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Phosphorylation of the Yeast Heat Shock Transcription Factor Is Implicated in Gene-Specific Activation Dependent on the Architecture of the Heat Shock Element

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Heat shock transcription factor (HSF) binds to the heat shock element (HSE) and regulates transcription, where the divergence of HSE architecture provides gene- and stress-specific responses. The phosphorylation state of HSF, regulated by stress, is involved in the activation and inactivation of the transcription activation function. A domain designated as CTM (C-terminal modulator) of the *Saccharomyces cerevisiae* HSF is required for the activation of genes containing atypical HSE but not typical HSE. Here, we demonstrate that CTM function is conserved among yeast HSFs and is necessary not only for HSE-specific activation but also for the hyperphosphorylation of HSF upon heat shock. Moreover, both transcription and phosphorylation defects due to CTM mutations were restored concomitantly by a set of intragenic suppressor mutations. Therefore, the hyperphosphorylation of HSF is correlated with the activation of genes with atypical HSE but is not involved in that of genes with typical HSE. The function of CTM was circumvented in an HSF derivative lacking CE2, a yeast-specific repression domain. Taken together, we suggest that CTM alleviates repression by CE2, which allows HSF to be heat-inducibly phosphorylated and presume that phosphorylation is a prerequisite for the activator function of HSF when it binds to an atypical HSE.

All organisms exhibit a conserved protective response to elevated temperatures and to a variety of chemical and physiological stresses. This response, designated as the heat shock response, is characterized by the rapid production of a set of proteins called heat shock proteins (Hsps). Hsps are molecular chaperones involved in the folding, trafficking, maturation, and degradation of proteins. An increased accumulation of Hsp in cells is essential for survival of cells exposed to various protein-damaging stresses (for a review, see reference 17). In eukaryotes, stress-inducible expression of *HSP* genes is regulated by heat shock transcription factor (HSF). The HSF protein, forming a homotrimer, binds to a regulatory sequence designated as the heat shock element (HSE) of the target genes, which consists of multiple, contiguous, and inverted repeats of the 5-bp sequence NGAAN (where N is any nucleotide). HSF proteins of various organisms share common structural motifs, including the helix-turn-helix DNA-binding domain, coiled-coil domain required for trimer formation, and C-terminal transcription activation domain (for reviews, see references 35, 37, and 58). The structural and functional conservation of HSFs among eukaryotes has also been supported by findings that human and *Drosophila* HSFs functionally substitute for those of the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, respectively (19, 34, 55). Therefore, the HSF-HSE interaction is a crucial regulatory mechanism for an evolutionarily well-conserved cellular stress response.

In *S. cerevisiae*, HSF is encoded by a single essential gene, *HSF1* (52, 57). Other yeasts, such as *S. pombe* (19) and *Kluyveromyces lactis* (26), also have a single *HSF* gene, whereas most vertebrates and higher plants possess multiple genes (for a review, see reference 37). The existence of multiple HSF species in higher eukaryotes suggests that the HSF isoforms may have specialized functions that can be triggered by distinct stresses; in addition, these isoforms may activate specific target genes. In yeasts, single HSFs are thought to play multiple roles that are shared with the isoforms in higher eukaryotes. The activator function of human HSF1 (hHSF1) is regulated at two steps: the first step involves trimerization and nuclear localization of monomeric hHSF1 in the cytosol, and the second step is the acquisition of the ability of HSE-bound hHSF1 to activate transcription (1, 13, 44, 64). By contrast, *S. cerevisiae* Hsf1 (ScHsf1) is localized in the nucleus and is bound to HSE, even under normal growth conditions (21, 25, 50); thus, the latter step is thought to be triggered by stresses. In both mammalian and yeast HSFs, the activator function is regulated by intra- and intermolecular interactions (1, 6, 9, 30, 46, 63). Deletion analysis of ScHsf1 has revealed that the central region contains regulatory domains, including the DNA-binding and trimerization domains, and a yeast-specific repression element referred to as CE2 (26, 51, 57). Two transcription activation domains designated as AR1 and AR2 (also known as NTA/AAD and CTA/CAD) are located at the N and C termini, respectively (10, 36, 49). The activator function of these domains is repressed under normal growth conditions by interaction with the central regulatory domains (5, 9, 23, 26, 36), and stresses such as heat shock may induce a conformational change to an active form. Moreover, ScHsf1 is inducibly phosphorylated in response to heat shock (23, 49, 50, 52) and oxidative stress (33). The roles played by hyperphosphorylation have been debated, in particular its putative involvement in the acquisition of activating ability (49, 50), as well as in the return of an activated protein to its inactive state (23). In human cells, the HSE binding of hHSF1 has been shown to precede inducible phos-

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phorylation, suggesting that the acquisition of transcription activity of the HSE-bound hHSF1 is linked to phosphorylation (13, 59). The phosphorylation of hHSF1 has also been implicated in both activation and inactivation, depending on the amino acid residues to be phosphorylated (11, 12, 14, 22, 24, 27, 28, 59).

The binding of HSF to the HSE is cooperative, and some deviations from the canonical NGAAN sequence are tolerated in functional HSEs (16, 20, 43, 53, 60). The number of the 5-bp repeating units in HSE varies with genes, but at least three units may be required for recognition by HSF, since it forms a homotrimer (3, 29, 43, 53, 56, 60). The divergence of HSE architecture may provide gene-specific responses to stresses. In mammalian cells, three HSF isoforms may share a distinct set of target genes. In support of this idea, in vitro DNA binding and footprinting studies using mammalian HSF proteins have revealed intriguing differences between HSF1 and HSF2; HSF1 prefers an array of four to five units of NGAAN, whereas HSF2 prefers two to three units (29). Mammalian HSF1 and HSF2, when expressed in yeast, activate different sets of target genes, depending on their respective preference for the number of the 5-bp unit. The HSE-specific activator function is determined by the slightly different amino acid sequences of the DNA-binding domains (2, 34). It has consistently been shown that point mutations in the DNA-binding domain of ScHsf1 alter its binding specificity to the HSE (8, 9, 45, 48, 61). In addition, the trimerization domain and linker region between the DNA-binding and trimerization domains affect preferences regarding the spacing, orientation, and number of the 5-bp unit, as well as the binding affinity to the HSE (15, 18).

Depending on the architecture of the HSE, two activation domains of ScHsf1 exhibit distinct properties in activation function. The presence of either the AR1 or the AR2 activation domain is sufficient for the induction of many *HSP* genes upon heat stress, i.e., of those that contain at least three contiguous NGAAN inverted repeats (typical, NTTCNNGAANNT TCN). By contrast, AR2 but not AR1 is responsible for the heat-induced activation through atypical HSE of genes such as *CUP1* (encoding copper metallothionein), *HSP82* (Hsp90), *HSC82* (Hsp90), and *MDJ1* (a DnaJ homologue) (39, 43, 49, 53, 54, 62). The HSE of *CUP1* consists of two consecutive pentamers followed by another pentamer unit after a gap of 5 bp [NTTCNNGAAN(5 bp)NGAGN] (43, 48, 54, 61).

We have recently identified a novel domain in ScHsf1 that is essential for *CUP1* activation (39). The region designated as CTM (C-terminal modulator) is located at the C terminus and is rich in basic amino acids. The requirement of CTM for *CUP1* activation has been ascribed to the HSE architecture, since CTM becomes dispensable for activation when an atypical HSE of *CUP1* is converted to the typical HSE. In addition, CTM is necessary for the heat-induced transcription of *HSP26* containing an atypical HSE but is dispensable for the activation of *SSA1* bearing the typical HSE. The CTM domain is not involved in the ScHsf1-HSE interaction, as judged by gel retardation assay, and when studied by the artificial recruitment technique, this domain fails to activate transcription by itself. Therefore, CTM is thought to modulate the activator function of ScHsf1 depending on the architecture of the HSE (39). It has also been suggested that CTM affects the function of the central regulatory domains upon heat shock, such that an active conformation of ScHsf1 is generated, which in turn enables AR2 to successfully transmit the activation signal to the transcription machinery (40).

To gain further insight into the mechanism underlying the gene-specific activation regulated by HSF, we focused on the role of CTM during the heat-induced activation of ScHsf1. Here, we identified CTM in the HSFs of *S. pombe* and *K. lactis* and demonstrated that the CTM of the former species is functional in *S. cerevisiae*, as the *S. pombe* CTM is necessary for the heat shock response of *CUP1* but not for that of *SSA4*. (*SSA4*, encoding Hsp70, contains the typical HSE.) Furthermore, heat-inducible hyperphosphorylation of ScHsf1 was dependent on the presence of functional CTM. The isolation and characterization of intragenic mutations that suppress CTM mutations disclosed an interaction between CTM and the repression domain CE2. In the Discussion section, we will discuss the relationships among the CTM-CE2 interaction, heat-inducible phosphorylation, and HSE-specific activation of transcription.

MATERIALS AND METHODS

Yeast strains. *S. cerevisiae* strain HS82 (*MAT*α *his3 leu2 trp1 ura3 hsf1*::*HIS3* YCp-*URA3-HSF1*) (39) was crossed with W303-1a (*MAT***a** *ade2 his3 leu2 trp1 ura3 can1*). The resulting diploid was sporulated, and a segregant (*MAT*α *ade2 his3 leu2 trp1 ura3 can1 hsf1*::*HIS3* YCp-*URA3-HSF1*) was isolated (HS126). The reporter gene *SSA4-lacZ′* (38) was integrated into the *leu2* locus of HS126 to create HS134. Strain HS155 was an *ace1*::*ADE2* derivative of HS134. The *hsf1* genes bearing deletion or point mutations listed in Table 1 were introduced into strains HS126, HS134, or HS155 by the plasmid shuffling technique to create *hsf1* mutant strains (4).

The *hsf1* derivatives (Table 1) were cloned into pRS-type centromeric vectors (47). Parental plasmid pSK906 was a *URA3*-marked plasmid (pRS316) bearing the *HSF1* gene from −729 to +2882 relative to the translation initiation site (39). A derivative lacking the C-terminal 15 amino acids of ScHsf1 (*hsf1*-ΔCTM) and fusion genes with *S. pombe hsf* + (*hsf1-Sp* and *hsf1-Sp*/ΔCTM) or human *HSF1* (*hsf1-Hs*) contained the *ADH1* terminator at the 3′ noncoding region (39). The DNA fragment encoding the C-terminal region of *S. pombe* HSF was obtained by reverse transcription-PCR (RT-PCR) of total RNA prepared from *S. pombe* strain JY742 (a kind gift from Y. Nogi). The cDNA clone encoding human *HSF1* was a kind gift from A. Nakai.

Isolation of intragenic suppressor mutations. Plasmid pK137 was a *TRP1*-marked plasmid bearing *hsf1*-ΔAR1/ba1. To construct a gene library containing mutations in the coding sequence for the N-terminal region of Hsf1-ΔAR1/ba1, a segment from −729 to +1545 of *hsf1*-ΔAR1/ba1 was amplified by PCR under an error-prone condition (31). The mutagenized fragments were isolated by digestion with SalI (5′ upstream site in the vector) and NheI (at +1444) and cloned into SalI-NheI-digested pK137. For the mutagenesis of the sequence encoding the C-terminal region, a segment from +1375 to +2882 of *hsf1*-ΔAR1/ba1 was subjected to error-prone PCR, and the NheI (at +1444)-PstI (3′ downstream site in the vector) fragments were cloned into NheI-PstI-digested pK137 as described above. The mutant libraries were introduced into strain HS133, which was a derivative of HS134 harboring YCp-*URA3-hsf1-ΔAR1/ba1* (pK136) instead of YCp-*URA3-HSF1*. Transformants were grown at 38°C to identify plasmids suppressing the temperature-sensitive growth phenotype of HS133. Plasmids recovered from the transformants were sequenced in order to identify suppressor mutations.

RNA analysis. Cells were grown in rich medium consisting of 1% yeast extract, 2% polypeptone, and 2% glucose (YPD) to an optical density at 600 nm (OD_{600}) of 1.0 under the conditions described in the figure legends. Total RNA was prepared from the cells and was quantified by A_{260} (38–40).

The relative amount of specific mRNA was determined by quantitative RT-PCR analysis. Briefly, the total RNA (5 μg) was subjected to RT in a 20-μl reaction mixture containing 3 U of avian myeloblastosis virus reverse transcriptase (Promega) and 0.3 μg of oligo(dT) for 1 h at 42°C. A portion of the mixture (5 μl) was then applied to the following amplification protocol of 10 cycles, each of which consisted of 94°C for 1 min, 52°C for 1 min, and 72°C for 1 min. The PCR products were separated by polyacrylamide gel electrophoresis (PAGE) and visualized by ethidium bromide staining. Band images were captured by a

TABLE 1. Plasmids used for the construction of *hsf1* mutant strains

Plasmid	Description
pSK906	Wild-type *HSF1* on pRS316 (YCp-*URA3*)[a]
pK136	*hsf1-ΔAR1/ba1* on pRS316
pK137	*hsf1-ΔAR1/ba1* on pRS314 (YCp-*TRP1*)
pK144	*hsf1-Hs* [*hsf1-ΔAR1/N583/hHSF1*(316–529)] on pRS315 (YCp-*LEU2*)
pK152	*hsf1-Sp* [*hsf1-ΔAR1/N583/SpHsf*(269–609)] on pRS315
pK154	*hsf1-Sp/ΔCTM* [*hsf1-ΔAR1/N583/SpHsf*(269–594)] on pRS315
pK157	Wild-type *HSF1* on pRS314
pK158	*hsf1-ΔAR1* on pRS314
pK159	*hsf1-ba1* on pRS314
pK176	*hsf1-Hs+CTM* [*hsf1-Hs+CTM*(784–833)] on pRS315
pIS4	E830K mutation in *hsf1-ΔAR1/ba1* (pK137)
pIS1, -2, -11	K491R mutation in *hsf1-ΔAR1/ba1* (pK137)
pIS3	Y537C mutation in *hsf1-ΔAR1/ba1* (pK137)
pIS9	Q535R and K833E mutations in *hsf1-ΔAR1/ba1* (pK137)
pIS12	Q535R and N636S mutations in *hsf1-ΔAR1/ba1* (pK137)
pIS15	Q535R mutation in *hsf1-ΔAR1/ba1* (pK137)
pN39	K491R mutation in *hsf1-ba1* (pK159)
pN40	Y537C mutation in *hsf1-ba1* (pK159)
pN41	Q535R mutation in *hsf1-ba1* (pK159)
pN42	K491R mutation in *hsf1-ΔCTM* (pN53)
pN43	Y537C mutation in *hsf1-ΔCTM* (pN53)
pN44	Q535R mutation in *hsf1-ΔCTM* (pN53)
pN53	*hsf1-ΔCTM* on pRS314
pN57	K491R mutation in *HSF1* (pK157)
pN58	Y537C mutation in *HSF1* (pK157)
pN59	Q535R mutation in *HSF1* (pK157)
pN61	*hsf1-ΔCE2/ba1* on pRS314
pN64	*hsf1-ΔCE2* on pRS314

[a] For details, see reference 39.

digital camera (C-3000zoom; Olympus) and analyzed with a GelPro analyzer (Media Cybernetics). The following regions of the genes relative to the transcription initiation site were amplified by PCR: +1372 to +1629 of *ACT1*, +54 to +261 of *CUP1*, and +1705 to +2012 of *SSA4*.

Preparation of anti-ScHsf1 antiserum. The NarI (at −6)-StuI (at +1747) fragment of pK157 (YCp-*TRP1-HSF1*) encoding the N-terminal 583 amino acids of ScHsf1 (N583/WT) was cloned into pGEX-6P1 (Amersham Biosciences). A fusion protein of glutathione *S*-transferase (GST) and N583/WT was expressed in *Escherichia coli* and captured on glutathione Sepharose 4B (Amersham Biosciences). The N583/WT polypeptide was then liberated from GST by treatment of the fusion protein with Precision protease according the protocol recommended by the manufacturer (Amersham Biosciences). The purified polypeptide was used for the immunization of the mice, and anti-ScHsf1 serum was prepared by standard methods.

Phosphorylation analysis. Cells were grown in YPD medium at 28°C to an OD_{600} of 1.0 and then at 39°C for 15 min, unless otherwise indicated. The cells were immediately collected by centrifugation, washed once with ice-cold water, and stored at −80°C until use. Cells were disrupted by vortexing with glass beads in sodium dodecyl sulfate (SDS) sample buffer (42). After boiling the samples in a water bath for 5 min, the extract was cleared by centrifugation. The protein concentration of each extract was determined by using a Bio-Rad dye staining kit. Proteins (50 μg) were subjected to SDS-PAGE and blotted on a membrane. The membrane was incubated with anti-ScHsf1 serum and subsequently with horseradish peroxidase-conjugated anti-mouse immunoglobulin G. Reacted proteins were visualized by ECL Plus (Amersham Biosciences) and analyzed by a FAS-1000 lumino imager (TOYOBO)

For the phosphatase treatment, cleared cell extract was prepared as above, with the exception that the buffer used for this treatment contained 0.5% SDS, 20 mM Tris-HCl (pH 7.6), 20 mM sodium phosphate, 2 mM EDTA, 20 mM NaF, and 1 mM phenylmethylsulfonyl fluoride. Proteins (100 μg) were incubated with anti-ScHsf1 serum for 1 h and then with protein A Sepharose CL-4B (Amersham Biosciences) on a rotating wheel for another 2 h. The resin was washed with radioimmunoprecipitation assay buffer (42), and half of the precipitate was treated with 5 U of calf intestine alkaline phosphatase in 10 μl of Tris-buffered saline buffer (42) for 30 min at 28°C. The samples were then subjected to SDS-PAGE and immunoblotting as described above.

In vivo labeling of ScHsf1 protein with [^{32}P]orthophosphate was carried out as described previously (33). Briefly, cells were grown at 28°C for 20 h in low-phosphate synthetic complete (SC) medium, which was prepared by the addition of potassium phosphate to phosphate-free SC medium (BIO 101, Inc.) at a 1/50 concentration of normal SC medium (33). After washing the cells with sterile water, they were inoculated into the same medium to an initial OD_{600} of 0.1 to 0.2 in a final volume of 5 ml and were grown for an additional 8 to 10 h to an OD_{600} of 1.0 to 1.5. Cells were harvested and resuspended in 5 ml of fresh medium, to which 250 μCi of [^{32}P]orthophosphate was added. The culture was incubated for 2 h at 28°C, and then the temperature was kept at 28°C or shifted to 39°C for 15 min. The ScHsf1 protein was immunoprecipitated from cell extracts, separated by SDS-PAGE, and blotted on a membrane as described above. Labeled proteins were visualized with a BAS-5000 imaging analyzer (Fuji Film). Total ScHsf1 protein was then detected by immunoblotting as described above.

Protein affinity chromatography. The N583/WT polypeptide was purified as described above. For the purification of the N583/ΔCE2, N583/K491R, N583/Y537C, and N583/Q535R polypeptides, the NarI-StuI fragments of pN64 (*hsf1-ΔCE2*), pN57 (*hsf1-K491R*), pN58 (*hsf1-Y537C*), and pN59 (*hsf1-Q535R*) were cloned into pGEX-6P1, from which the peptides were expressed. The DNA fragments downstream of the StuI site (at +1747) encoding the C-terminal 250 amino acids of Hsf1 or Hsf1-ba1 were cloned into pGEX-3X (Amersham Biosciences). Fusion proteins with GST (GST-250C/WT and GST-250C/ba1) were expressed from the constructs and immobilized on glutathione Sepharose 4B equilibrated with buffer (20 mM HEPES-KOH [pH 7.6], 1 mM EDTA, 0.05% Nonidet P-40, 20% glycerol) containing 50 mM potassium acetate. The N-terminal polypeptide was incubated with the resin for 30 min on ice. The resin was then washed with the same buffer containing 300 mM potassium acetate. The bound polypeptides were eluted by boiling in SDS sample buffer, electrophoresed on SDS-polyacrylamide gel, and subjected to immunoblotting as described above.

RESULTS

Functional significance of the C-terminal basic region of HSF. We have previously shown that the C-terminal basic region of ScHsf1, designated CTM, is necessary for the heat-inducible transcription of several genes such as *CUP1* and

HSP26, the HSEs of which diverge from the typical HSE (39). Alterations of two arginine residues (amino acid positions 826 and 830) in the CTM to glutamate (i.e., the ba1 mutation), which inactivate CTM function (39), led to the slow growth of cells at 38°C (Fig. 1B). Figure 1C shows the results of quantitative RT-PCR analyses of transcripts from the *CUP1* and *SSA4* genes, which contain gapped and contiguous repeats of NGAAN, respectively (7, 43). In *HSF1* wild-type cells, a temperature shift from 28°C to 39°C led to the rapid accumulation of *CUP1* mRNA. By contrast, transcription of *CUP1* was severely inhibited when the cells expressed Hsf1-ba1 instead of the wild-type protein. In these cells, *SSA4* underwent a heat shock response, as it did in *HSF1* cells, but the pattern of mRNA accumulation was slightly delayed in *hsf1-ba1* cells, peaking 30 min after the temperature shift. It should be noted in this context that CTM neither activates transcription by itself, nor does it affect the HSF-HSE interaction (39). Therefore, the above observations suggest that CTM possesses the ability to regulate the activator function of ScHsf1 when it binds to an atypical HSE but not to the typical HSE. Note that the temperature-sensitive growth of cells expressing Hsf1-ba1 is not ascribed to a loss of *CUP1* activation, since *CUP1* is not essential for the growth of yeast (48, 61). We suggest that the ba1 mutation inhibits the heat-induced transcription of an unknown essential gene or set of genes necessary for cells to survive at higher temperatures. In this study, the mRNA levels of *CUP1* and *SSA4* were analyzed as an index of the activation of CTM-dependent and -independent genes, respectively.

In contrast to the effects of the CTM mutations, the deletion of AR1 from ScHsf1 (Hsf1-ΔAR1) had little effect on the growth of yeast at 38°C (Fig. 1B) (49). The heat-induced transcription of *CUP1* and *SSA4* was observed in *hsf1*-ΔAR1cells, but the synthesis of each mRNA continued for a slightly longer period in *hsf1*-ΔAR1 cells than in *HSF1* cells (Fig. 1C) (39, 49, 62). We have previously shown that the deletion of AR2 also results in temperature-sensitive growth of the cell and in reduced activation of *CUP1* (39, 40). Thus, the presence of both AR2 and CTM is required for ScHsf1 function at higher temperatures.

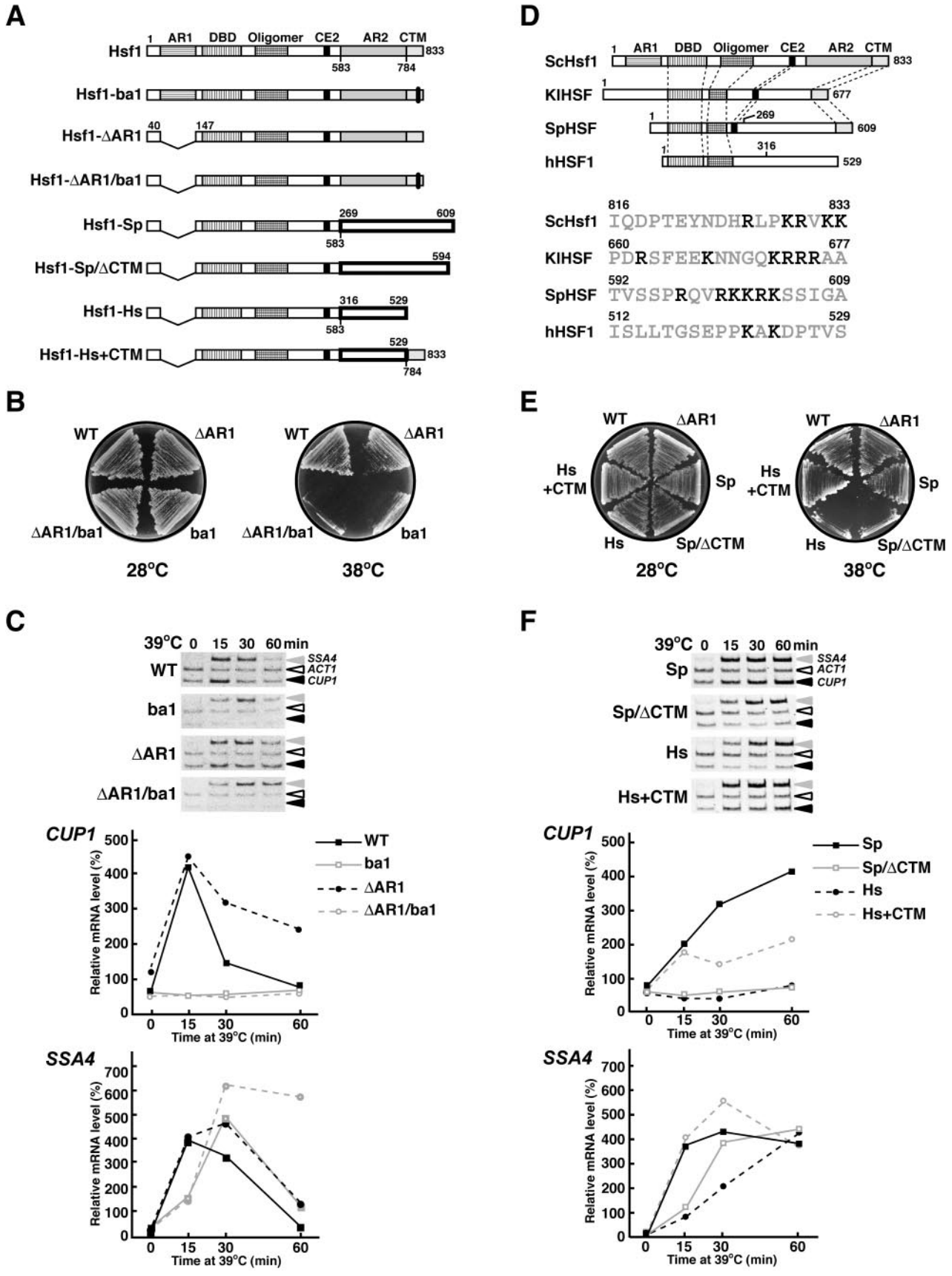
The heat shock factors of *S. pombe* and *K. lactis* also contain regions rich in basic amino acids at their C termini (Fig. 1D, SpHSF and KlHSF). To test the functional conservation of CTM in *S. pombe* HSF, Hsf1-ΔAR1 amino acids 584 to 833 (containing the AR2 and CTM domains) were replaced with SpHSF amino acids 269 to 609 in order to construct Hsf1-Sp (Fig. 1A). Although the deletion of all of AR1, AR2, and CTM from ScHsf1 renders the *S. cerevisiae* cells unable to grow above 33°C (49), cells expressing Hsf1-Sp were able to grow at 38°C (Fig. 1E). Transcription of both *CUP1* and *SSA4* was activated by Hsf1-Sp in response to heat shock (Fig. 1F). Thus, amino acids 269 to 609 of SpHSF substituted for the roles of AR2 and CTM of ScHsf1. As shown in Fig. 1B and C, the introduction of the ba1 mutation into Hsf1-ΔAR1 (Hsf1-ΔAR1/ba1) led to an unambiguous temperature-sensitive phenotype of cell growth, inhibition of the heat shock response of *CUP1*, and a sustained response of *SSA4*. When the CTM-like sequence of SpHSF (amino acids 595 to 609) was deleted from the Hsf1-Sp chimera (Hsf1-Sp/ΔCTM), similar effects to those of the *hsf1*-ΔAR1/ba1 mutation were observed (Fig. 1E and 1F). These results strongly suggest that the basic C-terminal region of SpHSF functions in the same manner as the CTM of ScHsf1.

Unlike yeast HSFs, hHSF1 lacks an apparent basic region in its C terminus (Fig. 1D). As shown in Fig. 1E and F, the expression of Hsf1-Hs, in which the AR2 and CTM domains of Hsf1-ΔAR1 were replaced with the C-terminal region of hHSF1, failed to support the growth of *S. cerevisiae* cells at 38°C. The heat shock response of *CUP1* was also abrogated in these cells, despite the hHSF1 segment containing a domain capable of activating heat-inducible genes in human cells (64). Interestingly, both defects were restored when the CTM of ScHsf1 (amino acids 784 to 833) was fused with the C terminus of Hsf1-Hs (Hsf1-Hs+CTM). This chimeric protein also mediated the heat shock response of *SSA4*. Therefore, CTM has the ability to regulate the activator function of a heterologous activation domain in the context of ScHsf1 fusion. The above observations clearly demonstrated that yeast CTM plays crucial roles in all of the following processes: cell growth at higher temperatures, the heat-induced transcription of *CUP1*, and the rapid heat shock response of *SSA4*.

Effects of CTM on the phosphorylation of HSF. The serine and, to a lesser extent, threonine residues of ScHsf1 are constitutively phosphorylated at normal growth temperatures, and heat shock induces further extensive phosphorylation (49). The phosphorylation of ScHsf1 is known to cause mobility shifts of the protein on gel (23, 33, 49, 50, 52). To analyze the phosphorylation state of the protein, extracts prepared from normal and heat-shocked cells were subjected to SDS-PAGE and to immunoblot analysis with an anti-ScHsf1 serum (Fig. 2A). The ScHsf1 proteins of wild-type cells grown at 20 or 28°C migrated in the gel as a sharp and discrete band (lanes 1 and 2). When the growth temperature was shifted upward from 28°C for 15 min, slowly migrating broad bands emerged (lanes 3 to 5). The mobility gradually decreased while the temperature increased. Phosphatase treatment of immunoprecipitates from the normal and heat-shocked extracts led to the disappearance of the bands converging at a fast-migrating band (Fig. 2B, lanes 1 to 4). In vivo labeling of the cells with [^{32}P]orthophosphate revealed that ScHsf1 protein is slightly phosphorylated at 28°C (Fig. 2C, lane 1). The ^{32}P incorporation increased fivefold when the cells were grown at 39°C, which coincided with the appearance of slowly migrating bands (Fig. 2C, lane 2). These results were consistent with previous observations, as shown by immunoblot (33, 49, 52), in vivo ^{32}P labeling (33, 49), and gel retardation analyses (50, 52).

Surprisingly, the heat-inducible mobility shifts were abolished when the ba1 mutation was introduced into ScHsf1 (Fig. 2A, lanes 6 to 10). The abolishment of the mobility shifts was confirmed by immunoprecipitation (Fig. 2B, compare lanes 5 and 7). In the ^{32}P-labeling experiment, a less than twofold increase in phosphorylation was observed in *hsf1-ba1* cells after shifting the temperature from 28°C to 39°C (Fig. 2C, lanes 3 and 4). When the cells were grown at 28°C, however, Hsf1-ba1 protein was phosphorylated as efficiently as the wild-type protein (Fig. 2C, lanes 1 and 3). These results strongly suggest that CTM is necessary for heat-inducible, but not for constitutive, phosphorylation of ScHsf1.

We next analyzed the electrophoretic patterns of the chimeric ScHsf1 proteins used for the above analyses (see Fig. 1A for the constructions). As shown in Fig. 2D, the mobility of the



control Hsf1-ΔAR1 protein from cells grown at 39°C was slower than that from cells grown at 28°C (lanes 3 and 4). In cells expressing Hsf1-Sp that bears the C-terminal region of SpHSF, heat-induced mobility shifts of the protein took place (lanes 5 and 6). However, deletion of the CTM-like region (Hsf1-Sp/ΔCTM) suppressed the heat-induced mobility shifts (lanes 7 and 8). The mobility of the ScHsf1-hHSF1 chimera (Hsf1-Hs) was not significantly affected by the heat shock, but fusion of CTM (Hsf1-Hs+CTM) resulted in a dramatic retardation of protein mobility (lanes 9 to 12). These results strengthened the idea that the function of CTM is conserved in yeast and further served to establish a novel function of CTM (i.e., the heat-inducible phosphorylation [mobility shift] of ScHsf1).

Isolation and characterization of intragenic suppressors of CTM mutations. The CTM is thought to affect the function of the central regulatory domain upon heat shock by allowing an active conformation of ScHsf1 that exposes AR2 to the transcription machinery (40). To identify the region(s) of ScHsf1 that functionally interact(s) with the CTM, we isolated intragenic mutations that suppress the temperature-sensitive growth defect of the Hsf1-ba1 substitution protein. Because cells expressing Hsf1-ΔAR1/ba1 are more sensitive to heat than *hsf1-ba1* cells (Fig. 1B), the intragenic suppressor screen was done in the *hsf1*-ΔAR1/ba1 background (see Materials and Methods). Several weak and strong suppressor mutants were mapped in the DNA-binding domain and in other regions of ScHsf1, respectively. Here we focused on strong suppressors, the properties of which are summarized in Fig. 3A and B. One intragenic suppressor designated as IS-4 was located in the CTM region, which contained lysine in place of glutamate at position 830 (E830K). The heat shock response of *CUP1* took place in cells harboring IS-4 (data not shown). Two arginine residues at positions 826 and 830 in the CTM had been converted to glutamate upon mutation to ba1 (39); the fact that reversion to a basic amino acid occurred in association with suppressor mutation reinforced the significance of the basic amino acids in CTM for function (39). Three other suppressor alleles (IS-1, IS-2, and IS-11) contained an amino acid alteration at the 491 position of lysine to arginine (K491R). Another allele, IS-3, contained a change of tyrosine to cysteine at position 537 (Y537C). Each of the two remaining isolates, harboring IS-9 and IS-12, contained mutations at two positions (Table 1), and the common mutation of the two was designated as IS-15, altering glutamine at position 535 to arginine (Q535R), which contributed to the suppressor effect.

We analyzed whether or not the intragenic suppressors were able to affect heat-induced transcription of *CUP1* and *SSA4* (Fig. 3C). Although the *hsf1*-ΔAR1/ba1 mutation caused the complete inhibition of *CUP1* activation, the additional mutation K491R (IS-1) restored accumulation of the mRNA to 70% of the wild-type level at 15 min after the temperature shift. Allele IS-3 (Y537C) mediated a weak heat shock response of *CUP1*, leading to lower-level, slower accumulation of the mRNA than was observed in *HSF1* cells. Low-level activation of *CUP1* was also observed in cells harboring IS-15 (Q535R). The delayed and sustained heat shock response of *SSA4* caused by the *hsf1*-ΔAR1/ba1 mutation was partially corrected by suppressor mutations. Thus, the intragenic mutations suppressed not only the growth defect at higher temperatures but also the transcription defect due to Hsf1-ΔAR1/ba1.

The loss of activation of *CUP1* by heat shock has been associated with the function of CTM but not AR1 (39), suggesting that the isolated suppressor mutations affected the function of CTM. To test this possibility, we combined each suppressor mutation with an ScHsf1 derivative, in which CTM function had been nullified by the deletion of C-terminal 15 amino acids (39). All of the K491R, Q535R, and Y537C mutations suppressed the temperature-sensitive phenotype due to the CTM deletion (ΔCTM, Fig. 4A). In addition, the heat-inducible transcription of *CUP1*, inhibited by ΔCTM mutation, was significantly restored; for example, the mRNA in *hsf1*-ΔCTM/K491R cells accumulated to a level comparable to that in *HSF1* cells (Fig. 4B). Therefore, these mutations circumvented the requirement of CTM for growth at higher temperatures and for the activation of *CUP1*.

Connection of CTM function with CE2. The suppressor mutation Y537C was located within the CE2 repression domain, while K491R and Q535R were located near the domain (Fig. 3A and see Fig. 5A). The CE2 domain, which consists of a heptapeptide sequence and an adjacent serine stretch, was previously identified as a conserved element between ScHsf1 and KlHSF (26). A slightly divergent sequence was found to exist in the corresponding region of SpHSF (Fig. 1D and 5A).

FIG. 1. Characterization of ScHsf1 derivatives. (A) Schematic representation of ScHsf1 protein and its derivatives. Each protein is illustrated as a rectangle, the designation of which is shown to the left. The structural motifs of ScHsf1 are indicated above ScHsf1; AR1 and AR2 refer to the N- and C-terminal activation domains, respectively; DBD, DNA-binding domain; Oligomer, oligomerization domain; CE2, conserved element 2; CTM, C-terminal regulatory domain. (26, 39, 49, 51, 57). The numbers represent amino acid positions. Bent lines connecting a pair of rectangles indicate AR1 deletion. The thick vertical line in CTM denotes the ba1 mutation (Arg826Glu and Arg830Glu). Thick-lined boxes represent the C-terminal regions of SpHSF and hHSF1, in which the amino acid numbers correspond to those in the original proteins. (B) Growth of cells expressing deletion and point mutants of ScHsf1. Cells expressing ScHsf1 (wild type [WT]), Hsf1-ΔAR1 (ΔAR1), Hsf1-ba1 (ba1), or Hsf1-ΔAR1/ba1 (ΔAR1/ba1) were streaked on a YPD plate and were incubated at 28 or 38°C for 2 days. (C) mRNA levels of *CUP1* and *SSA4* in cells expressing deletion and point mutants of ScHsf1. Cells expressing various Hsf1 derivatives were grown in YPD medium at 28°C, and then the temperature was shifted to 39°C. At the indicated times, aliquots of cells were removed and stored at −80°C before use. Total RNA prepared from each sample was subjected to RT-PCR analysis with sets of primers for *CUP1*, *SSA4*, and *ACT1* (upper panel). The lower panel shows the kinetic profiles of the *CUP1* or *SSA4* mRNA, in which the levels are normalized to the value of *ACT1* mRNA as 100%. (D) Carboxy-terminal sequences of various HSFs. The upper panel shows a schematic overview of the structures of ScHsf1, KlHSF, SpHSF, and hHSF1. Homologous regions are shaded equivalently. The lower panel shows their C-terminal amino acid sequences. The basic amino acids are indicated in bold letters. The numbers show amino acid positions. (E) Growth of cells expressing ScHsf1 chimeras. The growth of cells expressing ScHsf1 (WT), Hsf1-ΔAR1 (ΔAR1), Hsf1-Sp (Sp), Hsf1-Sp/ΔCTM, (Sp/ΔCTM), Hsf1-Hs (Hs), or Hsf1-Hs+CTM (Hs + CTM) was analyzed as described for panel B. (F) mRNA levels of *CUP1* and *SSA4* in cells expressing ScHsf1 chimeras. Total RNA samples prepared from cells shown in panel E were analyzed as described for panel C.

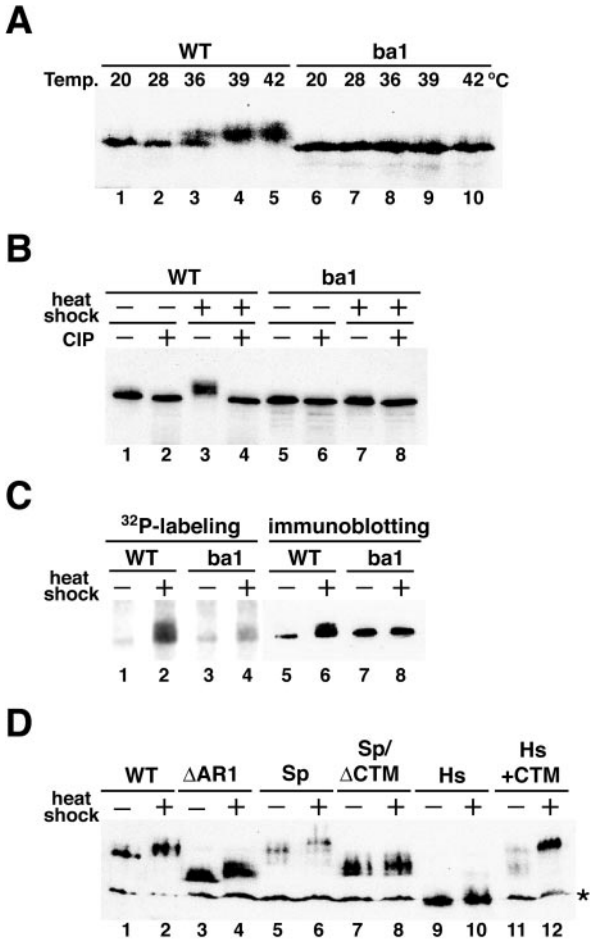


FIG. 2. Phosphorylation of ScHsf1 derivatives. (A) Immunoblot analysis of ScHsf1. Cells expressing ScHsf1 (wild type [WT]) or Hsf1-ba1 (ba1) were grown in YPD medium at 28°C (lanes 2 and 7) or at 36, 39, or 42°C (lanes 3 to 5 and 8 to 10) for 15 min. For lanes 1 and 2, the cells were grown at 20°C. The prepared cell extracts were subjected to SDS-PAGE and immunoblot analysis with anti-ScHsf1 serum. (B) Phosphatase treatment of ScHsf1. Cells expressing ScHsf1 (WT) or Hsf1-ba1 (ba1) were grown at 28°C (heat shock −) or 39°C (heat shock +) for 15 min. The cell extracts were subjected to immunoprecipitation with anti-ScHsf1 serum. The precipitates were divided into two portions, incubated in the absence (CIP −) or presence (CIP +) of calf intestine alkaline phosphatase, and subjected to immunoblotting. (C) In vivo ^{32}P labeling of ScHsf1. Cells expressing ScHsf1 (WT) or Hsf1-ba1 (ba1) were grown at 28°C (heat shock −) or 39°C (heat shock +) for 15 min in the presence of [^{32}P]orthophosphate. The cell extracts were separated by SDS-PAGE and subjected to phosphorimaging (lanes 1 to 4) and immunoblot (lanes 5 to 8) analyses. (D) Immunoblot analysis of ScHsf1 chimeras. Cells expressing ScHsf1 (WT), Hsf1-ΔAR1 (ΔAR1), or various chimeras (Sp, Sp/ΔCTM, Hs, and Hs+CTM) were grown at 28°C (heat shock −) or 39°C (heat shock +) for 15 min. The cell extracts were subjected to immunoblot analysis as described for panel A. In lanes 9 and 10, Hsf1-Hs proteins migrated close to an unknown cross-reacting protein indicated by an asterisk on the right.

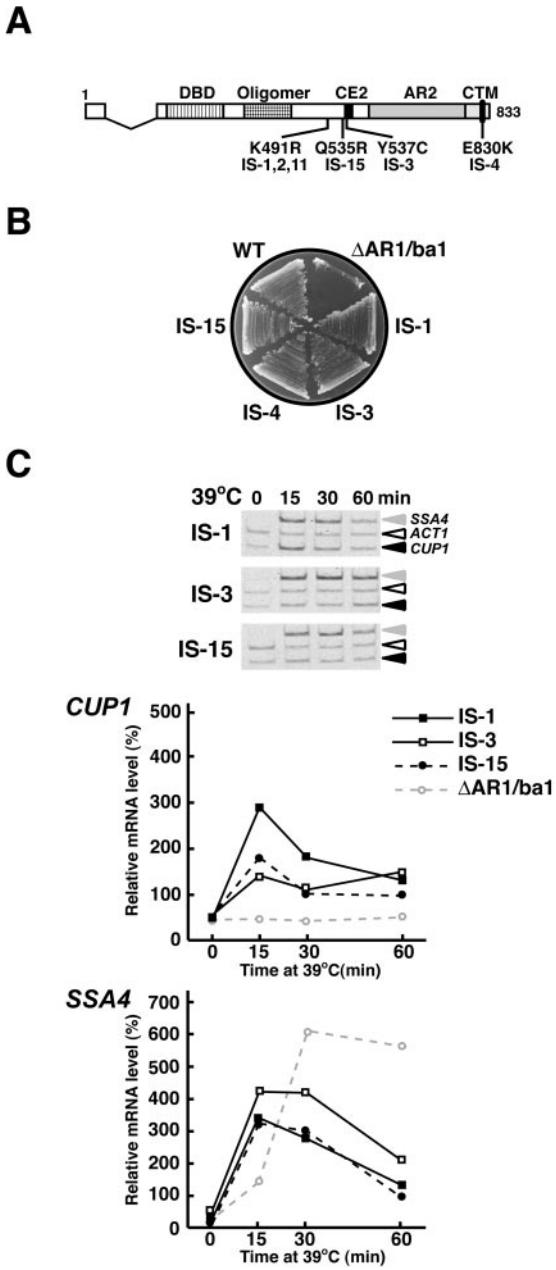


FIG. 3. Characterization of intragenic suppressors of CTM mutation. (A) Location of suppressor mutations. The suppressor genes and their mutations are summarized. The structural motifs of Hsf1-ΔAR1/ba1 are shown in Fig. 1A. (B) Growth of *hsf1*-ΔAR1/ba1 cells containing suppressor mutations. Cells harboring *HSF1* (WT), *hsf1*-ΔAR1/ba1 (ΔAR1/ba1), or various suppressor genes (IS-1, -3, -4, and -15) were streaked on a YPD plate and were incubated at 38°C for 2 days. (C) mRNA levels of *CUP1* and *SSA4* in *hsf1*-ΔAR1/ba1 cells containing suppressor mutations. Total RNA samples prepared from the cells shown in panel B were analyzed as described for Fig. 1C. For comparison, the kinetic profiles of the *CUP1* or *SSA4* mRNA in *hsf1*-ΔAR1/ba1 cells are also shown.

Deletion of CE2 from ScHsf1 or KlHSF has been shown to convert the inducible activation domains in a constitutive manner, such that transcription of target genes takes place even at the normal temperatures, suggesting that CE2 is involved in the repression of the activator function (26). To know whether or not the suppressor mutations would affect the basal transcriptional activity of ScHsf1, we analyzed the mRNA level of *CUP1* in cells grown at 28°C. Because both ScHsf1 and a copper-responsive activator, Ace1, mediate basal expression of

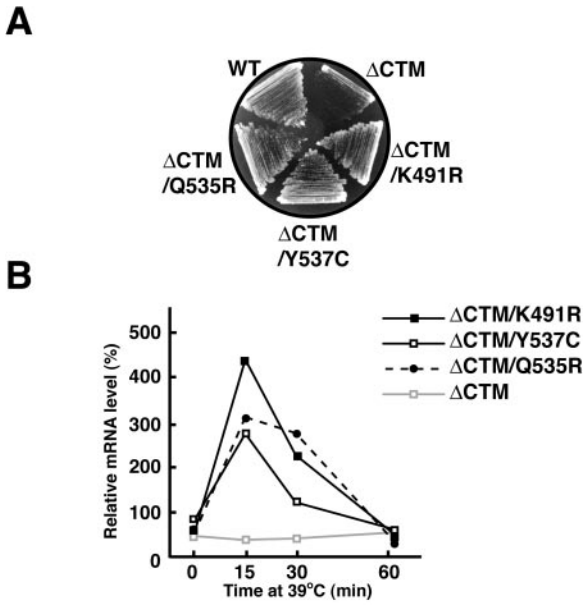


FIG. 4. Effect of suppressor mutations on CTM function. (A) Growth of *hsf1*-ΔCTM cells containing suppressor mutations. The growth of cells expressing ScHsf1 (wild type [WT]), Hsf1-ΔCTM (ΔCTM), or Hsf1-ΔCTM containing the suppressor mutations (K491R, Y537C, and Q535R) was analyzed as described for Fig. 3B. (B) mRNA levels of *CUP1* and *SSA4* in *hsf1*-ΔCTM cells containing suppressor mutations. Total RNA samples prepared from the cells shown in panel A were analyzed as described for Fig. 1C, and a kinetic profile of the *CUP1* mRNA is shown.

CUP1 (48, 61), we used *ace1* null mutant cells. As shown in Fig. 5B, introduction of the Y537C mutation into wild type *HSF1* caused a 1.5-fold increase in the *CUP1* mRNA at 28°C. Neither K491R nor Q535R affected the basal-level expression of *CUP1*. As a control, we analyzed the mRNA level in cells expressing an ScHsf1 derivative lacking CE2 (Hsf1-ΔCE2, see Fig. 5A for the construction) and found a twofold increase in amount over that of the *HSF1* control at 28°C.

The above observations imply that at least the Y537C mutation impairs CE2 function. Deletion of CE2 (Hsf1-ΔCE2) affected neither the growth of cells nor the heat shock response of *CUP1* and *SSA4* (Fig. 5C and D). To examine the functional relationship between CE2 and CTM, the CE2 deletion was combined with the ba1 mutation (Hsf1-ΔCE2/ba1). The resulting protein did support both growth at 38°C and the heat-inducible transcription of *CUP1* (Fig. 5C and D). In the absence of the CE2 domain, therefore, the ba1 mutation had no notable effect on either the heat sensitivity of the cells or the activator function of ScHsf1. These results show that CTM alleviates the repression function of CE2 in response to heat shock but becomes dispensable when CE2 is deleted.

Phosphorylation of Hsf1-ba1 derivatives containing suppressor mutations. As described above, the CTM domain is necessary for the heat-inducible phosphorylation of ScHsf1 (Fig. 2). Moreover, CTM is essential for the activation of *CUP1* but not *SSA4*. The requirement for gene activation was bypassed by the K491R, Q535R, Y537C, or ΔCE2 mutation (Fig. 4B and 5D). If the phosphorylation of ScHsf1 is indeed implicated in gene-specific activation, then the phosphorylation defect of Hsf1-ba1 would be restored by the suppressor mutations. As expected, the electrophoretic mobility of Hsf1-ba1 containing the K491R, Q535R, or Y537C mutation was retarded on the gel when the cells were grown at 39°C for 15 min (Fig. 5E, lanes 2, 4, and 6). Heat-inducible mobility shifts of ScHsf1 lacking CE2 occurred independently of the presence of functional CTM (Fig. 5E, lanes 8 and 10). The above results, when taken together, demonstrate that all known defects associated with CTM mutations can be restored by CE2 deletion, as well as by the suppressor mutations. We suggest that the CTM domain is involved in both phosphorylation and in the gene-specific activation ability of ScHsf1 by controlling the repression function of CE2 upon heat shock.

Intramolecular interaction mediated by CTM and CE2. It was thought that heat shock induces intramolecular interactions among HSF domains; this led the protein to undergo a conformational change, which in turn results in the conversion of the protein from an inactive form to an active form (5, 9, 30, 36, 49). We then analyzed the physical interaction between the N-terminal regulatory region and the C-terminal AR2-CTM domains of ScHsf1 by GST pull-down assay (Fig. 6). The C-terminal 250 amino acids of ScHsf1 were produced in *E. coli* as a fusion protein with GST (GST-250C/WT), and the protein was immobilized on glutathione Sepharose gel. The resin was incubated with the N-terminal peptide (N583/WT), which had been produced in *E. coli* and purified to homogeneity (Fig. 6A and B). After extensive washing, the bound proteins were subjected to immunoblot analysis with anti-ScHsf1 serum. As shown in Fig. 6C, the N583/WT peptide was detected in the bound fraction prepared from the resin immobilizing GST-C250/WT (row 1, lane 2). The involvement of both CTM and CE2 in binding was indicated by the result that the ba1 mutation in the GST fusion (GST-250C/ba1) inhibited the binding of N583/WT (row 1, lane 4) and also by the finding that the N-terminal peptide lacking CE2 (N583/ΔCE2) was not efficiently captured on the GST-C250/WT resin (row 2, lane 3). The binding between the N- and C-terminal peptides was also inhibited by the Y537C mutation, which would impair the CE2 repression function (row 4). It is therefore likely that physical interactions involving the CTM and CE2 domains are implicated in the formation of an active conformation upon heat shock. However, neither K491R nor Q535R, whose properties were different from Y537C in derepression of *CUP1*, affected binding of the peptides (rows 3 and 5). It remained unknown whether these mutations suppress a loss of CTM function through affecting the CE2 function or through different mechanism(s).

DISCUSSION

The present study of the role of the C-terminal modulator CTM revealed a novel link between the heat-inducible hyperphosphorylation and HSE-specific activator function of ScHsf1. We have demonstrated that CTM function is conserved in *S. pombe* HSF and is necessary for (i) heat-inducible but not constitutive phosphorylation, (ii) the activation of *CUP1* via an atypical HSE, and (iii) the growth of yeast at higher temperatures. Although CTM appeared to be involved in the rapid response of *SSA4* to heat, overall accumulation of the mRNA was not significantly affected by loss-of-function

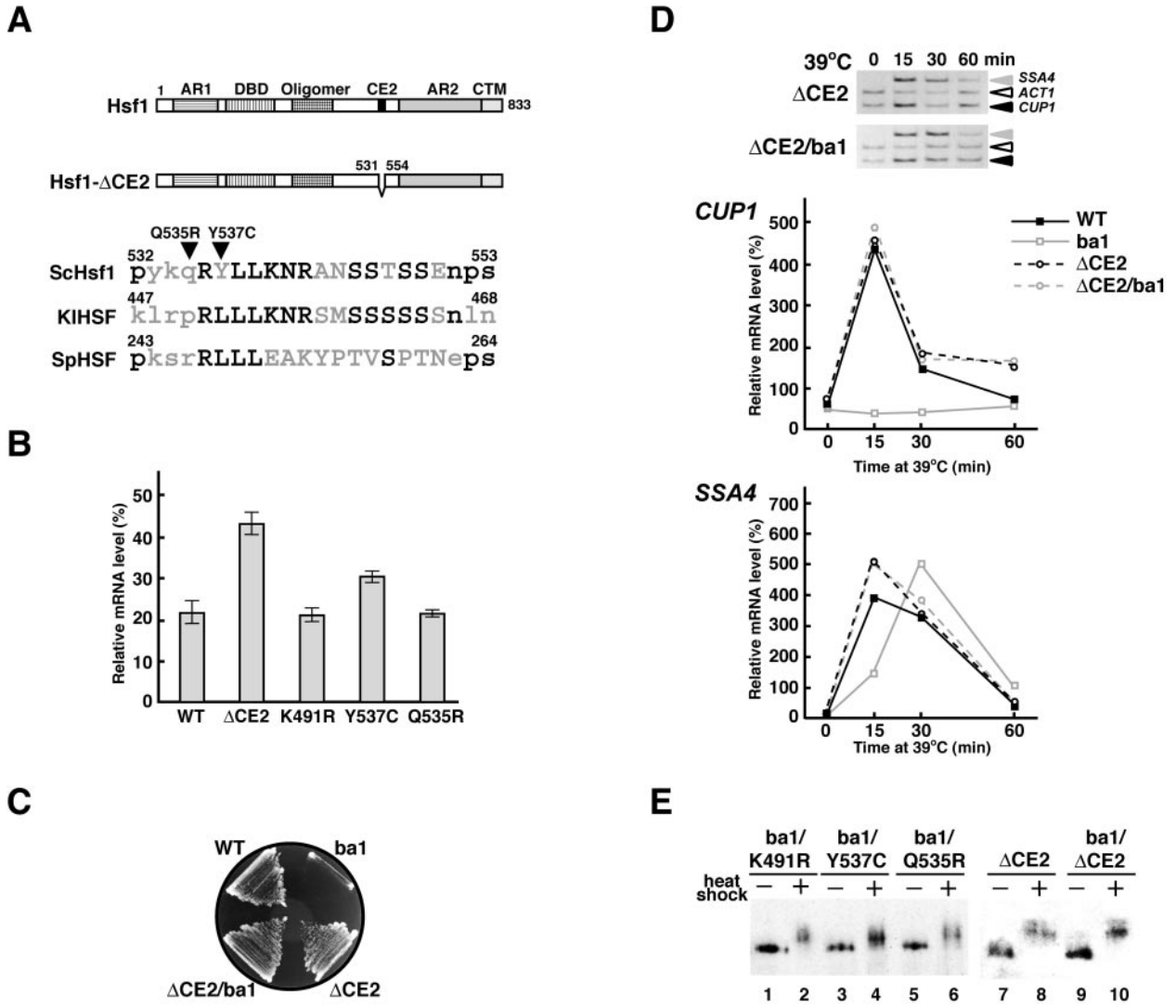


FIG. 5. Effect of CE2 deletion on CTM function. (A) Amino acid sequences of the CE2 regions. The upper panel shows the structures of the ScHsf1 derivatives. The structural motifs are shown in Fig. 1A. The lower panel shows the amino acid sequence of the deleted region in Hsf1-ΔCE2 in the top row. The positions of two suppressor mutations are also indicated by solid triangles. In the second and third rows, the conserved sequences found in KlHSF and SpHSF, respectively, are shown. The CE2 element consists of a heptapeptide sequence and a serine stretch (26), which are indicated in capital letters. The amino acids that are identical in at least two orthologs of the HSFs are indicated in bold letters. The numbers show the amino acid positions. (B) Basal level of *CUP1* mRNA in *hsf1*cells containing suppressor mutations. *ace1* null mutant cells expressing ScHsf1 (WT), Hsf1-ΔCE2 (ΔCE2), Hsf1-K491R, Hsf1-Y537C, or Hsf1-Q535R were grown at 28°C. Total RNA prepared from each sample was subjected to RT-PCR analysis with sets of primers for *CUP1* and *ACT1*. The relative levels of *CUP1* mRNA, which are normalized to the value of *ACT1* mRNA as 100%, represent the means ± standard errors of three independent experiments. (C) Growth of cells expressing Hsf1-ΔCE2 derivatives. The growth of cells expressing ScHsf1 (WT), Hsf1-ba1 (ba1), Hsf1-ΔCE2 (ΔCE2), or Hsf1-ΔCE2/ba1 (ΔCE2/ba1) was analyzed as described for Fig. 3B. (D) mRNA levels of *CUP1* and *SSA4* in cells expressing Hsf1-ΔCE2 derivatives. Total RNA samples prepared from the cells shown in panel C were analyzed as described for Fig. 1C. For comparison, the kinetic profiles of the *CUP1* or *SSA4* mRNA in *HSF1* and *hsf1-ba1* cells are also shown. (E) Immunoblot analysis of Hsf1-ba1 protein containing suppressors of the ba1 mutation. For lanes 1 to 6, the cells expressing Hsf1-ba1, which contains suppressor mutations (ba1/K491R, ba1/Y537C, and ba1/Q535R), were grown at 28°C (heat shock −) or 39°C (heat shock +) for 15 min. For lanes 7 to 10, the cells expressing Hsf1-ΔCE2 (ΔCE2) or Hsf1-ΔCE2/ba1 (ΔCE2/ba1) were used. The cell extracts were subjected to immunoblot analysis as described for Fig. 2A.

mutations of CTM. The dispensability of CTM function in *SSA4* induction indicates that the activator ability of ScHsf1 bound to the typical HSE is independent of hyperphosphorylation. In contrast, in order for *CUP1* to be activated, ScHsf1 must be hyperphosphorylated upon heat shock. Notably, both transcription and phosphorylation defects due to CTM mutations were concomitantly restored by single-amino-acid alterations. Therefore, phosphorylation of ScHsf1 is implicated specifically in the heat-induced transcription of genes containing atypical HSE, such as *CUP1*. The requirement of CTM was circumvented by the deletion of the repression domain CE2, suggesting that CTM is involved in the relief of the CE2 repression function in response to heat shock. It is therefore reasonable to assume that the CTM-CE2 interaction regulates

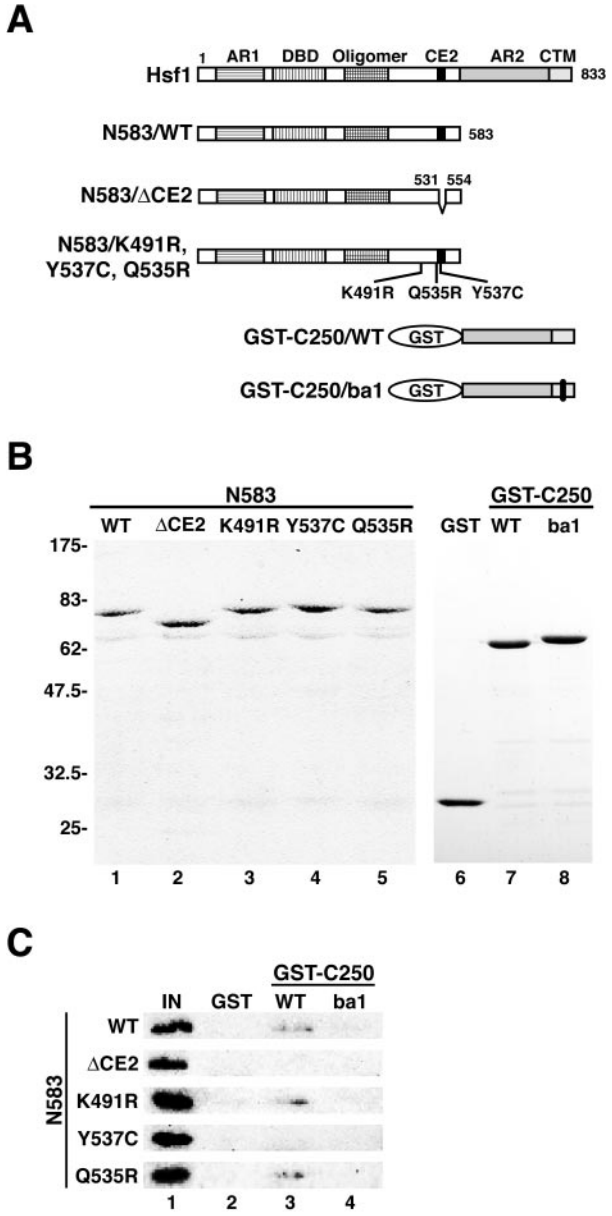


FIG. 6. Physical interaction mediated by CTM and CE2. (A) Schematic representation of the polypeptides used for the binding assay. The structures of the N-terminal polypeptides (N583/WT, N583/ΔCE2, and N583 containing the K491R, Y537C, or Q535R mutation) and C-terminal polypeptides fused with GST (GST-C250/WT and GST-C250/ba1) are shown with motifs that are explained in the legend to Fig. 1A. (B) Coomassie brilliant blue staining of purified polypeptides. Purified polypeptides N583/WT (lane 1), N583/ΔCE2 (lane 2), N583/K491R (lane 3), N583/Y537C (lane 4), N583/Q535R (lane 5), GST (lane 6), GST-C250/WT (lane 7), and GST-C250/ba1 (lane 8) were subjected to SDS-PAGE and stained with Coomassie brilliant blue. Molecular mass markers are indicated to the left in kilodaltons. (C) Immunoblot analysis of bound proteins. Purified protein GST (lanes 2), GST-C250/WT (lanes 3), or GST-C250/ba1 (lane 4) was immobilized on glutathione Sepharose and incubated with N583/WT, N583/ΔCE2, N583/K491R, N583/Y537C, or N583/Q535R polypeptide. After extensive washing, bound polypeptides were subjected to immunoblot analysis. Lane 1 contains input N-terminal polypeptides (IN).

the heat-inducible phosphorylation of ScHsf1, which is a prerequisite for the activator function when ScHsf1 binds to atypical HSE.

Roles of CTM and CE2 on the heat-inducible activation of ScHsf1. Saltsman et al. (41) previously demonstrated that deletion of the C-terminal region, including the CTM-like domain of SpHSF, caused increased heat sensitivity of the *S. pombe* cells for growth. In the present study, we provided further evidence that the CTM-like region of SpHSF, when integrated in ScHsf1 and expressed in *S. cerevisiae*, carries out the normal functions of CTM as regards phosphorylation, gene-specific transcription, and heat sensitivity of the cell. In addition, CTM was capable of restoring the function of the activation domain of hHSF1 in the context of Hsf1-Hs+CTM. In these chimeras, temperature-dependent activation function is repressed by the DNA-binding, trimerization, and CE2 domains of ScHsf1, indicating that CTM modulates the regulatory function of these domains upon heat shock.

The intragenic mutations suppressing the defects associated with a loss of CTM function were located within or near the yeast-specific element CE2, which is necessary to restrain the activity of HSF at low temperatures and/or to return the protein from the activated state to the inactive state (23, 26). One suppressor mutation, Y537C, lying in the CE2 element, derepressed the expression of *CUP1* at the normal temperatures, implying that this mutation impairs the function of CE2. In support of this idea, all phenotypes associated with CTM mutations were suppressed by the deletion of CE2. We therefore propose that an additional role is played by CE2, namely, the inhibition of heat-inducible phosphorylation. It is reasonable to assume that CTM relieves the inhibitory effects in response to heat shock, and therefore the requirement for CTM is circumvented in ScHsf1 derivatives that lack the CE2 function. We suggest that the CTM-CE2 interaction is conserved in yeast HSFs and is involved in the regulation of HSE sequence-specific transcription through the phosphorylation of HSFs.

Høj and Jakobsen (23) identified the serine stretch of CE2 as one of the phosphorylation sites. They argued that phosphorylation of CE2 serves as a regulatory mechanism to deactivate HSF. In their analysis, mutations of CE2 led to a reduction in the heat-induced retardation of the HSF-HSE complex, as determined by gel retardation assay. However, in the present study, we observed the slow migration of CE2-lacking ScHsf1 upon exposure of the cells to heat shock, as determined by immunoblot analysis (Fig. 5E). We performed phosphatase treatment analysis in order to confirm that the observed retardation was due to phosphorylation (data not shown). This apparent discrepancy may be explained by the fact that the mobility of a DNA-protein complex on nondenaturing gel is significantly affected by its conformation (30). Thus, the phosphorylation of the serine stretch might be sufficient to retard the migration of the HSF-HSE complex on nondenaturing gel due to changes in the conformation, but that would not be sufficient to achieve a mobility shift of HSF on denaturing gel. We assume that the phosphorylation of the serine stretch of CE2 and extensive phosphorylation of the other residues are involved in the deactivation and activation of HSF, respectively.

Putative mechanism of HSE-specific transcription regulated by phosphorylation. We have shown that CTM is re-

quired for the activation of genes containing atypical HSE but not the typical HSE (39). Our preliminary results supported this notion. In cells expressing Hsf1-ba1, exposure to heat shock caused an accumulation of transcripts from genes containing the typical HSE, such as *UBI4*, *SIS1*, *HSP104*, and *KAR2*, but not from genes containing atypical HSE, such as *CPR6* and *YDJ1* (H. Imazu and H. Sakurai, unpublished results). By using various ScHsf1 derivatives and intragenic suppressor mutations, it was shown here that CTM is also required for the heat-inducible phosphorylation of ScHsf1. In light of these findings, we suggest that the hyperphosphorylation of ScHsf1 is dispensable for the activation of genes containing the typical HSE but is indispensable for activation via atypical HSE. Since the ba1 mutation was unable to completely inhibit the heat-inducible phosphorylation of the protein (Fig. 2C), we have not ruled out the possibility that low-level phosphorylation, which does not cause mobility shifts on denaturing gel, serves a different regulatory mechanism.

In human cells, the binding of hHSF1 to the HSE precedes its phosphorylation (13, 59). This finding, taken together with the previous observation that a loss of CTM function has little effect on the ScHsf1-HSE interaction (39), suggests that CTM-dependent phosphorylation is necessary for the acquisition of the activator ability of ScHsf1 when it binds to an atypical HSE. We propose the involvement of phosphorylation in HSE architecture-dependent activation as follows. The activator function of ScHsf1 is repressed by the DNA-binding and trimerization domains at the normal growth temperatures (5, 9, 36), which is relieved by intra- and/or intermolecular interactions upon heat shock. Both domains make protein-protein contacts among neighboring monomers bound to an array of the NGAAN repeats of the HSE (32, 51). When ScHsf1 binds to the six contiguous repeats of NGAAN comprising the *SSA4* HSE, heat shock induces a conformational change of both domains to generate a transcriptionally active form, which is mediated and/or stabilized by the interactions among the regularly bound monomers without phosphorylation of the protein. In contrast, the ScHsf1 trimer, when bound to the gapped HSE of *CUP1* [NTTCNNGAAN(5 bp)NGAGN], may not be stably converted to the active form. In fact, the DNA-binding domain bound to the gapped NGAGN fails to interact with that bound to the first two 5-bp units, as judged from the cocrystal structure of the DNA-binding domain and the HSE (32). In this case, phosphorylation may be essential to stabilize the active form. Alternatively, phosphorylation may be involved in the interaction of ScHsf1 with as yet unknown protein(s) regulating the activity and/or conformation of ScHsf1 bound to the gapped HSE. Consistent with these ideas, the conformation of ScHsf1 bound to the gapped HSE of *CUP1* has been shown to be different from that bound to the typical HSE (43). To elucidate the exact role of phosphorylation, it will be necessary to identify the amino acid residue(s) that is phosphorylated upon heat shock.

In addition to heat shock, various environmental and intracellular stresses control HSF activity. It has been shown that oxidative stress induces ScHsf1 phosphorylation and transcription of *CUP1* (33). The *CUP1* activation by oxidative stress is dependent on the presence of functional CTM (39). Our preliminary observation that CTM was also involved in oxidative stress-induced phosphorylation (J. Ueda and H. Sakurai, unpublished results) implies that CTM plays a central role in both gene- and stress-specific transcription by regulating the phosphorylation of ScHsf1. Further genetic and biochemical studies will be required to elucidate the details of gene-specific activation by HSF in response to distinct stimuli. Nevertheless, by demonstrating that the phosphorylation of HSF is implicated in HSE-specific transcription, the present report will contribute important information to aid our understanding of the molecular mechanism(s) involved in the stress response.

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