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Self-Regulation of 70-Kilodalton Heat Shock Proteins in *Saccharomyces cerevisiae*

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To determine whether the 70-kilodalton heat shock proteins of *Saccharomyces cerevisiae* play a role in regulating their own synthesis, we studied the effect of overexpressing the *SSA1* protein on the activity of the *SSA1* 5′-regulatory region. The constitutive level of Ssa1p was increased by fusing the *SSA1* structural gene to the *GAL1* promoter. A reporter vector consisting of an *SSA1-lacZ* translational fusion was used to assess *SSA1* promoter activity. In a strain producing approximately 10-fold the normal heat shock level of Ssa1p, induction of β-galactosidase activity by heat shock was almost entirely blocked. Expression of a transcriptional fusion vector in which the *CYC1* upstream activating sequence of a *CYC1-lacZ* chimera was replaced by a sequence containing a heat shock upstream activating sequence (heat shock element 2) from the 5′-regulatory region of *SSA1* was inhibited by excess Ssa1p. The repression of an *SSA1* upstream activating sequence by the *SSA1* protein indicates that *SSA1* self-regulation is at least partially mediated at the transcriptional level. The expression of another transcriptional fusion vector, containing heat shock element 2 and a lesser amount of flanking sequence, is not inhibited when Ssa1p is overexpressed. This suggests the existence of an element, proximal to or overlapping heat shock element 2, that confers sensitivity to the *SSA1* protein.

The synthesis of a small set of proteins in response to elevated temperature and other stressful conditions (the heat shock response) has been observed in almost every species examined to date (21). Because of the ease with which these proteins can be induced and the near universality of this phenomenon, the response of cells to stress has long been used as a model for the regulation of gene expression. Much has been learned about one aspect of heat shock regulation, the induction of mRNA synthesis. The induction of heat shock transcripts in *Escherichia coli*, for example, is now known to depend on the product of the *rpoH* gene, a heat-shock-specific sigma factor. In eucaryotic cells, the heat shock factor (HSF) binds to heat shock elements (HSEs) in the promoters of heat-inducible genes and is thought to be responsible for the increase in transcription. The consensus sequence of HSEs is CNNGAANNT TCNNG. In contrast, little is known about how the heat shock response is terminated. Only two studies have dealt extensively with this question. Tilly et al. (36) have shown that DnaK, the hsp70 analog in *E. coli*, negatively regulates its own synthesis and that of other heat shock genes. Similarly, the repression of hsp synthesis and the resumption of normal protein synthesis have been postulated to require a critical level of hsp70 in *Drosophila* (11). This hypothesis is based on experiments in which *Drosophila* tissue culture cells were fed amino acid analogs, or treated with cycloheximide, and the level of functional hsp70 was found to be inversely correlated with its own synthesis. Although these results are suggestive of autoregulation, they do not demonstrate a cause-and-effect relationship between the level of hsp70 and its synthesis.

In *Saccharomyces cerevisiae*, the *HSP70* multigene family comprises nine genes, including the most recently identified member, *KAR2* (30). On the basis of sequence relatedness, common regulation, and functional equivalence, the other eight genes have been assigned to four subfamilies: *SSA*, *SSB*, *SSC*, and *SSD* (7, 40). The *SSA* group has four members, distinguishable by both their structure and their regulation. Although the DNA sequences of *SSA1* and *SSA2* are about 97% identical and both genes are expressed constitutively at 23°C, only *SSA1* is induced by heat shock. *SSA3* and *SSA4*, on the other hand, have diverged about 20% from each other and from *SSA1* and *SSA2*. They can be regarded as classical heat shock genes: their transcripts are undetectable at 23°C, but are strongly induced by a rapid shift to 37°C (8).

The expression of *SSA1* has been studied more extensively than that of the other yeast *HSP70* genes. The *SSA1* promoter region contains several HSEs, one of which, HSE2, is located 192 nucleotides from the 5′ end of the mRNA. HSE2 has been shown to be important for both basal and heat-inducible expression (33). In addition, Park and Craig have identified a sequence element that partially overlaps HSE2 and which inhibits the basal rate of *SSA1* transcription (29). A mutation in this upstream repression sequence (URS) increases the basal level of expression of *SSA1* two- to threefold. In a transcriptional fusion vector containing HSE2 and flanking regions, the effect of the URS is more dramatic, causing 25-fold repression of HSE2-driven expression.

The work presented here was undertaken to determine whether the 70-kilodalton (kDa) heat shock proteins of *S. cerevisiae* are autoregulatory. Because *SSA2* can compensate for mutations in *SSA1*, it is necessary to construct *ssa1 ssa2* double mutants to assess the effect of disrupting *SSA1* function on *SSA1* expression. Cells containing *ssa1* and *ssa2* insertion mutations exhibit the following phenotypes: they are enlarged and unable to form colonies at 37°C, and the levels of some heat shock transcripts are elevated, including those encoded by the *ssa1* and *ssa2* mutant alleles (5, 40; E. A. Craig, unpublished results). The increase in *ssa1* and *ssa2* expression can be explained in one of two ways: either the double mutation constitutes a stress that results in hsp induction, as suggested by the growth phenotype; or *SSA1* is

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negatively autoregulated. To distinguish between these possibilities, the effect of high constitutive expression of Ssa1p on its own synthesis was studied. Evidence that is consistent with the self-regulation of *SSA1* is presented.

MATERIALS AND METHODS

RNA isolation and RNA blots. RNA was isolated as described by Ingolia et al. (17), electrophoresed on denaturing 1% agarose gels (5), and blotted onto Gene Screen or nitrocellulose by following the instructions of the manufacturer. To assess the relative amounts of RNA loaded in each lane, the blots were soaked in 5% acetic acid for 15 min at room temperature and then stained with 0.04% methylene blue–0.5 M sodium acetate for 10 min, also at room temperature. After shaking the blots in water overnight, the rRNA bands appear as dark blue on a pale blue background. Hybridization was carried out at 45°C in 50% formamide, as described by Craig and Jacobsen (6).

Enzyme assays. β-Galactosidase activity was determined as described by Slater and Craig (33). The optical density of the growing yeast culture was measured at 600 nm (OD_{600}), and a measured volume (v, in milliliters) was pelleted in a siliconized 1.5-ml microcentrifuge tube. The supernatant was removed, and the cell pellet was frozen in a dry ice-ethanol bath (−70°C). For the enzymatic assay, the cell pellets were vigorously suspended in 800 μl of Z buffer (25)–20 μl of 0.1% sodium dodecyl sulfate–20 μl of chloroform. After incubation of the suspension for 5 to 10 min at 28°C, the reaction was initiated by the addition of 160 μl of a 4-mg/ml concentration of *o*-nitrophenyl-β-D-galactoside in A buffer (25). The reaction was terminated by addition of 400 μl of 1.0 M Na_2CO_3, and the incubation time (t, in minutes) in the presence of *o*-nitrophenyl-β-D-galactoside was noted. Cleavage of *o*-nitrophenyl-β-D-galactoside produces a colored compound, *o*-nitrophenol, that can be quantified by determining the A_{420}. The cell debris was then pelleted for 1 min in a microcentrifuge, and the A_{420} of supernatant was determined with a rapid-sampling spectrophotometer. Units of β-galactosidase activity were defined as $(A_{420} \times 1{,}000)/(OD_{600} \times t \times v)$.

Yeast transformations. Cells were transformed with centromeric and integrative vectors by the lithium acetate method, as described previously (18). Integrative vectors were linearized to stimulate recombination and direct the site of integration (28). Cells were transformed with vectors containing the *leu2*-d allele (13), according to the spheroplast method (2), after growth overnight in complete medium lacking an exogenous carbon source (16).

Media. The strains used in this study are given in Table 1. The media used in this study were as follows: complete dextrose medium (YPD) contained 1% yeast extract (Difco Laboratories), 2% Bacto-Peptone (Difco) and 2% glucose; minimal medium (SD) contained 0.7% yeast nitrogen base (Difco) without amino acids and 2% glucose, supplemented with adenine, uracil, lysine, histidine, leucine, methionine, tyrosine, phenylalanine, arginine, and tryptophan to the concentrations recommended by Sherman et al. (32). Selective media were made by omitting one or more of the amino acids, as necessary. For plates, 2% Bacto-Agar (Difco) was added. Complete and minimal galactose-based media (YPG and SG) were made by substituting galactose (G-0750; Sigma Chemical Co.) for glucose in the YPD and SD recipes.

Growth conditions and heat shock protocol. Yeast cultures were grown at 23°C with constant agitation (150 rpm) in Orbital water baths. To heat shock the cells, 10 ml of each culture was transferred to a glass flask prewarmed to 37°C in another shaking water bath.

TABLE 1. Strains used[a]

Strain	Genotype
Progenitor strain	
DS110	*MAT***a** *leu2-3,112 lys1 lys2 his3-11,15 Δtrp1 GAL2 ura3-52*
DS113	*MAT***a** *leu2-3*,112 lys1 lys2 his3-11,*15 Δtrp1 GAL2 ura3-52 cir*^{0}
DS114	*MAT*α *leu2-3,112 lys1 lys2 his3-11,15 Δtrp1 GAL2 ura3-52 cir*^{0}
DS110 derivatives	
DS111	*MAT***a** YIpGAL1-SSA1
DS112	*MAT***a** YIpGAL1/10
DS113/DS114 derivatives	
DS115	*MAT***a**/α pZK0 YEpGAL1-SSA1
DS116	*MAT***a**/α pZK0 YEpGAL1/10
DS117	*MAT***a**/α YCpCUP1-*lacZ* YEpGAL1-SSA1
DS118	*MAT***a**/α YCpCUP1-*lacZ* YEpGAL1/10
DS119	*MAT***a**/α YCpSSA2-*lacZ* YEpGAL1-SSA1
DS120	*MAT***a**/α YCpSSA2-*lacZ* YEpGAL1/10
DS121	*MAT***a**/α pZJHSE2-137 YEpGAL1-SSA1
DS122	*MAT***a**/α pZJHSE2-137 YEpGAL1/10
DS125	*MAT***a**/α pZJHSE2-40 YEpGAL1-SSA1
DS126	*MAT***a**/α pZJHSE2-40 YEpGAL1/10
DS127	*MAT***a**/α YCpSSA4-*lacZ* YEpGAL1-SSA4
DS128	*MAT***a**/α YCpSSA4-*lacZ* YEpGAL1/10
DS129	*MAT***a**/α YIpSSA1-*lacZ* YEpGAL1-SSA4
DS130	*MAT***a**/α YIpSSA1-*lacZ* YEpGAL1/10

[a] Strain construction is described in Materials and Methods. Since these strains differ only in the plasmids that they carry, their complete genotypes are omitted for the sake of brevity. Note that the lower-numbered strain of each pair is the experimental strain, that is, the cells containing the *GAL1p-SSA1* fusion.

Protein gels. Extraction and electrophoresis of yeast proteins were carried out as described previously (6). Two-dimensional polyacrylamide gel electrophoresis was performed as described by O'Farrell (26).

Plasmid construction. All bacterial transformations were performed as described previously (4), using strain MC1066 (23). The plasmids used in this study are shown in Fig. 1.

(i) YCpGAL1-SSA1 and YIpGAL1-SSA1. Plasmid YG 100BH,containing the complete *SSA1* coding sequence, was opened at the *Xba*I site (−350, where +1 marks the position of the ATG) and digested with *Bal* 31 exonuclease for various periods of time by the method of Maniatis et al. (22). Deletion endpoints were estimated by second cutting the samples and sizing them on polyacrylamide gels. Those in the target size range were ligated with *Hin*dIII linkers, reclosed, and amplified in *E. coli*. Three clones were sequenced by the chemical cleavage method (24), and the positions of the *Hin*dIII sites were determined to be 33, 30, and 16 base pairs (bp) 5′ to the ATG. The *GAL1p-SSA1* fusion was constructed by inserting the *SSA1*-containing *Hin*dIII-*Sph*I fragment of YG100Δ-16 into pBM272, a plasmid containing the divergent promoters *GAL1* and *GAL10*. The YIpGAL1-SSA1 integrative vector was created by cutting the YCpGAL1-SSA1 vector with *Ava*I and reclosing it, thus removing the centromere.

(ii) YEpGAL1/10. The *GAL1/10* promoter was isolated

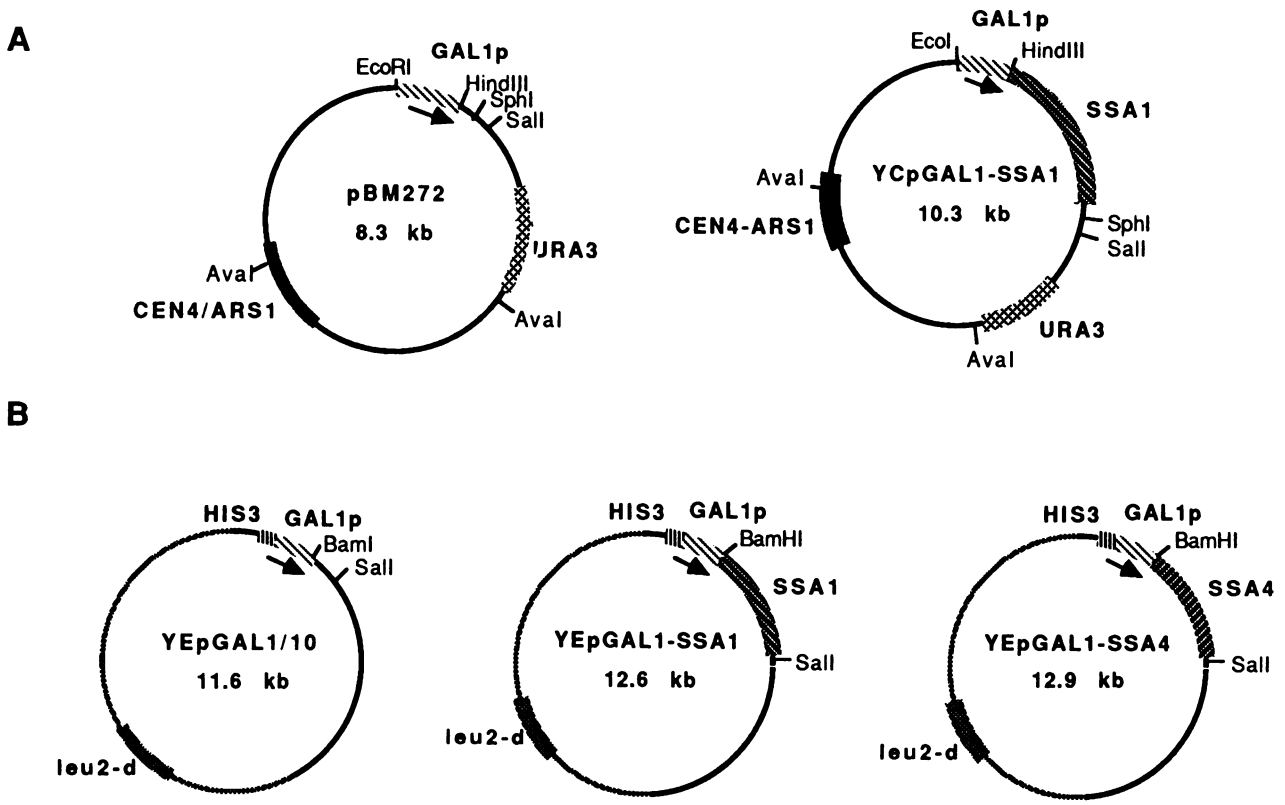

FIG. 1. Overexpression vectors. (A) *GAL1/10* expression vector, pBM272, and its Ssa1p overexpressing derivative, YCpGAL1-SSA1. (B) High-copy-number *GAL1/10* expression vector, YEpGAL1/10, and its Ssa1p- and Ssa4p-overexpressing derivatives, YEpGAL1-SSA1 and YEpGAL1-SSA4. Details of their construction are given in Materials and Methods. kb, Kilobases.

from pBM252 as a *Bam*HI-*Bgl*II fragment and inserted into the *Bam*HI site of the *leu2*-d (Beggs) vector pC1/1. Clones were screened for the orientation in which the *GAL1* promoter was proximal to the *Sph*I site of pC1/1. It should be noted that about 30 N-terminal codons of *HIS3* remain fused to the *GAL10* promoter in this vector.

(iii) YEpGAL1-SSA1. *SSA1* was isolated from the YCpGAL1-SSA1 vector as a *Bam*HI-*Sal*I partial digestion product and ligated with *Bam*HI-*Sal*I-cut YEpGAL1/10 DNA. Since the *Bam*HI site is immediately adjacent to the *Hin*dIII site in YCpGAL1-SSA1, the entire *SSA1* coding region is contained on the subcloned fragment.

(iv) YEpGAL1-SSA4. Taking advantage of a unique restriction site at −90, a *Stu*I-*Sph*I fragment containing the *SSA4* coding region was ligated with the small *Sca*I-*Sph*I fragment and the large *Sca*I-*Hin*cII fragment of pUC18, thus placing *SSA4* in the pUC18 polylinker, just downstream of *Bam*HI. *SSA4* was then moved into pBM272 as a *Bam*HI-*Sph*I piece, to create YCpGAL1-SSA4, which in turn was used as a source of a *Bam*HI-*Sal*I fragment containing *SSA4*. Finally, *SSA4* was ligated to *Bam*HI-*Sal*I-cut YEpGAL1/10, thereby placing it under *GAL1* control.

(v) pZK0. pZF0, the parent plasmid of pZK0, is a centromeric vector containing the *SSA1* regulatory region (−1200 to +30, where the coding sequence begins at +1) joined, in frame, to the *E. coli lacZ* gene. The modification of pZF0 was undertaken to change its selectable marker from *URA3* to *LEU2*. *LEU2* was removed from pYe(cen3)41 on a 2.2-kilobase *Pst*I fragment and ligated with single-*Pst*I-cut pZF0 DNA. The ligation mixture was used to transform MC1066, and Leu$^+$ Amp^r colonies were selected. Plasmid DNA was extracted and screened for clones containing the *LEU2* gene inserted into the *Pst*I site of *URA3*. To ensure the loss of *URA3* function, *Bal* 31 exonuclease was used to remove approximately 320 bp from the 3′ end of the *URA3* sequence.

Strain construction. Plasmid pC1/1 encodes a wild-type *LEU2* gene that is lacking most of its promoter and which is called *leu2*-d (2). The unusually high copy number of this plasmid (hundreds per cell) presumably compensates for the poor expression of *leu2*-d (13). Strain DS110 was transformed with pC1/1 to cure the endogenous 2μm plasmid. Transformants were grown selectively for 1 week, during which time the *leu2*-d vector was replicated at the expense of the endogenous plasmid (12, 37), and then in complete medium for an additional 2 months. After about 500 generations of nonselective growth, the cells were shown to have completely lost 2μm DNA by Southern blotting. The *cir*^{0} derivative of DS110 was then transformed with an HO plasmid to allow mating-type switching, and the resulting diploids were sporulated to give DS113 and DS114. This isogenic pair of strains was used in the construction of all other strains shown in Table 1 except DS111 and DS112, which are direct derivatives of DS110. The diploid strains were created by mating the DS113-derived cells (carrying the various *GAL1/10* vectors) with DS114 (carrying the various *lacZ* fusion vectors).

RESULTS

Moderate overexpression of Ssa1p reduces the basal and induced activity of an *SSA1-lacZ* translational fusion by about

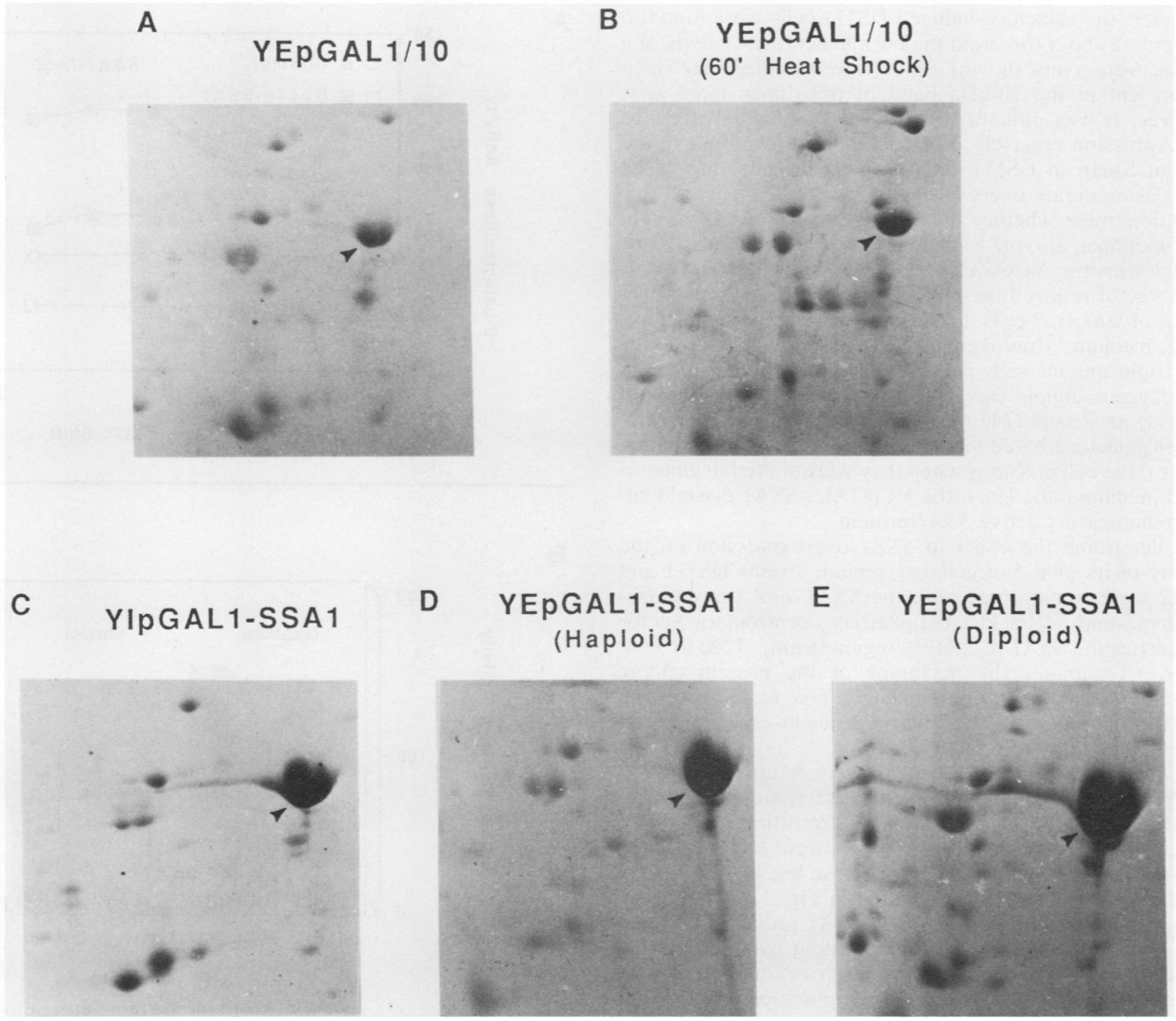

FIG. 2. Two-dimensional protein gels showing the relative abundance of the *SSA1* protein in strains carrying the *GAL1p-SSA1* fusion. Protein extracts were prepared and fractionated as described in Materials and Methods. All strains were grown in selective galactose-based media. (A) Haploid cells (strain DS10) carrying the YEpGAL1/10 vector and grown at 23°C. (B) Haploid cells (strain DS10) carrying the YEpGAL1/10 vector and shifted from 23 to 37°C 60 min prior to harvest. (C) Haploid cells (strain DS10) carrying an integrated copy of the *GAL1p-SSA1* fusion and grown at 23°C. (D) Haploid cells (strain DS10) carrying the YEpGAL1-SSA1 vector and grown at 23°C. (E) Diploid cells (strain DS15) carrying the YEpGAL1-SSA1 vector and grown at 23°C.

twofold. If the *SSA1* protein negatively regulates its own synthesis, then one might expect that heat shock should not induce *SSA1* expression in cells already producing a greater than heat shock level of Ssa1p. To test this idea, we measured the activity of a reporter vector consisting of the *SSA1* promoter fused to the *E. coli lacZ* gene in cells forced to overexpress *SSA1*. The *SSA1* protein was overproduced by placing the *SSA1* coding region under the control of the *GAL1* promoter. Using the site-directed integration method of Orr-Weaver et al. (28), the *GAL1p-SSA1* fusion vector was targeted to the *URA3* locus of strain DS110, to give strain DS111. This manipulation should produce a genomic copy of the full-length *GAL1p-SSA1* hybrid gene. An isogenic control strain, DS112, was created by directing the *GAL1/10* expression vector (used to construct the *GAL1p-SSA1* fusion) to the *URA3* locus of DS110. Since the *GAL1* promoter is highly active when cells are grown in galactose-based media, and repressed in glucose-based media (19), the level of the *SSA1* protein in yeast cells carrying the *GAL1p-SSA1* fusion can be raised or lowered by simply switching carbon sources in the growth medium. To estimate the degree of Ssa1p overexpression in a strain carrying an integrated copy of the *GAL1p-SSA1* fusion, strains DS111 and DS112 were grown to mid-log phase in complete glucose-based and complete galactose-based media, and protein extracts from these cultures were fractionated on two-dimensional polyacrylamide gels (Fig. 2A to C). The induction of Ssa1p is not readily observed in panel B of Fig. 2 because the *SSA1* and *SSA2* proteins are not resolved into distinct spots and because the basal rate of Ssa1p synthesis is only about one-third that of Ssa2p. It is apparent, however, that severalfold more hsp70 is present in the fusion-containing DS111 cells grown in galactose-based medium as compared with DS111 cells grown on glucose-based medium (not shown) or control cells grown on either sugar. When one-dimensional gels were stained and scanned with a den-

sitometer, the galactose-induced DS111 cells were found to accumulate about threefold the normal heat shock level of a 70-kDa protein (not shown). Since proteins other than Ssa1p are present in the 70-kDa band of one-dimensional gels, however, it was difficult to quantify the degree of Ssa1p overexpression precisely. For convenience, we refer to the level of Ssa1p in DS111 cells growing on galactose-based media as moderate overexpression.

To determine whether the vector-encoded *SSA1* protein was functional, an *ssa1 ssa2* double mutant strain was transformed with the YCpGAL1-SSA1 vector. The YCpGAL1-SSA1 vector relieved the temperature-sensitive growth phenotype of *ssa1 ssa2* cells on galactose-based, but not glucose-based, medium. Moreover, the otherwise inviable *ssa1 ssa2 ssa4* triple mutant cells can be recovered by sporulating a heterozygous diploid carrying the *GAL1p-SSA1* fusion (40). The *ssa1 ssa2 ssa4 GAL1p-SSA1* haploid cells formed colonies on galactose-based medium, but stopped dividing within two or three cell divisions when they were shifted to glucose-based medium (40). Thus, the YCpGAL1-SSA1 plasmid encodes biologically active *SSA1* protein.

To determine the effect of *SSA1* overexpression on the activity of its own 5′-regulatory region, strains DS111 and DS112 were transformed with the *SSA1-lacZ* translational fusion plasmid pZK0. Plasmid pZK0 is a centromeric vector that carries the *SSA1* regulatory region (from −1200 to +30, where +1 signifies the beginning of the protein coding sequence) joined in frame to the *E. coli lacZ* gene. The regulation of the *SSA1-lacZ* hybrid gene closely mimics that of the native copy of *SSA1* (33).

DS111(pZK0) and DS112(pZK0) were routinely grown to mid-log phase at 23°C in supplemented minimal galactose-based (or glucose-based) medium. At zero time, cells were shifted to 37°C, and duplicate samples were taken every 30 min for 2 to 4 h to assay β-galactosidase levels. The results of a typical experiment are pictured in Fig. 3. In numerous trials, moderate overexpression of *SSA1* reduced the basal and induced β-galactosidase activity about twofold, but had no effect on the kinetics of induction or on the relative increase in *SSA1-lacZ* expression upon heat shock (the induction ratio, calculated as β-galactosidase activity at 37°C/β-galactosidase activity at 23°C) (Fig. 3A). The absolute levels of β-galactosidase were 1.8- to 2.3-fold lower in DS111(pZK0) than in DS112(pZK0) at 23°C and 1.7- to 2.3-fold lower after heat shock. No differences in β-galactosidase levels were observed when the cells were grown in glucose-based medium (Fig. 3B).

High-level overexpression of Ssa1p slows cell growth and inhibits induction of the *SSA1-lacZ* translation fusion. Since moderate overexpression of Ssa1p decreased the low- and high-temperature steady-state expression of the *SSA1-lacZ* fusion, we asked whether higher levels of Ssa1p could block the heat inducibility of the *SSA1* promoter. To augment Ssa1p synthesis, the *GAL1/10* promoter fragment and the *GAL1p-SSA1* fusion were moved to the high-copy-number plasmid pC1/1, and the resulting vectors, YEpGAL1/10 and YEpGAL1-SSA1, were used to transform a strain carrying the integrated *SSA1-lacZ* translational fusion (33). As expected, increasing the gene dosage of the *GAL1p-SSA1* fusion resulted in a further elevation in the level of Ssa1p. When grown in galactose-based media, haploid strains carrying YEpGAL1-SSA1 accumulated roughly 10-fold the normal heat shock level of the *SSA1* protein, as estimated by densitometric analysis of one-dimensional protein gels stained with Coomassie brilliant blue and by examining Coomassie brilliant blue-stained two-dimensional protein gels (Fig. 2). Diploid strains carrying YEpGAL1-SSA1 seemed to accumulate somewhat more of the *SSA1* protein than the isogenic haploid strains (cf. panels D and E of Fig. 2). For this reason, diploid cells were used throughout this work. It is likely that GAL4, the positive regulator of the *GAL1* promoter, is limiting in cells carrying YEpGAL1-SSA1, since the level of Ssa1p does not increase linearly with increasing copy number of the *GAL1p-SSA1* fusion. For convenience, we refer to the level of Ssa1p in all cells

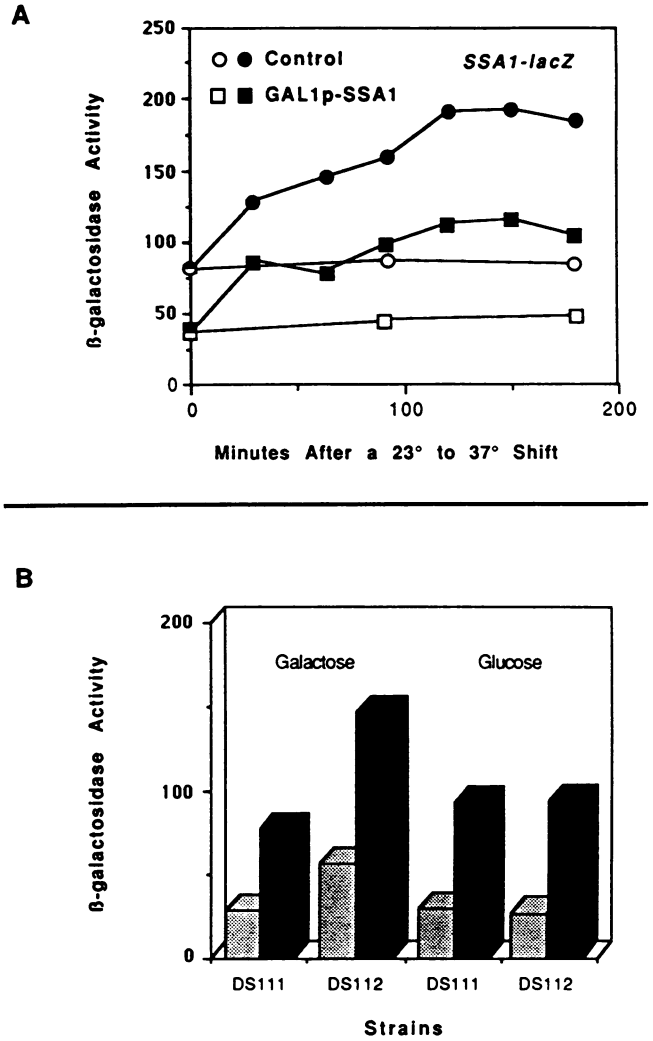

FIG. 3. Effect of a moderate excess of Ssa1p on expression of the *SSA1-lacZ* translational fusion. (A) Cells carrying the *SSA1-lacZ* translational fusion on a centromeric plasmid (pZK0) were grown to mid-log phase at 23°C in selective galactose-based media. At zero time, portions of each culture were shifted to 37°C, and duplicate aliquots were taken at 30-min intervals, while a portion was left at 23°C. DS111 is DS110 with an integrated copy of the *GAL1p-SSA1* vector (□, ■). DS112 is DS110 with an integrated copy of the control *GAL1/10* parent vector (○, ●). Open symbols indicate the 23°C cultures, and closed symbols indicate the heat-shocked cultures. The plotted β-galactosidase activity levels represent the average of two measurements at each time point. (B) Strains DS111 and DS112 were grown to mid-log phase at 23°C in selective glucose-based media. Duplicate samples were taken before (stippled bars; 23°C) and 90 min after (filled bars) a shift to 37°C. β-Galactosidase activity is plotted alongside data taken from the experiment shown in panel A.

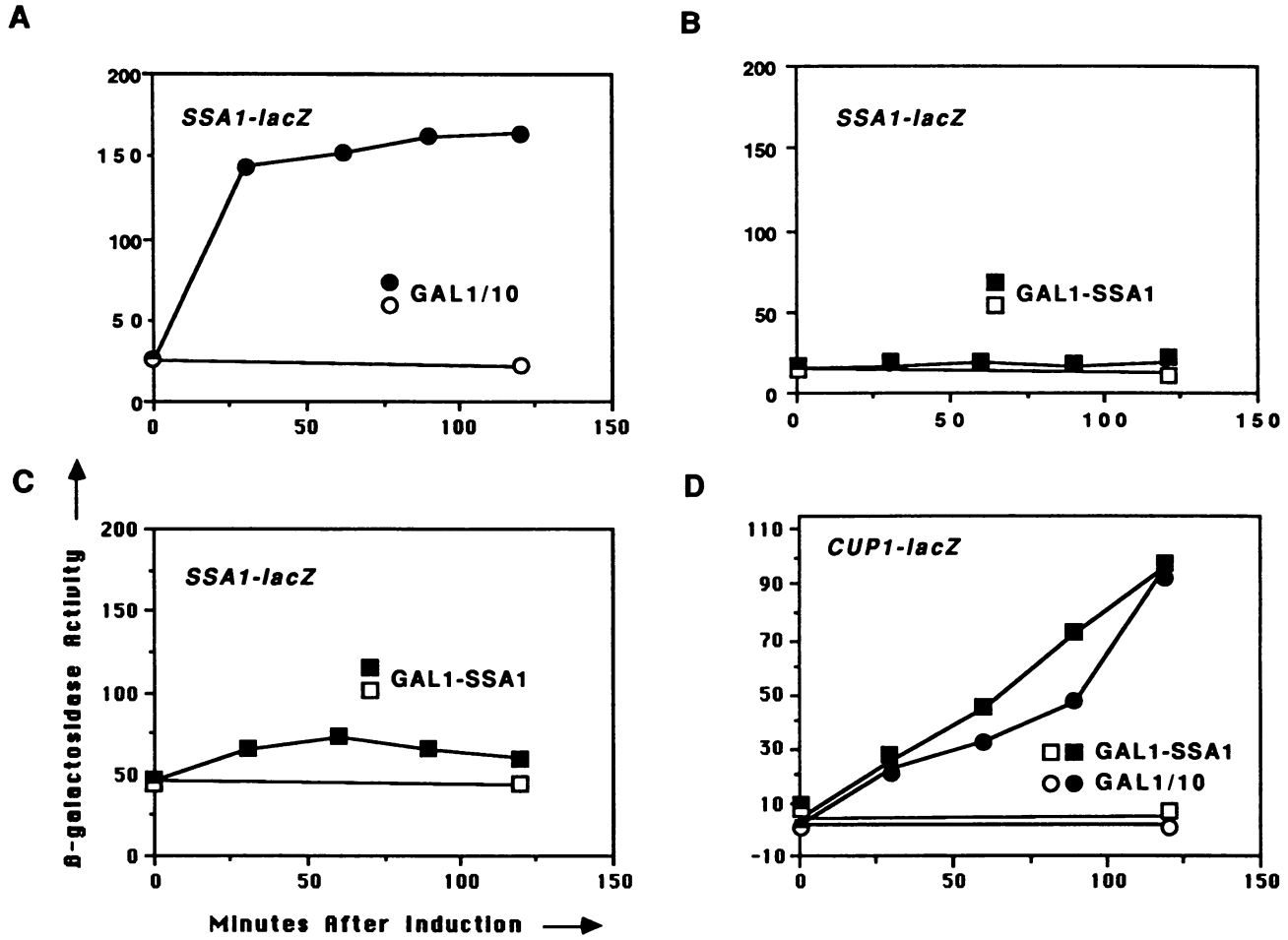

FIG. 4. Effect of a large excess of Ssa1p on expression of the *SSA1-lacZ* and *CUP1-lacZ* translational fusions. The strains used in the experiments shown in panels A, B, and C contain an integrated copy of the *SSA1-lacZ* translational fusion at the *SSA1* locus. Those in panel D contain a *CUP1-lacZ* translational fusion on a *TRP1*-containing centromeric vector. Cells were grown to mid-log phase at 23°C in selective galactose-based medium. At zero time, portions of each culture were shifted to 37°C, and duplicate aliquots were taken at 30-min intervals to measure β-galactosidase activity. In the experiment shown in panel D, cells were diluted 1% with 50 mM $CuSO_4$ at the time of the temperature shift to induce *CUP1-lacZ*. The graphs show the basal (□, ○) and induced (■, ●) levels of β-galactosidase activity, as defined in Materials and Methods, and represent the average of two measurements at each time point. (A) Diploid strain DS116, which carries the control YEpGAL1/10 vector. (B) Newly transformed diploid strain DS115, containing the YEpGAL1-SSA1 vector. (C) Diploid strain DS115 after repeated subculture. (D) DS118, which contains the control YEpGAL1/10 plasmid (○, ●), and DS117, which carries the YEpGAL1-SSA1 plasmid (□, ■). The stability of the *CUP1-lacZ-TRP1* centromeric vector was measured at the completion of the assay by spreading cells of each type on YPD plates and scoring the resulting colonies for tryptophan prototrophy. There was no apparent loss of the *CUP1-lacZ-TRP1* vector from either strain.

carrying YEpGAL1-SSA1 and growing on galactose-based media as high-level overexpression.

Yeast cells that are forced to express high levels of Ssa1p grow poorly. The doubling time of strain DS115 (carrying YEpGAL1-SSA1) in selective galactose-based medium is about 15 h as compared with a doubling time of about 6.5 h for the isogenic control strain, DS116 (carrying YEpGAL1/10). The poor growth of *SSA1*-overexpressing strains complicates the interpretation of *SSA1* regulatory studies in two ways. First, the effect of high constitutive levels of the *SSA1* protein on the expression of the *SSA1-lacZ* fusion must be distinguished from the indirect effects, if any, of poor growth. Second, the long doubling time of cells grossly overexpressing *SSA1* allows any cells that escape the forced overproduction of this protein, by gene conversion of the chromosomal *leu2* marker and loss of the *GAL1p-SSA1* vector, for example, to overgrow the culture. In fact, gene conversion of the genomic *leu2-3,112* allele to wild type did occur (not shown), allowing some DS115 cells to lose YEpGAL1-SSA1. For this reason, single-colony isolates of both the *SSA1*-overexpressing and control strains were used to inoculate fresh cultures at the beginning of each experiment, and the number of cell generations prior to the heat shock was kept to a minimum. Poor growth of the *GAL1p-SSA1*-containing cells was taken as evidence of continued *SSA1* overexpression.

When these precautions were used, strain DS115, which carries the high-level Ssa1p-overproducing plasmid, showed a nearly complete block of induction after heat shock (Fig. 4A and B), while the basal β-galactosidase activity was about 71% lower than that measured in the control strain (DS116). After a number of days in culture, however, the DS115 cells showed an increase in the basal β-galactosidase level and a slightly greater induction ratio. In the experiment

pictured in Fig. 4C, for example, the basal β-galactosidase activity of the subcultured DS115 cells was about twice that of the control and was induced about 64% by heat shock. This increase in inducibility as the cells were subcultured most likely reflected a decrease in the levels of Ssa1p, due to loss of YEpGAL1-SSA1. We believe the basal rate of β-galactosidase synthesis in the older DS115 culture is elevated because overexpression of *SSA1* constitutes a stress. Thus, the basal activity of the *SSA1* promoter reflects a balance between activation by stress and repression by Ssa1p. In support of this explanation, *SSA1* overexpression has been shown to stimulate transcription of its heat-inducible relatives *SSA3* (not shown) and *SSA4* (see Fig. 5B).

Excess Ssa1p does not have a global effect on gene expression. Because cells producing high levels of the *SSA1* protein grow poorly, it was necessary to determine whether the reduced expression of the *SSA1-lacZ* fusion was due to a nonspecific effect on transcription or translation. To test the specificity of *SSA1*-mediated negative regulation, the expression of an unrelated gene, *CUP1*, was analyzed in the presence and absence of excess Ssa1p. *CUP1* encodes copperthionein, a copper-binding metallothionein protein in yeasts. Like the endogenous *CUP1* gene, a reporter gene consisting of the *CUP1* 5′-regulatory region fused to the *E. coli lacZ* gene is induced by copper (35). Figure 4D shows the results of an experiment in which the β-galactosidase levels in the *SSA1*-overexpressing strain DS117 and in the control strain, DS118, were measured before and after induction of the *CUP1p-lacZ* fusion with $CuSO_4$. To mimic the conditions used in assaying the *SSA1-lacZ* fusion, the cells were shifted from 23 to 37°C when the inducer ($CuSO_4$) was added. Although the doubling time of the cells overexpressing *SSA1* was more than twice that of the control culture, the induction of the *CUP1p-lacZ* fusion was unaffected.

Similar experiments were performed to determine whether *SSA1* affects the expression of the closely related gene, *SSA2*. Although the coding regions of *SSA1* and *SSA2* are 97% identical, the 5′-regulatory regions of the two genes are not related, and, unlike *SSA1*, *SSA2* shows less than twofold induction by heat. It is not surprising, therefore, that the expression of a hybrid gene consisting of the *SSA2* promoter fused to the *E. coli lacZ* gene was not significantly affected by overexpression of *SSA1*: the level of β-galactosidase in the YEpGAL1-SSA1-containing strain, DS119, was only 6% lower at 23°C and only 21% lower at 37°C than the level of β-galactosidase in the YEpGAL1/10-containing strain, DS120 (not shown).

Additional evidence that the effect of the *SSA1* protein on its own expression is not due to a generalized effect on cellular physiology was provided by protein-labeling experiments (not shown). Similar patterns of expression, as determined by analysis of labeled proteins on a one-dimensional gel, were observed in the control and Ssa1p-overproducing strains, indicating that the synthesis of most proteins is not affected by *SSA1* overexpression. Quantitation of the β-galactosidase band revealed a 90% reduction in the *SSA1-lacZ* fusion protein, consistent with the 90% reduction in β-galactosidase enzymatic activity observed. This experiment, together with the behavior of the *CUP1p-lacZ* and *SSA2-lacZ* fusions in cells containing high levels of Ssa1p, demonstrates that the reduced expression of *SSA1-lacZ* is specifically due to Ssa1p overproduction and is not due to a general effect on RNA or protein synthesis.

Excess Ssa1p reduces the abundance of the *SSA1-lacZ* hybrid message and inhibits expression of a transcriptional

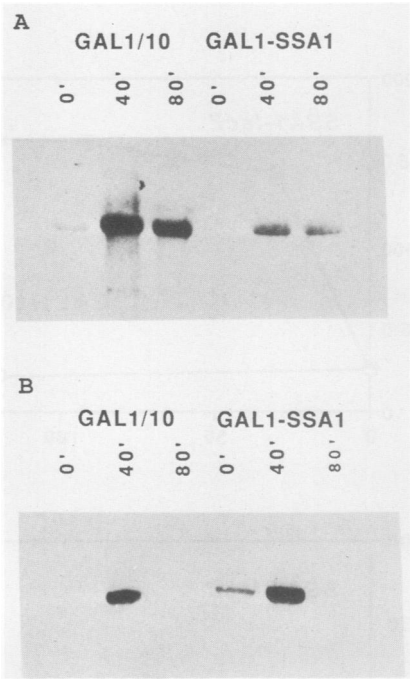

FIG. 5. Effect of high-level *SSA1* overexpression on synthesis of the *SSA1-lacZ* hybrid transcript and on the native *SSA4* and *SSA3* transcripts: RNA synthesis in strains DS115 and DS116. DS115 and DS116 cells were grown to mid-log phase at 23°C in selective galactose-based media. At zero time, portions of each culture were shifted to 37°C. Aliquots were taken at 40 and 80 min to extract RNA, which was done as described in Materials and Methods. The samples were separated on 1% denaturing agarose gels, blotted to nitrocellulose, and hybridized to an isolated piece of the *lacZ* coding region (A) or to a fragment from the coding region of *SSA4* (B). The lanes were shown to contain approximately equal amounts of RNA by staining the blots with methylene blue.

***SSA1-lacZ* fusion.** To determine whether *SSA1* self-regulation is mediated at the level of translation or RNA metabolism, the relative amounts of the *SSA1-lacZ* fusion mRNA in strains DS115 (carrying YEpGAL1-SSA1) and DS116 (carrying YEpGAL1/10) were assessed by Northern (RNA) blot analysis. Cells were heat shocked, and aliquots were removed for measurement of β-galactosidase activity and for RNA extraction. The *SSA1-lacZ* hybrid mRNA was about threefold less abundant in DS115 cells than in DS116 cells (Fig. 5A), while overexpression of *SSA1* resulted in a fivefold reduction of β-galactosidase activity. This result suggests that *SSA1* self-regulation is at least partially mediated at the level of transcription or message stability. Although excess Ssa1p had no effect on the induced levels of the *SSA3* (not shown) and *SSA4* mRNAs (Fig. 5B), the basal levels of these transcripts were increased. Apparently, *SSA1* overexpression constitutes a stress that results in the partial induction of some heat shock genes. Either the basal expression of *SSA3* and *SSA4* is not inhibited by Ssa1p, or the level of Ssa1p in these cells is not sufficient to counteract the Ssa1p-induced metabolic stress.

In an effort to clarify the effect of Ssa1p on *SSA1* transcription, we analyzed the expression of *SSA1* transcriptional fusions. Since HSE2 appears to be one of the primary elements involved in *SSA1* expression (33), *CYC1* promoter-*lacZ* gene fusions in which the *CYC1* upstream activating sequence (UAS) had been replaced by *SSA1* HSE2-containing sequences were used in these experiments. A fusion

containing HSE2 on a 137-bp piece of *SSA1* DNA (positions −262 to −131) in place of the *CYC1* UAS (pZJHSE2-137) (Fig. 6A) has been shown to be heat inducible (33) (Fig. 6B). In the experiment shown in Fig. 6B, β-galactosidase activity rose 63-fold after a heat shock in the control strain, whereas β-galactosidase activity increased only 12-fold in cells producing excess Ssa1p. In the course of numerous trials, some variability in the induced as well as in the basal β-galactosidase levels of DS121 was observed, probably due to the instability of the *GAL1p-SSA1* plasmid, but the induction of the HSE2-*CYC1-lacZ* hybrid was always two to five times greater in DS122 than in DS121. Thus, HSE2-137 contains a sequence element that causes a heterologous promoter to become sensitive to *SSA1* overexpression.

To delineate further the putative autoregulatory element, an additional transcriptional fusion vector, pZJHSE2-40, was assayed in cells transformed with the YEpGAL1-SSA1 and YEpGAL1/10 plasmids. This construct is identical to pZJHSE2-137, with the exception of the sequences that were used to replace the *CYC1* UAS (Fig. 6A). In pZJHSE2-40, a replica of HSE2 positions −203 to −168 was substituted for the UAS in the parent vector. This oligonucleotide, HSE2-40, comprises the 14-bp HSE core, plus 4 5′- and 18 3′-flanking nucleotides. HSE2-40 has been shown to confer both a heat-inducible activity on the *CYC1* promoter and a negative effect on basal transcription (29). The regulation of basal activity is due to the URS that partially overlaps HSE2. In cells expressing wild-type levels of Ssa1p, the basal and heat-inducible activity of plasmid pZJHSE2-40 is similar to that of pZJHSE2-137.

High-level overexpression of *SSA1* did not significantly affect the induced activity of HSE2-40 (Fig. 6C). In heat-shocked cells carrying YEpGAL1-SSA1, β-galactosidase encoded by pZJHSE2-40 increased to within 25% of the level measured in the control cells. Together, the results of the two experiments with the *SSA1* transcriptional fusions suggest the existence of an autoregulatory sequence element located between positions −262 and −131, but not contained within the region −203 to −168.

The *SSA4* protein inhibits its own expression and reduces the level of the *SSA1-lacZ* mRNA. The inhibition of *SSA1* expression by excess Ssa1p led us to ask whether self-regulation is a general property of 70-kDa heat shock proteins in *S. cerevisiae*. As a first step toward answering this question, we tested the effect of excess *SSA4* protein on its own expression by measuring β-galactosidase levels in strains carrying a centromeric *SSA4-lacZ* translational fusion vector and either the YEpGAL1-SSA4 or the YEpGAL1/10 high-copy-number plasmid. Although the basal activity of the *SSA4-lacZ* hybrid was not significantly affected by overexpression of *SSA4*, its induction was partially repressed (Fig. 7A). Sixty minutes after a shift to 37°C, the level of β-galactosidase was about 2.3-fold higher in DS127 than in DS128. Interestingly, the inhibition of *SSA4-lacZ* expression by excess Ssa4p was not as great when the cells were shifted to 39°C (not shown). The induction ratio of DS128 exceeded that of DS127 by a factor of 2.2 at 37°C, but only by a factor of 1.4 at 39°C. These results are consistent with the idea that the amount of hsp70 that must accumulate before its synthesis can be turned off is dependent on the severity of the stress (11). According to this hypothesis, a lesser inhibition of the *SSA4-lacZ* fusion would be expected under increasingly stressful conditions, given a fixed constitutive level of Ssa4p.

To determine the effect of excess *SSA4* protein on *SSA1*expression, a strain containing the *SSA1-lacZ* transla-

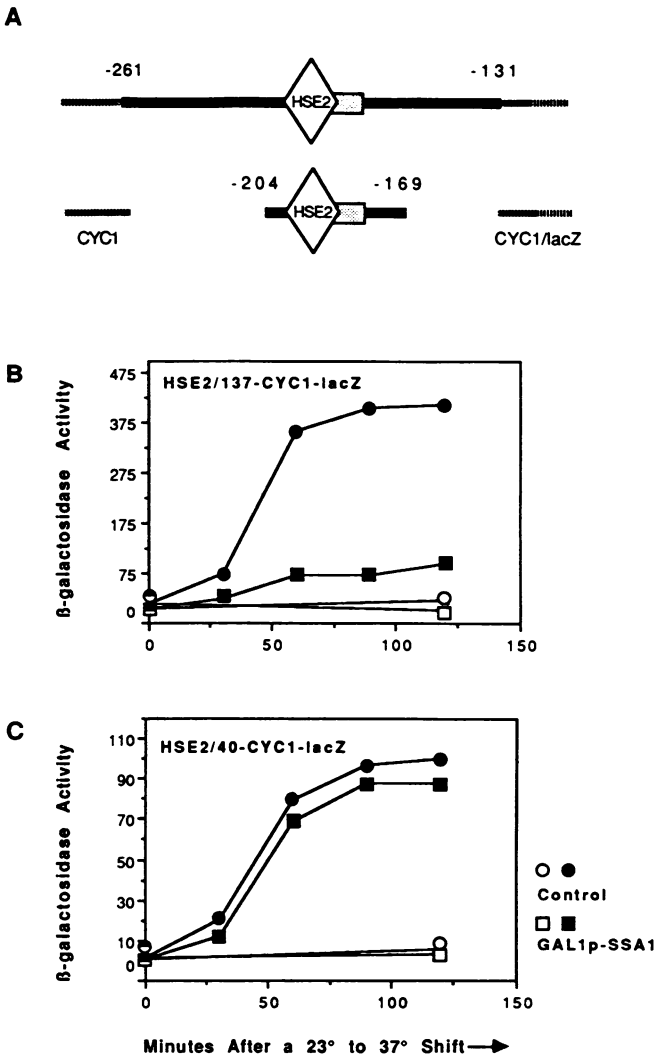

FIG. 6. Effect of a large excess of Ssa1p on expression of the HSE2/137-*CYC1-lacZ* and HSE2/40-*CYC1-lacZ* transcriptional fusions. (A) HSE2-*CYC1-lacZ* transcriptional fusion vectors. HSE2/137-*CYC1-lacZ*: The black line represents the 130-bp *Alu*I fragment isolated from the *SSA1* 5′-regulatory region, which contains HSE2, signified by the diamond, and the negative regulatory element, URS, pictured as a grey rectangle. HSE2/40-*CYC1-lacZ*: The black line represents the synthetic oligonucleotide containing a copy of HSE2 and a copy of the URS. The grey and striped lines represent the *CYC1* promoter sequence and the *lacZ* coding region, respectively. (B and C) All cells were grown to mid-log phase at 23°C in selective galactose-based media. At zero time, portions of each culture were shifted to 37°C, and duplicate samples were taken at 30-min intervals to measure β-galactosidase activity. Open symbols indicate the 23°C cultures, and closed symbols indicate the heat-shocked cultures. The plotted β-galactosidase activity levels represent the average of two measurements at each time point. (B) Diploid strains DS121 (□, ■) and DS122 (○, ●). Both strains carry an integrated copy of the HSE2/137-*CYC1-lacZ* transcriptional fusion at the *URA3* locus. In addition, DS121 carries the YEpGAL1-SSA1 vector and DS122 carries the YEpGAL1/10 vector. (C) Diploid strains DS125 (□, ■) and DS126 (○, ●). Both strains carry an integrated copy of the HSE2/40-*CYC1-lacZ* transcriptional fusion at the *URA3* locus. In addition, DS125 carries the YEpGAL1-SSA1 vector and DS126 carries the YEpGAL1/10 vector.

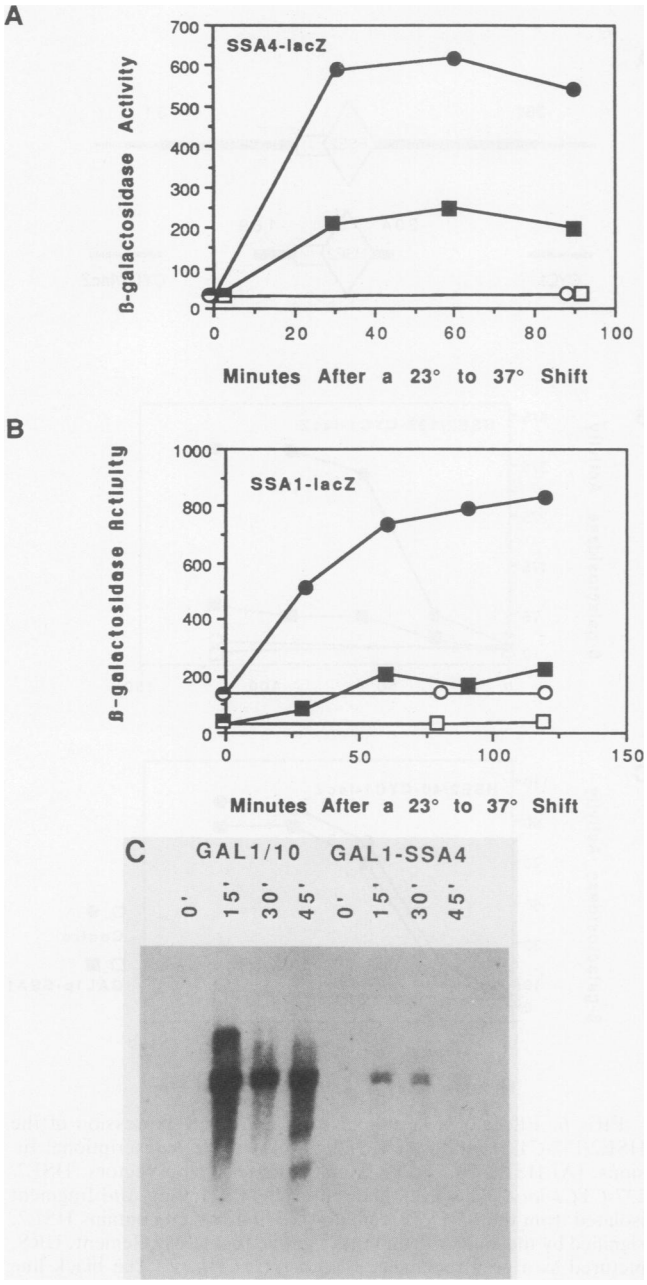

FIG. 7. Effect of the *SSA4* protein on its own synthesis and on *SSA1*. Cells were grown to mid-log phase at 23°C in selective galactose-based media. At zero time, portions of each culture were shifted to 37°C, and duplicate aliquots were taken at 30-min intervals to measure β-galactosidase activity. Open symbols indicate cells grown at 23°C, and closed symbols indicate the heat-shocked cultures. (A) Activity of an *SSA4-lacZ* translational fusion before and after heat shock in cells carrying the YEpGAL1-SSA4 vector, strain DS127 (□, ■), and in cells carrying the control YEpGAL1/10 vector, strain DS128 (○, ●). (B) Activity of an *SSA1-lacZ* translational fusion before and after heat shock in cells carrying the YEpGAL1-SSA4 vector, strain DS129 (□, ■), and in cells carrying the control YEpGAL1/10 vector, strain DS130 (○, ●). (C) Aliquots of DS129 and DS130 cultures were taken at 15, 30, and 45 min after a 23 to 37°C shift, and RNA was extracted as described in Materials and Methods. The RNA samples were separated on 1% denaturing agarose gels, blotted to nitrocellulose, and hybridized to an isolated piece of the *lacZ* coding region. The lanes were shown to contain approximately equal amounts of RNA by staining the blots with methylene blue.

tional fusion integrated at the *SSA1* locus was transformed with the YEpGAL1-SSA4 and the YEpGAL1/10 vectors to give strains DS129 and DS130, respectively. Log-phase cultures of DS129 and DS130 were shifted from 23 to 37°C, and samples were taken at 15-min intervals to extract RNA and at 30-min intervals to measure β-galactosidase activity. The results of this experiment are presented in Fig. 7B and C. Clearly, a high level of the *SSA4* protein inhibited the activity of the *SSA1-lacZ* fusion. Although the activity of the *SSA1-lacZ* fusion in the experimental cells increased upon heat shock, the basal and induced levels of β-galactosidase were 3.6- and 6.8-fold lower in the DS129 cells as compared with the control. A similar repression was observed in the synthesis of the *SSA1-lacZ* hybrid mRNA in DS129, as measured by Northern blot analysis (Fig. 7C). These observations suggest that the *SSA4* protein inhibits the expression message abundance.

DISCUSSION

Ssa1p plays a part in negatively regulating its own synthesis. In this study, we have shown that high constitutive levels of the *SSA1* protein inhibit the expression of an *SSA1-lacZ* translational fusion. Threefold overexpression of *SSA1* reduces both basal and induced activities of this fusion by about twofold, without affecting the relative increase in its activity after heat shock; higher levels of Ssa1p almost completely block the induction of the *SSA1-lacZ* translational fusion. Several lines of evidence indicate that this decrease in Ssa1p expression is specific. Since the *SSA2-lacZ* and *CUP1p-lacZ* translational fusions, in addition to the HSE2-40-*CYC1-lacZ* transcriptional fusion, were all unaffected by a large excess of Ssa1p, the repression of *SSA1-lacZ* by Ssa1p must not have been due to a general inhibition of gene expression. These results, along with the inhibitory effects of Ssa4p overproduction, suggest that the 70-kDa heat shock proteins of *S. cerevisiae* play some part in negatively regulating their own synthesis. The overexpression of the *ssa1* and *ssa2* mutant transcripts in *ssa1 ssa2* cells is consistent with this idea (E. A. Craig, unpublished results).

Evidence for transcriptional control of *SSA1* self-regulation: a *cis*-acting element that confers sensitivity to the *SSA1* protein is located near HSE2. The decreased abundance of the *SSA1-lacZ* hybrid mRNA in cells overexpressing Ssa1p suggests that *SSA1* self-regulation is mediated at the level of transcription, message stability, or both. The repression of the transcriptional HSE2-137-*CYC1-lacZ* fusion by excess Ssa1p supports the idea that *SSA1* self-regulation is at least partially controlled at the transcriptional level. Since expression of the HSE2-137-*CYC1-lacZ* fusion was inhibited by high levels of Ssa1p, while a fusion containing the core HSE2 plus the URS (pZJHSE2-40) was not, the 137-bp segment must contain a sequence element that confers sensitivity to Ssa1p repression and which is absent from the 40-bp fragment containing HSE2 and the URS. We have tentatively named this proposed element SRS1, for self-regulating sequence, number one. SRS1 may overlap HSE2 and the adjoining element, URS, but it must not be contained within them, since pZJHSE2-40 is insensitive to *SSA1* overexpression. Alternatively, SRS1 may be located in the proximal or distal regions of the 137-bp DNA fragment containing HSE2.

There are a number of mechanisms by which SRS1 might confer sensitivity to the *SSA1* protein. Although SRS1 might affect the secondary structure of the DNA in the HSE2

region, thus affecting the binding of HSF, we think it is more likely that it is a binding site for a *trans*-acting regulatory factor. The regulatory factor might bind to SRS1 and thereby block the binding of HSF to HSE2, much as the simian virus 40 T antigen, the polyomavirus T antigen, and the initiator protein of plasmid R6K are thought to exclude RNA polymerase from their respective promoters (9, 10, 20). Alternatively, it might prevent some other regulatory factor from binding to SRS1 via a protein-protein interaction. The dependence of *SSA1* self-regulation on a sequence distinct from HSE2 and the HSE2-associated URS, however, argues against models in which a *trans*-acting protein inactivates HSF, or modifies the URS binding factor, and thus affects the affinity of these regulatory proteins for their binding sites. Of course, the data reported here do not allow us to identify such a *trans*-acting factor. A likely candidate, however, is the *SSA1* protein itself.

Physiological significance of *SSA1* self-regulation. The simplest model for the *SSA1* self-regulation, similar to that proposed by DiDomenico and colleagues for *Drosophila HSP70* (11), is that Ssa1p accumulates to a particular threshold level following heat shock, whereupon it represses its own synthesis. If this hypothesis is correct, the *SSA1-lacZ* fusion should not be heat inducible in cells expressing a moderately high constitutive level of Ssa1p. Our results are not entirely consistent with this idea. Although the basal and induced activities of the *SSA1-lacZ* fusion are reduced in cells constitutively producing an approximately threefold excess of the *SSA1* protein, the induction ratio is unaffected. To block the induction of the *SSA1-lacZ* translational fusion completely, approximately 10 times the normal heat shock level of the *SSA1* protein is required.

Complete repression of the *SSA1* promoter by moderately high levels of Ssa1p may not be observed for a number of reasons. First, the *SSA1-lacZ* translational fusion used in this study consists of the 5′-regulatory region of *SSA1* and its first eight codons, joined to *lacZ*. This hybrid gene may not contain all of the signals that are required for *SSA1* self-regulation. Additional *cis*-acting regulatory elements might lie within the *SSA1* coding region, as found in the mammalian β-tubulin gene (14), or downstream of its 3′ terminus, as in the case of the lambda *int* gene (15).

A second point to consider is the intracellular location of the overproduced protein. In stressed *Drosophila* cells, hsp70 migrates to the nucleus after it is synthesized in the cytoplasm. If the cells are allowed to recover, hsp70 returns to the cytoplasm, but moves back to the nucleus upon a second heat shock (39). Although the intracellular location of Ssa1p is not known, it is reasonable to presume that mislocalization of the excess protein would lessen its self-regulating activity. Indeed, the intracellular location of hsp26 in yeasts is thought to depend on the physiological state of the cell (31).

A requirement for high-level overexpression of *SSA1* might also be expected if its autoregulatory function depends on a modification or a conformational shift of Ssa1p. Perhaps heat shock causes both the induction of Ssa1p synthesis and the activation of its inhibitory function. Raising the level of *SSA1* protein does not ensure that the pool of molecules capable of acting as repressors will also increase. Alternatively, the negative regulation of *SSA1* may require a factor, or factors, in addition to the *SSA1* gene product. A number of self-regulating proteins that depend on corepressors have been described (1, 3, 38). If a second factor is required for the negative regulation of *SSA1*, it is not surprising that moderate overexpression of *SSA1* alone is not sufficient to repress the system fully. Although our data do not bear on this question, there are some good candidates for a second variable. In *Drosophila* cells, the higher-order chromatin structures containing heat shock genes are disrupted upon temperature elevation (42), and the transcriptional activity of the HSF is thought to be induced (34, 41). Either of these factors could influence *SSA1* expression during recovery from heat shock.

Finally, it may be necessary to modify the simplest model of *SSA1* self-regulation. The heat shock genes in yeasts, as in other organisms, are set for a rapid induction. It may be impossible to block that induction without greatly perturbing the system. Ogden et al. (27) have reported results that suggest that the autoregulation of *araC* is greatly reduced or nonexistent during the derepression of the system. Similarly, the mechanism of Ssa1p self-regulation may be transiently inoperative in the first minutes following heat shock.

***SSA4* overexpression affects the expression of *SSA1*.** Overproduction of Ssa4p inhibits the abundance of the *SSA1-lacZ* hybrid message as well as the expression of its own gene. This finding underlines the complexity of the regulation of the *SSA* subfamily. It is possible that the inhibition of Ssa1p synthesis after heat shock depends on the accumulation of Ssa3p or Ssa4p and that overexpression of Ssa1p merely mimics the activity of the natural regulator. Our finding that overexpression of Ssa4p inhibits *SSA1* expression is consistent with this idea, but we cannot yet determine whether overproduction of Ssa4p allows this protein to usurp the role of Ssa1p in *SSA1* regulation, whether a large excess of Ssa1p allows it to mimic the regulatory activity of Ssa4p, or whether both proteins perform regulatory functions. Further analysis will be required to clarify the interactions among the members of this subfamily and their effects on the expression of other heat-inducible genes.

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