Mutated Yeast Heat Shock Transcription Factor Exhibits Elevated Basal Transcriptional Activation and Confers Metal Resistance*

(Rceived for publication, June 1, 1995, and in revised form, August 4, 1995)

Andrew K. Sewell†, Fumihiko Yokoyan‡, Wei Yu†, Terumi Miyagawa‡, Tetsuo Murayama‡, and Dennis R. Winge‡†

From the †Departments of Medicine and Biochemistry, University of Utah Health Sciences Center, Salt Lake City, Utah 84132 and the ‡Department of Biology, Faculty of Science, Ehime University, Matsuyama 790, Japan

Cadmium-resistant Saccharomyces cerevisiae strain 301N exhibits high basal as well as cadmium-induced expression of the CUP1 metallothionein gene. Since regulation of CUP1 is usually restricted to copper ions, our goal was to identify the factor responsible for the high metallothionein levels in strain 301N. The gene responsible for the observed phenotype is a spontaneously mutated heat shock transcription factor gene (HSF1). A double, semidominant HSF1 mutant with substitutions at codons 206 and 256 within the DNA-binding domain of the heat shock factor (HSF) confers two phenotypes. The first phenotype is elevated transcriptional activity of the HSF mutant (HSF301), which results in constitutive thermotolerance. A second HSF301 phenotype is enhanced binding affinity for the heat shock element (HSE) within the CUP1 5′-sequences, resulting in high basal transcription of metallothionein. The CUP1 HSE is a minimal heat shock element containing only two perfectly spaced inverted repeats of the basic nGAAn block. Cells containing HSF301 are resistant to cadmium salts. The single R206S mutation is responsible for the high affinity binding to the CUP1 HSE. In addition, the R206S HSF substitution exhibits constitutive transcriptional activation from a consensus HSE (HSE2). The F256Y substitution in HSF attenuates the effects of R206S on the consensus HSE2, but not on the CUP1 HSE.

All cells are capable of coping with changes in their environment, such as exposure to elevated temperatures, toxins, and oxidants. In response to certain stress conditions, activation of stress gene expression occurs, resulting in an elevated synthesis of stress proteins, commonly called heat shock proteins (hsp)3 (1, 2). That these hsp genes are induced by a variety of stress conditions implies that they have broadly protective functions.

The induction of heat shock protein(s) occurs at the level of transcription (3–5). Genes encoding the various hsp molecules contain a conserved promoter element, designated a heat shock element (HSE) (1, 6). The induction of hsp70 in animal cells by heat or metal ions requires only the HSE in the promoter (7, 8). HSEs contain multiple 5-bp inverted repeats of the sequence nGAAn (9–12). The number of 5-bp boxes may range from three to six (9–11). A perfect consensus array of three boxes would be the sequence 5′-nGAAnTTCCnGAAn-3′. Not all HSEs have perfect inverted repeats, but it appears that they have at least two perfect nGAAn boxes (9–12). A compilation of 40 naturally occurring HSEs from different organisms revealed that seven contained only three nGAAn blocks, and in each case, these three nGAAn boxes were in combination with additional HSE units, permitting cooperative interactions (12).

Transcriptional activation of genes containing heat shock promoter elements is mediated by the heat shock factor (HSF). Saccharomyces cerevisiae has one HSF encoded by the HSF1 locus (13, 14). Yeast HSF is a trimeric protein reported to bind HSE sequences constitutively at low temperature (5, 15–17). Within the N-terminal region of the 633-residue yeast HSF polypeptide is a conserved sequence of 89 residues that is important in binding to the 5-bp HSE boxes (18, 19). It is likely that each subunit of trimeric HSF contacts a separate 5-bp box within a HSE, but the actual HSF-HSE complex appears to contain multiple HSE trimers (12, 17–19). Furthermore, most stress genes contain multiple HSEs, so additional interactions can exist between adjacent HSF-HSE complexes. The interaction of Drosophila HSF with HSEs is known to be highly cooperative (9, 20), although in yeast, cooperativity is not essential as a minimal HSE of three perfect nGAAn units is functional (21).

In yeast, the bound HSF-HSE complex is transcriptionally silent until stress activation (16, 22–25). HSF undergoes a conformational change upon stress activation, but the mechanism of stress-induced conformational dynamics is unclear (16, 22–25). In contrast, the activation process in animal cells initially involves oligomerization to the trimeric state, which already exists in yeast (1). Yeast HSF contains domains that function as constitutive transcriptional activation domains when fused to heterologous DNA-binding domains (22, 23).

Since these activation domains are not constitutive in HSF at low temperature, it appears that the normal mode of action of HSF is to hinder the effectiveness of these domains. This hindrance is relieved upon change to stress conditions (16). The regulatory domains of HSF can even repress the activity of a heterologous transcriptional activation domain fused in place of its own C-terminal activation domain (24).

Repression of the activation domain(s) appears to involve the DNA-binding domain, the trimerization domain, and the C-terminal conserved sequence. Deletion of the N-terminal 146 codons in HSF1 results in loss of low temperature repression (22). Constitutive activity is also observed in HSF1 mutations within the DNA-binding domain (residues 167–256), deletions within the oligomerization domain (residues 350–402), or deletions within a C-terminal conserved region (residues 535–551) (22–25). A mutation at codon 232 in the DNA-binding domain was shown to yield a 200-fold increase in activity at
26 °C (24).

In addition to the effects of HSF on expression of hsp genes, HSF is known to affect the expression of the gene CUP1, which encodes metallothionein (26–28). The heat shock transcription factor (HSF) is responsible for high basal expression of CUP1 in cells starved for glucose (28). The 5′-sequence of the CUP1 gene contains a minimal heat shock promoter element (HSE) that mediates both a limited heat shock induction of CUP1 expression with wild-type HSF and CUP1 transcription observed in glucoselimitation (26, 28). In addition, a point mutation within HSF1 can suppress the copper-sensitive phenotype of an ACE1 deletion strain by enhancing basal transcription of CUP1 in glucose-grown cultures (26, 27).

CUP1 expression is normally regulated by Cu(II) ions through the ACE1 transcription factor (29–31). Unlike animal cells, in which metallothionein biosynthesis is regulated by a variety of metal ions, the metalloregulation of yeast metallothionein is copper-specific (30). However, cadmium-mediated CUP1 expression was observed in one cadmium-resistant strain of S. cerevisiae (32). This strain, designated 301N (32), arising from spontaneous mutation, exhibited both high basal CUP1 transcription and cadmium-induced CUP1 expression (33–35). The observed cadmium resistance was a consequence of C(M) buffering by metallothionein (33).

To elucidate the basis for the cadmium metalloregulation in strain 301N, we set out to identify the factor responsible for CUP1 expression in this yeast. In this study, we demonstrate that the regulatory factor conferring constitutive expression of CUP1 and cadmium metalloregulation in strain 301N is HSF. A double mutation within HSF1 in sequences encoding the DNA-binding domain leads to elevated basal transcription of CUP1 and constitutive thermotolerance. One dramatic result is that a single mutation at codon 206 (R206S) results in constitutive transcriptional activity of HSF on the consensus HSE-containing promoter, but not on the CUP1 HSE. It is noteworthy that the mutation at codon 256 suppresses the transcriptional activity of the R206S HSF mutant on the consensus HSE, but is without effect on transcriptional activation of the CUP1 HSE. This work clearly demonstrates differential activity of HSF on various HSE sequences.

MATERIALS AND METHODS

Strains—Strains DTY22 (MATα, ura3-52, his3, LEU2::YipCL, CUP1) and DTY23 (MATα, his3, ura3-52, acl-1, LEU2::YipCL, CUP1) were gifts from Dr. J. M. Thiele. Strains DTY22 and DTY23 contain a CUP1/αα2 fusion gene integrated at LEU2. Strain PS145 (ade2-1, can1-100, his3-11,15, leu2-3,112, trp1-1, ura3-1, his3A::LEU2, YCPGal1:HSF-URA3) (14) was a gift of Dr. C. H. M. Nelson. In all experiments using transformed PS145 cells, the YCPGal1:HSF-URA3 vector was shed by growth on medium containing 5-fluoroorotic acid. Strain 301N is MATa, ura3-52, CUP1. Strain CL7 (MATα, ura3, LEU2::YipCL) was isolated from segregants of tetrad spores of a cross between YI574 (MATα, leu2, ura3, his4, lys7) and TE22-2AE (MATα, ura3, LEU2::YipCL).

HSF Cloning—DNA partially digested with Sau3A1 from strain 301N was cloned into YCP50 (36) at the BamHI site, and over 50,000 total Escherichia coli transformants were recovered. Of the 2.4 × 106 yeast transformants, 29 colonies exhibiting a blue hue on 5-bromo-4-chloro-3-indolyl β-o-galactosidase plates were selected as candidates for enhanced constitutive expression of CUP1/ααZ.

Vectors—A 3.9-kb EcoRI fragment containing the entire HSF1 locus was cloned into YCPlac22, YEplac112, YEpplac33, and YEplac195 (37) to create a series of vectors containing either the wild-type or 301N mutant HSF1 on both 2µ-based high copy and centromere-based low copy vectors with either TRP1 or URA3 selection. TRP1-based vectors were used to transform PS145 cells, and URA3-based vectors were used to transform both DTY23 and DTY113 cells.

Mutagenesis—Mutagenesis was performed on a 3.9-kb EcoRI fragment containing wild-type HSF1 in vector pA1H1 (Promega) as described previously (38). Mutated sequences were removed as an NsiI/Stul fragment and cloned into the wild-type sequence. Sequencing of the 2-kb NsiI/Stul region confirmed that only the desired mutations were introduced.

Transformations—Yeast cells were transformed using the lithium acetate procedure (39). Standard yeast cultivating conditions were used. β-Galactosidase Assays—β-Galactosidase enzyme assays were performed to quantify lacZ expression. Assays were carried out on extracts from cells grown to an A600nm of 0.7–0.85 as described previously (40).

Thermotolerance—One-hundred microtiter cultures of growths grown to an A600nm of 0.7–0.75 at 23 °C were added to 10 ml of fresh culture medium at either 23 °C (control) or 52 °C. Heat shock survival was measured by the ability to form colonies after further dilution and plating on yeast extract/petitone/dextrose plates, which were incubated at 30°C.

Gal Shift Analysis—Extracts from cells from log-phase cultures grown at 23 °C were prepared by cell disruption with glass beads in 20 mM Tris-Cl, pH 8, 10 mM MgCl2, 1 mM EDTA, 5% glycerol, 0.1% β-mercaptoethanol, 0.3 mM KCl. Cells were vortexed for 5 min with 0.5 volume of 425–600 µM glass beads (Sigma). Extracts were diluted with buffer to a final protein concentration of 3 mg/ml. Aliquots of 4 µl of this protein were incubated for 20 min with 8 µl of reaction mixture (20 mM Tris-Cl, pH 8.0, 2 mM dithiobitol, 2 mM EDTA, 12.5% glycerol, 0.2% Nonidet P-40, 0.2 mg/ml bovine serum albumin containing the specified amount of poly(dI-dC) and the 32P-labeled, double-stranded oligonucleotide probe (14 pm) before being run on a 4% polyacrylamide gel in 1 × TBE (41). The CUP1 probe contained the sequence CTCAGGACCTATTAGAGCGAAAAGAG, and the HSE2 oligonucleotide probe contained the sequence CTAGAAGCCTTCAAGAGCGATTCCCCC. The underlined nucleotides represent the conserved nGAAn inverted repeats characteristic of HSE sequences. The boldface GAG in the CUP1 HSE is a third candidate 5-bp block. The sequence of the truncated CUP1 HSE probe was GCTGCGATTATTCTAGAGCGAAAAT. Two other CUP1 HSE variants were synthesized. One variant contained a NgAAn box in place of the nGAAn block. The second variant contained three NgAAn boxes in the sequence GCTGCAGCTTCTAGAGCGAAAAGAG. The competition gel shift assays involved adding both radiolabeled DNA probe and unlabeled DNA prior to addition of protein.

Protein Inductions—The identification of heat shock proteins in S. cerevisiae cells was carried out as described previously (42).

RESULTS

Cloning of HSF—The cadmium tolerance of S. cerevisiae strain 301N was previously shown to arise from enhanced basal expression of CUP1 (35). Mating of 301N with a yeast strain containing an integrated CUP1/ααZ fusion gene resulted in a diploid that maintained limited cadmium-induced lacZ expression, implying that this phenotype is partially dominant (26, 35). To determine the identity of the factor conferring the Cd2+ phenotype, a DNA library was constructed from 301N. Transformation of the DNA library into S. cerevisiae strain CL7 yielded 29 colonies exhibiting high constitutive expression of CUP1/ααZ as a reporter for CUP1 expression. Plasmids recovered from these colonies exhibiting the most intense blue coloration on 5-bromo-4-chloro-3-indolyl β-o-galactosidase plates revealed inserts of 5.7, 6, and 14 kb. Restriction mapping of these plasmids suggested that the inserts in the three plasmids were related.

The 5.7-kb insert was subcloned into the integrating plasmid, YIp5. This vector was linearized and transformed into strain CL7. The resulting strain was crossed with a DTY22 variant. Both haploid strains contained CUP1/ααZ fusion genes. Tetrad analysis revealed a 2:2 segregation of enhanced lacZ expression, implying that the 5.7-kb insert contains sequences capable of up-regulation of CUP1 expression.

The restriction map of each plasmid was unrelated to maps of CUP1 or ACE1, but was similar to that of yeast HSF1. DNA primers specific for S. cerevisiae HSF1 were successful in priming sequencing for each of the three plasmids. From sequence analysis of the entire HSF1 open reading frame, two mutations at codons 206 and 256 (R206S and F256Y) were found. These two codons occur within sequences encoding the DNA-binding domain of HSF. Two other nucleotide differences exist at
codons 522 and 831 relative to the HSF1 sequence reported by Wiederrecht et al. (13), but are identical to the HSF1 sequence reported by Sorger and Pelham (14).

Characterization of hsf1-301—The EcoRI fragments of both the wild-type and mutant HSF genes were cloned into YEp- and YCp-based vectors. The mutant HSF gene was designated as hsf1-301. To probe the effect of hsf1-301 on metal resistance, we elected to use a yeast strain carrying a disrupted chromosomal HSF1 locus (4, 14). HSF1 is an essential gene in yeast, so the strain with the disrupted chromosomal HSF1 was kept viable with a HSF1 gene carried on a URA3-containing vector (14). This strain, PS145, was transformed with HSF1 or hsf1-301 contained on TRP1-based plasmids. Selection on 5-fluoroorotic acid allowed for shedding of the URA3-containing HSF1 plasmid, leaving cells with only HSF1 genes on the TRP1 vectors. These Ura− Trp+ transformants were used in subsequent studies. Transformants with hsf1-301 as the only functional HSF gene were viable. Thus, HSF301 must be capable of the essential housekeeping functions of HSF. In addition, HSF301 was also able to induce expression of heat shock proteins after heat shock (data not shown).

The presence of episomal hsf1-301 in yeast containing wild-type CUP1 conferred resistance to cadmium salts (Fig. 1A). Cells harboring YCpHSF301 were markedly more cadmium-resistant than either control cells or cells harboring YCpHSF1 (Fig. 1A). This effect is observed in cells at 30 °C, a temperature at which the HSF-mediated stress response is limited. We were unable to determine whether hsf1-301 conferred copper tolerance in these cells as they were wild-type in ACE1, which specifically mediates copper-induced expression of CUP1. To test the effect of hsf1-301 on copper tolerance, the vectors were transformed into ace1-1 cells (DTY23) (Fig. 1B). ace1-1 cells are unable to couple CUP1 expression with the copper concentration. Cells harboring YCpHSF301 were more copper-tolerant than control cells or cells transformed with YCpHSF1. When the HSF genes were present on high copy YEp plasmids, wild-type HSF1 conferred limited tolerance, and as expected, hsf1-301 cells exhibited marked copper tolerance.

Effect of HSF301 on CUP1 Expression—The mechanism of HSF301-induced metal resistance is expected to involve activation of CUP1 transcription, although CUP1 contains a single HSE with only two perfectly spaced inverted repeats of the basic nGAAAn unit that defines a HSE (10, 28). Functional HSEs consist of three or more nGAAAn units, and promoters containing three nGAAAn inverted repeats tend to have additional HSEs (9–12).

To quantify the transcriptional effect of HSF, PS145 cells harboring the episomal HSF genes were transformed with a YEp vector containing the CUP1/lacZ fusion gene. Transformants harboring YCp-based hsf1-301, but not YCp-based HSF1, revealed high β-galactosidase levels at 23 °C, consistent with high constitutive expression of CUP1/lacZ (Fig. 2A). Cells were maintained at 23 °C to minimize heat activation of HSF. Thus, activity is a measure of constitutive transcriptional activity of HSF. The difference between YCpHSF and YCpHSF301 was over 100-fold. Wild-type HSF was able to transactivate CUP1 expression to an appreciable extent only when expressed on a high copy plasmid. The presence of hsf1-301 in PS145 cells mimics two key phenotypes of strain 301N, namely enhanced metal tolerance and high basal CUP1 expression.

Effect of HSF301 on General Stress Responsiveness—The elevated transcription activation activity of HSF301 raised the question of whether general stress responsiveness was elevated in cells harboring hsf1-301. Cells with episomal HSF genes were tested for their ability to withstand a transient 52 °C heat shock. Normally, cell survival after a transient 52 °C heat shock is enhanced if cells are incubated at 37 °C prior to exposure to the high temperature. The activation of HSF at 37 °C enables a greater percentage of cells to survive the 52 °C shock. In the absence of the 37 °C activation step, survival of a direct 52 °C shock is dependent on basal activity of HSF. After 4 min at 52 °C, over 60% of the cells harboring hsf1-301 survived the heat shock compared with <10% of the cells with HSF1. The presence of hsf1-301 must therefore yield higher basal expression of one or more genes that are critical for heat tolerance.

A second test of the effects of hsf1-301 on general stress responsiveness was to measure the activity of HSF301 on a consensus HSE sequence. PS145 cells containing either episomal HSF1 or hsf1-301 were transformed with a vector containing a synthetic consensus HSE sequence in place of the upstream activating sequence of a CYC1/lacZ fusion gene (4). The fusion gene contains a HSE consisting of four nGAAAn inverted repeats, the sequence of which is a consensus of HSEs found in

![Fig. 1. Metal resistance of cells harboring episomal HSF genes.](Image)

![Fig. 2. HSF-mediated expression of CUP1/lacZ (A) and HSE2/CYC1/lacZ (B) in PS145 cells transformed with different HSF plasmids.](Image)
a myriad of hsp genes (4). Both HSF1 and hsf1-301 were able to drive the expression of lacZ from the consensus HSE2 promoter, but hsf1-301 yielded 2-3-fold higher levels of expression (Fig. 2B). No lacZ expression was observed in cells containing a HSE12/CYC1/lacZ fusion gene with a mutated HSE sequence (HSE12) that matched the consensus in only six of eight positions (4, 17) (data not shown). Thus, both HSF and HSF301 transactivate through HSE sequences. Since yeast HSF is known to be constituтивy bound to the consensus HSE2 promoter (17), the enhanced basal transcription observed with HSF301 must arise from elevated transcriptional activation rather than an increased DNA binding avidity.

Metal ions are known to stimulate the general stress response pathway (1), and this effect may contribute to cadmium-induced CUP1 expression in strain 301N (32). Cd(II) stimulation of CUP1 was found to be strain-specific. No Cd(II) stimulation of CUP1/lacZ expression was observed in PS145 cells harboring either hsf1-301 or HSF1. In contrast, significant Cd(II) stimulation of CUP1/lacZ expression was observed in DTY23 cells harboring episomal hsf1-301 (Fig. 3) or in cells harboring HSF1 on a high copy plasmid (data not shown). Cells were incubated with CdSO$_4$ for 2 h prior to cell harvest. Since DTY23 cells lack a functional ACE1, the ACE1-dependent, copper-induced expression of CUP1 is precluded. Transformation of these cells with YEpHSF301 resulted in appreciable copper stimulation of CUP1/lacZ. Maximal metal-induced expression of CUP1 occurred at metal ion concentrations that are inhibitory to cell growth, so the effect may be a general stress response. It is unclear why the metal effect is temperature-dependent and shows a different temperature dependence for copper and cadmium ions (Fig. 3).

Mapping of HSF Mutations—As mentioned above, hsf1-301 contains two codon differences from the published yeast HSF1 DNA sequence. To limit the observed phenotype to just these two changes and to rule our possible differences in the unsequenced 5′- and 3′-sequences, a 2013-bp Nsi/Stul fragment containing both mutations was removed from hsf1-301 and swapped into the wild-type HSF1 gene. This chimera faithfully duplicated the phenotype of hsf1-301. Subsequently, the Nsi/Stul fragment of the wild-type HSF1 sequence was mutated to create the two individual mutations, R206S and F256Y. Individually, the R206S HSF mutant, but not the F256Y HSF mutant, conferred cadmium tolerance (Fig. 4). Two previous studies identified a V203A mutation in HSF1, which resulted in elevated CUP1 basal transcription (26, 27). We created the V203A mutation in HSF1 to permit direct comparison with R206S HSF. Cells containing V203A HSF were equally resistant compared with cells with R206S HSF (Fig. 4).

The individual HSF mutants were tested for their ability to transactivate the expression of CUP1/lacZ and HSE2/CYC1/lacZ fusion genes. Studies with PS145 cells containing CUP1/lacZ revealed high constitutive expression of lacZ at 23 °C conferred by HSF301 and both R206S and V203A HSF mutants (Fig. 5A). Whereas the HSF double mutant imparted slightly higher metal tolerance to cells compared with R206S HSF, there was no significant difference between HSF301 and R206S HSF in activating lacZ expression from the CUP1/lacZ fusion gene. Thus, the single Arg → Ser mutation at codon 206 is largely responsible for the phenotype of HSF301. The individual F256Y HSF mutant was without effect on lacZ expression from a CUP1/lacZ fusion gene compared with wild-type HSF (Fig. 5A).

In contrast, cells containing the HSE2/CYC1/lacZ fusion gene exhibited marked enhanced expression of lacZ with R206S HSF compared with HSF301 (Fig. 5B). This is unlike the results with CUP1/lacZ, in which the transcriptional activity of R206S HSF was equivalent to that of HSF301 (Fig. 5A). To confirm that the R206S results were not a result of a secondary mutation, PS145 cells were retransformed with HSE2/CYC1/lacZ and HSF genes. Quantitation of β-galactosidase levels revealed similarly high activity as in the original transformants. In contrast to R206S HSF, V203A HSF was without effect on HSE2/CYC1/lacZ expression.

Yeast transformants with the various individual mutant HSF genes and the HSE2/CYC1/lacZ fusion gene were tested for heat shock-induced lacZ expression to determine whether R206S HSF was fully active at 23 °C (Fig. 6). Whereas heat shock conditions resulted in induction of lacZ expression for wild-type HSF and F256Y HSF, in excess of 10-fold, cells containing R206S lacZ exhibited a ~2-fold heat shock response. It is also curious that V203A HSF responds so poorly to heat shock (Fig. 6).

Mechanism of HSF301 Activity—As mentioned above, the CUP1 promoter contains a minimal heat shock element consisting of two perfectly spaced nGAAAn inverted repeats and a third nGAGn block separated from the two perfect inverted repeats by a missing block (28). An oligonucleotide duplex of 26 bp encompassing the candidate CUP1 HSE was used in gel retardation assays with protein extracts from yeast cells with different HSF genes. A specific DNA–protein complex was seen in extracts of cells with hsf1-301, but not with the wild-type
gene (Fig. 7). To rule out the possibility that this complex consisted of a protein induced by HSF301 but not by the factor itself, gel retardation assays were performed in the presence of antiserum to yeast HSF (lane 6). A “supershift” was observed in incubations containing the HSF antiserum, whereas no change in the mobility of the HSF DNA complex occurred in incubation with preimmune serum (lane 7).

Gel retardation assay of extracts from cells carrying singly mutated HSF genes showed evidence of a HSF DNA complex with the CUP1 HSE sequence with the individual R206S HSF mutant, but not with the F256Y HSF mutant (Fig. 8A). Using a HSE2 oligonucleotide, both the wild-type and mutant HSF molecules formed DNA-protein complexes as judged by the gel retardation assay (Fig. 8B). Each individual mutant HSF protein formed a HSF-DNA complex. A reproducible difference existed in the extent of electrophoretic mobility of the HSF-DNA complex for the different HSF molecules. It appears that the actual HSF-DNA complex consists of multiple HSF molecules (12, 22), so differences in mobility may arise from variations in the oligomeric complexes that form or from differences in the individual monomers.

Since gel retardation studies were carried out with crude yeast extracts in which the actual HSF protein concentration was undefined, it was important to verify the conclusion that wild-type HSF did not bind with equal affinity to the CUP1 HSE oligonucleotide. The HSF antiserum did not work in Western analysis, so competition binding gel shift experiments were carried out to substantiate this result (Fig. 9). Radiolabeled HSE2 probe was shifted with extracts from cells expressing either wild-type HSF or HSF301. Using extracts containing HSF301, the addition of either unlabeled CUP1 HSE DNA or HSE2 DNA to the binding reaction prior to addition of protein resulted in a concentration-dependent competition in binding. The competition diminished the amount of radiolabeled probe in the retarded HSF DNA complex (Fig. 9A). In multiple experiments with HSF301, unlabeled HSE2 or the CUP1 HSE oligonucleotides were nearly equivalent in competition. In contrast, with extracts containing wild-type HSF, HSE2 was 5–10-fold more effective than CUP1 HSE DNA as a competitor as determined by densitometry (Fig. 9A). This is further evidence that HSF301 forms a more avid complex with the CUP1 HSE sequence than wild-type HSF.

Previous footprinting results on CUP1 suggested that the CUP1 HSE may consist of the sequence CTTC TAGAA G-CAAAAAGAG, with the underlined nucleotides being the conserved GAA inverted repeats (28). HSE sequences do not typically exhibit gapped nGAAn blocks, and the candidate gapped box in the CUP1 HSE is a nonconsensus nGAGn box (10, 12). To address whether the nGAGn block is a critical part of the CUP1 HSE, three oligonucleotides consisting of altered CUP1 HSE sequences were tested as competitors in a gel shift assay with a cell extract containing HSF301 protein and radiolabeled HSE2 DNA as probe. The first CUP1 HSE oligonucleotide variant contained the gapped GAG trinucleotide substituted by TTT. The gapped nTTTn box duplex failed to compete even at a concentration 100-fold greater than the concentration of CUP1 HSE DNA that exhibited 75% competition (HSE(M1) in
Fig. 9. Competition of wild-type HSF and HSF301 binding to the radiolabeled HSE2 DNA oligonucleotide by unlabeled oligonucleotides. Only the position of the HSF-DNA complex is shown in the competition studies, and densitometry was used for quantitation. In A, protein (20 μg) from clarified extracts of PS145 cells containing either YEpHSF or YEpHSF301 was incubated with the radiolabeled HSE2 oligonucleotide in the presence of increasing amounts of unlabeled DNA as specified. In B, the HSF301 extract was used with radiolabeled HSE2 DNA and increasing quantities as shown of unlabeled CUP1 HSE, two mutated CUP1 HSEs, or an oligonucleotide duplex containing the binding site for AMT1. The CUP1 HSE(M1) contained the gapped nTTTn box. The two consensus nGAAn boxes in the CUP1 HSE were changed to nGAGn boxes in the HSE(M2) duplex.

**DISCUSSION**

The cadmium tolerance observed in S. cerevisiae strain 301N was found to arise from a semidominant mutation within the DNA-binding domain of HSF. The dominant effect of HSF301 on Cd(II) tolerance arises from HSF301-induced transcription of CUP1-encoding metallothionein. HSF301 confers minimal metal tolerance in S. cerevisiae cells lacking a functional CUP1 (data not shown). Elevated metallothionein levels enable efficient Cd(II) sequestration as a cadmium-metallothionein complex.

A second phenotype of hsf1-301 cells is enhanced transcription activation activity. Wild-type HSF mediates both transient and sustained effects in relationship to stress (16). The elevated basal activity of HSF301 at low temperatures is observed with a consensus HSE cloned upstream of the CYC1/lacZ fusion gene that was originally used to demonstrate constitutive binding of HSF (17). In addition, high basal activity of HSF301 at low temperature is indicated by the thermotolerance of cells harboring hsf1-301. The mutations in hsf1-301 appear to result in high basal expression of one or more genes that are critical for thermal tolerance. As mentioned, a V203A substitution in HSF was reported previously to enhance CUP1 expression, but curiously not to give heat tolerance (26, 27).

Enhanced basal activity of HSF301 is not unique to the 301N mutations in HSF1. Other mutations or deletions within the DNA-binding domain of HSF, the trimerization domain, and the HSF conserved element CE2 result in loss of low temperature repression (23–25). Sequences within these three regions of HSF are important in maintaining HSF in a repressed state at 23 °C. Another mechanism for enhanced low temperature activity of HSF301 may be increased efficiency of assembly of the transcription complex (43). Certain HSF1 mutants are constitutively active (23–25). Other mutantshave intermediate effects on HSF deregulation in that transactivation is further enhanced by heat shock (23–25). The elevated activity of HSF301 is of intermediate effect as transcription of CUP1 is further enhanced by heat shock and metal ions.

The two distinct phenotypes observed with HSF301, namely enhanced low temperature transactivation of multiple genes containing HSE elements and enhanced CUP1 binding, arise from the R206S mutation. There are two dramatic results in
the experiments with HSE2/CYC1/αACZ. First, R206S HSF activates HSE2/CYC1/αACZ (but not CUP1/αACZ) expression constitutively. The clear implication is that the R206S HSF molecule is capable of discriminating between HSEs. The second novel result is that HSF301 has reduced transcription activation activity compared with R206S HSF on the HSE2/CYC1/αACZ fusion gene, yet equivalent activity on CUP1/αACZ expression. It appears that the second mutation in HSF301 at codon 256 (F256Y) suppresses the effects of the R206S mutation on HSE2, but not on the CUP1 HSE.

Based on the known structures of Kluyveromyces lactis and Drosophila HSFs (18, 44), Arg-206 is expected to be a moderately solvent-exposed residue situated near the start of the highly irregular second α-helix within the DNA-binding domain. Although the function of this helix in HSF/DNA interaction remains unresolved, the structure of HSF resembles a family of DNA-binding proteins with a helix-turn-helix structural motif (18, 44). Based on structural comparisons, HSF helix 3 is predicted to function as the recognition helix (18, 44). Residues corresponding to Arg-206 in CAP, Oct1, λ-repressor, and 434 Cro form hydrogen bonds to phosphates in the DNA backbone (44). A Glu → Lys mutation in this codon of the λ-repressor resulted in a 600-fold enhancement in DNA binding affinity, which was attributed to the formation of a new salt bridge (45).

The constitutive activity of R206S HSF was attenuated in the context of the double hsf1-301 mutation. The F256Y substitution suppresses the activity of the R206S mutation on HSE2. Phenylalanine is found at codons corresponding to codon 256 in all known HSF molecules (44). The distance of Arg-206 and Phe-256 α-carbons in non-DNA-bound HSF is 12 Å, and side chains are separated by 8.9 Å. The presence of a buried Tyr-256 may alter the HSF tertiary fold to compromise the effects of the R206S mutation. Final understanding of the relationship of Arg-206 and Phe-256 in the HSF structure will have to await elucidation of the HSF-DNA costructure.

HSF301 exhibits enhanced binding to CUP1 as reported previously for the V203A HSF mutant (26, 27). Band shift analysis with the CUP1 HSE oligonucleotide revealed formation of a specific complex with HSF301, but not with wild-type HSF. The absence of a complex formation with wild-type HSF under normal conditions implies that wild-type HSF is not constitutively bound to the CUP1 HSE. A recent report demonstrated that wild-type HSF forms a complex with the CUP1 HSE sequence (28). To observe a complex, the investigators used an unusually high concentration (30 g) of partially purified HSF. In the gel shift experiments reported here, we used 12–20 μg of total protein from a crude yeast extract, and under these conditions, wild-type HSF does not bind the CUP1 HSE. In addition, the CUP1 HSE is an inferior competing oligonucleotide to the HSE2 oligonucleotide containing a HSE sequence with four nGAAn inverted sequence repeats.

Most hsp genes contain multiple HSE sequences, with each HSE containing at least three conserved nGAAn inverted repeats (9–12). The number of nGAAn repeats usually ranges from three to six (9–12). The presence of multiple HSEs within a promoter sequence permits cooperative binding of HSF (9, 12, 17, 20). A perfect consensus array of three boxes would be the sequence 5′-nGAAnTTcnGAnA−3′ (9–11). Two types of variations of the consensus sequence exist in naturally occurring HSEs. First, limited sequence variation is permitted, and second, interrupted periodic arrangement of nGAAn blocks is tolerated (9–11). For example, in Drosophila, three nGAAn blocks that include a one-block gap can be a functional HSE provided that the three blocks are properly oriented and spaced (11). The CUP1 5′-sequences contain one consensus HSE, but with only two perfectly spaced nGAAn boxes (26, 27). Footprinting results indicate that HSF contacts a larger segment of the sequence; suggesting that the actual HSE may consist of the sequence TTCTAGAAGCGAAAAG (28). We verify here that an oligonucleotide containing the two perfect nGAAn repeats, but lacking the gapped nGAAn box, failed to compete with the HSE2 DNA in binding assays with HSF301. GAG is not a common trinucleotide HSE repeat in yeast, but changes in the third position are the least detrimental (10). The gapped nGAAn box is not a critical factor for the enhanced HSF301 binding to the CUP1 HSE as a gapped nGAAn HSE variant appears equivalent to the wild-type CUP1 HSE sequence in HSF301 binding. Furthermore, the enhanced DNA binding affinity of HSF301 for the CUP1 HSE does not relate to altered specificity as a HSE with all nGAAn boxes fails to bind HSF301.

Two aspects of the minimal CUP1 HSE appear to be optimal. First, the arrangement of nGAAn units in the CUP1 HSE appears to be favorable for activity as it was recently demonstrated that (nTTCnnGAAn)2 elements are more active than (nGAAnTTcn)2 elements (12). Second, the adenine upstream of the GAA block in CUP1 is clearly the preferred nucleotide for this position in S. cerevisiae HSEs (10). Most naturally occurring HSEs from different organisms contain more than three nGAAn blocks, and those containing three nGAAn blocks were typically in combination with additional HSEs, permitting cooperative interactions (12). The presence of a single gapped three-nGAAn block HSE in CUP1 is consistent with this HSE being a minimal element, and HSF binding may be expected to be weak. The high affinity binding of HSF301 to this minimal non-consensus HSE is striking.

The R206S mutation in HSF301 may result in enhanced DNA affinity by one of several mechanisms. First, the mutation may permit additional HSF-DNA contacts, which stabilize the interaction. Second, the mutation may confer greater flexibility in HSF301, permitting enhanced binding avidity to the CUP1 HSE element with the candidate gapped nGAAn-like array. Third, the R206S mutation may contribute to higher order assembly beyond the trimeric state, which may stabilize DNA/protein interaction. The arginine at codon 206 is not conserved in all HSF molecules, and in fact, a serine residue exists at this position in one tomato HSF (44). Structural studies on the HSF301 interaction with the CUP1 HSE may be insightful in understanding how HSF contacts HSEs differentially.

Acknowledgments—We are grateful to Jose Bonner for the wild-type HSF1 gene, a series of HSF1-specific oligonucleotides for sequencing, and comments on this manuscript. We are grateful to Peter Sorger for the HSE2/CYC1/αACZ fusion gene; Hillary Nelson for strain PS145, antisera to HSF, and x-ray coordinates for the HSF structure; Dennis Thiele for strains DTY23 and DTY113; and Elizabeth Craig, Susan Lindquist, and Hillary Nelson for helpful discussions.

REFERENCES
1. Morimoto, R. I., Sarge, K. D., and Abravaya, K. (1992) J. Biol. Chem. 267, 21987–21990
2. Lindquist, S. (1986) Annu. Rev. Biochem. 55, 1111–1191
3. Parker, C. S., and Topol, J. (1984) Cell 37, 273–283
4. Sorger, P. K., and Pelham, H. R. B. (1987) EMBO J. 6, 3035–3041
5. Jakobsen, B. K., and Pelham, H. R. B. (1988) Mol. Cell. Biol. 8, 5040–5042
6. Pelham, H. R. B. (1982) Cell 30, 517–528
7. Moos, D. D., Theodorakis, N. G., and Morimoto, R. I. (1988) Mol. Cell. Biol. 8, 4736–4744
8. Williams, G. T., and Morimoto, R. I. (1990) Mol. Cell. Biol. 10, 3125–3136
9. Xiao, H., Periic, S., and Lis, J. T. (1991) Cell 64, 585–593
10. Fernandez, M., Xiao, H., and Lis, J. T. (1994) Nucleic Acids Res. 22, 167–173
11. Amin, J., Ananthan, J., and Voellmy, R. (1988) Mol. Biol. Cell. 8, 3761–3769
12. Bonner, J. J., Balou, C., and Fackenthal, D. L. (1994) Mol. Cell. Biol. 14, 501–508
13. Wiedenheft, G., Seto, D., and Parker, C. S. (1988) Cell 54, 841–853
14. Sorger, P. K., and Pelham, H. R. B. (1988) Cell 54, 855–864
15. Sorger, P. K., and Nelson, H. C. M. (1989) Cell 59, 807–813
16. Sorger, P. K. (1991) Cell 65, 363–366
17. Sorger, P. K., Lewis, M. J., and Pelham, H. R. B. (1987) Nature 329, 81–84
18. Harrison, C. J., Bohm, A. A., and Nelson, H. C. M. (1994) Science 263, 224–227
19. Flick, K. E., Gonzalez, L., Jr., Harrison, C. J., and Nelson, H. C. M. (1994) J. Biol. Chem. 269, 12475–12481
20. Topol, J., Ruden, D. M., and Parker, C. S. (1985) Cell 42, 527–537
21. Siater, M. R., and Craig, E. A. (1987) Mol. Cell. Biol. 7, 1906–1916
22. Nieto-Sotelo, J., Wiederrecht, G., Okuda, A., and Parker, C. S. (1990) Cell 62, 807–817
23. Jakobsen, B. K., and Pelham, H. R. B. (1991) EMBO J. 10, 369–375
24. Bonner, J. J., Heyward, S., and Fackenthal, D. L. (1992) Mol. Cell. Biol. 12, 1021–1030
25. Høj, A., and Jakobsen, B. K. (1994) EMBO J. 13, 2617–2624
26. Yang, W., Gahl, W., and Hamer, D. (1993) Mol. Cell. Biol. 13, 3676–3681
27. Silar, P., Butler, G., and Thiéle, D. J. (1991) Mol. Cell. Biol. 11, 1232–1238
28. Tanai, K. T., Liu, X., Silar, P., Sosinski, T., and Thiéle, D. J. (1994) Mol. Cell. Biol. 14, 8155–8165
29. Thiéle, D. J. (1988) Mol. Cell. Biol. 8, 2745–2752
30. Furst, P., Hu, S., Hackett, R., and Hamer, D. (1988) Cell 55, 705–717
31. Welch, J., Fogel, S., Buchman, C., and Karin, M. (1989) EMBO J. 8, 255–260
32. Tohoyama, H., and Murayama, T. (1977) Agric. Biol. Chem. 41, 1523–1524
33. Inouhe, M., Hiyama, M., Tohoyama, H., Jho, M., and Murayama, T. (1989) Biochim. Biophys. Acta 993, 51–55
34. Tohoyama, H., Tomoyasu, T., Inouhe, M., Jho, M., and Murayama, T. (1992) Curr. Genet. 21, 275–280
35. Tohoyama, H., Inagawa, A., Keiko, H., Inouhe, M., Jho, M., and Murayama, T. (1992) FEMS Microbiol. Lett. 95, 81–86
36. Rose, M. D., Novick, P., Thomas, J. H., Botstein, D., and Fink, G. R. (1987) Gene (Amst.) 60, 237–243
37. Gietz, R. D., and Sugino, A. (1988) Gene (Amst.) 74, 527–534
38. Sewell, A. K., Jensen, L. T., Erickson, J. C., Palmier, R. D., and Winge, D. R. (1994) Biochemistry 34, 4740–4747
39. Elbe, R. (1992) Biotechniques 13, 18–20
40. Thorsvaldsen, J. L., Sewell, A. K., McCowen, C. L., and Winge, D. R. (1993) J. Biol. Chem. 268, 12512–12518
41. Sorger, P. K., Ammerer, G., and Shore, D. (1989) in Protein Function: A Practical Approach (Creighton, T. E., ed) pp. 199–224, IRL Press, New York
42. Nicolet, C. M., and Craig, E. A. (1991) Methods Enzymol. 194, 710–717
43. Giardina, C., and Lis, J. T. (1995) Mol. Cell. Biol. 15, 2737–2744
44. Vuister, G. W., Kim, S.-J., Orosz, A., Marquardt, J., Wu, C., and Bax, A. (1994) Nature Struct. Biol. 1, 605–613
45. Nelson, H. C. M., and Sauer, R. T. (1985) Cell 42, 549–558
