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Suppression of an Hsp70 Mutant Phenotype in *Saccharomyces cerevisiae* through Loss of Function of the Chromatin Component Sin1p/Spt2p

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The Ssa subfamily of Hsp70 molecular chaperones in the budding yeast *Saccharomyces cerevisiae* has four members, encoded by *SSA1*, *SSA2*, *SSA3*, and *SSA4*. Deletion of the two constitutively expressed genes, *SSA1* and *SSA2*, results in cells which are slow growing and temperature sensitive. In this study, we demonstrate that an extragenic suppressor of the temperature sensitivity of *ssa1 ssa2* strains, *EXA1-1*, is a loss-of-function mutation in *SIN1*/*SPT2*, which encodes a nonhistone component of chromatin. Loss of function of Sin1p leads to overexpression of *SSA3* in the *ssa1 ssa2* mutant background, at a level which is sufficient to mediate suppression. In a strain which is wild type for *SSA* genes, we detected no effect of Sin1p on Ssa3p expression except under conditions of heat shock. Existing data indicate that expression of *SSA3* in the *ssa1 ssa2* mutant background as well as in heat-shocked wild-type strains is mediated by the heat shock transcription factor HSF. Our findings suggest that it is HSF-mediated induction of *SSA3* which is modulated by Sin1p. The *EXA1-1* suppressor mutation thus improves the growth of *ssa1 ssa2* strains by selectively increasing HSF-mediated expression of *SSA3*.

The Hsp70s (heat shock proteins of 70 kilodaltons) comprise a highly abundant and well-conserved family of molecular chaperones, involved in facilitating a wide variety of cellular processes. In the budding yeast *Saccharomyces cerevisiae*, one of the most abundant subfamilies of Hsp70 is the cytosolic Ssa subfamily. There are four Ssa proteins, which have more than 80% amino acid identity with each other. Together, the Ssa proteins are essential for vegetative growth; at least one must be expressed at high levels in order for cells to be viable (39). The four subfamily members have distinct patterns of regulation. During exponential growth, only Ssa1p and Ssa2p are detectable; expression of Ssa3p and Ssa4p requires induction by heat shock or other stressors (38). Processes in which the Ssa proteins have been implicated include translocation of substrates into mitochondria and the endoplasmic reticulum (3, 11, 13), thermotolerance (33), and protein folding (24). Ssa proteins also function in the autoregulation of the heat shock response, as was demonstrated by the observation that overexpression of either Ssa1p or Ssa4p interferes with induction of a reporter gene under the control of a heat shock promoter element (HSE) (37).

The importance of Ssa protein in the regulation of heat shock genes has also become apparent in the analysis of *ssa1 ssa2* double mutant strains. *ssa1 ssa2* strains, lacking functional copies of the two constitutively expressed subfamily members, are unable to form colonies at elevated temperatures and exhibit slow growth at permissive temperatures (12). The physiology underlying this phenotype is likely to be complex. The presence of a wild-type copy of *SSA4* allows the strain to survive, as indicated by the inviability of an *ssa1 ssa2 ssa4* triple mutant (39). Neither *SSA3* nor *SSA4* would ordinarily be expressed during exponential growth; their expression in *ssa1 ssa2* mutant strains (which appears to be stronger for Ssa4p than for Ssa3p) is mediated by a generalized, constitutive induction of HSE-mediated transcription (5, 7). This induction is not specific to *SSA3* and *SSA4*, as an HSE-regulated marker gene is also induced (7) and a number of heat shock-regulated genes are constitutively expressed in this background (12, 12a; this report). *ssa1 ssa2* cells can therefore be thought of as constitutively heat-shocked.

The complexity of this situation became apparent in the previous analysis of an extragenic suppressor of the slow growth of *ssa1 ssa2* cells, a suppressor called *EXA3-1* (for extragenic suppressor of Hsp70 subfamily A). *EXA3* is allelic to *HSF1*, which encodes the heat shock transcription factor HSF (28). The *EXA3-1* mutation creates an amino acid substitution in the DNA-binding domain of HSF, a change which reduces HSF-mediated expression under both basal and heat-shock conditions. Ssa protein is thus lower in *ssa1 ssa2 EXA3-1* cells than in the parental *ssa1 ssa2* strain. Furthermore, increasing HSF-mediated expression by introducing an extra copy of *HSF1* into *ssa1 ssa2* cells causes further impairment of growth rather than suppression (16). These data clearly demonstrate that a cytosolic deficiency in Ssa protein in just one aspect of the phenotype of the *ssa1 ssa2* strain. The constitutive expression of heat shock genes which allows these cells to survive (by enabling expression of *SSA3* and *SSA4*) introduces a secondary problem: likely the overexpression of another heat-inducible protein(s) which is detrimental for growth. The *EXA3-1* mutation represents a delicate solution to this problem, adjusting heat shock gene expression so that the toxicity of the response is minimized while Ssa protein is maintained at levels which, though reduced, are apparently sufficient for growth.

Here we report the characterization of another extragenic suppressor of the temperature sensitivity of *ssa1 ssa2* strains, *EXA1* (28). We show that *EXA1* is allelic to *SIN1*/*SPT2*, which encodes a nonhistone component of chromatin. Sin1p/Spt2p is an abundant, nonessential, nucleus-localized protein with the ability to bind DNA nonspecifically in vitro and to affect ex-

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TABLE 1. Yeast strains used in this study

Strain	Shorthand genotype	MAT	Genotype or description[a]	Reference
MW116	*a1 a2 a3*	α	*ssa1::HIS3 ssa2::LEU2 ssa3::TRP1*	39
MW163	*a1 a2 a4 [A2pA3]*	**a**	*ssa1::HIS3 ssa2::LEU2 ssa4::URA3 [pYe(CEN3)30::SSA2pSSA3]*	39
BB127[b]		**a**/α	*ssa1-3::HIS3/ssa1-3::HIS3 ssa2-2::URA3/ssa2-2::URA3 EXA1-1/+*	This study
BB346[c]		**a**/α	*ssa1-3::HIS3/+ ssa2-2::URA3/+*	This study
BB358[d]		**a**/α	*ssa1-3::HIS3/+ ssa2-2::URA3/+ Δsin1::TRP1/+*	This study
BB359[e]	*a1 a2 EXA1-1*	α	*ssa1-3::HIS3 ssa2-2::URA3 EXA1-1*	This study
BB360[e]		**a**	*ssa1-3::HIS3 ssa2-2::URA3 EXA1-1*	This study
BB361[f]	*a1 a2 Δsin1*	α	*ssa1-3::HIS3 ssa2-2::URA3 Δsin1::TRP1*	This study
BB363[f]	*a1 a2*	α	*ssa1-3::HIS3 ssa2-2::URA3*	This study
BB364[f]	*Δsin1*	**a**	*Δsin1::TRP1*	This study
BB365[f]	*WT*	**a**	WT at *SSA1*, *SSA2*, *SSA3*, *SSA4*, and *SIN1*	This study
BB366[g]		**a**/α	*ssa1-3::HIS3/ssa1-3::HIS3 ssa2-2::URA3/ssa2-2::URA3 Δsin1::TRP1/EXA1-1*	This study

[a] All strains also carry the following markers: *his3-11,15 leu2-3,112 ura3-52 trp1-Δ1*.
[b] Diploid product of haploid strains JN14 and JN37 (28).
[c] Diploid product of haploid strains JN49 and JN54 (28).
[d] Derived from BB346 by integrative transformation.
[e] Derived from BB127 by sporulation and dissection.
[f] Derived from BB358 by sporulation and dissection.
[g] Diploid product of haploid strains BB360 and BB361.

pression of a variety of loci in vivo (21). *SIN1* (for Swi-independent transcription) is so named because loss of Sin1p function allows constitutive expression of a group of genes normally dependent on the multiprotein Swi/Snf complex (36). The Swi/Snf complex from both yeast and human cells has the ability to alter nucleosomal structure in chromatin, rendering promoter templates more accessible to activators such as Gal4p and general transcription factors such as TATA-binding protein (8, 18, 19, 22, 30). Conversely, Sin1p is thought to act at some promoters to maintain chromatin in a repressive state, inaccessible to the transcription machinery. Mutations in *SIN1* also allow increased expression of marker genes whose promoters have been disrupted by the transposable element Ty; hence, its alternate name is *SPT2* (for suppressor of Ty insertion) (32, 40). For simplicity, we will use the name *SIN1* throughout this report.

We show that loss of function of Sin1p mediates the suppression conferred by *EXA1-1* and increases the HSF-mediated expression of Ssa3p. Expression of other heat-shock-regulated genes, including Ssa4p and Hsp104, is not increased. In fact, overexpression of Ssa3p in the *EXA1-1* suppressor strain may serve to downregulate other heat shock-responsive genes, thus simultaneously increasing levels of Ssa protein while decreasing expression of any heat-inducible proteins which are detrimental for growth.

MATERIALS AND METHODS

Strains, media, and genetic techniques. *Escherichia coli* cells were grown in LB (0.5% yeast extract, 1% tryptone, 1% NaCl) supplemented with 100 μg of ampicillin per ml as necessary for plasmid selection. *E. coli* cells were transformed by electroporation with a Gene Pulser apparatus (Bio-Rad Laboratories, Hercules, Calif.) according to the manufacturer's instructions or by a $CaCl_2$-based protocol (25). Restriction enzymes and buffers were from New England Biolabs (Beverly, Mass.), Promega (Madison, Wis.), or Boehringer Mannheim (Indianapolis, Ind.) and were used according to manufacturer's instructions.

Yeast strains were grown in YPD (1% yeast extract, 2% peptone, 2% dextrose) or in selective medium (0.67% yeast nitrogen base without amino acids, 2% dextrose, supplemented with required amino acids as necessary). Liquid selective medium used for immunoblot analysis and determination of growth curves contained an elevated level of dextrose (6%) to delay the diauxic shift. Yeast strains were transformed by electroporation using the Gene Pulser apparatus (Bio-Rad Laboratories) or according to a modified lithium acetate protocol (14). Matings were done on YPD, with selection of zygotes by micromanipulation. Sporulation plates contained 1% potassium acetate, 0.1% yeast extract, 0.05% dextrose, and amino acid supplements.

Yeast strains are shown in Table 1. *ssa1* and *ssa2* disruptions in MW116 and MW163 were constructed by inserting an auxotrophic marker gene into the coding region, replacing codons 307 to 386; *ssa3* and *ssa4* disruptions in these strains are insertions of the markers indicated at positions near the 5′ end of each gene. *ssa1-3::HIS3* and *ssa2-2::URA3* mutants each carry a replacement of codons 10 to 160 with the respective marker gene. A comparison of strains carrying the original *ssa1* and *ssa2* disruptions (those used in MW116 and MW163) with those carrying the more extensive deletions, *ssa1-3* and *ssa2-2*, found them to be phenotypically indistinguishable (39).

Isolation of extragenic suppressors of the *ssa1 ssa2* high-temperature growth defect, including *EXA1-1*, has been previously described (28). To identify and clone *EXA1-1*, a high-copy-number genomic library was prepared from an *ssa1-3 ssa2-2 EXA1-1* strain (28) by *Sau*3A partial digestion and ligation into *Bam*HI-digested YEp351 (17). This library was transformed into an *ssa1 ssa2* strain, and transformants were screened for improved high-temperature growth.

Disruption of *SIN1* by linear transformation was conducted with plasmid construct WB51 (21), a generous gift of Ira Herskowitz. This construct replaces an 805-bp *Pst*I-*Hin*dIII fragment of *SIN1* (which encodes amino acids [aa] 48 to 315 of 333 aa) with the marker gene *TRP1*. Disruption was confirmed by PCR and restriction analysis.

RNA analysis. Cells were either grown to mid-log phase at 32°C or grown to mid-log phase at 23°C and subjected to a 30-min heat shock at 39°C as indicated and harvested by centrifugation. Total cellular RNA was isolated by the heat-freeze extraction method as previously described (34) and quantitated spectrophotometrically.

S1 nuclease protection assays were carried out essentially as described previously (2). For detection of *SSA3* mRNA, an oligonucleotide was synthesized which is complementary to nucleotides (nt) +29 to −23 (where the AUG start codon is nt +1 to +3) and which carries an 8-nt 3′ overhang. (Synthesis was by Genosys, Inc., The Woodlands, Tex.) The oligonucleotide was purified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), eluted into 0.3 M sodium acetate, phenol extracted, ethanol precipitated, and spectrophotometrically quantitated before labeling with polynucleotide kinase and hybridization to total RNA. For standardization, a second oligonucleotide, which is complementary to nt 295 to 258 of the mature actin RNA and which carries a 26-nt 3′ overhang, was used in parallel. Data from four separate sets of S1 digestion reactions gave similar results and were combined to give mean values (see Fig. 4A).

Primer extension analysis was performed essentially as described previously (2), with an 18-nt primer complementary to nt 52 to 35 of *SSA4* to generate a 106-nt product. For standardization, an oligonucleotide complementary to the snRNA U4 was used to generate a 44-nt product. Three sets of similar primer extension results were combined to give mean values (see Fig. 4B).

***SSA3* promoter fusion plasmids.** The promoter fusion vectors used to express *SSA3* at various levels were constructed by Mumberg et al. (26). PCR was used to create an *Spe*I site immediately upstream of the start codon of *SSA3* for fusion to the various promoters. All PCR-generated sequences were confirmed by dideoxy sequencing using Sequenase (United States Biochemical, Cleveland, Ohio) to verify that no PCR-induced mutations had been introduced. Ligation of a 2.2 kb *Spe*I-*Hin*dIII *SSA3* fragment into the expression vectors put *SSA3* under the control of the *ADH1*, *TEF2*, or *GPD* promoter on either a low or a high-copy-number vector, as indicated in Table 2. The plasmids are ordered from 1 to 6 (Table 2) according to the level of β-galactosidase activity expressed from a similar fusion of *lacZ* to each construct, as reported by Mumberg et al. (26). These expression levels correlated well with our observations for Ssa3p (see Fig. 5A).

Growth tests. Growth tests on agar plates were routinely conducted by growing cells overnight at permissive temperatures and then diluting them into sterile

TABLE 2. *SSA3* expression plasmids

Plasmid no.	Full plasmid name	Copy no. (description)[a]
1	p415ADH:SSA3	Low (CEN)
2	p415TEF::SSA3	Low (CEN)
3	p425ADH:SSA3	High (2μ)
4	p415GPD:SSA3	Low (CEN)
5	p425TEF:SSA3	High (2μ)
6	p425GPD:SSA3	High (2μ)

[a] CEN, centromere containing; 2μ, 2μ based.

water as a series of 10-fold serial dilutions. Aliquots of each dilution were spotted onto agar plates which were incubated at the temperatures indicated. Liquid growth tests were conducted by growing cultures overnight at the temperature indicated, verifying that they were in exponential growth, and monitoring their growth through optical density (OD) measurements (at 600 nm) taken approximately every 45 to 75 min over a period of 6 to 12 h. These measurements were used to construct an exponential growth curve, which was used to calculate doubling time. As indicated in the figure legends, each doubling time calculation was repeated for multiple transformants and multiple cultures, and these data were combined to obtain an average population doubling time and the standard error of measurement for each strain.

Anti-Ssa3p/Ssa4p antibodies. PCR was used to generate a *Bam*HI site immediately upstream of codon 539 and an *Eco*RI site immediately downstream of codon 603 of both *SSA3* and *SSA4*. Each *Bam*HI-*Eco*RI fragment was cloned into pGEX-KT (15) to create a fusion of glutathione-*S*-transferase to a 65-aa peptide of either Ssa3p or Ssa4p. Each fusion protein was expressed in *E. coli* and purified by adsorption to glutathione-agarose beads (Sigma Chemical Co., St. Louis, Mo.). Eluate from the beads was used to inoculate rabbits.

Rabbit antisera generated against the Ssa3 fusion protein and the Ssa4 fusion protein had indistinguishable reactivities. Neither showed any reactivity against Ssa1p or Ssa2p, and both showed a similar reactivity with Ssa3p and Ssa4p. This result is not surprising, given that 49 of the 65 Ssa amino acids in the fusion constructs are identical between Ssa3p and Ssa4p and many of the remaining residues are conservative substitutions. The antiserum raised against the Ssa3 fusion protein was arbitrarily chosen for use in these experiments.

Immunoblot analysis. For immunoblot analysis, protein extracts were prepared by vortexing cells in the presence of glass beads in a buffer containing 2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris (pH 8.0), and 1 mM EDTA; boiling the lysates in Laemmli sample buffer (4); and clarifying the extracts by centrifugation in a microcentrifuge at top speed for 5 min. For each experiment, a preliminary polyacrylamide gel was run and stained with Coomassie blue, and the intensity of Coomassie staining in each lane was quantitated by densitometric scanning and used to normalize loadings. Equivalent amounts of each extract were then loaded on a second polyacrylamide gel and transferred to nitrocellulose. Membranes were blocked in Tris-buffered saline (20 mM Tris [pH 7.4], 150 mM NaCl) containing 0.5% polyoxyethylene-sorbitan monolaurate (Tween 20; Sigma Chemical Co.) as a blocking agent; this solution was also used for incubations with both primary and secondary antibodies. Detection utilized secondary anti-rabbit immunoglobulin G antibody conjugated to horseradish peroxidase and the ECL Western blotting detection kit (Amersham Corp., Arlington Heights, Ill.)

Ssa3p and Ssa4p are similar in predicted size (70,554 versus 69,657 Da) and are not always separated by SDS-PAGE. To achieve reliable separation, we used 7.5% polyacrylamide gels which contained 0.3% bisacrylamide, rather than the more standard 0.2%. Gels (11 cm by 16 cm by 0.75 mm) were electrophoresed at 30 mA for approximately 4 h, at which point the Ssa proteins had migrated about halfway through the separating gel. When this procedure was used, Ssa3p consistently migrated more slowly than Ssa4p (see in Fig. 2 to 4). Use of this protocol allowed us to determine that the protein which was originally observed to accumulate in *ssa1 ssa2 EXA1-1* strains (28) is in fact Ssa3p and that this protein comigrates with the Ssa3p expressed in strains which are wild-type at the *EXA1*/*SIN1* locus.

RESULTS

Identification of *EXA1-1*. *EXA1-1* (Extragenic suppressor of Hsp70 subfamily A) was isolated as a dominant suppressor of the temperature sensitivity of an *ssa1 ssa2* double deletion strain (28). To clone the gene responsible for suppression, a genomic library was constructed from an *ssa1 ssa2 EXA1-1* haploid strain by using a high-copy-number yeast vector (see Materials and Methods). Library plasmids were transformed into an *ssa1 ssa2* strain and screened for the ability to confer improved high-temperature growth. One class of plasmids identified in this screen were those carrying either of the remaining two *SSA* gene family members, *SSA3* or *SSA4*, indicating that when present in high copy number, either *SSA3* or *SSA4* is able to functionally substitute for the lack of *SSA1* and *SSA2*. Suppression by another library plasmid, p24, is shown in Fig. 1A.

As diagrammed in Fig. 1B, suppressor plasmid p24 contains a 4.77-kb insert which includes the 3′ end of *RAD4* (approximately 50% of the coding region), the entire *SIN1*/*SPT2* open reading frame, and approximately 24% of an adjacent full-length Ty1 transposable element. Truncation of the p24 insert fragment at a *Hin*dIII site at the 3′ end of *SIN1*/*SPT2* had no effect on suppression (Fig. 1B), indicating that the Ty1 element is not relevant to suppression. Similarly, disruption of the *RAD4* coding region at an internal *Nde*I site had no effect. By contrast, disruption of the *SIN1*/*SPT2* coding region at a *Pst*I

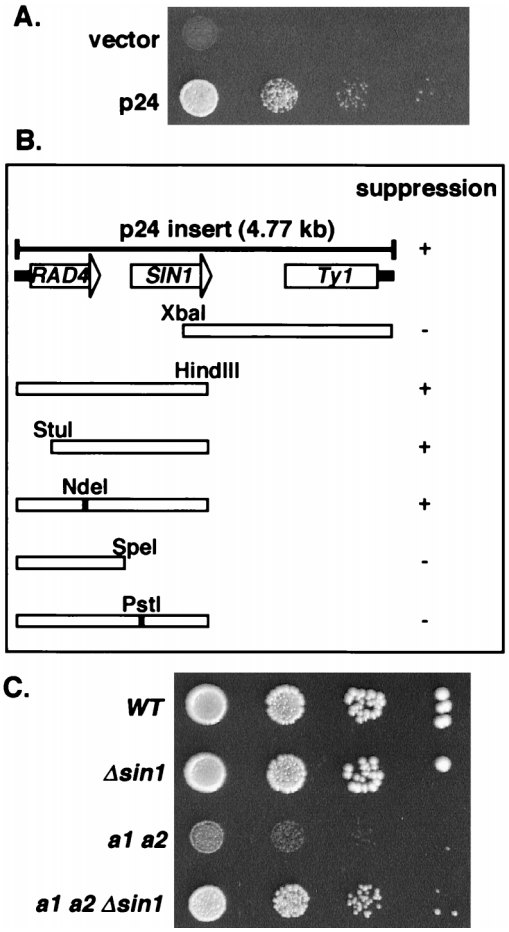

FIG. 1. Suppression of the *ssa1 ssa2* growth defect by library plasmid p24. (A) *ssa1 ssa2* strain BB363 was transformed with either a vector control or with library plasmid p24. Transformants were selected, grown at permissive temperatures, and then tested for improved growth by spotting 10-fold serial dilutions onto an agar plate containing a medium selective for the plasmid. The plate shown was incubated at 34°C for 3 days. (B) Subclone analysis of p24. The yeast insert contained on each plasmid tested is represented schematically by a rectangular box, with the ability (+) or inability (−) of each construct to cause suppression indicated to the right. Filled-in boxes represent disruption of the *Nde*I site in *RAD4* (by filling in the 2-nt 5′ overhang) or the *Pst*I site in *SIN1* (by deletion of the 4-nt 3′ overhang). Both disruptions alter the reading frame of the affected gene. (C) Deletion of *SIN1* causes suppression. Haploid progeny of diploid strain BB358 were isolated, tested for the indicated mutations by replica plating, and spotted as 10-fold serial dilutions to test growth. The YPD plate shown was incubated at 34°C for 3 days. In both panels A and C, approximately equal numbers of cells were spotted for each strain. *WT*, wild type.

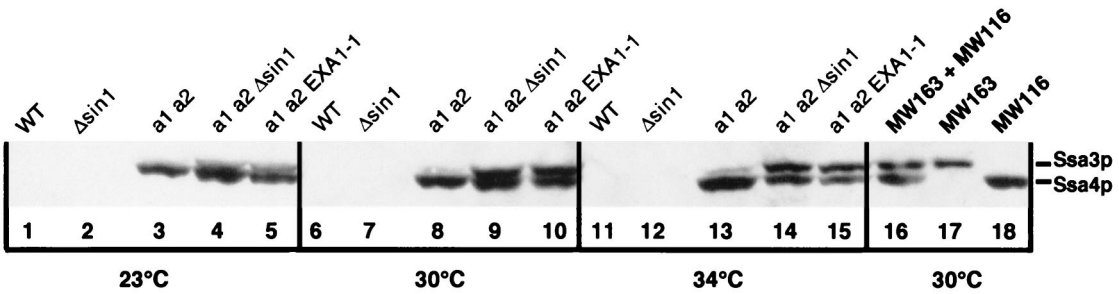

FIG. 2. Loss of function of Sin1p increases Ssa3p expression in *ssa1 ssa2* strains. Cells were grown in YPD to mid-log phase at the temperature indicated. Extracts were probed by immunoblot analysis using an antiserum reactive against Ssa3p and Ssa4p (see Materials and Methods). Extracts from MW163 and MW116 were used as markers and were adjusted to give equivalent signals, requiring approximately 1/15 as much MW163 extract as MW116 extract. WT, wild type.

site near the 5′ end eliminated suppression, demonstrating that it is *SIN1*/*SPT2* which is responsible for the ability of p24 to confer improved growth. Interestingly, truncation at the *Hin*dIII site removes the last 17 codons of *SIN1*/*SPT2* without affecting suppression. This result was explained when the *SIN1*/*SPT2* region of the *EXA1-1* library plasmid was subcloned and sequenced and a nonsense mutation which introduces a stop codon 5′ of the *Hin*dIII site, at codon 227 of 333, was revealed.

We predicted that a nonsense mutation at codon 227 of *SIN1* would generate a dominant negative allele, since a previous genetic analysis of *SIN1* found that truncation of the protein coding region at positions corresponding to aa 179, 213, 271, 303, 318, or 324 results in a dominant negative phenotype (23). To verify that suppression of the *ssa1 ssa2* growth phenotype resulted from a loss of Sin1p function, we generated a chromosomal deletion of *SIN1* in a diploid strain already heterozygous for deletion of both *SSA1* and *SSA2*, creating strain BB358 (see Materials and Methods for details). Haploid progeny of BB358 were then isolated and tested for growth. Deletion of *SIN1* has no discernible effect on growth of an otherwise wild-type strain, as previously reported (40). However, growth of *ssa1 ssa2* strains is significantly improved by deletion of *SIN1* (Fig. 1C). When compared side by side, the level of growth improvement achieved by deletion of *SIN1* was indistinguishable from that generated by introduction of suppressor plasmid p24 (data not shown), strongly suggesting that the allele of *SIN1* carried on the suppressor plasmid has a dominant negative phenotype.

To confirm that *EXA1* is allelic to *SIN1*, *ssa1 ssa2 EXA1-1* strain BB360 was crossed to *ssa1 ssa2* Δ*sin1* strain BB361. The resulting diploid strain, BB366, was sporulated and dissected, and haploid progeny were tested for growth at 34°C. All progeny of 22 four-spore tetrads showed growth comparable to the parental strains and significantly better than that of *ssa1 ssa2* strains, indicating that *EXA1-1* is tightly linked to *SIN1*. We conclude that the *EXA1-1* suppressor mutation is the mutation identified on library plasmid p24: the introduction of a premature stop codon in *SIN1* in place of codon 227.

Loss of function of Sin1p causes overexpression of *SSA3*. Sin1p is an abundant nuclear protein with nonspecific DNA-binding activity whose function affects the expression of a wide variety of genes (21, 31, 36, 40). We surmised that loss of Sin1p function causes improved growth of *ssa1 ssa2* strains by altering expression of a gene or genes whose product is important for growth of this strain. Given our isolation of both *SSA3* and *SSA4* as high-copy-number suppressors of the *ssa1 ssa2* strain, altered regulation of one or both of these loci through loss of function of Sin1p was an obvious hypothesis.

To facilitate analysis of Ssa3p/Ssa4p expression, antibodies were raised against a protein fusion of glutathione-*S*-transferase and a peptide from the carboxyl terminus of Ssa3p (aa 539 to 603; see Materials and Methods). This region of Ssa3p has 75% amino acid identity with Ssa4p but only 48 and 45% identity with Ssa1p and Ssa2p, respectively. The resulting antibodies do not cross-react with Ssa1p or Ssa2p, as indicated by the absence of reactive bands in an extract from a wild-type strain in exponential growth (e.g., Fig. 2, lane 1). Previous studies using [^{35}S]methionine and two-dimensional gel electrophoresis have shown that under these conditions, Ssa1p and Ssa2p are abundant, while Ssa3p and Ssa4p are undetectable (39). The antibodies do react with both Ssa3p and Ssa4p, as demonstrated by analysis of extracts from strains MW163 and MW116, in which Ssa3p or Ssa4p, respectively, is the only Ssa protein expressed (lanes 17 and 18). This analysis also demonstrated that Ssa3p and Ssa4p are separable under the conditions used, with Ssa3p migrating slightly more slowly than Ssa4p (lanes 16 to 18).

Our immunoblot analysis confirmed previous observations (39) that Ssa3p and Ssa4p expression is induced in *ssa1 ssa2* cells compared to wild-type cells (for example, compare lane 8 to lane 6). This effect is considerably more dramatic for Ssa4p, but Ssa3p is clearly detectable in *ssa1 ssa2* cells upon longer film exposures. Comparison of an *ssa1 ssa2* strain (lane 8) with either an *ssa1 ssa2* Δ*sin1* strain or an *ssa1 ssa2 EXA1-1* strain (lanes 9 and 10) demonstrated that loss of function of Sin1p leads to further elevated expression of Ssa3p. This effect was evident during steady-state growth at all temperatures tested, but was only detectable in strains carrying deletions of *ssa1* and *ssa2*. Ssa3p was not detectable under steady-state growth conditions in strains which were wild-type at *SSA1* and *SSA2*, regardless of the presence or absence of function of Sin1p (for example, compare lane 7 with lane 6). It is possible that loss of Sin1p increases the expression of Ssa3p under these conditions, and yet the level of Ssa3p remains below the limit of detection. Alternatively, expression of Ssa3p in wild-type cells during exponential growth may be repressed by a mechanism that does not require Sin1p. This finding thus led us to the question of whether we could detect an influence of Sin1p on *SSA3* expression in cells which are wild-type for Ssa.

In wild type cells, Ssa3p expression is normally induced in response to two distinct physiological signals: heat shock and the transition into stationary phase. Heat shock induction is mediated by an HSE in the *SSA3* promoter, a binding site for the heat shock transcription factor HSF (5). During the transition to stationary phase, induction of *SSA3* is mediated by an adjacent element, the post-diauxic shift upstream activation sequence, whose activity is increased by the presence of the HSE. No induction occurs without this upstream activation sequence, but the final level of stationary-phase expression is increased by the presence of the HSE. (6). To assess whether

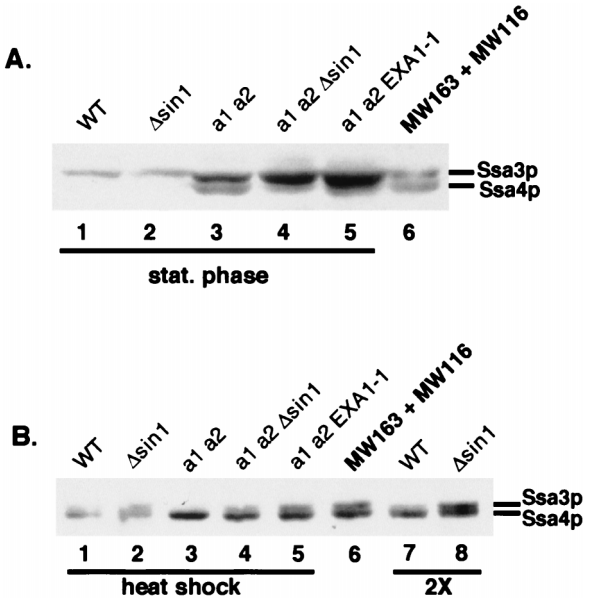

FIG. 3. The effect of Sin1p function on Ssa3p expression is apparent in heat-shocked but not stationary-phase cells. (A) As in Fig. 2, except cultures were grown at 30°C for 4 days to stationary (stat.) phase. Combined extracts from MW116 and MW163 (log-phase cultures [shown in Fig. 2]) were used as markers for Ssa3p and Ssa4p. (B) Cultures were grown to mid-log phase at 23°C and then transferred to 39°C for 75 min before harvest. Lanes 7 and 8 are the same as lanes 1 and 2, except with twice as much protein (2×) loaded. WT, wild type.

Sin1p plays a role in modulating Ssa3p expression in wild-type as well as *ssa1 ssa2* cells, we used extracts from stationary-phase and heat-shocked cultures.

In stationary-phase extracts, Ssa3p was detectable in all strains tested (Fig. 3A). In an *ssa1 ssa2* background, loss of function of Sin1p led to increased levels of Ssa3p, just as was seen in extracts from exponentially growing cultures (compare lanes 4 and 5 to lane 3). In cells which were wild-type for *SSA*, however, loss of Sin1p function had no detectable effect (compare lane 2 to lane 1). In heat-shocked extracts, the picture was different (Fig. 3B). Ssa3p expression was again higher in *ssa1 ssa2* Δ*sin1* and *ssa1 ssa2 EXA1-1* strains than in an *ssa1 ssa2* strain, as was seen for the other conditions tested (compare lanes 4 and 5 to lane 3). Under conditions of heat shock, however, Ssa3p expression was also higher in a Δ*sin1* strain than in a wild-type strain (compare lane 2 to lane 1 and lane 8 to lane 7). Since both the elevated *SSA3* expression seen in an *ssa1 ssa2* strain and the induction of *SSA3* by heat shock are mediated by the HSE (5), these findings suggest that loss of Sin1p function potentiates the ability of the heat shock transcription factor HSF to stimulate expression of *SSA3*. Interestingly, although induction of *SSA4* is also mediated by HSF (7), no increase in Ssa4p levels due to loss of function of Sin1p was detected under any conditions tested (Fig. 2 and 3).

Given existing data about Sin1p and its role in transcriptional regulation and chromatin structure (21, 31, 36, 40), we predicted that alteration of *SSA3* expression by Sin1p occurs at the level of transcription. To test this prediction, we monitored *SSA3* mRNA levels by S1 nuclease protection analysis in *ssa1 ssa2* strains with or without a functional copy of *SIN1* (Fig. 4A). As expected, loss of Sin1p function correlates with elevated levels of *SSA3* message: *SSA3* mRNA levels were 3- to 4.5-fold higher in *ssa1 ssa2* Δ*sin1* or *ssa1 ssa2 EXA1-1* strains compared to an *ssa1 ssa2* strain. In contrast, *SSA4* mRNA levels are decreased approximately twofold by loss of Sin1p function, as assessed by primer extension analysis (Fig. 4B).

Elevated Ssa3p expression is sufficient to explain suppression. To study the effects of Ssa3p expression on the growth of an *ssa1 ssa2* strain more closely, we constructed a set of expression constructs in which the *SSA3* protein coding region was regulated by a series of three heterologous promoters, each of which could be expressed from either a low- or a high-copy-number plasmid (26). For simplicity, we will refer to these plasmid constructs by the numbers 1 to 6, in order of the level of expression they provide (Table 2). To assess Ssa3p expression from these constructs in comparison with that generated by loss of Sin1p function, cultures were grown to early log phase in a selective liquid medium at 34°C. Extracts from these cultures were used in immunoblot analysis, as shown in Fig. 5A. As expected, Ssa3p expression increased progressively through the series of promoter constructs. Promoter constructs 2 and 3 gave levels of Ssa3p which were comparable to that generated by loss of Sin1p function, while constructs 4 to 6 gave much higher levels of expression.

To assess the effect of Ssa3p overexpression, growth tests were performed on liquid cultures, and the cultures' optical densities at 600 nm (OD) were used to monitor growth. OD measurements were taken throughout early log phase and used to calculate the doubling time of each strain. Combined measurements taken from multiple transformants are presented in Fig. 5B. In these assays, the plasmid constructs conferred varying degrees of growth, ranging from no significant improvement over the vector control for construct 1 to growth approaching that of the wild type for construct 6. Plasmid construct 2, which generated a level of Ssa3p expression similar to that conferred by lack of Sin1p function (Fig. 5A), also

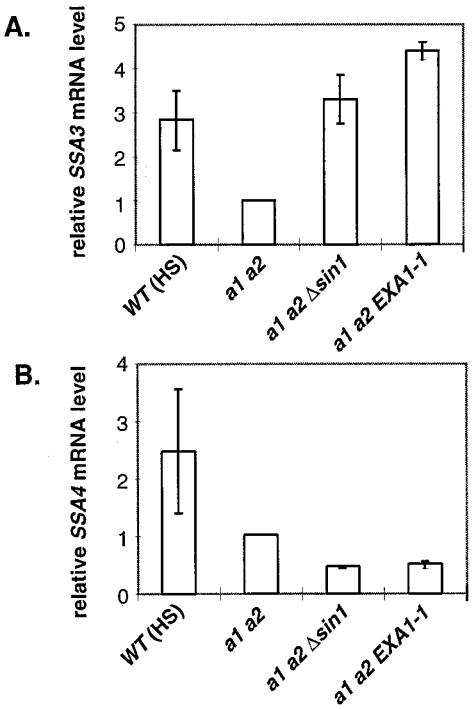

FIG. 4. Sin1p affects *SSA3* expression at the level of mRNA. Cultures were grown to mid-log phase at 32°C. For comparison, a wild-type culture was grown to mid-log phase at 23°C and subjected to a 30-min heat shock at 39°C [*WT*(HS)]. RNA was prepared and analyzed by S1 nuclease protection (A) or primer extension (B) assay as described in Materials and Methods. Results were normalized to those with strain *ssa1 ssa2* and are reported as a mean of three to four experiments, with error bars representing ± 2 standard errors.

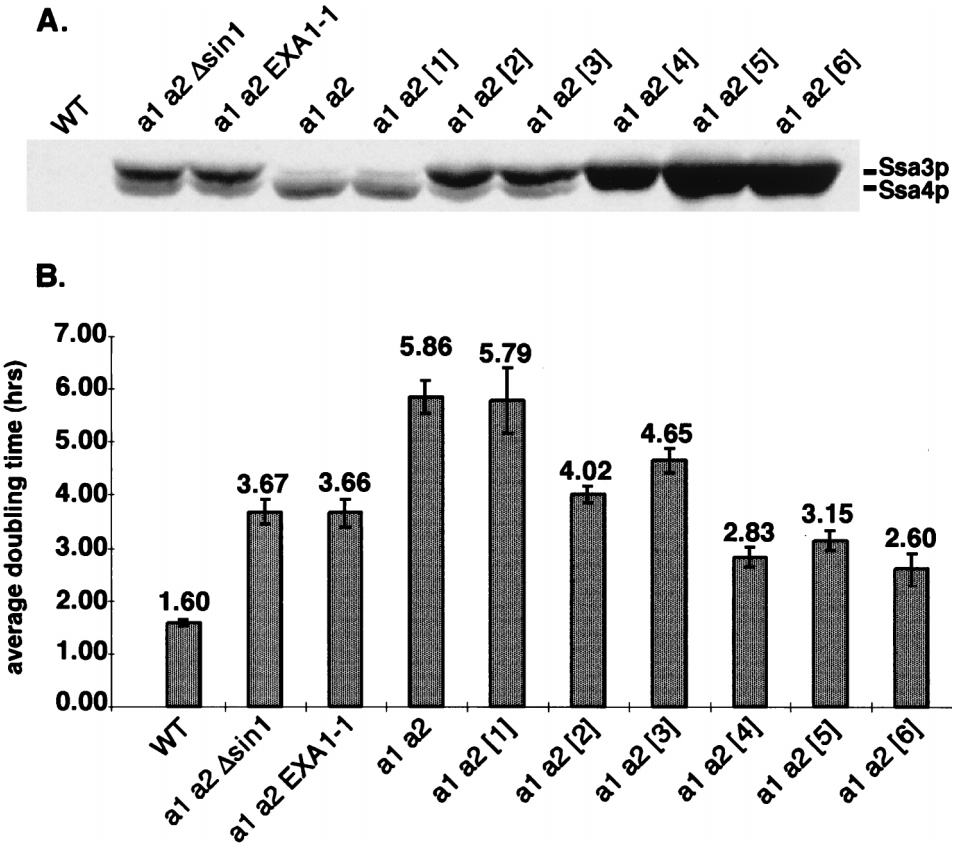

FIG. 5. Expression of varying levels of Ssa3p in an *ssa1 ssa2* strain. Cultures were grown at 34°C in a liquid medium selective for plasmids 1 to 6 (Table 2). Plasmid numbers are given in brackets; where no plasmid is indicated, strains were carrying the vector plasmid pRS315. (A) Anti-Ssa3/4p immunoblot. Cells were harvested in early log phase. Extracts were prepared and subjected to SDS-PAGE and immunoblot analysis using antibodies reactive against Ssa3p and Ssa4p. (B) Growth at 34°C throughout early log phase was monitored by OD measurements. For each strain, average doubling time was determined from at least two independent plasmid transformants and from a total of at least three separate cultures. Error bars represent ±2 standard errors. WT, wild type.

conferred a degree of growth improvement similar to that seen in *ssa1 ssa2* Δ*sin1* or *ssa1 ssa2 EXA1-1* strains (Fig. 5B). This result demonstrates that the overexpression of Ssa3p caused by lack of Sin1p function is sufficient to mediate significant improvement of growth.

The improvement of growth conferred by each *SSA3* plasmid to a population of cells in liquid culture under selective conditions (Fig. 5B) does not correlate directly with the average level of Ssa3p expressed (Fig. 5A). This finding likely reflects the lower stability of plasmids containing the 2μ origin of replication compared to centromere-containing plasmids (27) and a resultant underestimate of the growth improvement conferred by 2μ-based plasmids 3, 5, and 6.

In the course of these experiments, we noted that overexpression of Ssa3p from the promoter fusion constructs correlates with lowered expression of Ssa4p (Fig. 5A). This effect, although more subtle, is also detectable upon loss of function of Sin1p: Ssa4p expression is decreased slightly as Ssa3p expression is increased. Immunoblot analysis of extracts from the promoter fusion series demonstrated that Hsp104 expression is also inversely correlated with Ssa3p expression (data not shown). These observations suggest that Ssa3p, like Ssa1p and Ssa4p (37), can serve as a negative regulator of the heat shock response.

Effect of loss of function of Sin1p on expression of chaperones functionally related to Ssa proteins. The data shown in Fig. 5 demonstrate that elevated expression of Ssa3p is sufficient to explain suppression of the high-temperature growth defect of *ssa1 ssa2* cells by *EXA1-1*. However, as reported in the original characterization of the *EXA* suppressors (28), *EXA1-1* is able to confer improved growth to *ssa1 ssa2* mutant cells in the absence of a functional copy of *SSA3*. Given this finding, we decided to investigate the regulation of other genes which might be important to the growth of *ssa1 ssa2* strains. As an initial search for such genes, we analyzed the expression of various proteins which are known to cooperate with the Ssa proteins in cellular functions.

Hsp104 and Sti1p are both heat-inducible proteins with demonstrated functional interactions with Ssa. Hsp104 is important for the acquisition of thermotolerance in yeast. Genetic studies have demonstrated that the Ssa proteins are important for thermotolerance in the absence of Hsp104, and that Hsp104 is important for the vegetative growth of cells deficient in Ssa protein (33). Similarly, deletion of *sti1* causes a severe growth defect in combination with deletions of *ssa1* and *ssa2* (29). Sti1p is the yeast homolog of mammalian Hsp70 organizing protein, or Hop (for a review, see reference 20). Both Hsp104 and Sti1p showed elevated expression in strains lacking *ssa1* and *ssa2*, but expression was not further elevated by loss of function of Sin1p (Fig. 6A and B). In fact, Hsp104 is sensitive to feedback regulation of the heat shock response by Ssa3p (see above), and its levels, if anything, are slightly decreased upon loss of function of Sin1p.

In contrast, immunoblot analysis revealed that expression of Ydj1p was somewhat elevated in an *ssa1 ssa2* Δ*sin1* strain and an *ssa1 ssa2 EXA1-1* strain compared to an *ssa1 ssa2* strain, a

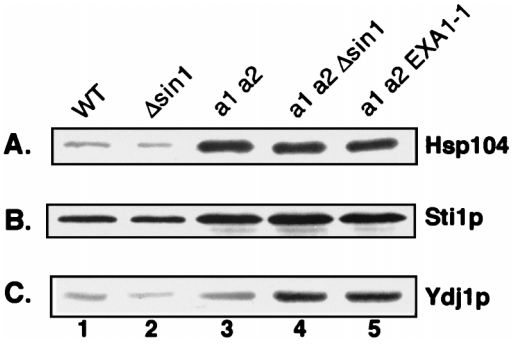

FIG. 6. Effect of loss of Sin1p function on expression of various chaperones. Cells were grown to mid-log phase at 30°C in YPD, separated by SDS-PAGE, and subjected to immunoblot analysis using antibodies reactive against Hsp104 (A), Sti1p (B), or Ydj1p (C). WT, wild type.

pattern similar to that seen for Ssa3p (Fig. 6C, compare lanes 4 and 5 to lane 3). Ydj1p is an *S. cerevisiae* homolog of the *E. coli* protein DnaJ, which cooperates with the Hsp70 DnaK in facilitating protein refolding and in lambda replication (24, 41). Deletion of *YDJ1* causes poor growth in otherwise wild-type cells (1, 9, 10) and is synthetically lethal with the quadruple mutation *ssa1-45* (Ts) *ssa2 ssa3 ssa4* (3). Compared to Ssa3p, the effect of loss of function of Sin1p on Ydj1p expression was minor and varied from experiment to experiment; the result shown in Fig. 6C is from an experiment in which the effect was particularly clear.

We wanted to determine whether overexpression of Ydj1p could improve the growth of an *ssa1 ssa2* strain, either alone or in combination with an elevated level of Ssa3p. To test this possibility, we began with two *ssa1 ssa2* strains. One of these was carrying the vector plasmid pRS315 and the other was carrying *SSA3* promoter fusion plasmid 2 (Table 2), which leads to Ssa3p expression comparable to that seen in the context of *EXA1-1-1* (Fig. 6A). We transformed each of these strains with either a vector control or a high-copy-number plasmid carrying *YDJ1* under the control of its own promoter. Introduction of the *YDJ1* plasmid into either strain caused a significant increase in expression of Ydj1p as assessed by immunoblot (Fig. 7A) and yet had no discernible effect on growth (Fig. 7B and C). We conclude that while expression of Ydj1p may be slightly affected by loss of function of Sin1p in *ssa1 ssa2* strains, elevated Ydj1p expression does not play a role in improved growth.

DISCUSSION

We have shown that the previously identified extragenic suppressor of *ssa1 ssa2* strain temperature sensitivity, *EXA1-1*, is a dominant loss-of-function mutation in *SIN1*. Loss of Sin1p function causes overexpression of *SSA3* in the context of HSF-mediated induction. *SSA3* is able to functionally complement the loss of *SSA1* and *SSA2*, and overexpression of *SSA3* is sufficient for suppression.

Sin1p limits HSF activity at *SSA3* but not at related loci. Sin1p is a highly abundant protein (~10,000 molecules per cell) which binds DNA nonspecifically in vitro and is known to affect a wide variety of loci in vivo, including *HO*, *INO1*, and Ty-disrupted promoters (21, 31, 36, 40). We have added *SSA3* to this growing list. In addition, Sin1p apparently modulates expression of other cellular components important in Ssa-deficient strains, as loss of function of Sin1p is able to improve the growth of an *ssa1 ssa2 ssa3* strain (28). Intriguingly, however, Sin1p's effect is not completely general. Our data show that loss of Sin1p function increases HSF-mediated expression of *SSA3*, and yet HSF-mediated expression of related genes, including *SSA4*, *HSP104*, and *STI1* is not similarly affected. This degree of specificity suggests the involvement of other regulatory factors which remain to be identified.

Our data suggest an interplay between HSF and Sin1p in the regulation of *SSA3* in which Sin1p limits the extent to which HSF can activate transcription. In yeast, HSF binds constitutively to its recognition sequence, the HSE. In promoters such as *SSA1*, this constitutive binding is important to basal expression (35). Upon heat shock, HSF is further activated, elevating expression of promoters such as *SSA1* and allowing expression of promoters such as *SSA3*. In the case of *SSA3* (but not *SSA4*, *HSP104*, or *STI1*), this activation may be limited by a compact nucleosomal structure maintained by Sin1p.

The Ssa proteins have a critical role in heat shock gene regulation. Our finding that the *EXA1-1* mutation leads to elevated expression of *SSA3* is in striking contrast to data regard-

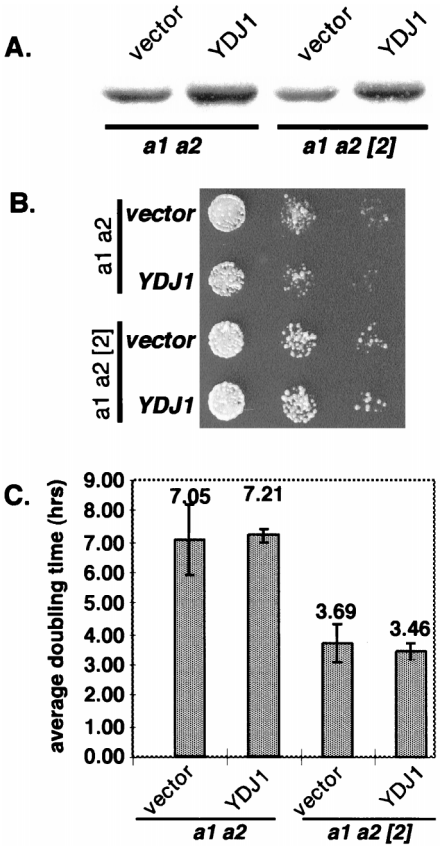

FIG. 7. Overexpression of Ydj1p does not improve growth. *ssa1 ssa2* strain BB363 carrying either a vector plasmid (*a1 a2*) or promoter fusion plasmid p415TEF:SSA3 (plasmid 2 [Fig. 5]; *a1 a2* [2]) was transformed with either pRS424 (vector) or pYW2, a multicopy plasmid carrying *YDJ1* (*YDJ1*). Transformants were selected at a permissive temperature, 23°C. (A) Immunoblot analysis. Cultures were grown at 34°C, and extracts were prepared and subjected to immunoblot analysis using antibody reactive against Ydj1p. (B) Colony growth tests. Transformants were grown at 23°C and then spotted as 10-fold serial dilutions onto an agar plate containing a medium selective for pRS424 or pYW2. The plate shown was incubated for 3 days at 34°C. Approximately equal numbers of cells were spotted for each strain. (C) Growth tests in liquid. Cultures were grown overnight in a medium selective for pRS424 or pYW2 at 34°C, and their growth throughout early log phase was monitored by OD measurements. For each strain, the average doubling time (indicated atop each bar) was determined from three independent plasmid transformants, and from a total of nine separate cultures. Error bars represent ±2 standard errors.

ing *EXA3-1*, the other extragenic suppressor of *ssa1 ssa2* cells identified in the same screen. *EXA3-1*, a mutation which alters the DNA-binding domain of HSF and thus lowers its ability to activate transcription, lowers the expression of heat shock-responsive genes, including *SSA3*. While *EXA3-1* and *EXA1-1* both exert their effects through altered regulation of HSF-mediated transcription, their effects are different: *EXA3-1* lowers HSF-activated transcription in a generalized fashion, while *EXA1-1* elevates HSF-induced expression of a particular gene, *SSA3*. These findings demonstrate that at least two possible mechanisms for improving the growth of *ssa1 ssa2* strains exist: compensating for loss of Ssa expression by selectively upregulating *SSA3* or alleviating the consequences of chronic heat shock gene overexpression by downregulating HSF activity. However, these two mechanisms of suppression may not be as disparate as they first appear. The Ssa proteins are important in autoregulation of the heat shock response (37). In the course of the present work, we have noted that overexpression of Ssa3p from heterologous promoters has the effect of lowering heat shock gene expression in an *ssa1 ssa2* mutant background, as evidenced by immunoblot analysis of Ssa4p and Hsp104. Thus, it is possible that the relevant effect of *EXA1-1* is not elevated levels of Ssa3p per se but is the ability of Ssa3p to autoregulate the heat shock response, decreasing HSF-mediated expression of some other component. The effect of Ssa3p on heat shock gene regulation is extremely subtle at the level of Ssa3p overexpression caused by *EXA1-1*, but a delicate alteration in expression may be just what is required.

The original motivation for characterizing extragenic suppressors of *ssa1 ssa2* strain temperature sensitivity was to identify the essential role of the Ssa proteins among the many roles which have been proposed. By increasing expression of Ssa3p, *EXA1-1* may alleviate deficiencies in any or all of the cellular pathways which require Ssa protein. However, *EXA1-1* has only one obvious characteristic in common with *EXA3-1*: both serve to downregulate the constitutive expression of heat shock genes in an *ssa1 ssa2* background. *EXA3-1* does so directly, by lowering the activity of HSF. *EXA1-1* does so indirectly, by increasing the expression of Ssa3p, which can in turn downregulate heat shock gene expression. In both cases, the effect on steady-state levels of heat shock proteins is extremely subtle, and yet the improvement of growth is quite clear. Whatever other functions Ssa protein may have in the cell, its role in regulation of the heat shock response is clearly both critical and exquisitely precise.

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