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Positive and Negative Regulation of Basal Expression of a Yeast HSP70 Gene

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The *SSA1* gene, one of the heat-inducible HSP70 genes in the yeast *Saccharomyces cerevisiae*, also displays a basal level of expression during logarithmic growth. Multiple sites related to the heat shock element (HSE) consensus sequence are present in the *SSA1* promoter region (Slater and Craig, Mol. Cell. Biol. 7:1906–1916, 1987). One of the HSEs, HSE2, is important in the basal expression of *SSA1* as well as in heat-inducible expression. A promoter containing a mutant HSE2 showed a fivefold-lower level of basal expression and altered kinetics of expression after heat shock. A series of deletion and point mutations led to identification of an upstream repression sequence (URS) which overlapped HSE2. A promoter containing a mutation in the URS showed an increased level of basal expression. A URS-binding activity was detected in yeast whole-cell extracts by a gel electrophoresis DNA-binding assay. The results reported in this paper indicate that basal expression of the *SSA1* promoter is determined by both positive and negative elements and imply that the positively acting yeast heat shock factor HSF is responsible, at least in part, for the basal level of expression of *SSA1*.

When cells are exposed to an increase in temperature or a variety of other environmental stresses, the synthesis of a set of proteins, the heat shock proteins (HSPs), is increased. This gene activation is rapid and transient and, in most cases, appears to be regulated primarily at the transcriptional level (2). Initial studies of the sequences necessary for inducibility of the heat shock genes of eucaryotes revealed a highly conserved sequence element, referred to as a heat shock element (HSE; 5′-CnnGAAnnTTCnnG-3′, where n is any nucleotide). Studies in monkey COS cells, *Xenopus laevis* oocytes, and the yeast *Saccharomyces cerevisiae* indicated that a single copy of an HSE was sufficient for heat-inducible expression (26, 27, 30). In contrast, analysis of an HSP70 in *Drosophila melanogaster* suggested that multiple HSEs act in a cooperative way to mediate transcriptional activation (13, 36). Recent in vitro binding studies with HeLa cell extracts indicated that multiple HSEs are stronger sites for binding of a transcription factor than is a single HSE (31).

The specific binding of putative transcription factors to HSEs has been reported for flies, yeast cells, and HeLa cells. A factor referred to as a heat shock factor (HSF) has been shown by footprinting and gel binding assays not only to bind to HSEs (20, 24, 25, 31, 39) but also to enhance transcription from heat shock promoters (25, 36). Exonuclease digestion studies of chromatin also revealed a protein interacting with HSEs which is presumably identical to HSF (41–43). Recently, the purification of the putative HSF from *Drosophila* and yeast cells (32, 39, 44) and the isolation of the gene encoding yeast HSF (33, 38) have been reported. Some important information concerning the mechanism of heat shock gene induction has come from studies on these diverse organisms. HSF is present in noninduced cells in an inactive form. In human and *Drosophila* cells, binding studies indicate that HSF is able to bind to DNA only after a heat shock (20, 31, 45). Recent studies have indicated a two-step mechanism of activation of human HSF: induction of sequence-specific binding ability followed by phosphorylation, suggesting that phosphorylation causes activation of the factor (22). In contrast, yeast HSF can bind to DNA whether or not the cells have been heat shocked. However, HSF isolated from heat-shocked yeast cells is more highly phosphorylated than that isolated from control cells, consistent with the modulation of HSF activity by phosphorylation (31, 33).

S. cerevisiae contains at least five heat-inducible HSP70 genes. Expression of three of these genes (*SSA1*, *SSA3*, and *SSA4*) has been studied in some detail. *SSA3* and *SSA4* are not expressed significantly before heat shock, while *SSA1* has a significant basal level of expression. The upstream promoter region of each of these genes contains multiple sites closely related to the HSE consensus sequence. It has been shown that each of these genes contains HSEs which are able to activate a heterologous promoter after heat shock (8, 30).

Although there are a number of HSEs in the *SSA1* promoter, deletion analysis of the *SSA1* promoter indicated that one of the HSE-homologous sites (denoted HSE2) is most important for heat-inducible expression. Deletions that removed upstream promoter-distal regions, including the perfect match with the HSE consensus sequence (HSE3), did not significantly influence heat-inducible expression. However, deletion of a promoter-proximal region containing HSE2 eliminated most of the heat-inducible expression of *SSA1*. HSE2 was able to activate the *CYC1* promoter in a heat-inducible manner when it was cloned into a *CYC1-lacZ* fusion vector in place of the *CYC1* upstream activating sequence (UAS). Moreover, this hybrid promoter displayed a substantial level of expression even before heat shock. However, when HSE2 was surrounded by its native flanking sequences in a *CYC1* fusion vector, the basal level of expression of a reporter gene was greatly decreased. Thus, it has been suggested that negative regulation is involved in modulating expression of the *SSA1* gene (8, 30).

Although the exact functions of the HSP70 proteins are unknown, some recent studies revealed possible roles of SSA proteins in translocation into the endoplasmic reticulum and mitochondria in yeast cells (6, 12). Deletions of these genes have deleterious effects on growth even at normal temperatures, indicating that these HSP70 proteins play an

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important role in normal cellular function as well as under conditions of stress (9, 10). These results imply that cells must express the HSP70 proteins at a certain level to be viable during normal growth. Therefore, the elements necessary for basal expression of the HSP70 genes are most likely to be important for cell viability.

In this paper, we have focused on the basal expression of *SSA1*. We present evidence that the basal expression of *SSA1* is determined primarily by HSE2 and an upstream repression sequence (URS) which overlaps HSE2. We also report detection of a URS-binding factor in yeast whole-cell extracts by a gel electrophoresis DNA-binding assay.

MATERIALS AND METHODS

DNA manipulations. All cloning strategies relied on standard methods (23) and have been described previously (30). Oligonucleotides were obtained from the University of Wisconsin Biotechnology Center. Cloning of oligonucleotides was performed as described previously (30) and verified by double-stranded plasmid sequencing (5). A series of deletions from pZJHSE2-137 (see Fig. 2) was generated by using Bal31 exonuclease. Both slow and mixed forms of Bal31 enzyme (International Biotechnologies, Inc.) were used as described in the manufacturer's protocol. The extent of each deletion was determined by double-stranded plasmid sequencing (5) with an oligonucleotide from the *CYC1* promoter (from −207 to −224) as a primer.

Site-directed mutagenesis. Point mutants were constructed by the method of Kunkel et al. (21) with slight modifications. DNA fragments containing the *SSA1* promoter from pZJHSE2-137 and pZD0-2 (30) were cloned into M13mp19 and used for preparation of uracil-containing templates in a *dut ung* strain, RZ1032 (HfrKL16 PO/45 [*lysA(61-62)*], *dut1 ung1 thi1 relA1 Zbd-279*::Tn*10 supE44*) (21). In vitro synthesis with the mutant primer was done as described by Zoller and Smith (47). The sequence of each mutant was confirmed by dideoxy sequencing analysis (29).

Yeast strains and transformations. Strain T69 (*MATα ura3-52 lys2-801 ade2-101 Δtrp1*) was used as a host for all plasmids for the β-galactosidase assay. Transformation was performed with lithium acetate by the published method (18). Protease-deficient strain BJ2168 (*MATa prb1-1122 pep4-3 prc1-407 leu2 trp1 ura3-52 gal2*) was used for extract preparation.

Enzyme assays. β-Galactosidase activity was assayed in yeast cells permeabilized with chloroform and sodium dodecyl sulfate as previously described (17, 30) except that phenylmethylsulfonyl fluoride (PMSF) was added to a final concentration of 1 mM. Units were calculated as $(OD_{420} \times 1{,}000)/(OD_{600} \times t \times v)$, where t is incubation time in minutes and v is volume in milliliters.

Extract preparation. Yeast whole-cell extracts were prepared by published methods (4, 28) with slight modifications. Cells grown to an OD_{600} of 1.0 at 23°C were harvested by centrifugation, suspended in extraction buffer [200 mM Tris hydrochloride (pH 8.0), 400 mM $(NH_4)_2SO_4$, 10 mM $MgCl_2$, 1 mM EDTA, 10% glycerol, 1 mM PMSF, 2 mM 2-mercaptoethanol], and broken with glass beads (0.45-mm diameter) with eight 30-s pulses of a bead beater (Biospec Products, Bartlesville, Okla.) at 4°C. The proteins were precipitated by the addition of $(NH_4)_2SO_4$ to a final concentration of 40%. The protein pellet was suspended in protein buffer (20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, pH 8.0], 5 mM EDTA) with 7 mM 2-mercaptoethanol, 1 mM PMSF, and 20% glycerol, followed by dialysis in protein buffer for 2 h at 4°C. Protein concentrations were determined by the Bradford method (3) with the Bio-Rad reagent.

Gel electrophoresis DNA-binding assay. DNA probes were end labeled with either Klenow enzyme and [α-^{32}P]dCTP or polynucleotide kinase and [γ-^{32}P]ATP by standard methods (23). Binding reactions were carried out in 20-μl volumes containing 1 mM $MgCl_2$, 120 mM NaCl, 20 mM HEPES (pH 8.0), 5% glycerol, 0.1% Nonidet P-40, 1 mM dithiothreitol, 0.1 to 0.5 ng of radiolabeled double-stranded DNA, 3 μg of poly(dI-dC) · poly(dI-dC), and 20 to 32 μg of protein. Reaction mixes were incubated for 20 min at 23°C and loaded immediately onto 4 or 6% polyacrylamide gels (acrylamide-bisacrylamide, 40:1) in 10 mM Tris hydrochloride–10 mM H_3BO_3–1 mM EDTA, pH 8.3. Gels were preelectrophoresed for 1 h and electrophoresed for 1.5 h at 200 V at room temperature. The gels were then dried onto Whatman 3MM paper and autoradiographed.

RESULTS

Basal level of expression of *SSA1*. Previous deletion analysis of the *SSA1* promoter suggested that sequences important for most of the basal level of expression are dispersed in the region upstream from the transcription initiation site. *CYC1* promoter fusion studies showed that one of the HSE-homologous sites (HSE2) and its adjoining nucleotides can promote transcription during normal growth as well as after heat shock (30). To test a role of HSE2 more directly in its native promoter, a double point mutation of HSE2 was generated (Fig. 1a), and the activity of a fusion of the mutant promoter linked to the reporter *lacZ* gene was measured. Two base substitutions in HSE2 caused a decrease in the basal activity of the *SSA1* promoter to 18% of the level of the wild-type promoter (Fig. 1a). Therefore, HSE2 is important in determining the basal level of expression of *SSA1*.

The kinetics of β-galactosidase activity induction of the wild-type and HSE2 point mutant promoter fusions after heat shock were measured to determine whether the mutations in HSE2 affected the heat-inducible expression of the *SSA1* promoter. Little difference between the activity of the wild-type and the mutant promoter was seen up to 1 h after temperature upshift. However, β-galactosidase activity from the wild-type promoter kept increasing up to 3 or 4 h after heat shock, while the activity from the HSE2 mutant peaked at 1 h after heat shock (Fig. 1b). These results indicate that other HSEs as well as HSE2 are involved in heat-inducible expression of *SSA1*.

Identification of a URS in the *SSA1* promoter. *CYC1* promoter fusion studies suggested that the *SSA1* promoter is also subject to negative regulation (30). As shown in Fig. 2, the 26-base-pair (bp) fragment containing HSE2 and its immediately adjoining nucleotides (from −203 to −182 with respect to the transcription start site, in pZJHSE2-26) conferred a substantial basal level of expression to a *lacZ* reporter gene. However, a larger DNA fragment (137 bp) containing the same HSE and its flanking sequences (from −261 to −131, in pZJHSE2-137) conferred about a 30-fold-lower basal level of expression, while the heat-inducible expression was almost the same. This result suggested that the 137-bp DNA fragment contains a sequence, called here an upstream repression sequence (URS), which negatively regulates the basal expression of *SSA1* (30). To identify the sequences involved in the negative regulation, a series of deletions were generated from both ends of the 137-bp DNA fragment (Fig. 2). Deletions (D4 and D5) which removed

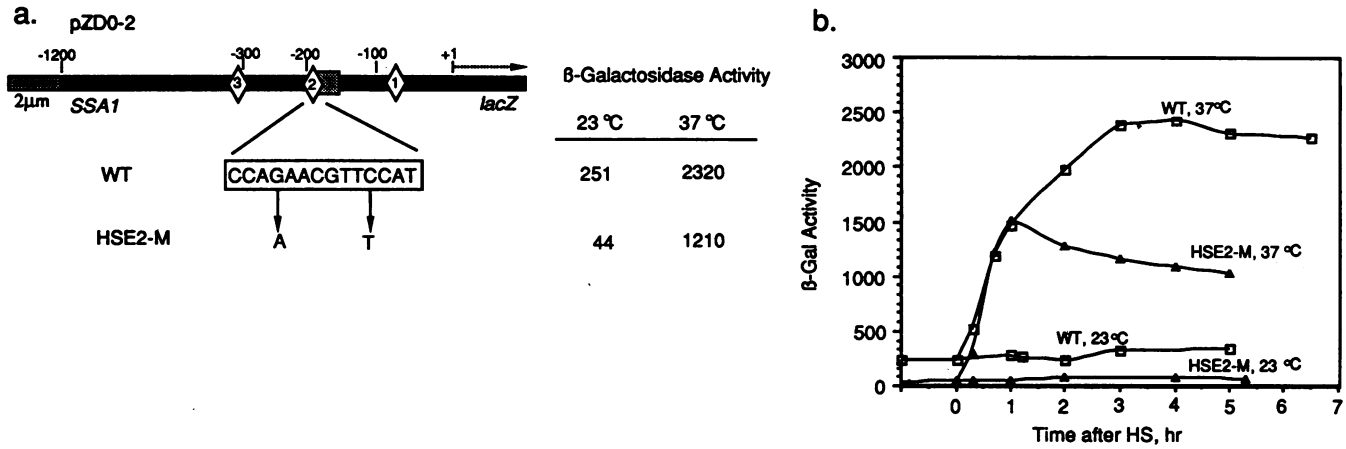


FIG. 1. β-Galactosidase activity of an *SSA1p-lacZ* fusion and its derivative which carries a double point mutation in HSE2. (a) Schematic representation of the *SSA1* promoter on a 2μm *SSA1p-lacZ* fusion plasmid, pZD0-2 (30). Each diamond represents an HSE-homologous site. The enlarged region shows the sequence of HSE2. +1 in this figure and throughout the paper indicates the 5′ end of the *SSA1* mRNA. The base substitutions in HSE2 are shown below the wild-type (WT) sequence. The β-galactosidase activity, in this figure and throughout the paper, in yeast cells transformed with each plasmid was determined before (23°C) and after (37°C) heat shock during logarithmic growth. Units of activity are defined in the text. The values shown represent the average for 12 samples from three independent transformants; heat shock samples were taken 3 h after a temperature upshift. The effect of the HSE2 mutation was also tested in a CEN vector carrying the entire *SSA1* promoter fused to the *lacZ* gene. The β-galactosidase activities in strains carrying the wild-type and HSE2 mutant (HSE2-M) promoter were reduced about fourfold compared with the levels in strains harboring the wild-type and mutant promoter fusions, respectively, on 2μm vectors. (b) Kinetics of β-galactosidase activity induction of the wild-type (WT) and HSE2 mutant (HSE2-M) *SSA1* promoter. Cells containing the *SSA1p-lacZ* fusion, pZD0-2, or the HSE2 point mutant were grown at 23°C. At time zero the culture was split; one half was maintained at 23°C, and the other half was shifted to 37°C. Samples were taken at the times indicated, and the β-galactosidase (β-Gal) activity was determined. The enzymatic activity at each time point represents the average for six samples from three independent transformants. All assays shown in this figure and throughout the paper were performed in duplicate on a few independent transformants; in all cases, each value deviated less than 15% from the mean. HS, Heat shock.

DNA sequences immediately downstream of the HSE2 showed an increased basal level of expression, indicating that these deletions destroyed the URS. To test directly whether HSE2 3′-proximal sequences are able to inhibit the basal level of expression, a 40-bp oligonucleotide containing HSE2 and the putative URS was synthesized and inserted in place of the *CYC1* UAS. As shown in Fig. 2, the plasmid containing the 40-bp fragment (pZJHSE2-40) showed a lower

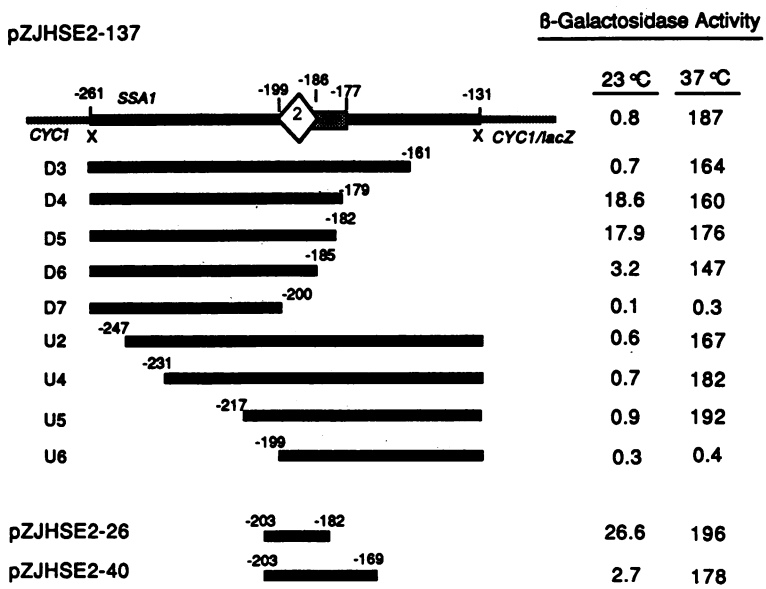


FIG. 2. β-Galactosidase activity of cells containing a series of deletion mutants of an *SSA1-CYC1* promoter fusion. The top line shows the promoter region from a 2μm-borne plasmid, pZJHSE2-137. Segments of the *SSA1* promoter (from positions −261 to −131) were inserted into the *Xho*I site (X) in the *CYC1-lacZ* fusion vector (pLG670Z) (17). The diamond represents HSE2, and the shaded box represents the putative URS overlapping HSE2. Below that are shown some representative deletion mutants generated from the 5′ and 3′ ends of the original 137-bp insert. Several other deletion mutants which are not shown with endpoints between −161 and −131 and between −261 and −231 displayed similar levels of expression with D3 and U2, respectively. The solid bars represent the portions of the *SSA1* promoter sequences that remain. Each deletion endpoint was determined by plasmid sequencing. pZJHSE2-26 and pZJHSE2-40 contain the 26-bp (HSE2) and 40-bp (HSE2 and URS) nucleotides, respectively, inserted into pLG670Z in place of the *CYC1* UAS.

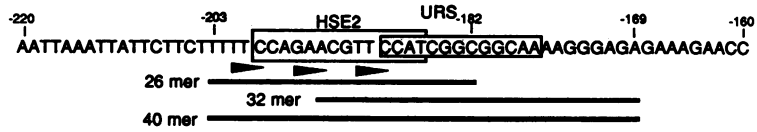


FIG. 3. DNA sequence of each oligonucleotide used in this study. Sequences near the HSE2 region in the *SSA1* promoter are shown, and each oligonucleotide is indicated as a heavy line. The sequences in the right-hand box show the URS, and sequences in the box overlapping URS are HSE2. Arrowheads mark the component GAA repeats with 2-nucleotide relative spacing, which has been shown to be necessary for heat-inducible expression (1). The 32-mer contains an extra C on both the 5′ and 3′ ends of the sequence. The 40-mer contains an extra C on the 3′ end of the sequence. All three oligonucleotides carry additional nucleotides TCGA at the 5′ end for cloning purposes.

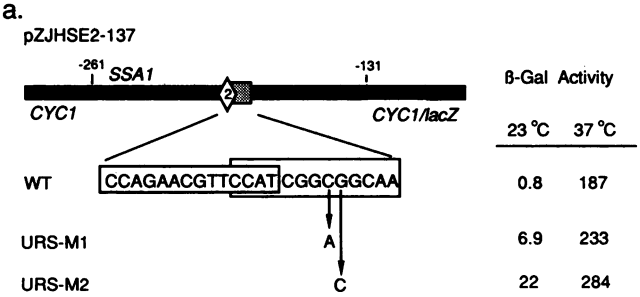
basal level of expression than the construction carrying the 26-bp fragment (pZJHSE2-26). This result also indicates that the URS resides within a small region 3′ of HSE2. The basal expression of pZJHSE2-40 was threefold higher than that of pZJHSE2-137 or the deletion mutant D3. This difference could be due to spacing differences or sequence context effects. However, we cannot completely rule out the possibility that other sequences in addition to the 40 bp are necessary for the level of repression observed with the 137-bp fragment. The 40-bp fragment contains a 13-bp sequence that has two mismatches, in the inverted orientation, to a consensus sequence, $TAGCCGCCGPu_4$, where Pu is any purine, proposed by Cooper and his colleagues to be a negative control site in yeast promoters (35). In the *SSA1* promoter, the 13-bp sequence partially overlaps HSE2 (Fig. 3).

Point mutational analysis of the URS. To test directly whether the 13-bp homologous sequence actually acts as a URS in the *SSA1* promoter, we made point mutations in the consensus sequence. In an *SSA1-CYC1* promoter fusion vector, separate changes of G to C or C to A in the core sequence of the putative URS caused a 30-fold or 9-fold increase in the basal level of expression, respectively (Fig. 4a). The same point mutations in the *SSA1* promoter-*lacZ* fusion (in pZD0-2) increased the basal level of expression two- to threefold (Fig. 4b). The results of the analysis of the two point mutants and the homology to the 13-bp consensus sequence of a URS found in other yeast promoters strongly suggest that the sequence acts as a URS in the *SSA1* promoter.

URS can also repress a heterologous promoter. To determine whether the URS can act independently from HSE, we tested whether the URS can also repress a heterologous promoter. To address this question, we inserted the 137-bp fragment of the *SSA1* promoter containing the URS (from −261 to −131; Fig. 4a) into the intact *CYC1* promoter, 3′ of the *CYC1* UAS. Insertion of the wild-type fragment repressed the activity of the *CYC1* promoter about fivefold (Fig. 5a). Insertion of the 137-bp DNA fragment carrying a mutation in HSE2 but a wild-type URS abolished *CYC1* UAS activity almost completely, suggesting that the URS represses *CYC1* transcription. In contrast, two constructions carrying point mutations in the URS did not significantly decrease the activity of the *CYC1* promoter when the mutant fragments were inserted into the same site in either orientation. The failure of the fragments containing the URS mutations to repress *CYC1* transcription clearly shows that the repressive effect of the 137-bp fragment is due not to a change in spacing between the *CYC1* UAS and the transcription initiation site but to the presence of the URS in the 137-bp fragment.

To test more directly whether the URS can act independently from HSE2, we constructed a 32-base oligonucleotide which carries only the URS and its flanking sequences (from −194 to −169; Fig. 3). The 32-bp oligonucleotide was inserted both downstream and upstream of the *CYC1* UAS. The 32-bp fragment repressed the activity of the *CYC1* UAS about 15-fold when it was inserted downstream of the *CYC1* UAS (Fig. 5b). When inserted in an upstream position, the 32-bp fragment repressed the *CYC1* UAS only about twofold. While insertion of the 32-bp fragment caused a decrease in expression of the *CYC1* promoter, the effects were less than those observed with the 137-bp fragment. These differences may be due to differences in spacing between the URS

a.



b.

Vector	Mutation	ß-Galactosidase Activity 23 °C	37 °C
pZD0-2	WT	251	2320
	HSE2-M	44	1210
	URS-M1	696	2228
	URS-M2	576	2242

FIG. 4. β-Galactosidase activity of URS point mutants before and after heat shock. (a) The top line shows the promoter region of pZJHSE2-137, a *CYC1-lacZ* fusion vector shown in Fig. 2. The enlarged region shows the sequence of HSE2 (left-hand box) and the putative URS (right-hand box) partially overlapping HSE2. The two point mutants of the URS (URS-M1 and URS-M2) have the same structure as pZJHSE2-137 except for the base substitutions shown below the wild-type (WT) sequence. The β-galactosidase activity of heat shock samples of the yeast cells transformed with pZJHSE2-137 and its derivatives reached a maximum level at 1 to 1.5 h after the temperature upshift and then slowly decreased; the heat shock samples were taken 1 h after the temperature upshift. (b) The same URS mutations were generated in pZD0-2, an *SSA1p-lacZ* translational fusion vector shown in Fig. 1a. Heat shock samples were taken 3 h after the temperature upshift. The effect of the URS mutation was also tested in a CEN vector. The β-galactosidase activities of both the wild-type and mutant promoters were decreased about fourfold compared with the levels of the wild-type and mutant promoters on 2μm vectors. HSE2-M, HSE2 mutant.

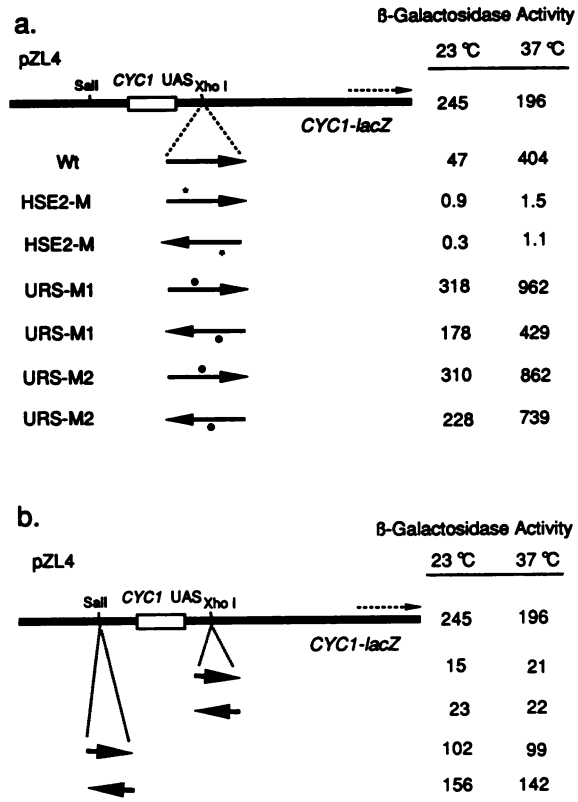


FIG. 5. Effect of the URS on expression of a heterologous promoter. (a) The top line shows the promoter region of a *CYC1-lacZ* fusion vector, pZL4 (30), carrying the *CYC1* UAS. The 137-bp wild-type (Wt) *SSA1* promoter fragment (from −261 to −131) and its mutant derivatives were inserted downstream of the *CYC1* UAS in either its native or inverted orientation (arrows). (b) The 32-bp synthetic oligonucleotide containing the URS was inserted either upstream or downstream of the *CYC1* UAS in both orientations. The basal (23°C) and heat-induced (37°C) β-galactosidase activity is given for each construction. Cells harboring each of these constructions were grown on glucose-based medium. The heat shock samples were taken 1 h after the temperature upshift. The asterisk and dot indicate the HSE2 mutations and the URS mutations, respectively.

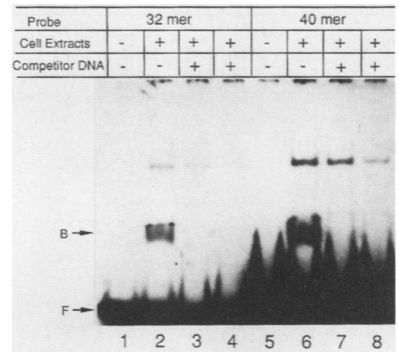


FIG. 6. Detection of a URS-binding protein in yeast whole-cell extracts. The gel-binding assays were performed with 0.2 ng of ^{32}P-labeled 32-bp double-stranded oligonucleotide (lanes 1 to 4) or 40-bp double-stranded oligonucleotide (lanes 5 to 8), shown in Fig. 3, 3 μg of poly(dI-dC) · poly(dI-dC), and whole-cell extracts prepared from yeast cells grown at 23°C. The reaction mixtures were loaded on a 6% polyacrylamide gel after incubation at 23°C for 20 min. Lanes 1 and 5, Probe only; lanes 2 and 6, probe and 20 μg of protein; lanes 3 and 7, probe, 20 μg of protein, and 100-fold molar excess of unlabeled 32-bp oligonucleotide duplex as a competitor; lanes 4 and 8, same as lanes 3 and 7, respectively, except 500-fold molar excess of unlabeled 32-bp oligonucleotide was added in each reaction. F and B indicate free DNA and protein-bound DNA, respectively.

and the *CYC1* UAS or between the URS and the transcription initiation site. However, we cannot completely rule out the possibility that the additional flanking sequences of the 32-bp fragment are also required for full repression. The *CYC1* promoter with a downstream URS sequence was not derepressed after a temperature upshift, suggesting a heat shock-independent mode of action of the URS-mediated repression. The URS still repressed the *CYC1* promoter even after induction with heme (data not shown).

Identification of a URS-binding activity in yeast cell extracts. In an attempt to detect proteins in yeast whole-cell extracts that interact with the URS, we used a gel electrophoresis DNA-binding assay. This technique is based on the slower migration of DNA-protein complexes than of free DNA on nondenaturing polyacrylamide gels (14, 15). The 32-bp double-stranded oligonucleotide carrying the URS and the 40-bp oligonucleotide carrying HSE2 and the URS (Fig. 3) were used as probes in a gel-binding assay. Both the 32-bp and 40-bp oligonucleotides formed a stable protein-DNA complex which was specifically inhibited by addition of the 32-bp oligonucleotide as a competitor DNA (Fig. 6). This protein-DNA complex was stable at moderately high salt concentrations (up to 12 mM $MgCl_2$ and 180 mM NaCl; data not shown). To confirm that this binding is specific to the URS, we carried out a series of competition experiments (Fig. 7a). Addition of either the wild-type 137-bp fragment (from −261 to −131) containing HSE2, the URS, and its flanking sequences (lane 3) or the 32-bp fragment (lane 6) significantly decreased the protein-DNA interaction with the labeled DNA. However, the 137-bp fragment containing point mutations in the URS (lane 4 and 5) or the 26-bp fragment carrying HSE2 only (lane 7) did not compete for the URS-binding activity. These results demonstrate the sequence specificity of the DNA-protein interaction. Interestingly, the 32-bp fragment competed more efficiently for binding of the URS-binding factor than the 40-bp fragment did (Fig. 7b; see Discussion). The higher band in Fig. 6 does not seem to be due to a specific interaction because it was not observed in all assays and was competed with by a number of random DNAs tested.

To test whether the mutation in HSE2 described previously eliminates binding of HSF in vitro, we used the 137-bp *Xho*I fragment (shown in Fig. 2) as a probe for a gel electrophoresis DNA-binding assay. We were able to detect a protein(s), presumably HSF detected by other groups (31, 39), which specifically bound to HSE2 in crude yeast cell extracts (Fig. 8, lane 2). The HSF-DNA complex was detected when the 137-bp DNA fragment carrying a mutation in the URS but wild-type HSE2 was used as a probe (lane 4). However, the binding was not detected when the 137-bp DNA fragment carrying two base substitutions in HSE2 was used as a probe (lane 3). Addition of the 26-bp or 40-bp oligonucleotide (carrying HSE2 or HSE2 plus URS, respectively) to the binding reaction also significantly decreased the specific interaction (lanes 7 and 6), but addition of the 32-bp oligonucleotide carrying only the URS did not compete for the specific binding (lane 5). These results indicate that HSF cannot bind to the mutant HSE2, which is consistent with expression of the mutant in vivo. The result

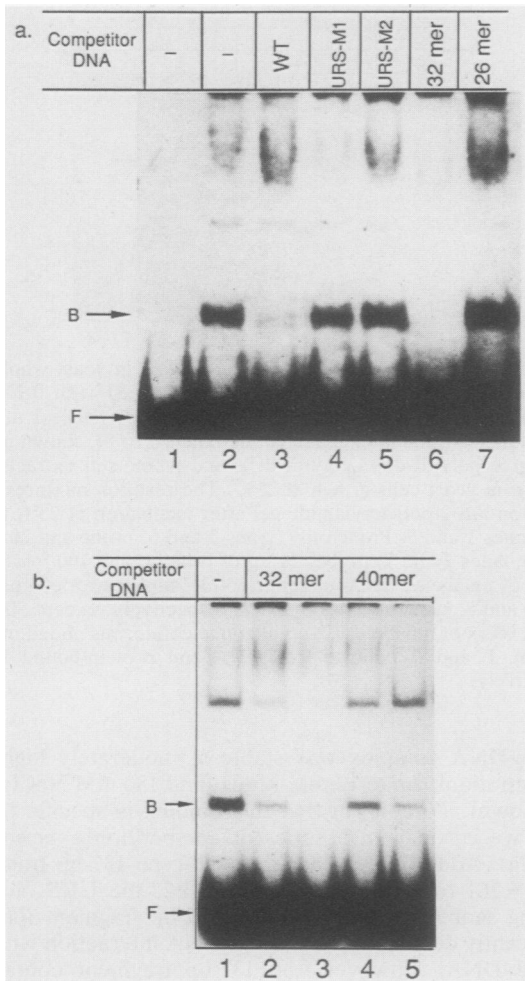


FIG. 7. Gel-binding competition studies with wild-type (WT) and mutant DNA fragments. The ^{32}P-labeled 32-bp oligonucleotide duplex was incubated with yeast cell extracts (containing 32 μg of protein) and 3 μg of poly(dI-dC) · poly(dI-dC). (a) Lane 1, Probe only; lane 2, probe and cell extracts; lanes 3 to 7, 400-fold molar excess of unlabeled competitor DNA added as indicated above each lane in addition to probe and cell extracts. (b) Lane 1, Probe and cell extracts only; lanes 2 to 5, 100-fold (lanes 2 and 4) or 500-fold (lanes 3 and 5) molar excess of unlabeled competitor DNA added as indicated above each lane in addition to probe and cell extracts. B and F, Bound and free DNA, respectively.

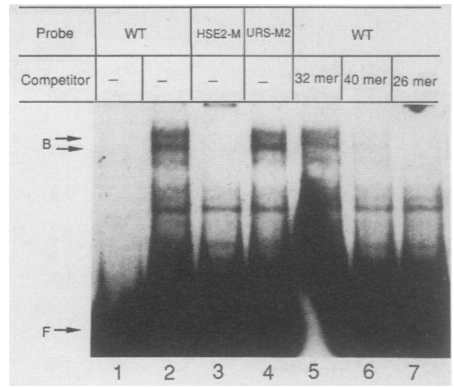


FIG. 8. Detection of HSE-binding activity in crude yeast cell extracts. Either ^{32}P-labeled 137-bp wild-type (WT) or mutant DNA fragment was incubated with yeast cell extracts (containing 24 μg of protein) and poly(dI-dC) · poly(dI-dC) in the presence or absence of competitor DNA. The reaction mixtures were loaded on a 4% polyacrylamide gel, and the gel was run for 1 h at 200 V. Lane 1, Wild-type probe only; lane 2, wild-type probe and cell extracts; lane 3, HSE2 mutant (HSE2-M) probe and cell extracts; lane 4, URS-M2 mutant probe and cell extracts; lanes 5 to 7, wild-type probe, cell extracts and 500-fold molar excess of competitor DNA as indicated above each lane. B and F, Bound and free DNA, respectively.

also indicates that HSF or the URS-binding factor can bind to the 40-bp fragment (Fig. 6 and 8). Interestingly, the protein-DNA complexes specific to HSE2 were always detected as two bands, suggesting that at least two forms of HSF are present in the extracts. It is also possible that two different proteins with similar DNA-binding specificity are responsible for the two bands (see Discussion). It is unlikely, however, that one is an HSF-DNA complex and the other an HSF-URS-binding protein-DNA complex because neither band was affected by addition of the 32-bp fragment carrying the URS as a competitor DNA.

The addition of the 32-bp fragment as a competitor DNA did not affect any specific band when the wild-type 137-bp fragment was used as a probe (Fig. 8, lane 5). Also, no specific band was missing when the 137-bp fragment carrying the URS mutation was used as a probe (lane 4; compare with lane 2, where wild-type probe was used). The bands in the middle of the gel seem to be due to nonspecific binding because they were not inhibited by addition of any specific DNA fragments and the binding was not stable at moderately high salt concentrations (data not shown). Thus, the URS-binding protein was not detectable with the 137-bp DNA fragment used as a probe. The 40-bp DNA showed a smaller band shift than the 32-bp DNA upon binding of the URS-binding factor (Fig. 6). It is likely that retardation of the DNA after binding of the URS-binding factor is quite small, so that a relatively large DNA fragment does not show any detectable shift after binding of the factor.

DISCUSSION

HSE2 is important for basal expression of the *SSA1* gene. The results presented here indicate that an HSE is important for basal expression of a heat shock gene. A double point mutation of HSE2 caused a fivefold reduction in the basal expression of *SSA1*, implying that a transcription factor binds to the HSE in the absence of stress. We cannot completely rule out the possibility that a distinct basal element overlaps HSE2. However, we think that is unlikely, because a *CYC1-lacZ* fusion vector carrying only HSE2 displayed a substantial level of expression before heat shock (30). The interpretation that an HSE is responsible for basal expression is also consistent with results from other laboratories. Unlike that isolated from human or *Drosophila* cells, yeast HSF isolated from non-heat-shocked cells has been shown to bind to DNA (31). We have also detected HSF binding to HSE2 in cell extracts from yeast cells grown at 23°C. It has been reported that HSF from heat-shocked cells is more highly phosphorylated than that from unstressed cells, suggesting that HSF activity is modulated by phosphorylation (33). Moreover, it has been shown that disruption of the HSF gene is lethal even in the absence of heat shock, indicating that HSF is essential for viability at normal growth temperatures (33, 38). Some heat shock and heat shock-related genes are essential for growth at normal temperatures (11, 37). Therefore, the inviability of *hsf* strains could be due to the lack of expression of these genes under normal conditions.

Deletion analysis of the *SSA1* promoter indicated that sequences 5′ of HSE2 are also important for basal expression (30). Therefore, it is likely that HSE2 contributes to the basal expression of *SSA1* in conjunction with sequences further upstream, such as poly(dA-dT) sequences or upstream HSEs. The results reported here also indicate that HSE2 is important for heat-inducible expression. An HSE2 mutant promoter was heat inducible but showed a lower level of expression and altered kinetics, suggesting that other HSEs as well as HSE2 play a role in heat-inducible expression. Although the current data do not allow us to fully understand the mechanism of *SSA1* gene regulation, it is possible to explain the kinetics of the heat-inducible expression of the HSE2 mutant as follows. HSF bound to HSE3 is responsible for most of the heat-inducible expression during the initial stage of temperature upshift. After the URS-binding factor dissociates from the partially overlapping binding site, HSF bound to HSE2 also contributes to the heat-inducible expression. It will be of interest to know whether all HSEs in the *SSA1* promoter are functionally equivalent in their contributions to basal and heat-inducible expression. Promoters containing point mutations in each of these HSEs are being constructed in order to test HSE functions.

Negative regulation of the *SSA1* gene. We localized a negative regulatory region overlapping the 3′ side of HSE2 by deletion mapping and point mutagenesis. Using a gel electrophoresis DNA-binding assay, we also detected a URS-binding activity in yeast cell extracts. These results strongly suggest that the expression of the *SSA1* gene is under negative control mediated by a repressor.

Negative regulatory sequences have been described within 5′-flanking sequences of several yeast genes. The URS in the *SSA1* promoter shares strong homology with URS1 in the *CAR1* gene. A similar sequence is present in several other yeast promoters, and in some cases, such as *CAR1* and *ENO1*, it has been shown that the sequence is contained in a region associated with negative control of the genes (7, 35). Two point mutations within the URS in the *SSA1* promoter resulted in an increase of basal level of expression. An analogous mutation within the URS1 of the *CAR1* gene was also shown to lead to constitutive expression of that gene (34). In the *SSA1* promoter, the URS partially overlaps a positively acting HSE2. In contrast, the homologous sequence is separated from the positively acting element in both *CAR1* and *ENO1* (7, 34).

We detected a protein which can interact with a DNA fragment carrying the *SSA1* URS in vitro. This protein is presumably a *trans*-acting regulator acting through the URS. It is therefore possible that this putative *trans*-acting regulator of the *SSA1* URS also mediates repression of *CAR1* or *ENO1*, similar to the coordinate regulation of **a**-specific genes by the α2 protein. It has been shown that the α2 protein interacts with a negative *cis*-acting sequence in the *STE6* promoter (19, 40), and similar sequence of the α2 operator was found in several **a**-specific genes in yeast cells. However, *CAR1*, *ENO1*, and *SSA1* are genes of diverse function and do not show any obvious similarities in expression. It is possible that there exists a family of repressors that share a portion of a DNA-binding domain, but not the physiological specificity that is responsible for the negative regulation of these genes. It is also possible that specific sequences flanking the consensus sequence in each gene distinguish a different repressor binding to each gene.

The structure and regulation of the *SSA1* promoter are complex. Although there are other HSEs in the *SSA1* promoter, the results of deletion and point mutational analysis indicate that HSE2 and the URS play key roles in the control of *SSA1* gene expression. However, from the data obtained thus far, it is not possible to ascertain the exact mechanism of HSE2-URS regulation. It is clear that disruption of the URS in the *SSA1* promoter does not lead to full induction of the gene in the absence of an inducing stimulus. This is because *SSA1* is also under the control of the positive regulatory factor HSF, which is activated by heat shock. Similar results were obtained in a study of the human beta interferon promoter, which contains a constitutive transcription element and a negative regulatory sequence that prevents enhancer activity prior to induction (16). In vivo DNase I footprinting suggested that repressor molecules bind to the beta interferon regulatory region under noninducing conditions. After induction, the repressor molecules dissociate from the DNA, and a positive transcription factor binds and activates the beta interferon promoter (46).

Although the putative binding sites, the URS and HSE2, in the *SSA1* promoter overlap, we have not established whether binding to the URS prevents binding of HSF to HSE2. We detected an HSF-DNA complex and a URS-binding factor-DNA complex, but not an HSF–URS-binding factor–DNA complex in our gel-binding studies. The 32-bp fragment, which carried only the URS, competed more efficiently for binding of the URS-binding factor than the 40-bp fragment, which carried both the URS and HSE2 (Fig. 7b). Also, the 26-bp fragment carrying only HSE2 competed more efficiently for HSF binding than the 40-bp fragment (data not shown). These findings are consistent with the idea that HSF competes with the URS-binding protein for binding to the partially overlapping sites. Therefore, we favor the following model. In the *SSA1* promoter, the negative factor modulates HSE2 activity by competing with the activator HSF for binding to the partially overlapping sites, thus decreasing the basal level of expression, which is dependent on HSE2. After heat shock, activated HSF can bind to HSE2, either displacing the URS-binding factor or competing more effectively for binding to the overlapping sites.

According to the model, it is possible to interpret the effect of mutations in HSE2 and URS as follows. A mutation in HSE2 prevents binding of HSF, directly resulting in a decrease in basal expression. In addition, an HSE2 mutation could have an indirect effect on basal expression. Without competition with HSF binding to the overlapping site, URS binding could increase, thus increasing its negative effect on other, more distant, HSEs or other components of the transcription machinery. Similarly, in the URS mutants, the basal level of expression is higher due to increased HSF binding to the partially overlapping site in the absence of competition with URS-binding factor. Also, URS-binding factor cannot bind to the mutated site, resulting in derepression. Future analysis of the interactions between HSF and the URS-binding factor and their binding sites should lead to a better understanding of the role of these two factors in modulating the basal and heat-inducible expression of *SSA1*.

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