domain of either LexA or GAL4 is fused to HSF1, reveals that the transcriptional activation domain remains repressed despite the ability of chimeric HSF1 to bind DNA constitutively (13, 35, 40, 48). In these chimeric HSF1 constructs, the transactivation domain is derepressed either by exposure to heat shock or upon deletion of the negative regulatory domain (40). These results reveal that chimeric HSF1 retains the ability to regulate the transactivation domain in a manner similar to that of endogenous HSF1. In contrast to the negatively regulated state of in vivo-expressed HSF1, recombinant HSF1 expressed in *Escherichia coli* exists in a trimeric state that exhibits constitutive DNA binding activity (8, 24, 37, 39). These results suggest that certain regulatory events which are specific to the eukaryotic cell may be necessary to maintain HSF1 in a repressed state. Among the differences between the recombinant and in vivo forms of HSF1 is that recombinant HSF1 is not constitutively phosphorylated.

HSFs undergo reversible inducible phosphorylation upon exposure of yeast, *Drosophila*, and mammalian cells to a variety of stresses. However, a direct role for phosphorylation remains uncertain. Activation of DNA binding can occur in the absence of inducible phosphorylation; furthermore, the HSF1 induced by amino acid analogs is not inducibly phosphorylated (39). A role for phosphorylation has been suggested in the regulation of the transactivation domain and during attenuation of the activated form of HSF (10, 15). Phosphorylation represents one of the best-studied examples of posttranslational modifications which influence the activities of the respective protein. A number of transcription factors utilize phosphorylation to provide a potent regulatory step in the processes of nuclear import, DNA binding activity, transcriptional activity, association with other factors, and degradation (16, 17, 22). In many of these examples, the negative regulation of transcription factor activity by phosphorylation involves predominantly regulation at or prior to the level of DNA binding. One exception is the cyclic AMP response element-binding protein, which is negatively regulated by phosphorylation of the activation domain by calmodulin-dependent protein kinase type II (43).

In this study, we addressed the role of constitutive phosphorylation in the regulation of HSF1 transcriptional activity. To accomplish this, we identified the site(s) of phosphorylation and examined how the loss of these phosphoamino acids influences the transcriptional activity of HSF1. We utilized an approach in which a collection of chimeric GAL4-HSF1 deletion mutants were used to identify serine residues 303 and 307 as targets of constitutive phosphorylation. Replacement of these residues with alanine results in derepression of HSF1 at control temperatures. Thus, constitutive phosphorylation negatively regulates HSF1 transcriptional activity under control

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temperatures at a step following the acquisition of DNA binding.

MATERIALS AND METHODS

HeLa S3 cell culture, heat shock, and [^{32}P]orthophosphate labeling conditions. HeLa S3 cells were grown in Joklik's medium containing 5% calf serum. Cell growth and heat shock conditions were as previously described (1). [^{32}P]orthophosphate (ICN) was added to cells at 50 μCi/ml at 6 h prior to heat shock following centrifugation (600 × *g* for 5 min) and resuspension in prewarmed phosphate-deficient Dulbecco's modified Eagle medium (DMEM) at a density of 5×10^5 cells/ml. Unlabeled cells were treated similarly. At the indicated time points, aliquots of 5×10^6 cells were collected and frozen.

Gel mobility shift assay, Western blotting, immunoprecipitation, and Hsp70 transcription analysis. HSF DNA binding activity was measured as previously described (33). Quantitation of HSF-HSE complexes was performed with a PhosphorImager 400A (Molecular Dynamics). Western blot analysis was performed with 10 μg of whole cell extract and rabbit polyclonal anti-HSF1 serum (39), and immune complexes were visualized with the ECL detection system (Amersham). The immunoprecipitation assays used approximately 5×10^6 ^{32}P-labeled cells, which were harvested, washed with ice-cold phosphate-buffered saline, and lysed in ice-cold RIPA buffer (10 mM Tris [pH 7.4], 150 mM NaCl, 1% sodium deoxycholate, 1% Triton X-100 containing 50 mM NaF, 0.2 mM $NaVO_4$, 5 mM PP_i, 1 mM phenylmethylsulfonyl fluoride, 2 μg of leupeptin A per ml, 2 μg of pepstatin A per ml). The lysates were cleared by centrifugation and 3 μl of rabbit anti-HSF1 polyclonal serum was added to the cell lysate. After agitation for 1 h at 4°C, 30 μl of a 1:1 slurry of phosphate-buffered saline–protein A–Sepharose was added and the mixture was mixed for another hour at 4°C. Beads were washed five times with ice-cold RIPA buffer containing 0.1% sodium dodecyl sulfate (SDS). Following the last wash, the beads were resuspended in 25 μl of Laemmli sample buffer and boiled for 5 min. Proteins were separated by SDS–8.5% polyacrylamide gel electrophoresis (PAGE) and visualized by autoradiography. Nuclei from 8×10^6 cells were isolated and frozen for use in transcription run-on assays (33). ^{32}P-labeled RNA was hybridized to nitrocellulose-immobilized plasmids corresponding to the Hsp70 gene (45), the glyceraldehyde 3-phosphate dehydrogenase gene, and the β-actin gene (14). Plasmid pBR322 was used as a nonspecific hybridization control.

NIH 3T3 cell culture, [^{32}P]orthophosphate labeling, phosphoamino acid analysis, and tryptic phosphopeptide mapping. NIH 3T3 cells were grown in DMEM containing 5% calf serum and maintained in a controlled 5% CO_2 incubator at 37°C. For steady-state labeling, cells were incubated in phosphate-deficient DMEM containing 5% dialyzed serum and 1 mCi of [^{32}P]orthophosphate per ml for 10 h prior to heat shock and/or harvesting. Immunoprecipitation and SDS-PAGE were performed as described above. Phosphoamino acid analysis was performed as previously described (5). Tryptic phosphopeptide analysis was performed on HSF1 eluted from SDS-PAGE gel slices. The eluted HSF1 was trichloroacetic acid precipitated, oxidized with performic acid, and digested with tolylsulfonyl phenylalanyl chloromethyl ketone (TPCK)-trypsin (Worthington) as previously described (5). Digested protein was desalted on a 10-ml G-25 Sephadex column, and phosphopeptide mapping was performed in two dimensions on thin-layer chromatography (TLC) plates (EM Science). The protein samples were separated in the first dimension by electrophoresis with an HTLE-7000 apparatus (CBS Scientific) in pH 4.7 buffer (1-butanol–pyridine–glacial acetic acid–water [2:1:1:36]) at 800 V and 18°C for 50 min. The plates were dried, and chromatography was performed in the second dimension in 1-butanol–pyridine–glacial acetic acid–water (15:10:3:12). The plates were visualized by using a PhosphorImager 400A (Molecular Dynamics) and autoradiography.

COS-7 cell culture, transfection, and [^{32}P]orthophosphate labeling. COS-7 cells were grown in DMEM containing 7.5% calf serum and maintained in a controlled 5% CO_2 incubator at 37°C. Cells were transfected by using Lipofectamine reagent (GIBCO Bethesda Research Laboratories) in accordance with the manufacturer's instructions. mHSF1 deletion constructs were cloned into plasmid pSG424, originally constructed in Mark Ptashne's laboratory (gift of N. Jones). pSG424 contains both the simian virus 40 origin of replication and promoter and amino acids 1 to 147 of the yeast GAL4 DNA binding domain. At 30 h posttransfection, cells were either harvested or heat shocked for 1 h at 42°C. mHSF1 was immunoprecipitated and analyzed as described above.

In vitro phosphorylation of recombinant HSF1 with p42 mitogen-activated protein (MAP) kinase. A 100-ng sample of purified recombinant HSF1 was incubated in 18 μl of kinase buffer (50 mM KPO_4 [pH 6.5], 10 mM dithiothreitol, 10 mM $MgCl_2$), and the reaction was initiated by addition of 1 μl of [γ-^{32}P]ATP (7,000 Ci/mmol; ICN) and 1 μg of p42 MAP kinase (Santa Cruz Biotechnology). After incubation for 15 min at 30°C, the reaction was terminated by addition of Laemmli sample buffer (25), after which the sample was boiled and resolved by SDS-PAGE.

NIH 3T3 cell transfection, primer extension, and CAT analysis. For primer extension analysis, NIH 3T3 cells were transfected by using Lipofectamine (GIBCO Bethesda Research Laboratories) reagent in accordance with the manufacturer's instructions. Cells were harvested 30 h posttransfection or heat shocked for 3 h at 43°C prior to harvest. A 10-μg sample of RNA was hybridized with an end-labeled chloramphenicol acetyltransferase (CAT) primer, and primer extension analysis was performed as previously described (40). For analysis of full-length (wild-type or mutant) HSF1, cells were transfected by the calcium phosphate method as previously described (3). HSF1 was cloned into pCDNA3 (Invitrogen) for expression, and 500 ng of the plasmid was used per transfection along with 7.5 μg of plasmid pHBCAT (45) and 7.5 μg of plasmid pCH110 (Pharmacia) for a β-galactosidase internal control. The total DNA amount per transfection was brought to 20 μg by using plasmid pCDNA3. Cells were harvested 30 h posttransfection for measurement of CAT activity.

RESULTS

HSF1 activation is characterized by rapid DNA binding, inducible phosphorylation, and transcriptional activity. The activation properties of HSF1 were studied in HeLa cells exposed to a 42°C heat shock for 4 h. HSF1 DNA binding activity is detected within minutes of temperature elevation, achieves maximal levels between 5 and 15 min, and is maintained at this level for 60 min (Fig. 1A). Thereafter, HSF1 DNA binding activity attenuates to control levels over a period of 2 h, even though the cells remain exposed to the original heat shock temperature. The appearance and loss of HSF1 DNA binding activity is not due to any fluctuation in the levels of HSF1 protein (Fig. 1B), although there are striking differences in electrophoretic mobility due to inducible phosphorylation of HSF1 (4, 10, 15, 26, 39, 41).

Within 2 to 3 h after the initial shift to the heat shock temperature, most of the HSF1 returns to the faster-migrating species or control form of HSF1. The phosphorylation state of HSF1 before or during heat shock was examined directly by steady-state labeling of HeLa cells with [^{32}P]orthophosphate followed by immunoprecipitation with polyclonal anti-HSF1 antibodies (Fig. 1C). HSF1 is constitutively phosphorylated (Fig. 1C, 37°C control lane) and undergoes inducible phosphorylation upon heat shock coincident with a mobility shift as detected by SDS-PAGE. To further analyze HSF1 properties during heat shock, transcription of the *hsp70* gene was measured with a nuclear run-on assay. Quantitation of all three activities reveals that the rapid and transient increase in *hsp70* transcription (Fig. 1D) parallels the kinetics of HSF1 DNA binding and inducible phosphorylation. It is, however, evident that activation of HSF1 DNA binding activity precedes both *hsp70* gene transcription and the inducibly phosphorylated state of HSF1 (Fig. 1E). Furthermore, the rates of *hsp70* transcription and HSF1 inducible phosphorylation increase similarly, whereas during attenuation, *hsp70* transcription rates decline rapidly prior to loss of DNA binding activity and the inducibly phosphorylated state (Fig. 1D). The similarity in the activation profile for inducible phosphorylation and *hsp70* transcription suggests that these events are linked. If we consider a predicted 1- to 2-min lag time for elongation of *hsp70* transcripts, the DNA binding activity still precedes *hsp70* transcription; however, the inducible phosphorylation begins to lag behind *hsp70* transcription (36). These results are in agreement with those of previous studies with nonsteroidal anti-inflammatory drugs which have been shown to uncouple HSF1 DNA binding and transcriptional activity (10, 18–20, 27).

HSF1 is constitutively and inducibly phosphorylated at multiple serine residues. We further examined the phosphorylation state of HSF1 by Western blot and immunoprecipitation assays by using extracts from control and heat-shocked NIH 3T3 cells which were steady state labeled with [^{32}P]orthophosphate. Consistent with previous findings, HSF1 is constitutively phosphorylated and upon heat shock treatment acquires additional phosphorylation which coincides with slower electrophoretic mobility on SDS-PAGE (Fig. 2A and B). The immunoprecipitated HSF1 was also subjected to phosphoamino acid analysis. As shown in Fig. 2C, HSF1 from control cells, as well as that from stressed (heat-shocked and cadmium-treated)

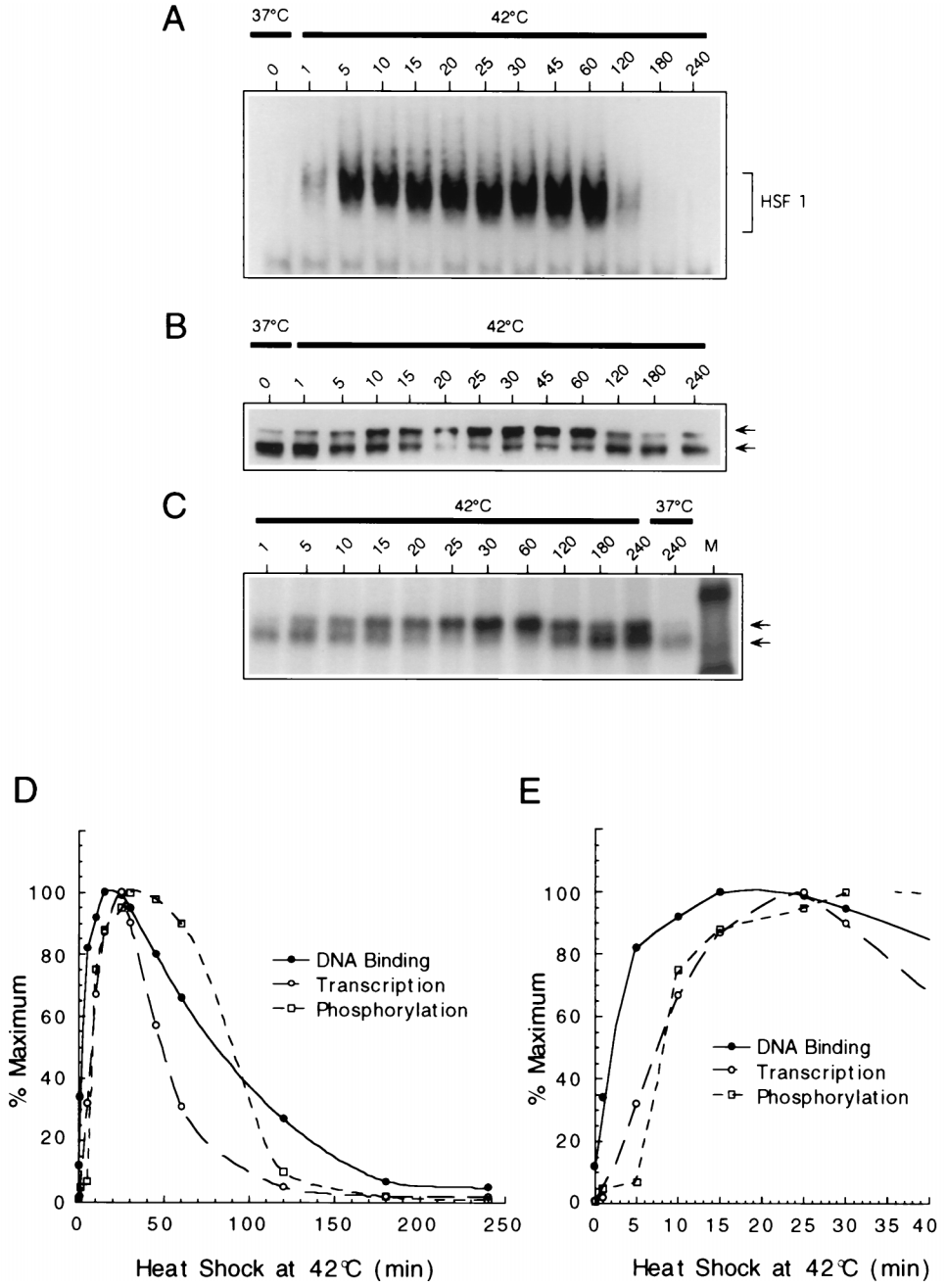

FIG. 1. Kinetics of HSF1 DNA binding, phosphorylation, and transcription during activation and attenuation of the heat shock response. HeLa S3 cells were subjected to a sustained 42°C heat shock for 4 h. Cells were harvested at the indicated times and processed as described in Materials and Methods. (A) Gel mobility shift analysis indicating HSF1 DNA binding activity at selected time points (minutes). The complex representing HSF1 DNA binding activity is indicated. (B) Western blotting with polyclonal anti-HSF1 serum. Arrows indicate two HSF1 populations with mobilities of approximately 70 and 80 kDa which differ in phosphorylation state. (C) Immunoprecipitation analysis of HSF1 from HeLa cells steady state labeled with 50 μCi of [^{32}P]orthophosphate per ml. Lane M contained molecular size markers. (D) Quantitative analysis of HSF1 DNA binding, inducible phosphorylation, and *hsp70* gene transcription. The data is representative of three separate experiments. Transcription of the endogenous *hsp70* gene was measured by using nuclear run-on analysis (data not shown). (E) Graph showing the kinetics of activation on an expanded scale through 30 min of heat shock.

cells, is phosphorylated upon serine residues. These results are consistent with those of previous studies on HSF from *Saccharomyces cerevisiae* and human cells (10, 41). This analysis also revealed that a trace amount of threonine is present in the control HSF1 which is not seen in HSF1 from stressed cells, suggesting that heat shock causes threonine dephosphorylation.

The phosphorylation pattern of HSF1 from control and heat-shocked cells was examined by using two-dimensional tryptic phosphopeptide mapping. Analysis of HSF1 immunoprecipitated from extracts of control cells labeled with [^{32}P]orthophosphate revealed a consistent pattern of five phosphopeptides (numbered and underlined in Fig. 3), of which phosphopeptide 2 is the most prominent. Examination of the tryptic phos-

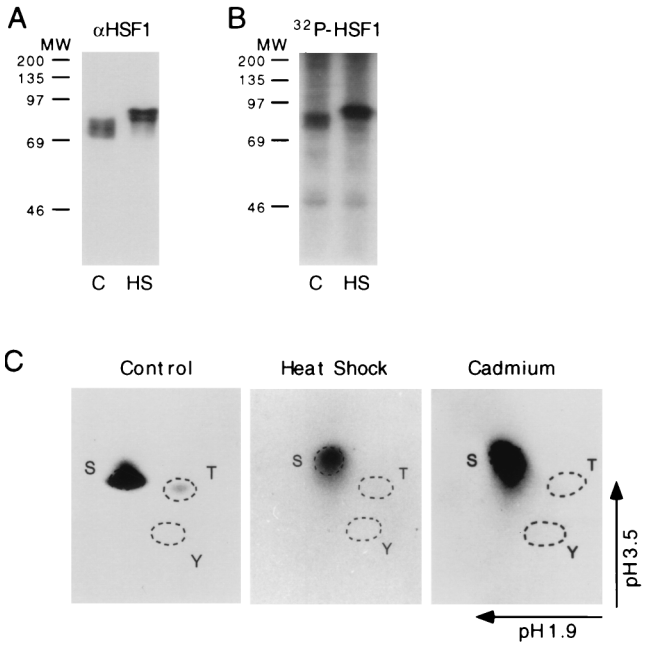

FIG. 2. HSF1 is constitutively and inducibly phosphorylated at serine residues. Immunoprecipitation and phosphoamino acid analysis of HSF1. (A) Western blotting of extracts prepared from NIH 3T3 cells maintained at 37°C (lane C) or cells exposed to a 1-h 42°C heat shock (lane HS). MW, molecular weight in thousands. (B) Immunoprecipitation of HSF1 from control (lane C) and heat-shocked (lane HS) NIH 3T3 cells steady state labeled for 10 h with 1 mCi of [^{32}P]orthophosphate per ml. (C) Phosphoamino acid analysis of HSF1 immunoprecipitated from steady-state-labeled NIH 3T3 cells maintained at 37°C, heat shocked at 42°C for 1 h, or exposed to 90 mM cadmium sulfate for 2 h. HSF1 was hydrolyzed in 6 N HCl at 110°C for 1 h and separated by two-dimensional electrophoresis on a TLC plate as described in Materials and Methods. The relative positions of phosphoserine, phosphothreonine, and phosphotyrosine are indicated by the letters S, T, and Y, respectively.

phopeptide pattern of HSF1 isolated from heat-shocked cells reveals the presence of control phosphopeptides and additional heat shock-induced peptides (indicated by letters). The intensity of spot 1 decreases upon heat shock; this may be due to dephosphorylation, further phosphorylation, or changes in protease accessibility. These control and heat shock peptide patterns were used as references to identify sites of HSF1 phosphorylation.

Deletion mapping of HSF1 identifies a region that is constitutively phosphorylated. The serine residues in HSF1 which are constitutively phosphorylated were mapped through the use of a collection of chimeric HSF1 proteins in which the yeast GAL4 DNA binding domain was fused to various segments of HSF1. These chimeric HSF1 proteins have proven to be effective tools for dissection of domains involved in the regulation and activity of HSF1 in control and heat-shocked cells (13, 35, 40, 48). The rationale was to use the GAL4-HSF1 chimeras retaining various segments of HSF1 to map the region(s) of constitutive phosphorylation (Fig. 4A). Additionally, it was necessary to demonstrate whether the GAL4-HSF1 chimeras would exhibit the same phosphopeptide pattern as observed for endogenous HSF1. To examine this, the GAL4/124-503 construct was transfected into COS-7 cells, after which the cells were steady state labeled with [^{32}P]orthophosphate. The cells were exposed to heat shock so that constitutive and inducible phosphorylation of HSF1 could be analyzed simultaneously. Immunoprecipitated HSF1 was then subjected to tryptic phosphopeptide analysis. As seen in Fig. 4B, the pattern of tryptic phosphopeptides observed for chimeric GAL4/124-503 is similar to the phosphopeptide pattern established for endogenous HSF1; however, we noted consistent differences in the relative intensities of certain phosphopeptides.

The origin of the constitutively labeled phosphopeptides in HSF1 was subsequently established by analysis of GAL4-HSF1 deletion constructs. For example, the phosphopeptide pattern of constructs 288-425 and 295-498 revealed that peptides 1 to 5 are retained. The identity of these peptides was confirmed by comigration with endogenous HSF1 (data not shown). Further deletion to residue 308 (mutant 308-503) results in the loss of peptides 1 to 3 and 5 and retention of only peptide 4 (Fig. 4B). Taken together, these results suggest that phosphopeptides 1 to 3 and 5 originate from within amino acid residues 295 to 308. Within these boundaries is a single predicted tryptic peptide which contains two serine residues, at positions 303 and 307 (Fig. 4C). The identification of this region of HSF1 as the primary site(s) of constitutive phosphorylation was corroborated by analysis of another HSF1 deletion construct in which only residues 203 to 311 are expressed. Based on the previous deletion mutants of HSF1, we predict that constitutively phosphorylated peptides 1 to 3 and 5 should be present within these boundaries. As shown in Fig. 4B, construct 203-311 retains phosphopeptides 1 to 3 and 5 and lacks peptide 4. Therefore, based on an analysis of HSF1 deletion mutants from both the amino and carboxyl termini, we conclude that a single tryptic peptide located between residues 299 and 309 contains sites for constitutive serine phosphorylation.

A perplexing issue is the origin of multiple phosphopeptides from this region of HSF1. We suggest that this is likely to result from partial trypsin cleavage, perhaps affected by the presence of proximal basic residues at positions 296 and 298. Multiple phosphopeptides could also arise from phosphorylation on either serine residue 303 or 307 or both. Therefore, we conclude that the primary site of phosphorylation of HSF1 maps to the region between amino acid residues 295 and 310. Comparison of the sequences surrounding serines 303 and 307 in other HSFs reveals that these residues are highly conserved in mouse, chicken, and human HSF1s (Fig. 4C). Additionally, comparison to the sequence of *Drosophila* HSF reveals a single potential phosphorylation site in which the serine residue is flanked by proline residues (Fig. 4C). Although our analysis of HSF1 deletion mutants focused on the mapping of the prominent constitutive phosphopeptides, our data also reveals that

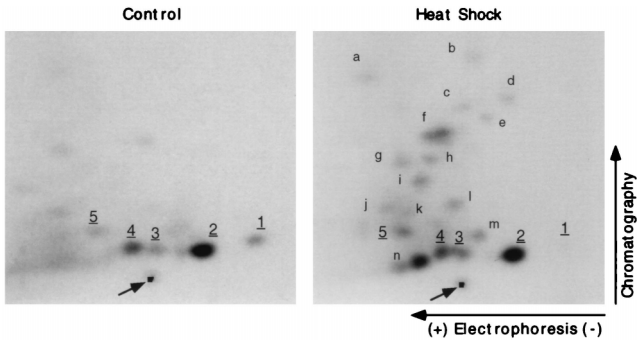

FIG. 3. Comparison of the two-dimensional tryptic phosphopeptide patterns of HSF1 from control and heat-shocked NIH 3T3 cells. HSF1 immunoprecipitated from steady-state-labeled cells was digested with tolylsulfonyl phenylalanyl chloromethyl ketone-trypsin and electrophoretically separated by TLC in the horizontal dimension, followed by ascending chromatography as described in Materials and Methods. Constitutive phosphopeptides are numbered 1 to 5 and underlined. Inducible phosphopeptides are labeled with letters. The small arrows indicate the positions of the origins.

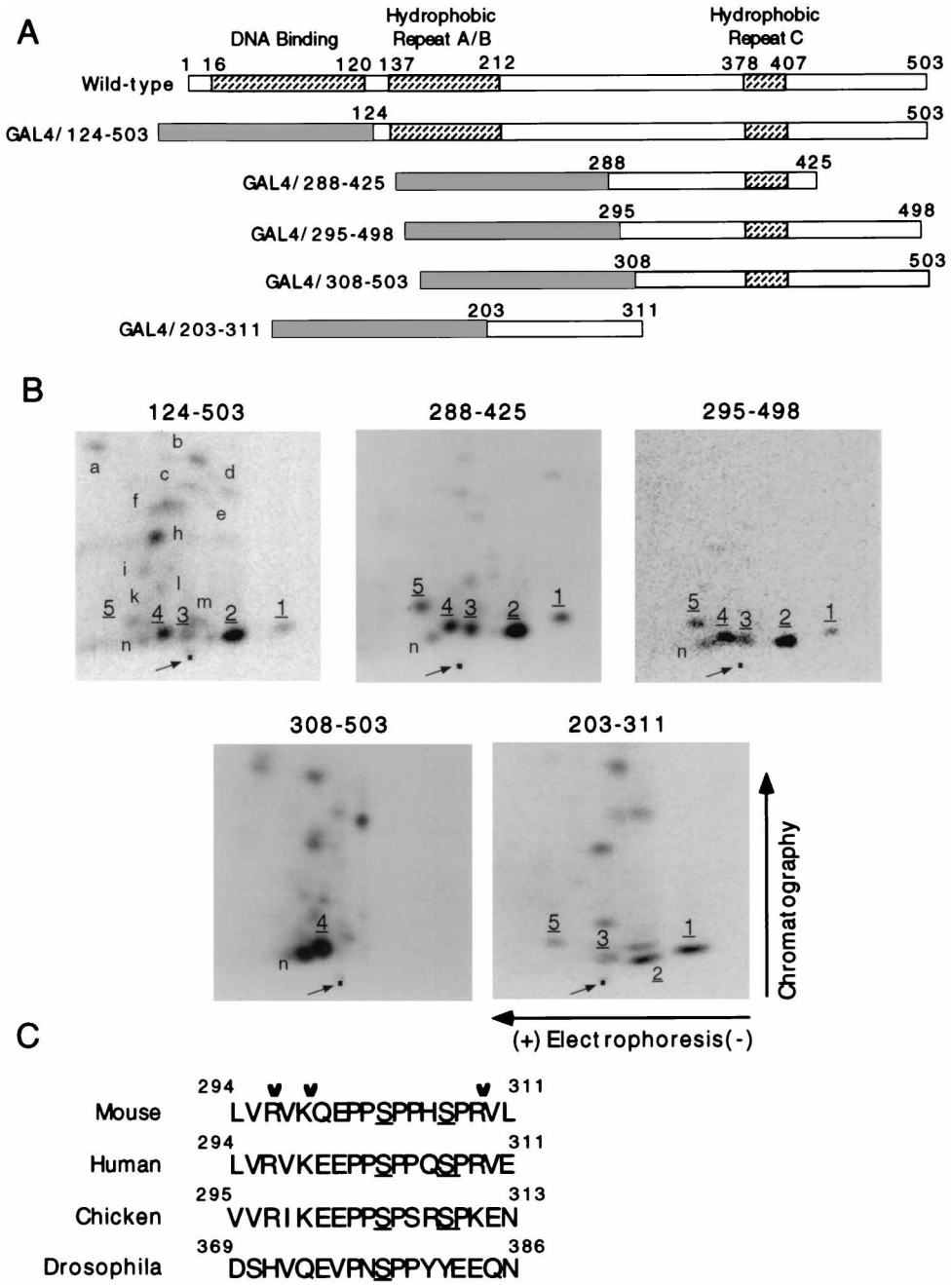

FIG. 4. Mapping of a region of constitutive phosphorylation in HSF1 by deletion mapping with GAL4-HSF1 chimeras. (A) Schematic diagram indicating the boundaries of GAL4-mHSF1 chimeras. GAL4 DNA binding domain residues 1 to 147 are indicated by a stippled box. (B) Two-dimensional tryptic phosphopeptide patterns of GAL4-HSF1 chimeras transfected into COS-7 cells. Chimeras were immunoprecipitated from steady-state [^{32}P]orthophosphate-labeled COS-7 cells upon exposure to a 1-h 42°C heat shock. Constitutive phosphopeptides are numbered and underlined. Inducible peptides are indicated with letters. The small arrows indicate the positions of the origins. (C) Amino acid sequence of the mHSF1 region between residues 294 and 311. Predicted trypsin cleavage sites are indicated by downward-pointing arrows, and serine residues are underlined. Also indicated are the corresponding regions of human HSF1 (37) and chicken HSF1 (34) and a segment of *Drosophila* HSF (8).

constitutive peptide 4 is likely to originate from distal residues located between positions 310 and 425 of HSF1. Furthermore, multiple inducible peptides are lost upon deletion to residue 288, and peptide *n* appears to map between residue 308 and the C terminus.

HSF1 is constitutively phosphorylated on serine residues 303 and 307. To directly corroborate the results obtained from the deletion mutant forms of HSF1, serine residues 303 and 307 were changed by site-directed mutagenesis to alanines and analyzed in the context of the GAL4/124-503 construct. Mutation of both serines 303 and 307 (S303A and S307A) eliminates phosphopeptides 1 to 3 and 5, thus resembling the pattern of the mutant construct GAL4/308-503, in which serines 303 and 307 were deleted (Fig. 5). The tryptic phosphopeptide pattern obtained with mutation of serine 303 (S303A) or 307 (S307A) reveals that peptides 2, 3, and 5 are no longer de-

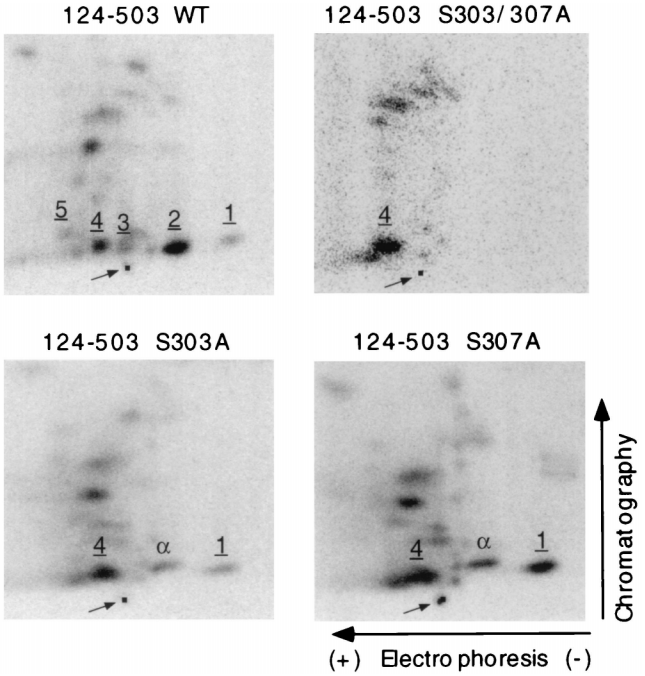

FIG. 5. Identification of serines 303 and 307 as sites of constitutive phosphorylation in HSF1. Two-dimensional tryptic phosphopeptide patterns of GAL4-mHSF1 chimera 124-503 with alanine substitutions at both residues 303 and 307 (S303/307A), at residue 303 (S303A), or at residue 307 (S307A). Constitutive peptides are numbered and underlined. A novel peptide which appears in S303A and S307A is labeled α. The small arrows indicate the positions of the origins. WT, wild type.

tected and peptides 1 and 4 are retained (Fig. 5). We note the presence of a novel peptide α with an electrophoretic mobility distinct from that of peptide 2 as confirmed by comigration of the phosphopeptides generated from wild-type GAL4/124-503 and GAL4/124-503 S303A (data not shown). These results establish that both serine residues 303 and 307 are constitutively phosphorylated in HSF1.

p42 and p44 MAP kinases efficiently phosphorylate recombinant HSF1. The residues flanking serines 303 and 307 (Fig. 4C) correspond to a consensus sequence for proline-directed kinases including members of the MAP (ERK1/2) kinase family. Therefore, we examined the in vitro phosphorylation pattern of recombinant HSF1 by using p42 MAP kinase (ERK-2). The tryptic phosphopeptide pattern of in vitro-phosphorylated HSF1 reveals four major tryptic peptides (M1 to M4), of which M1 and M2 comigrate with peptides 1 and α, respectively (Fig. 6). Interestingly, p42 MAP kinase-phosphorylated peptide M3 may correspond to phosphopeptide 1 of HSF1 from heat-shocked cells. Similar results were obtained with p44 MAP kinase (ERK1) (12). These results reveal that although HSF1 is a suitable in vitro substrate for proline-directed kinases, including the p42 and p44 MAP kinases, the pattern of phosphorylation has only one phosphopeptide in common with HSF1 from control cells. Whether this is due to differences in the conformation of recombinant HSF1 or the kinase activities remains to be determined.

Mutation of S303 results in loss of negative regulation of HSF1 transcriptional activity. We next examined whether phosphorylation of HSF1 at serine 303 or 307 influences the activity of the repressed transactivation domain. To address this, the transactivation properties of site-directed S303A and S307A mutants were assessed in the context of GAL4-HSF1 by cotransfection with a reporter construct containing GAL4 DNA binding motifs upstream of the CAT gene (G_5BCAT) and an internal control (Rous sarcoma virus-CAT). Total RNA was isolated from cells maintained under control or heat shock conditions, and the expression of both CAT reporter constructs was measured by primer extension analysis. The properties of wild-type construct GAL4/203-503 are observed as repression of basal activity, which is induced sevenfold upon heat shock (Fig. 7B). In contrast, the S303A and S303A/S307A constructs were constitutively active. These results reveal that constitutive phosphorylation acts to repress the transactivation domain. Additionally, the consequence of derepression, obtained by alanine substitution, is that the chimeric GAL4-HSF1 construct is not significantly responsive to heat shock. We then examined whether the negative regulation mediated by phosphorylation at S303 and S307 was due to the presence of negative charges at these residues. To address this, we replaced both serine residues with aspartic acid residues (S303/S307D) and found that the resultant HSF construct was constitutively repressed and heat shock responsive in a manner similar to that of wild-type HSF (Fig. 7C).

The data presented thus far implicates constitutive serine phosphorylation in the negative regulation of HSF1 transcriptional activity. To determine whether this conclusion also holds for intact, full-length HSF1, we transfected NIH 3T3 cells with a plasmid expressing wild-type and mutant full-length HSF1 molecules. As seen in Fig. 8A, mutation of S303 and S307 to alanines in the context of full-length HSF1 results in sixfold derepression of activity compared to wild-type HSF1. This value was obtained following subtraction of the expression level of the reporter construct in the mock transfection compared to the wild-type or mutant HSF1 values. Furthermore, replacement of the S303 and S307 residues with aspartic acid residues maintains the repressed state of the HSF1 transactivation domain, thus providing evidence that the aspartic acid residues can functionally substitute for the phosphoserine residues. The levels of transfected wild-type or mutant HSF1

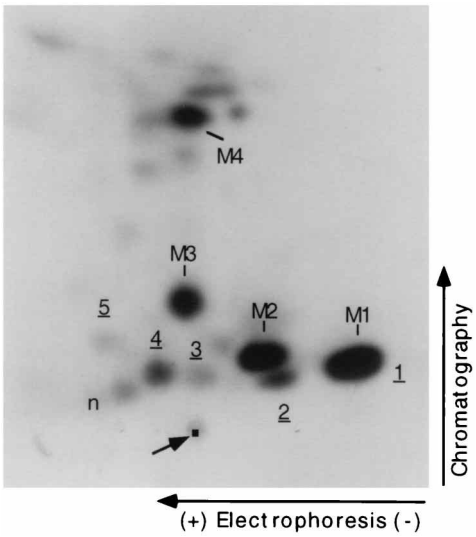

FIG. 6. In vitro phosphorylation of HSF1 by p42 MAP kinase and comigration with HSF1 immunoprecipitated from NIH 3T3 cells. HSF1 from heat-shocked NIH 3T3 cells and recombinant HSF1 incubated with p42 MAP kinase were separated by SDS-PAGE prior to phosphopeptide analysis. Peptides originating from NIH 3T3 HSF1 are indicated, and peptides originating from purified HSF1 incubated with p42 MAP kinase are labeled M1 to M4. The origin is indicated by the small arrow.

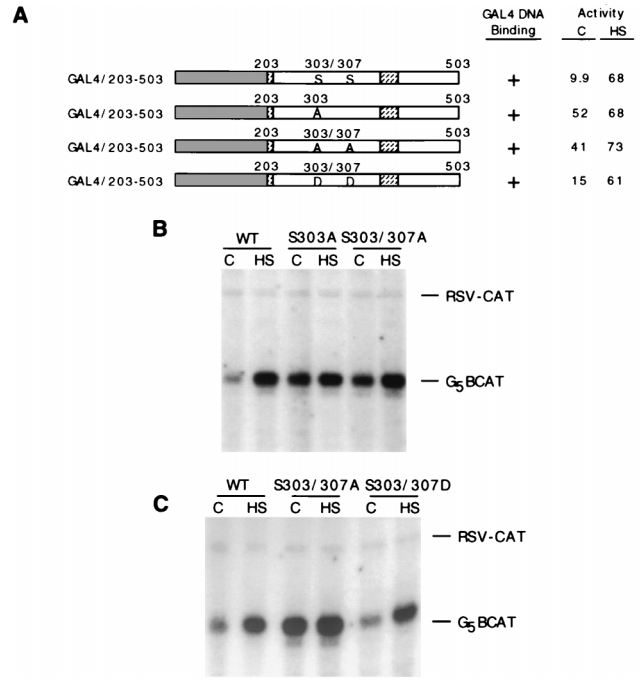

FIG. 7. Replacement of S303 or both S303 and S307 with alanine results in derepression of the transactivation domain and constitutive transcriptional activity. (A) Schematic illustration of the GAL4-HSF1 chimeras and their activities as shown in panels B and C. (B) Primer extension analysis of GAL4-HSF1 residues 203 to 503. NIH 3T3 cells were harvested 30 h posttransfection (lanes C) or subsequently heat shocked at 43°C for 3 h before harvesting (lanes HS). (C) Replacement of S303 and S307 with aspartic acid (S303/307D). Primer extension analysis was performed as described in Materials and Methods. The Rous sarcoma virus (RSV)-CAT and G_5BCAT extension products are indicated. WT, wild type.

proteins were very similar (Fig. 8B). Not surprisingly, the alanine mutant in Fig. 8B reveals an increased electrophoretic mobility on SDS-PAGE, due to lack of phosphorylation, which is reversed upon replacement with aspartic acid residues. As an additional control for transfection efficiency, we compared the levels of HSF1 DNA binding activity by using the gel mobility shift assay. The results shown in Fig. 8C demonstrate that the DNA binding ability of HSF1 is the same for the wild-type and mutant proteins. These results provide independent evidence that S303 and S307 have a critical role in the negative regulation of the HSF1 transactivation domain and corroborate our results obtained by using GAL4-HSF1 constructs.

DISCUSSION

Our analysis of the regulation of HSF1 transcriptional activity provides direct evidence for a role of constitutive phosphorylation at serine residues 303 and 307 in the repression of the carboxyl-terminal transactivation domain. These serine residues correspond to the major sites of phosphorylation of HSF1 in cells maintained under control conditions. Substitution of these serine residues with alanine derepresses the transactivation domain such that the S303A and S307A mutants exhibit increased transcriptional activity.

HSF1 has a complex regulatory mechanism in which both DNA binding and transcriptional activities are negatively regulated. The regions in HSF1 implicated in the regulation of these activities are spatially separated, as the DNA binding domain is located in the extreme amino terminus and the transactivation domain is located at the extreme carboxyl terminus. Despite the evidence that both activities are coordinately induced in the endogenous HSF1 protein in response to heat shock and other stresses, it is clear that these activities can be uncoupled. For example, exposure of mammalian cells to nonsteroidal anti-inflammatory drugs, such as salicylate or indomethacin, stably activates an intermediate trimeric state of HSF1 which is capable of DNA binding yet is transcriptionally inert (10, 18, 19, 27). Another example in which uncoupling has been observed is chimeric HSF1 proteins in which the DNA binding domain has been replaced with a heterologous GAL4 or LEXA DNA binding domain, resulting in a factor that exhibits constitutive DNA binding while maintaining the transcriptional activation domain in a repressed state. Taken together, these observations support a proposal that the transactivation domain and the DNA binding domain are negatively regulated and activated by distinct mechanisms.

Our in vivo phosphopeptide mapping studies have identified the major sites of constitutive phosphorylation of HSF1. Analysis of the sequences surrounding the critical serine residues reveals that the immediate and flanking sequences are highly conserved among vertebrate HSFs and contain a perfect consensus for phosphorylation by proline-directed kinases. Additionally, there are a number of observations to support a role for MAP (ERK) kinase activities in the regulation of heat shock factor. For example, heat shock among other environmental and physiological conditions induces both MAP kinase (6, 11, 21) and MAP kinase phosphatase (2, 9, 28) activities. More specifically, overexpression of dominant-negative ERK1

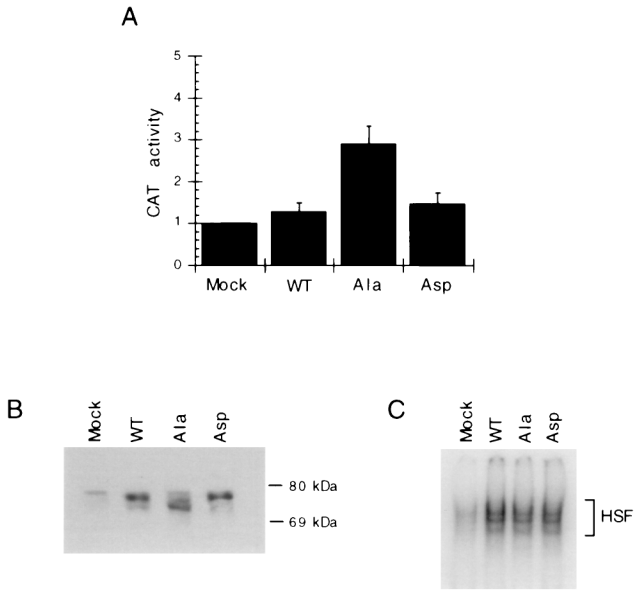

FIG. 8. Serine phosphorylation negatively regulates the transcriptional activity of full-length HSF1: effects of substitution with alanine or aspartic acid residues. (A) NIH 3T3 cells were transfected with the indicated full-length HSF1 constructs (wild type [WT], alanine substitution, and aspartic acid substitution) or mock transfected with the plasmid alone. pHBCAT was used as the heat shock element-containing reporter plasmid, and pCH110 (expressing β-galactosidase) was used as an internal control for transfection efficiency. The level of derepression of the alanine mutation was calculated after subtraction of the mock transfection value (1.0), resulting in a wild-type HSF1 value of 0.3, an alanine mutant HSF1 value of 1.9, and an aspartic acid mutant HSF1 value of 0.5. Cells were harvested 30 h after transfection for measurement of CAT activity. (B) Western blotting with an anti-HSF1 monoclonal antibody indicating the relative expression levels of wild-type HSF1 and the mutant constructs represented in panel A. (C) Gel mobility shift analysis indicating the DNA binding activities of wild-type HSF1 and the mutant constructs represented in panel A. The results in panels A, B, and C are representative of three independent experiments.

increases Hsp70 transcription, while vanadate treatment increases phosphorylation of HSF1 and delays activation of HSF1 by heat shock, suggesting a role for MAP kinases as negative regulators of the heat shock response (30). One question which remains unanswered is whether MAP kinases phosphorylate HSF1 in vivo. Two recent studies demonstrate a possible role of MAP kinases in the regulation of HSF1 by overexpressing Raf-MAPK pathway kinases upstream of MAP kinase (7, 23). Although our study does not directly address the issue of which kinases phosphorylate HSF1 in vivo, we note that the tryptic peptides of HSF1 phosphorylated in vitro with p42 and p44 MAP kinases are not identical to those generated in vivo. Therefore, we suggest either that these kinases are not the corresponding in vivo kinase activities for HSF1 or that the recombinant form of HSF1 is not the appropriate substrate. Clearly, additional studies are required to identify the kinase activities responsible for both constitutive and inducible serine phosphorylation of HSF1.

In conclusion, our studies reveal a role for constitutive serine phosphorylation in the regulation of HSF1. Consistent with previous findings, our data suggests that this level of regulation occurs after the acquisition of DNA binding activity. Similar studies of HSF1 regulation (13, 35, 40, 48) have identified a distinct *cis* element in HSF1 which overlaps the hydrophobic repeat (HR-A/B) and is also involved in negative regulation of the transactivation domain. Thus, HSF1 has at least two distinct regulatory domains involved in repression, one which requires constitutive phosphorylation at residues S303 and S307 and a distinct amino-terminal domain. Although our studies do not address the mechanisms by which negative regulation of the transactivation domain is derepressed upon heat shock, we suggest that intramolecular interactions occur between these regulatory regions and the transactivation domain.

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