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Identification of a Novel Class of Target Genes and a Novel Type of Binding Sequence of Heat Shock Transcription Factor in *Saccharomyces cerevisiae**

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In response to hyperthermia, heat shock transcription factor (HSF) activates transcription of a set of genes encoding heat shock proteins (HSPs). The promoter regions of HSP genes contain the HSF binding sequence called the heat shock element (HSE), which consists of contiguous inverted repeats of the sequence 5′-nGAAn-3′ (where n is any nucleotide). We have constructed an *hsf1* mutant of *Saccharomyces cerevisiae* and analyzed genome-wide changes in heat shock response in the mutant cells. The results have revealed that Hsf1 is necessary for heat-induced transcription of not only HSP but also genes encoding proteins involved in diverse cellular processes such as protein degradation, detoxification, energy generation, carbohydrate metabolism, and maintenance of cell wall integrity. Approximately half of the Hsf1-regulated genes lacked the typical HSE in their promoter regions. Instead, several of these genes have a novel Hsf1 binding sequence that contains three direct repeats of nTTCn (or nGAAn) interrupted by 5 bp. The number and spacing of the repeating units are critical determinants for heat-induced transcription as well as for recognition by Hsf1. In the yeast genome, the presence of the sequence is enriched in Hsf1-regulated genes, suggesting that it is generally used as an HSE in the Hsf1 regulon.

All organisms respond to elevated temperatures by changing transcription programs and expressing a set of proteins called heat shock proteins (HSPs),[1] many of which are chaperones involved in protein folding, trafficking, maturation, and degradation (1). In eukaryotes, heat shock transcription factor (HSF) activates transcription of HSP genes under a variety of chemical and physical stresses as well as heat shock. The target genes of HSF contain a cis-acting sequence designated the heat shock element (HSE). The HSF-HSE interaction is conserved from yeasts to humans (2–4). The HSF proteins have common structural motifs, including a winged helix-turn-helix DNA-binding domain (DBD), a hydrophobic repeat region essential for formation of a three-stranded coiled-coil, and a C-terminal transactivation domain (2–4). For binding to the HSE, HSF forms a homotrimer through the coiled-coil domains and the DBD recognizes the sequence 5′-nGAAn-3′. The HSE is composed of at least three contiguous inverted repeats of the nGAAn unit (perfect type, nTTCnnGAAnnTTCn) (5–8). A variation of the HSE, two consecutive nGAAn units followed by another unit after a gap of 5 bp (gap type, nTTCnnGAAn(5 bp)nGAAn), is tolerated by HSFs (9, 10). The mammalian HSF isoforms, HSF1 and HSF2, which may respond to distinct stresses and regulate different target genes, exhibit differences in their abilities to bind to variations in the HSE (11, 12). Therefore, the divergence of HSE architecture is believed to provide gene-specific responses to stress.

The baker's yeast, *Saccharomyces cerevisiae*, has one HSF encoded by the *HSF1* locus (13, 14). Yeast Hsf1 is indispensable for viability because it binds constitutively to the HSE and maintains basal levels of transcription of genes even at normal growth temperatures (15–18). Heat shock leads to cooperative interactions of Hsf1 with the HSE and to acquisition of higher level transcriptional activity of Hsf1 (19, 20). Various regulatory domains have been identified in Hsf1, including the DBD, coiled-coil trimerization domain, and an N-terminal (AR1/NTA/AAD) and a C-terminal (AR2/CTA/CAD) activation domain (13, 21–23). In addition, Hsf1 contains yeast-specific domains termed CE2 and CTM; the former negatively regulates the activator function and the latter alleviates the CE2 repression function in response to heat shock (24, 25). Interestingly, CTM is necessary for heat-induced hyperphosphorylation of Hsf1 and for activation of genes containing the gap-type HSE but not the perfect-type HSE (25). The AR2, but not the AR1, activation domain is responsible for transcription through the gap-type HSE (9, 10, 24), suggesting that the CTM-CE2 interaction regulates phosphorylation, which in turn activates the AR2 function of Hsf1 bound to the gap-type HSE.

Hsf1 is thought to activate transcription of HSP genes in response to heat shock, but its involvement in regulation of other heat-inducible genes has not been investigated. Using chromatin immunoprecipitation together with a DNA microarray analysis, Hahn *et al.* (20) have recently shown that putative Hsf1 targets represent nearly 3% (165 genes) of the genome. It is not known, however, whether Hsf1 directly regulates transcription of all these genes in response to heat shock. To gain further insight into the Hsf1 regulon of yeast, we have constructed a new *hsf1* mutant that fails to mediate heat-induced transcription of various known target genes. We have used this mutant to perform a genome-wide survey to identify genes whose expression is dependent on normal Hsf1 function. We found that some of the Hsf1-regulated genes contain a novel

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The expression data of this work can be accessed through the NCBI Gene Expression Omnibus (GEO) under GEO accession number GSE2103.

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[1] The abbreviations used are: HSP, heat shock protein; HSF, heat shock factor; HSE, heat shock element; DBD, DNA-binding domain; ORF, open reading frame; YPD, rich glucose medium; WT, wild type.

type of HSE. These results provide important information for understanding the transcriptional network regulated by HSF upon various chemical and physiological stresses as well as heat shock.

EXPERIMENTAL PROCEDURES

Yeast Strains and Media—Strains were derived from HS126 (*MATα ade2 his3 leu2 trp1 ura3 can1 hsf1*::*HIS3* YCp-*URA3-HSF1*) or HS134 (HS126 containing *SSA4-lacZ* reporter gene in the chromosomal *leu2* locus) (25). Strain HS164 was an *msn2*::*LEU2 msn4*::*ADE2* derivative of HS126. Strain HS170T was a derivative of HS126 containing pK157 (YCp-*TRP1-HSF1*) instead of YCp-*URA3-HSF1*. The nTTCn repeat sequence of the *FSH1* promoter was changed to the "m1" and "m2" sequences (see Fig. 4*A*) in the chromosome of HS170T to create HS218 and HS219, respectively. Rich glucose medium (YPD), enriched synthetic glucose medium, and 5-fluoroortic acid-containing medium were prepared as described previously (24–26).

Isolation of Temperature-sensitive hsf1 Mutations—Plasmid pK157 was a *TRP1*-marked plasmid bearing the wild type *HSF1* gene from −729 to +2882 relative to the translation initiation site (24). To construct a gene library containing mutations in the coding sequence of the Hsf1 N-terminal region, a segment from −729 to +1545 of *HSF1* was amplified by PCR under error-prone conditions (25). The mutagenized fragments were digested with BamHI (at +195) and NheI (at +1444) and cloned into BamHI-NheI-digested pK157. The mutant library was used to transform strain HS134 to tryptophan prototrophy. Transformants were replica plated on 5-fluoroorotic acid-containing medium to shuffle out the resident *URA3*-marked plasmid bearing the wild type *HSF1*. The plates were incubated at 38 °C to identify plasmids bearing mutant *hsf1* genes that confer temperature-sensitive growth. Plasmids recovered from the cells were sequenced. One of these, designated pAY9, contained a substitution of phenylalanine to serine at the 256 position in *HSF1* (YCp-*TRP1-hsf1-F256S*).

Plasmids—Site-directed mutagenesis was carried out by the megaprimer PCR method (27). A substitution of valine to alanine at the 203 position (V203A) was introduced into *HSF1* (pK157) and *hsf1-F256S* (pAY9) to create pAY15 and pAY18, respectively. Similarly, plasmids pAY19 and pAY22 contained an R206S substitution in *HSF1* and *hsf1-F256S*, respectively. These plasmids were transformed into HS134, and transformants were streaked on a 5-fluoroorotic acid-containing plate to isolate cells expressing mutant Hsf1 proteins.

The *FSH1-lacZ* reporter gene was constructed as follows. The 5′-region of *FSH1* from −350 to +45, in which the SalI and BamHI sites were created at the 5′- and 3′-ends, respectively, was amplified by PCR from yeast chromosomal DNA. The SalI-BamHI-digested fragment was substituted for the *CYC1* promoter region of the *CYC1-lacZ* reporter (pLG670Z; YEp-*URA3-CYC1-lacZ*) (28). Point mutations were introduced by the megaprimer PCR method. To construct *SSC1/CYC1-lacZ* reporters, an oligonucleotide of the TTC repeating sequence of *SSC1* (from −285 to −256) or its derivatives was inserted into the XhoI site lying upstream of the *CYC1* promoter of pLG670Z.

RNA Analysis—Cells were grown in YPD medium at 28 °C to an optical density of 1.0 at 600 nm, and then the temperature was shifted to 39 °C. Total RNA was prepared from the cells and analyzed by reverse transcription-coupled PCR as described previously (25). The amounts of PCR products, which represent relative amounts of mRNA, were compared after normalizing RNA samples with the levels of control *ACT1* mRNA (encoding actin). The nucleotide sequences of gene-specific primers are available upon request.

GeneFilter Hybridization and Data Analysis—Mini-array filter hybridization analysis was carried out as described previously (29). Briefly, *HSF1* and *hsf1-R206S/F256S* cells grown in YPD medium at 28 °C were incubated at 39 °C for 15 min. Total RNA was prepared, 10 μg was mixed with 1 μg of oligo(dT), and cDNA was synthesized by Superscript II (Invitrogen) in the presence of [α-^{33}P]dCTP. The mini-array genomic filters (GeneFilters®; Invitrogen) were hybridized with the labeled cDNA in a MicroHyb hybridization solution (Invitrogen) at 42 °C for 16 h. The filters were washed, exposed to an imaging plate, and scanned at a resolution of 25 microns on a BAS5000 image analyzer (FujiFilm, Tokyo, Japan). The intensity of signals was quantified by ArrayGauge, an image analyzing application (FujiFilm).

The hybridization of each sample was performed in duplicate. To facilitate comparisons, the raw hybridization intensity of each open reading frame (ORF) on a single array was normalized as follows. First, the local background intensity was subtracted from each ORF, and then each ORF value was normalized to equalize the total intensity of all ORFs on each array. The normalized intensities from duplicate experiments were averaged, and the average -fold changes were calculated. ORFs that displayed at least a 2.5-fold decrease in *hsf1-R206S/F256S* cells compared with *HSF1* cells are listed in Table I. Data have been deposited at the NCBI gene expression omnibus (GEO; www.ncbi.nlm.nih.gov/geo/) with GEO accession number GSE2103. The presence of the HSE in each gene was determined by searching the following sequences in both strands within 1000 bp of the 5′-untranslated regions of yeast genes using the Pattern Matching program (Saccharomyces Genome Database; www.yeastgenome.org/): perfect type (GAAnnTTCnnGAR, GARnnTTCnnGAA, and GAAnnYTCnnGAA), gap type (YTCnnGAAnnnnnnnGAA, TTCnnGARnnnnnnnGAA, and TTCnnGAAnnnnnnnGAR), and step type (nTTCnnnnnnnTTCnnnnnnnTTCn).

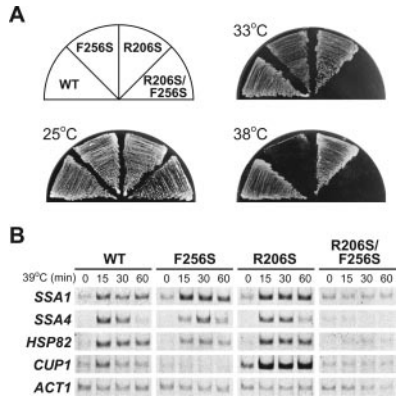

FIG. 1. **Characterization of *hsf1* mutant cells.** *A*, cells expressing wild type *HSF1* (*WT*), *hsf1-F256S* (*F256S*), *hsf1-R206S* (*R206S*), or *hsf1-R206S/F256S* (*R206S/F256S*) were streaked on a YPD medium plate and incubated at 28, 33, or 38 °C for 3 days. *B*, cells were grown in YPD medium 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. Total RNA prepared from each sample was subjected to reverse transcription-coupled-PCR analysis with sets of primers for the heat-inducible genes (*SSA1*, *SSA4*, *HSP82*, and *CUP1*) and control *ACT1* gene (encoding actin).

DNase I Footprint Analysis—The N-terminal Hsf1 polypeptide (amino acids 1–583 (N583/WT)) was produced in *Escherichia coli* and purified as described previously (25). The polypeptide was incubated with 5′-end-labeled DNA in 8 μl of buffer containing 20 mM Hepes-KOH (pH 7.6), 2 mM $MgCl_2$, 0.5 mM EDTA, 25 mM potassium acetate, 0.025% Nonidet-P40, 10 μg/ml poly(dI-dC), and 10% glycerol. After incubating on ice for 15 min, 2 μl of DNase I solution (0.1–0.2 units of DNase I in 20 mM Hepes-KOH, pH 7.6, plus 10 mM $CaCl_2$) was added and incubated for an additional 2 min. The reaction was stopped by adding 10 μl of stop solution (95% formamide, 22 mM Tris borate, 10 mM EDTA, 0.05% bromphenol blue, and 0.05% xylene cyanol) followed by boiling for 3 min. The samples were electrophoresed on a polyacrylamide-urea sequencing gel and subjected to phosphorimaging with a Typhoon9200 image analyzer (Amersham Biosciences). The probe DNAs were the *FSH1* fragment from −350 to +45, the *SSC1* fragment from −454 to −79, and the *CTH1* fragment from −463 to −52.

Gel Retardation Analysis—The reaction mixture, which was the same as above except containing 25 μg/ml poly(dI-dC), was incubated at room temperature for 15 min. The samples were electrophoresed on an agarose gel in 45 mM Tris borate plus 1 mM EDTA at 4 °C and subjected to phosphorimaging. The probe DNA fragments were *SSC1*wt (see Fig. 4*C*), 3P (5′-TCGACTTCTAGAAGCTTCCACTAATTAGTGCTACTCGA-3′), and 6P oligonucleotides (5′-TCGACTTCTAGAAGCTTCCAGAAATTTCTGGAACTCGA-3′).

Assay of β-Galactosidase Activity—The reporter genes *FSH1-lacZ*, *SSC1/CYC1-lacZ*, and their derivatives were introduced into strain HS170T. Transformants were grown in enriched synthetic glucose medium lacking uracil and tryptophan at 28 or at 39 °C for 90 min. The β-galactosidase activity was determined as described previously (28).

RESULTS

Isolation and Characterization of Temperature-sensitive hsf1 Mutants—From an *hsf1* library, we isolated temperature-sensitive mutants that failed to grow at 38 °C (Fig. 1*A*). One of these mutant strains contained an amino acid alteration of phenylalanine to serine at the 256 position (F256S) in *HSF1*. The Phe-256 residue is located in the fourth β-strand of the DBD and is conserved among HSFs from various organisms

(30, 31). In cells expressing the Hsf1-F256S protein, accumulation of transcripts from *SSA1* and *SSA4* (encoding Hsp70) was observed after the temperature upshift, but the kinetics were slightly delayed in comparison with the *HSF1* control (Fig. 1*B*). The mRNA levels of *HSP82* increased moderately, and *CUP1* (encoding copper metallothionein) mRNA decreased rather than increased after heat shock. Thus, the F256S mutation clearly altered the heat shock response of a subset of genes.

The *SSA1* and *SSA4* genes contain the perfect-type HSE, whereas *HSP82* and *CUP1* are the gap-type HSE (10). It has been reported that Hsf1 containing a substitution of valine to alanine at amino acid 203 (V203A) in the DBD elevates transcription of *CUP1* at both normal and higher temperatures (32, 33). A substitution of arginine to serine at 206 (R206S) causes constitutive activation of both *CUP1* and a reporter gene containing the perfect-type HSE (34). These mutant proteins bind to the *CUP1* HSE with higher efficiency than the wild type protein (32–34). Although we did not know why the F256S mutation led to the temperature-sensitive growth phenotype, we reasoned that it might be possible to restore transcriptional activation by counterbalancing the F256S mutation with the V203A or R206S substitution. We combined F256S with the V203A or R206S substitution, but, unexpectedly, the combinations exacerbated, rather than rescued, the growth defect of *hsf1-F256S* cells.

The *HSF1* gene containing both V203A and F256S led to growth failure of yeast at any temperature (data not shown). The cells expressing the Hsf1-R206S/F256S protein were able to grow slowly at 28 °C, but not at 33 °C, a temperature at which *hsf1-F256S* cells were able to grow (Fig. 1*A*). Heat-induced accumulation of various mRNAs in *hsf1-R206S/F256S* cells is shown in Fig. 1*B*. In agreement with the previous report (34), the mRNA levels of *CUP1* were higher in *hsf1-R206S* cells than in the *HSF1* control. Remarkably, combination of R206S with the F256S mutation abrogated the *CUP1* activation. Furthermore, heat shock response of *SSA1*, *SSA4*, and *HSP82*, which was slightly up-regulated in *hsf1-R206S* cells, was almost completely inhibited in *hsf1-R206S/F256S* cells. It should be noted that R206S was originally identified as one of two mutations in *hsf1–301* cells; the other is a substitution of Phe-256 to tyrosine (F256Y) (34). In contrast to the F256S isolated in this work, the substitution to tyrosine exhibited no significant transcriptional effects. The F256Y mutant attenuated the stimulatory effects of R206S on a reporter gene containing the perfect-type HSE but had no effect on R206S up-regulation of *CUP1* expression (34). The residue Phe-256 anchors the fourth β-strand to the hydrophobic core in the DBD (31), and the substitution to serine, a small hydrophilic residue, may destabilize formation of the fourth β-strand more than the substitution to tyrosine does.

Genome-wide Transcriptional Changes in Cells Bearing the hsf1-R206S/F256S Mutation—Various temperature-sensitive *hsf1* mutations have been isolated and characterized. Deletion of the C-terminal half of Hsf1 results in growth defects of cells at higher temperatures and inefficient heat shock response of genes containing the gap-type HSE (such as *HSP82* and *CUP1*), but not genes containing the perfect-type HSE (such as *SSA4*) (10, 24, 35). In cells containing an *hsf1* mutation termed *mas3*, heat-induced transcription of several genes is almost completely inhibited but, notably, activation of *HSP82* is observed (36, 37). In contrast, Hsf1-R206S/F256S failed to mediate activation of various genes including *SSA4* and *HSP82*, indicating more serious impairment of the activator function than the previous mutations. We therefore took advantage of this mutant to conduct a genome-wide survey of Hsf1-regulated genes.

We compared the gene expression profiles in *HSF1* and *hsf1-R206S/F256S* cells under heat shock conditions using mini-array filters of yeast ORFs. Table I is a list of genes that displayed at least a 2.5-fold lower signal intensity in mutant cells compared with wild type cells, following heat shock at 39 °C for 15 min. Transcription of all 72 genes was heat-inducible, as judged from previous genome-wide expression analysis (38, 39). Approximately 36% of the genes (26 of 72 genes) contained the perfect-type HSE, whereas 7 genes possessed the gap-type HSE (Table I). The majority of the genes (54 of 72 genes) have been identified as putative Hsf1 targets by genome-wide Hsf1 binding analysis (20). In Fig. 2*A*, the expression levels of these genes were independently verified by reverse transcription-coupled PCR analysis. Heat-induced transcription of the genes was observed in *HSF1* wild type cells but was severely inhibited in mutant cells. We suggest that transcription of at least 59 genes that contain the HSE, and/or have been shown to bind Hsf1, is directly activated by Hsf1 upon heat shock (Table I). The Hsf1-regulated genes included not only HSP but also genes encoding proteins involved in detoxification, protein degradation, energy generation, carbohydrate metabolism, and cell wall organization.

However, the remaining 13 genes neither contain an HSE-like sequence nor belong to the known Hsf1-binding genes. The previous genome-wide expression analysis showed that the heat shock response of several of these genes decreased in cells bearing defects in the *MSN2* and *MSN4* genes, which encode a pair of transcription activators involved in general stress responses (38). Furthermore, Msn2 and Msn4 are known to activate *DDR2*, *PGM2*, and *CTT1* upon heat shock (37, 40–42). We analyzed transcription of these 13 genes in *msn2/msn4* null cells as well as in *hsf1-R206S/F256S* cells. As shown in Fig. 2*B*, the *hsf1-R206S/F256S* mutation, but not the *msn2/msn4* double mutations, abrogated the heat shock response of *TIP1*, *SIP4*, and *MHP1*. Thus, Hsf1 has an essential role in activation of these genes, although an HSE was not readily identified in their promoter regions. On the other hand, activation of the remaining genes was reduced moderately in *hsf1-R206S/F256S* cells and almost completely in *msn2/msn4* cells (Fig. 2*B*, data not shown). Therefore, transcription of these genes is activated principally by Msn2 and/or Msn4. It is possible that Hsf1 binds to unknown recognition sequence(s) in these genes and regulates transcription in concert with Msn2 and Msn4. Alternatively, Hsf1 may modulate activator functions of Msn2 and Msn4. Further analysis is necessary to examine these possibilities.

Transcriptional Regulation through a Novel Hsf1 Binding Sequence—A relatively large number of the Hsf1-regulated genes (26 of 59 genes) lacked an apparent HSE in the 5′-upstream regions. To ascertain the direct involvement of Hsf1 in activation of these genes, we tested binding of Hsf1 protein to these genes using an *in vitro* DNase I footprint analysis. We prepared probe DNA from the 5′-region of the Hsf1-regulated gene, *FSH1*, and Hsf1 polypeptide containing the DBD and coiled-coil domain from recombinant *E. coli*. As shown in Fig. 3*A*, *right panel*, a region from −166 to −121 (relative to the translation initiation site) of the lower strand was protected against DNase I digestion in the presence of Hsf1. On the upper strand, the footprint was not apparent, but hypersensitive sites were observed at −114, −119, and −163, which flanked the protected region of the lower strand (*left panel*). As summarized in Fig. 3*D*, this region contained three nTTCn direct repeats that were interrupted by 5-bp blocks (nTTCn(5 bp)-nTTCn(5 bp)nTTCn). The same sequence was found in the 5′-upstream regions in 11 of 25 genes that lacked other apparent HSEs (Fig. 3*D*). We then tested the binding of Hsf1 to

TABLE I
Genes whose heat-induced expression is reduced in hsf1-R206S/F256S cells

ORF	Gene	Description and product[a]	HSE[b]	Hsf1-binding[c]
Chaperone				
YOR020C	*HSP10*	Chaperonin, groES homolog	Perfect	Yes
YFL014W	*HSP12*	Heat shock protein 12	Step	Yes
YBR072W	*HSP26*	Heat shock protein 26	Perfect	Yes
YCR021C	*HSP30*	Heat shock protein 30	Unknown	Yes
YDR171W	*HSP42*	Heat shock protein 42	Perfect	Yes
YDR258C	*HSP78*	Heat shock protein 78	Perfect	Yes
YPL240C	*HSP82*	Heat shock protein 82, mammalian HSP90 homolog	Gap	Yes
YLL026W	*HSP104*	Heat shock protein 104	Perfect	Yes
YAL005C	*SSA1*	HSP70 family	Perfect	Yes
YLL024C	*SSA2*	HSP70 family	Perfect	Yes
YBL075C	*SSA3*	HSP70 family	Step	Yes
YER103W	*SSA4*	HSP70 family	Perfect	Yes
YPL106C	*SSE1*	HSP70 family	Perfect	Yes
YBR169C	*SSE2*	HSP70 family	Unknown	Yes
YJL034W	*KAR2*	HSP70 family, mammalian BiP (GPR78) homolog	Perfect	Yes
YJR045C	*SSC1*	Mitochondrial HSP70-related protein	Step	Yes
YNL007C	*SIS1*	HSP40 family chaperone	Perfect	Yes
YNL064C	*YDJ1*	Yeast dnaJ homolog	Step	Yes
YFL016C	*MDJ1*	Mitochondrial dnaJ homolog	Step	Yes
YBR101C	*FES1*	HSP70 nucleotide exchange factor	Perfect	Yes
YDR214W	*AHA1*	HSP90 system co-chaperone	Perfect	Yes
YOR027W	*STI1*	Heat shock protein	Perfect	Yes
YML130C	*ERO1*	Required for oxidative protein folding in the ER	Step	Yes
YDR155C	*CPH1*	Cyclophilin 40, peptidyl-prolyl cis-trans isomerase	Step	Yes
YLR216C	*CPR6*	Cyclophilin 40, peptidyl-prolyl cis-trans isomerase	Gap	Yes
Detoxification and protein degradation				
YHR053C	*CUP1-1*	Copper binding metallothionein	Gap	Yes
YHR055C	*CUP1-2*	Copper binding metallothionein	Gap	Yes
YLR109W	*AHP1*	Thioredoxin peroxidase	Unknown	Yes
YBR082C	*UBC4*	Ubiquitin conjugating enzyme e2	Step	Yes
Energy generation				
YCR012W	*PGK1*	3-Phosphoglycerate kinase	Perfect	Yes
YHR174W	*ENO2*	Enolase	Unknown	Yes
YKL141W	*SDH3*	Succinate dehydrogenase cytochrome *b*	Perfect	No
YML054C	*CYB2*	L-(+)-lactate-cytochrome *c* oxidoreductase	Perfect	No
YOR344C	*TYE7*	bHLH/leucine-zipper protein	Perfect	Yes
Carbohydrate metabolism				
YML100W	*TSL1*	Trehalose-6-phosphate synthase/phosphatase	Perfect	Yes
YKL035W	*UGP1*	Uridinephosphoglucose pyrophosphorylase	Gap	Yes
YHR104W	*GRE3*	Aldose reductase	Unknown	Yes
Cell wall integrity				
YKL096W	*CWP1*	Cell wall mannoprotein	Unknown	Yes
YER150W	*SPI1*	Stationary phase-induced cell wall protein	Unknown	Yes
YMR251W-A	*HOR7*	HyperOsmolarity-responsive protein	Gap	Yes
YNL160W	*YGP1*	Secreted glycoprotein	Perfect	No
YOL109W	*ZEO1*	ZEOcin resistance	Step	Yes
Unclassified				
YCL050C	*APA1*	Diadenosine tetraphosphate phosphorylase	Perfect	Yes
YDR313C	*PIB1*	Phosphatidylinositol(3)-phosphate-binding protein	Unknown	Yes
YHR049W	*FSH1*	Family of serine hydrolases	Step	Yes
YGL037C	*PNC1*	Nicotinamidase/pyrazinamidase	Unknown	Yes
YDR151C	*CTH1*	Putative transcription factor	Step	Yes
YGR211W	*ZPR1*	Zinc finger protein	Perfect	Yes
YGR141W	*VPS62*	Vacuolar protein sorting	Unknown	Yes
YGR142W	*BTN2*	Expression is elevated in a btn1 minus yeast strain	Perfect	Yes
YOR007C	*SGT2*	Small glutamine-rich TPR-containing protein	Step	Yes
YPR028W	*YOP1*	Ypt-interacting protein	Perfect	No
YBR025C		Unknown	Perfect	No
YGR250C		Unknown	Unknown	Yes
YJL144W		Unknown	Unknown	Yes
YLL023C		Unknown	Unknown	Yes
YLR064W		Unknown	Gap	Yes
YLR327C		Unknown	Unknown	Yes
YOL032W		Unknown	Perfect	Yes
Genes that have not been recognized as Hsf1-regulated genes[d]				
msn2/4-independent genes				
YBR067C	*TIP1*	Cell wall mannoprotein	Unknown	No
YJL089W	*SIP4*	Involved in Snf1-regulated transcriptional activation	Unknown	No
YJL042W	*MHP1*	Microtubule-associated protein	Unknown	No
msn2/4-dependent genes				
YFR053C	*HXK1*	Hexokinase I	Unknown	No
YGR088W	*CTT1*	Catalase T	Unknown	No
YHR096C	*HXT5*	Hexose transporter	Unknown	No
YMR105C	*PGM2*	Phosphoglucomutase	Unknown	No

TABLE I—*continued*

ORF	Gene	Description and product[a]	HSE[b]	Hsf1-binding[c]
YMR169C	*ALD3*	Aldehyde dehydrogenase	Unknown	No
YMR170C	*ALD2*	Aldehyde dehydrogenase	Unknown	No
YOL053C-A	*DDR2*	DNA damage response	Unknown	No
YOR161C	*PNS1*	pH nine sensitive	Unknown	No
YER067W		Unknown	Unknown	No
YHR087W		Unknown	Unknown	No

[a] Derived from the *Saccharomyces* Genome Database and/or the Comprehensive Yeast Genome Database (Munich Information Center for Protein Sequences).
[b] See "Experimental Procedures."
[c] Data from Ref. 20.
[d] 13 genes that neither contain the HSE nor belong to the Hsf1-bound genes are classified based on the effect of *msn2/msn4* null mutations on the heat shock response (Fig. 2*B*, data not shown).

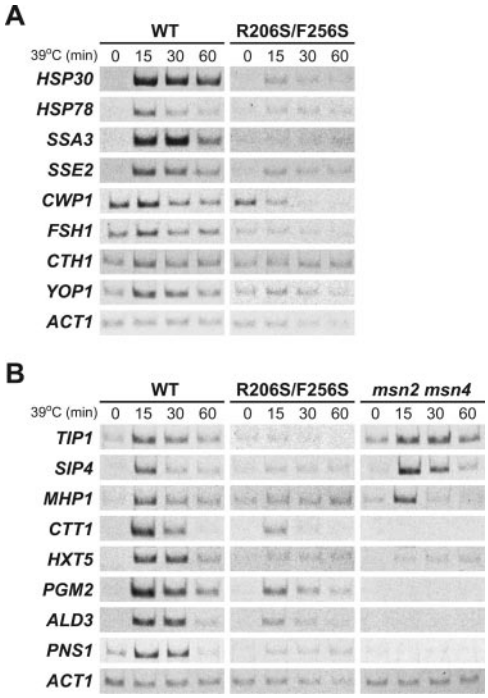

FIG. 2. **mRNA levels of heat shock-regulated genes in *hsf1-R206S/F256S* cells.** Total RNA prepared from wild type (*WT*) and *hsf1-R206S/F256S* (*R206S/F256S*) cells was analyzed as described for Fig. 1*B*. *A*, expression of genes that contain the HSE and/or belong to the known Hsf1-binding genes is shown. *B*, expression of genes that neither contain the HSE nor belong to the known Hsf1-binding genes is shown. Total RNA prepared from *msn2/msn4* null cells was analyzed in parallel.

several of these genes and found protection of the regions encompassing the nTTCn repeats of *SSC1* and *CTH1* and the inverted nGAAn repeats of *SSA3* (Fig. 3, *B* and *C*, data not shown).

To verify that three nTTCn repeats of the *FSH1* promoter are involved in heat-induced transcription, we constructed an *FSH1-lacZ* reporter gene in which the nTTCn units were altered (Fig. 4*A*). The β-galactosidase activity of cells carrying the wild type reporter increased 1.5-fold upon heat shock (*FSH1*wt-*lacZ*). Alteration of the central TTC to an unrelated sequence (AGA) abrogated the increase of β-galactosidase activity (*FSH1*m1-*lacZ*). When the spacing between the nTTCn units was elongated to 6 bp by inserting nucleotides, the heat shock response of the reporter was inhibited (*FSH1*m2-*lacZ*). Therefore, the three repeating units separated by 5-bp blocks compose an element that is responsible for heat-induced transcription. At the normal growth temperature, β-galactosidase activities of *FSH1*m1-*lacZ* and *FSH1*m2-*lacZ* were significantly lower than the *FSH1*wt-*lacZ* control. This result is consistent with the observation that Hsf1 is also involved in basal levels of transcription of heat-inducible genes (18, 19). We then constructed cells containing the m1 and m2 mutations in the chromosomal *FSH1* gene. As shown in Fig. 4*B*, analysis of the *FSH1* mRNA levels showed again an essential role of the nTTCn repeat sequence in basal and heat-induced transcription.

To further evaluate the function of the nTTCn units as an HSE, an oligonucleotide encompassing the three repeating units of the *SSC1* promoter was inserted upstream of the *CYC1* promoter in a *CYC1-lacZ* reporter (Fig. 4*C*). Insertion of the oligonucleotide increased the β-galactosidase activity from the reporter under non-heat shock conditions and transcription increased an additional 1.5-fold under heat shock conditions (*SSC1*wt/*CYC1-lacZ*). The 5′-nGAAn-3′ units, even when placed in the opposite orientation, mediated basal as well as heat-induced transcription (*SSC1*rv/*CYC1-lacZ*). However, expression of the reporters was significantly inhibited when the first TTC was altered to an unrelated sequence (TAG) or an inverted sequence (GAA) or when the spacing between the units was changed to 4 and 5 bp, 6 and 5 bp, or 4 and 4 bp (*SSC1*m1 to *SSC1*m5/*CYC1-lacZ*).

We tested the ability of the mutated oligonucleotides to bind Hsf1 using a gel retardation competition assay. As shown in Fig. 4*D*, *lane 2,* the labeled *SSC1*wt oligonucleotide migrated more slowly in the presence of the Hsf1 polypeptide. The formation of the DNA·Hsf1 complex was inhibited by addition of increasing amounts of unlabeled *SSC1*wt fragment (*lanes 3* and *4*) but was not significantly affected by fragments containing the mutated sequences (*lanes 5–14*). *In vitro*, *Saccharomyces* Hsf1 and *Drosophila* HSF are able to bind to a sequence consisting of two nTTCn tandem units with the 5-bp spacing but with a binding affinity significantly lower relative to the perfect-type HSE (8, 43, 44). *In vivo*, various derivatives of two tandem units were not able to mediate a heat shock response (Fig. 4, *A–C*). Taken together, we conclude that the sequence composed of three nTTCn repeats with the 5-bp spacing functions as an HSE in yeast. Because the repeats are separated like "stepping stones," we have named this sequence the "step-type" HSE.

Binding of Hsf1 to the Step-type HSE—The interactions of HSF and HSE are highly cooperative, and some deviations from the consensus nGAAn sequence are tolerated in functional HSEs (8, 10, 19). Structural analysis of co-crystals of the HSF DBD complexed with HSE DNA has shown that the DBD binds to the nGAAn sequence and interacts with neighboring DBDs. These data suggest an involvement of the DBD in cooperative binding of adjacent HSF monomers to the contiguous inverted repeats of the HSE (45). It is conceivable that two DBDs that do not make specific contacts with bases are placed over the two 5-bp gaps of the step-type HSE to stabilize interactions by filling the spaces between the nTTCn-bound DBDs. In this case, five Hsf1 monomers, which form two Hsf1 trimers, should bind to the sequence. Alternatively, three nTTCn units are recognized by each DBD of one Hsf1 trimer because the

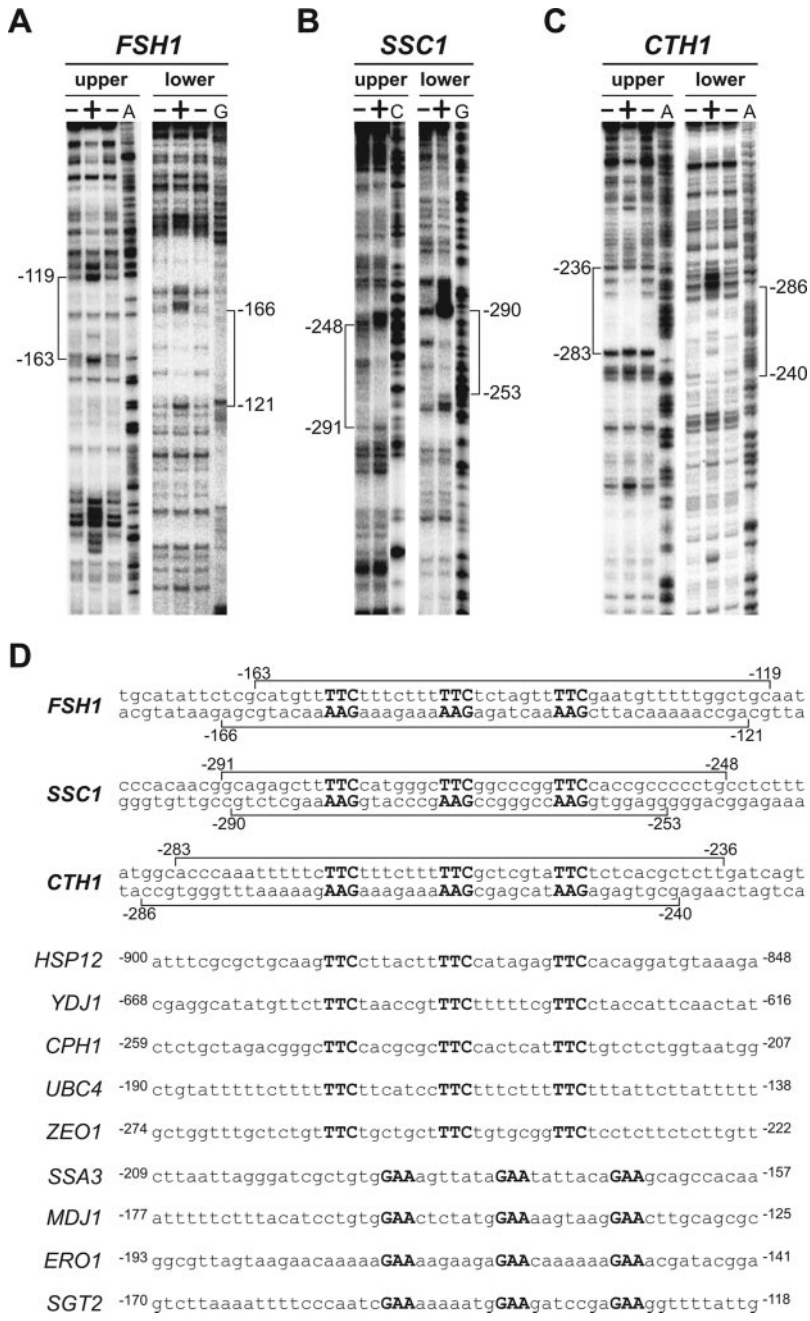

FIG. 3. **Binding of Hsf1 to the 5′-regions of various Hsf1-regulated genes.** The upper and lower strands of *FSH1* (*A*), *SSC1* (*B*), and *CTH1* (*C*) fragments were 5′-end-labeled with ^{32}P. The probe fragments (4 ng) were incubated without (− *lanes*) or with (+ *lanes*) 8 ng of recombinant polypeptide containing the N-terminal 583 amino acids of Hsf1. After treatment with DNase I, samples were subjected to polyacrylamide-urea gel electrophoresis and phosphorimaging. The *right lane* of each panel (marked *A*, *C*, or *G*) is a sequencing ladder. *D*, regions protected against DNase I digestion and nTTCn repeat sequences found in various genes are shown. Protected regions are indicated by *brackets*. *Numbers* show nucleotide positions relative to the translation initiation sites. *Bold uppercase letters* show the TTC repeating sequence.

linker region connecting the DBD and coiled-coil domain is thought to be relatively flexible (46).

To distinguish these two possibilities, we compared the mobility of the DNA·Hsf1 complex formed on the *SSC1*wt oligonucleotide with standards of one and two trimers formed on three and six inverted repeats of the nGAAn unit, respectively (7). As shown in Fig. 5, the step-type HSE·Hsf1 complex exhibited the same mobility as the complex formed on the three-repeat probe, suggesting that one Hsf1 trimer binds to the step-type HSE. The binding of Hsf1 to the probe of six units was cooperative, and the affinity of the binding to the step-type HSE was roughly equal to that of the three-repeat perfect-type HSE.

DISCUSSION

In yeast cells containing two substitution mutations in *HSF1*, R206S and F256S, the level of heat-induced transcription of 72 genes was lowered at least 2.5-fold compared with the wild type control as judged by mini-array filter analysis. We suggest that Hsf1 directly induces the heat shock response of at least 59 genes that contain the perfect- or gap-type HSE and/or have been assigned as Hsf1 targets by the previous genome-wide Hsf1 binding analysis (20). We have further analyzed the 5′-flanking regions of genes lacking the known types of HSE and identified a novel HSE consisting of three nTTCn direct repeats separated by 5-bp blocks. Some of the previously identified Hsf1 targets, such as *HSP60*, *HSC82*, *UBI4*, *EDC2*, and *RPN4* (37, 42, 47–49), were not recognized as Hsf1-regulated genes in the present work. The number of Hsf1-regulated genes identified was smaller than the number of Hsf1-bound genes, the latter being ~165 (20). Although the *hsf1-R206S/F256S* mutation has a severe inhibitory effect on the heat shock response of many genes, the mutant protein may still contain some residual activity allowing activation of the remaining Hsf1-regulated genes. It is also possible that the binding of Hsf1 does not cause heat-inducible transcription of the genes and that transcription of some Hsf1-bound genes is activated upon stresses other than heat shock (20, 49, 50).

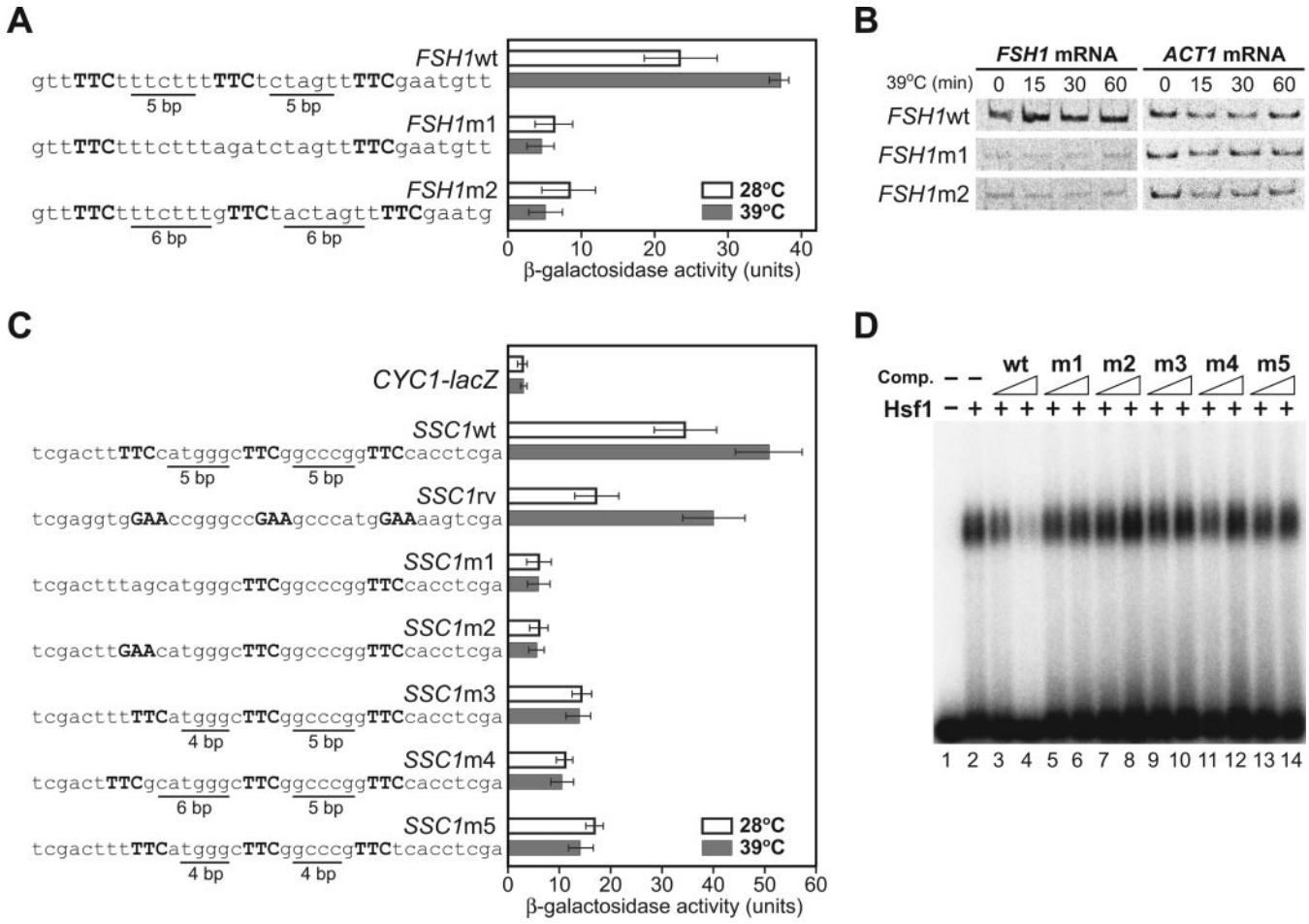

FIG. 4. **Heat shock response mediated by nTTCn repeating sequence and Hsf1.** *A*, wild type *HSF1* cells carrying *FSH1*wt-*lacZ*, *FSH1*m1-*lacZ*, or *FSH1*m2-*lacZ* reporter were grown at 28 (*open bar*) or 39 °C (*shaded bar*) for 1.5 h. The β-galactosidase activity is expressed as the mean ± S.D. of three independent experiments. Nucleotide sequences from −160 to −127 of *FSH1*wt, *FSH1*m1, and *FSH1*m2 are shown to the *left*. The TTC units are indicated by *bold uppercase letters*. *B*, *HSF1* cells containing wild type *FSH1* (*FSH1*wt) and its isogenic strains with a mutation in the promoter (*FSH1*m1 and *FSH1*m2) were grown and total RNA was prepared as described for Fig. 1*B*. The relative levels of *FSH1* and *ACT1* mRNAs were analyzed by reverse transcription-coupled-PCR method. *C*, the *SSC1*/*CYC1-lacZ* reporters containing various oligonucleotides were introduced into *HSF1* cells and β-galactosidase activity was determined as above. Sequences of oligonucleotides are shown to the *left*. The TTC (and GAA) units are indicated by *bold uppercase letters*. *D*, ability of the mutated oligonucleotides to bind Hsf1 was analyzed by gel retardation competition analysis. The end-labeled 38-bp fragment of *SSC1*wt (0.2 ng) was incubated without (*lane 1*) or with (*lanes 2–14*) 3 ng of Hsf1 polypeptide and subjected to agarose gel electrophoresis. Unlabeled oligonucleotide competitors (*Comp.*) were added to *lanes 3*, *5*, *7*, *9*, *11*, and *13* (6 ng of competitor) and *lanes 4*, *6*, *8*, *10*, *12*, and *14* (20 ng of competitor). Competitors were *SSC1*wt (*lanes 3* and *4*), *SSC1*m1 (*lanes 5* and *6*), *SSC1*m2 (*lanes 7* and *8*), *SSC1*m3 (*lanes 9* and *10*), *SSC1*m4 (*lanes 11* and *12*), and *SSC1*m5 (*lanes 13* and *14*).

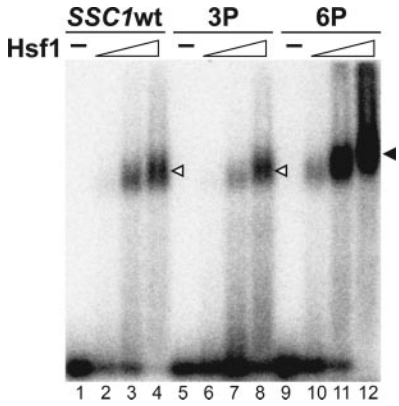

FIG. 5. **Formation of the DNA·Hsf1 complex on the step-type HSE.** The end-labeled 38-bp fragment (0.8 ng) was incubated without (*lanes 1*, *5*, and *9*) Hsf1 polypeptide or with 3 ng (*lanes 2*, *6*, and *10*), 10 ng (*lanes 3*, *7*, and *11*), or 30 ng (*lanes 4*, *8*, and *12*) of Hsf1 polypeptide and subjected to agarose gel electrophoresis. Probe DNA fragments were *SSC1*wt oligonucleotide (*lanes 1–4*), 3P (*lanes 5–8*, fragment containing perfect-type HSE), and 6P (*lanes 9–12*, fragment containing two copies of perfect-type HSE). *Open* and *closed* arrowheads indicate positions of one and two Hsf1 trimers, respectively.

The present work has identified new classes of Hsf1-regulated genes in addition to the known set of genes encoding proteins that function in protein folding, protein degradation, and detoxification. Thus, we found that Hsf1 activates transcription of genes involved in cell wall construction, whose heat shock response is known to be induced by Rlm1, a transcription factor controlled by the signal transduction pathway of the cell integrity mitogen-activated protein kinase (51). In addition, Hsf1 may regulate the energy generation process through enzymes in the process and through a Tye7 transcriptional activator of glycolytic genes (52). The other class of Hsf1-regulated genes encoded Ugp1 and Tsl1, which are enzymes in the metabolic pathway for synthesis of trehalose, a storage sugar that accumulates upon heat shock and during starvation (53). Interestingly, Msn2 and Msn4 activate transcription of other trehalose metabolism genes (*TPS1*, *TPS2*, and *TPS3*) and also of the Hsf1-regulated genes (*HSP26* and *HSP104*) under various stress conditions, including heat shock (53, 54). The heat shock response of several genes, which is induced principally by Msn2 and Msn4, was suppressed in *hsf1-R206S/F256S* cells (Fig. 2*B*). Taken together, we suggest that a group of transcriptional activators such as Hsf1, Rlm1, Tye7, Msn2, and Msn4

constitute a network with shared target genes that enable cells to survive in a high temperature environment.

For 20 years, the known HSEs have consisted of contiguous inverted repeats of nGAAn. Based on several lines of evidence, we have demonstrated that a sequence composed of three direct repeats of nTTCn (or nGAAn) separated by 5 bp constitutes a functional HSE, which we have called the step-type HSE. First, the sequence is recognized by the Hsf1 polypeptide, as judged by DNase I footprint and gel retardation analyses. Second, the sequence mediates heat-induced transcription of artificial reporter genes. Third, there is a close correlation between the Hsf1 binding and transcriptional activation abilities of the repeating nTTCn units, because both abilities are concurrently lost when the number and spacing of the units are altered. Additionally, a search to locate the nTTCn(5 bp)nTTCn(5 bp)nTTCn sequence within 1000 bp of the 5′-untranslated regions of yeast genes using the Pattern Matching program (Saccharomyces Genome Database) found that 235 of 6055 genes (3.9%) contain the sequence. The notable enrichment of the sequence in the Hsf1-regulated genes (20%, 12 of 59 genes), relative to the entire genome, supports the functional role as the HSE. It was previously reported that *MDJ1* and *YDJ1* (containing the step-type HSE) have a non-conventional HSE comprising nTTCn(11 bp)nGAAn(5 bp)nGAAn (47). We suggest that interaction with the two sequences leads to cooperative binding of the Hsf1 trimers. The remaining 14 genes, whose heat-induced transcription is regulated by Hsf1, possess none of the perfect-, gap-, or step-type HSE. Because the linker region of Hsf1 is flexible (46), it is reasonably assumed that Hsf1 is capable of interacting with more divergent sequences with different spacing and orientation of the nGAAn unit.

One Hsf1 trimer binds to the step-type HSE, and the spacing between the nTTCn units is important for recognition. The 5-bp spacing causes positioning of the nTTCn units on one surface of the DNA double helix. Although a sequence containing two tandem nTTCn units binds eukaryotic HSFs (8, 43, 44), the presence of the third unit with the 5-bp spacing is essential for efficient recognition and for heat-induced transcription by Hsf1. Two DBDs may bind asymmetrically to the two neighboring tandem nTTCn units (8, 43–45), although it remains to be resolved how the trimeric Hsf1 binds simultaneously to the three nTTCn units. Interactions with direct or inverted recognition sequences are well characterized in transcription factors that form dimers. For example, the orientation and spacing of the recognition sequences are critical determinants for nuclear hormone receptor dimers to bind to a response element in mammalian cells (55). In yeast, Gal4, Put3, and Ppr1, which are the transcriptional activators of the galactose, proline, and pyrimidine pathways, respectively, form homodimers and bind to inverted repeats of the triplet 5′-CGG-3′ with different spacings. In this case, the linker region and the beginning of the dimerization domain direct the protein to its preferred spacing (56). It is conceivable that the binding of trimeric Hsf1 to three stepping units is similarly determined by the linker region and coiled-coil domain. Deletion of the Hsf1 linker has been shown to affect the viability of yeast and the affinity of trimeric Hsf1 binding to the HSE *in vitro* (46).

In addition to the function as a stress-responsive transcriptional activator, analysis of an *hsf1* knock-out mouse revealed that mammalian HSF1 has roles in extra-embryonic development, postnatal growth, protection during inflammatory responses, female fertility, and cardiac redox homeostasis (57–59). *Drosophila* HSF is required for oogenesis and early larval development (60), and *Caenorhabditis elegans* HSF-1 is involved in aging (61). The survey of the yeast Hsf1-regulated genes shown here has uncovered various unexpected targets that may provide important clues for understanding how HSFs of multicellular organisms regulate diverse cellular processes. We have also discovered a novel Hsf1 recognition sequence. It will be of interest to investigate whether the different types of HSEs are implicated in differential gene- and stress-specific transcription by eukaryotic HSFs.

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