Isolation and Characterization of Extragenic Suppressors of Mutations in the *SSA* hsp70 genes of *Saccharomyces cerevisiae*

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ABSTRACT

Saccharomyces cerevisiae strains that contain null alleles of two hsp70 genes, *SSA1* and *SSA2*, are temperature sensitive for growth. In this study, extragenic suppressors of *ssa1 ssa2* have been isolated. Suppression is due to mutations at nuclear loci designated *EXA1*, *EXA2* and *EXA3* for *EX*tragenic suppressor hsp70 subfamily *A*. Two of the four *EXA1* alleles are dominant as is *EXA3-1*. The other two *EXA1* alleles as well as the sole *EXA2* allele are recessive. *EXA1* mutations lead to accumulation of a previously uncharacterized form of hsp70. *EXA2* and *EXA3* mutations affect the regulation of the stress response. In *exa2-1 ssa1 ssa2* strains the gene products of the remaining *SSA* hsp70 genes, *SSA3* and *SSA4* (Ssa3/4p), accumulate to higher levels. The *EXA3-1* mutation results in increased accumulation of both Ssa3/4p and the hsp70s encoded by the *SSB1* and *SSB2* genes (Ssb1/2p), suggesting that the *EXA3* gene product plays a central role in the yeast stress response. Consistent with this hypothesis, *EXA3-1* is tightly linked to *HSF1*, the gene encoding the transcriptional regulatory protein known as "heat shock factor." All of the genes identified in this study seem to be involved in regulating the expression of *SSA3* and *SSA4* or the activity of their protein products.

THE 70-kD heat shock proteins (hsp70) are highly conserved. In the eukaryotic cell, several different species of hsp70 are encoded by a family of highly related genes. hsp70s have been found in mitochondria (CRAIG *et al.* 1989; ENGMAN, KIRCHHOFF and DONELSON 1989; LEUSTEK *et al.* 1989; MIZZEN *et al.* 1989), chloroplasts (MARSHALL *et al.* 1990), and the endoplasmic reticulum (ER) (ROSE, MISRA and VOGEL 1989; NORMINGTON *et al.* 1989), as well as the cytoplasm.

A combination of *in vivo* and *in vitro* experiments indicates a role for hsp70 in several cellular processes. These include the translocation of proteins into the ER (DESHAIES *et al.* 1988; CHIRICO, WATERS and BLOBEL 1988) and mitochondrium (DESHAIES *et al.* 1988; MURAKAMI, PAIN and BLOBEL 1988) as well as participation in a number of specific protein-protein interactions. For example, the DnaK protein of *Escherichia coli* acts to loosen the association between the DnaB and lambda P proteins at the lambda origin of replication allowing DnaB to function in subsequent DNA synthesis (YAMAMOTO *et al.* 1987). Also, the mammalian protein hsc70, has been extensively studied as a protein involved in the ATP-dependent disruption of clathrin cages surrounding coated vesicles. A possible enzymatic basis for these types of activities has been studied *in vitro*. A mammalian ER localized hsp70, grp78, and a mammalian cytoplasmic hsp70, hsc70, were shown to bind different short oligo peptides with distinct binding affinities (FLYNN, CHAPPELL and ROTHMAN 1989). This binding was disrupted by ATP hydrolysis which is consistent with studies that have shown that hsp70s have ATP binding and ATPase activities. Thus, as a general rule, hsp70s can be thought of as peptide binding proteins that can be released from their substrate by ATP hydrolysis.

Eight different hsp70 genes have been isolated from *Saccharomyces cerevisiae*. The DNA sequence similarity of any two of these genes ranges from 50% to 96% (CRAIG 1985). Phenotypic analysis of *in vitro* constructed "knock out" mutations in these eight genes indicates that they can be placed in four functional subfamilies. Kar2p which is localized in the ER lumen (ROSE, MISRA and VOGEL 1989), and Ssc1p, which is localized in the mitochondrial matrix (CRAIG *et al.* 1989), are both encoded by unique, essential genes. However, strains containing single "knock out" mutations of members of the *SSA* and *SSB* subfamilies, which have four and two members respectively, are viable. When the *SSB1* and *SSB2* genes are both disrupted, cells grow much slower than wild type, especially at lower temperatures. This suggests that Ssb1p and Ssb2p have similar if not identical function (CRAIG and JACOBSEN 1985). For lack of any information indicating a functional difference between Ssb1p and Ssb2p they will be referred to collectively as Ssb1/2p throughout this report.

There are four members of the *SSA* hsp70 subfam-

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ily. The functional relationship of these four predominantly cytoplasmically localized proteins is complex. Strains in which the *SSA1* gene and the *SSA2* gene have been inactivated grow more slowly than wild-type strains at all temperatures and are unable to form colonies at 37° (Ts) (CRAIG and JACOBSEN 1984). The viability of *ssa1 ssa2* strains at temperatures lower than 37° is due to high expression of the *SSA4* gene which is not normally expressed under optimal growth conditions. Thus, inactivation of the *SSA4* gene in an *ssa1 ssa2* strain results in lethality (WERNER-WASHBURNE, STONE and CRAIG 1987). Ssa3p can rescue the viability of *ssa1 ssa2 ssa4* mutants only when the *SSA3* gene is expressed at high levels (WERNER-WASHBURNE, STONE and CRAIG 1987). Ssa3p and Ssa4p will be referred to as Ssa3/4p throughout this work for lack of any information indicating a functional difference between them.

Members of the *SSA* hsp70 subfamily are found in high abundance in the yeast cytosol at all temperatures. The whole spectrum of activities and the overall role of these essential proteins is not well understood. The main goal of this study is to isolate extragenic suppressor mutations that may reveal the essential role of cytosolic hsp70. Also, mutants that cause increased Ssa3/4p expression could suppress the *ssa1 ssa2* Ts phenotype. Study of this type of mutation could lead to insights into the regulation of the stress response. Here we report the isolation and characterization of mutations at three loci that suppress the growth defect of *ssa1 ssa2* strains. These loci have been designated *EXA1*, *EXA2* and *EXA3* for *EX*tragenic suppressor hsp70 sub family *A*.

MATERIALS AND METHODS

Yeast strains: All yeast strains used in this study are listed in Table 1. All of the mutant alleles used in this work were constructed previously. The *ssa1-3* and *ssa2-2* alleles were constructed by *in vitro* deletion of the DNA encoding amino acids 10–160 of both Ssa1p and Ssa2p. In the place of the deleted coding sequence, auxotrophic marker genes were inserted; *ssa1-3* carries the *HIS3* gene, *ssa2-2* carries the *URA3* gene. Disruption alleles of *SSA3* and *SSA4* were constructed by the insertion of the *TRP1* and *URA3* genes respectively (WERNER-WASHBURNE, STONE and CRAIG 1987). The *ssa2-1* allele was constructed by insertion of the *LEU2* gene into the coding sequence of *SSA2* (CRAIG and JACOBSEN 1984).

Isolation of revertants: Single colonies of *ssa1-3 ssa2-2* strain, JN49, were used to inoculate culture flasks containing 10 ml of YPD. The cultures were incubated for 2 days at 23°, at which point cell density was 1.2×10^8cells per ml. One half of each culture was mutagenized with ethyl methanesulfonate (EMS) to 50% viability and spread onto both YPD and complete synthetic media at 37°. The remaining cells were plated out onto YPD or complete synthetic media at 37° without mutagenesis. Revertant colonies were patched onto YPD, allowed to grow up at 23°, then replica plated onto histidine and uracil omission media at 23°, and YPD that was prewarmed to 37°. Isolates that were able to grow under all three of these conditions were purified by single-colony isolation.

Plasmids, bacterial strains and recombinant DNA manipulations: *E. coli* JM109 was the primary strain used for molecular cloning procedures. This genotype of this strain is *E. coli* K-12 *recA1, endA1 gyrA96, thi, hsdr17, supE44, relA1,* lambda$^-$. *Δ(lac-proAB), (F$^-$, traD36, proAB lacI/9 ZΔM15*). All cloning steps were carried out under the conditions recommended by the manufacturers. Small scale plasmid preparations were performed by the boiling method (MANIATIS, FRITSCH and SAMBROOK 1982). The construction of *pGAL1-SSA1* which contains the *SSA1* structural gene fused 3′ to the *GAL1* promoter has been described (WERNER-WASHBURNE, STONE and CRAIG 1987). The *SSC1* containing plasmid pKT12 contains the *SSC1* gene inserted into YCp19 which has had the *URA3* gene disrupted by filling in and religation at the *Apa*1 site (KIT TILLY, unpublished). pMR713 is a centromere based plasmid containing the *KAR2* and *LEU2* genes (MARK ROSE, unpublished). Plasmid YIpHSF-3 was constructed as follows. YIp351 (HILL *et al.* 1986) was cut to completion with *Sph*I and then a partial digest with *Eco*RI was performed to create a 4.4-kb *Sph*I/*Eco*RI fragment. This fragment was ligated to a 2.8-kb *Sph*I-*Eco*RI fragment from pHF309 (SORGER 1990) which contains the 5′ region of *HSF1*. YIpHSF-3 was integrated into the yeast genome by transformation of strain JN14 following digestion with *Bam*HI. Proper integration at the *HSF* locus was determined by Southern analysis done as in (CRAIG and JACOBSEN 1984). The resultant strain was designated MH293.

Genetic techniques and scoring of phenotypes: Standard genetic techniques were used in this study (SHERMAN, FINK and HICKS 1986). Except when scoring temperature sensitivity or selecting for temperature resistant revertants, the routine growth temperature for all other manipulations was 23°. Temperature sensitivity was scored by light replica plating of colonies or patches of cells from a 23° YPD plate to a YPD plate equlibrated to 37°. The most clear and consistently reproducible scoring of temperature sensitivity was accomplished by double replica plating. Transformation of yeast was by the method of ITO *et al.* (1983).

Media: Standard yeast media were used in this study (SHERMAN, FINK and HICKS 1986). Potassium acetate media used to induce sporulation in the presence of galactose contained galactose substituted for dextrose.

Immunological techniques: Whole cell lysate for immunoblotting was prepared by bead beating cells utilizing a mini bead beater (Biospec Products, Bartlesville, Oklahoma) in sodium dodecyl sulfate (SDS) sample buffer then boiling for 5 min. For immunoprecipitations, labeling was accomplished by the addition of [^{35}S]methionine to cells in synthetic media lacking methionine. Protein was prepared by bead beating cells in 400 μl of IP lysis buffer (125 mM Tris-HCl, pH 6.8, 0.1% SDS and 0.01% β-mercaptoethanol) and then boiling for 5 min. Labeled lysate was added to 1 ml of IP buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1 mM EDTA, 0.5% Tween-20) and centrifuged in a microfuge for 15 min. A 0.95-ml sample of supernatant was removed, combined with antibody and incubated on ice. After 90 min, 40 μl of a 10% slurry of protein A-Sepharose CL-4B (Pharmacia) in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1 mM EDTA, 0.5% Tween-20, were added and incubation continued at 4° for 90 min with inversion. Two washes were done with IP buffer followed by two washes with IP wash buffer (100 mM Tris-HCl, pH 7.5, 200 mM NaCl, 0.5% Tween-20,. 0.1% SDS). To release antigen, immunoprecipitates were boiled in SDS sample buffer. Assay of total protein was done using the Bio-Rad dye reagent (Bio-Rad, Rich-

TABLE 1

Yeast strains

Strain[a]	Genotype
JN9	*MATα his3-11,3-15 leu2-3,2-112 lys2 trp1-Δ1 ura3-52 exa2-1 ssa1-3 ssa2-2*
JN10	*MATa his3-11,3-15 leu2-3,2-112 lys2 trp1-Δ1 ura3-52 exa2-1 ssa1-3 ssa2-2*
JN14	*MATa his3-11,3-15 leu2-3,2-112 lys2 trp1-Δ1 ura3-52 ssa1-3 ssa2-2*
JN23	*MATa his3-11,3-15 leu2-3,2-112 lys2 trp1-Δ1 ura3-52 exa1-3 ssa1-3 ssa2-2*
JN29	*MATa his3-11,3-15 leu2-3,2-112 lys2 trp1-Δ1 ura3-52 EXA1-2 ssa1-3 ssa2-2*
JN31	*MATa his3-11,3-15 leu2-3,2-112 lys2 trp1-Δ1 ura3-52 EXA1-2 ssa1-3 ssa2-2*
JN35	*MATα his3-11,3-15 leu2-3,2-112 lys2 trp1-Δ1 ura3-52 EXA1-1 ssa1-3 ssa2-2*
JN37	*MATα his3-11,3-15 leu2-3,2-112 lys2 trp1-Δ1 ura3-52 exa1-4 ssa1-3 ssa2-2*
JN47	*MATa his3-11,3-15 leu2-3,2-112 lys2 trp1-Δ1 ura3-52 ssa1-3 ssa2-2*
JN49	*MATα his3-11,3-15 leu2-3,2-112 lys2 trp1-Δ1 ura3-52 ssa1-3 ssa2-2*
JN54	*MATa his3-11,3-15 leu2-3,2-112 lys2 trp1-Δ1 ura3-52*
JN55	*MATα his3-11,3-15 leu2-3,2-112 lys2 trp1-Δ1 ura3-52*
JN66	*MATα his3-11,3-15 leu2-3,2-112 lys2 trp1-Δ1 ura3-52 ssa1-3 ssa2-2 ssa3-1 EXA3-1*
MW141[b]	*MATα his3-11,3-15 leu2-3,2-112 lys2 trp1-Δ1 ura3-52 GAL2 ssa1-3 ssa2-1 ssa4-1 [pGAL-SSA1]*
JN130	*MATa his3-11,3-15 leu2-3,2-112 lys2 trp1-Δ1 ura3-52 ssa1-3 ssa2-2 EXA3-1*
JN131	*MATa his3-11,3-15 leu2-3,2-112 lys2 trp1-Δ1 ura3-52 ssa1-3 ssa2-2 ssa3-1*
JN134	*MATa his3-11,3-15 leu2-3,2-112 lys2 trp1-Δ1 ura3-52 ssa1-3 ssa2-2 EXA3-1*
JN314-8b	*MATa his3-11,3-15 leu2-3,2-112 lys2 trp1-Δ1 ura3-52 ssa1-3 ssa2-2*
RSY130[c]	*MATα mas2-10 pep4 his3*
MH293	*MATa his3-11,3-15 leu2-3,2-112 lys2 trp1-Δ1 ura3-52 ssa1-3 ssa2-2 HSF1::LEU2*

[a] All strains except indicated otherwise are from this study.
[b] WERNER-WASHBURNE, STONE and CRAIG (1987).
[c] Source RANDY SCHEKMAN, strain constructed by M. YAFFE.

mond, California). Production of serum 535 raised against the conserved hsp70 amino terminal region and western analysis have been described (CRAIG *et al.* 1989). ^{125}I-Conjugated protein A was supplied by Amersham. The anti-F_1 β-subunit serum was a gift from Dr. Michael Yaffee. The anti-ppαf serum was a gift from RANDY SCHEKMAN.

RESULTS

Isolation of revertants: Ts$^+$ revertants strain JN49 of genotype *ssa1-3 ssa2-2* were selected at 37°. Revertants capable of growth at 37° arose spontaneously at a frequency of approximately 10^{-7}. EMS mutagenesis of the cells to 50% viability resulted in a 13-fold increase in the reversion frequency. A total of 397 Ts$^+$ strains were isolated and retested for non-temperature sensitivity and the prototrophic marker genes inserted into *SSA1* and *SSA2*. Eleven of the most Ts$^+$ isolates that were also His$^+$ Ura$^+$ were out-crossed to reduce background genetic variation and to determine the genetic basis of suppression. The first of these crosses was to the highly related *ssa1-2 ssa2-3* strain JN314-8b. The successive two crosses were to the parental strain, JN49, or to the isogenic strain JN47. Only five of the above eleven revertants contained a mutation that segregated 2:2 through all three of these crosses. These isolates were designated AMR5, AMS4, DMR3, GMS4 and ASR3. Four of these five revertants were from EMS-mutagenized cultures. Two of these four, AMR5 and AMS4, were from the same single colony, raising the possibility that they may carry the same mutation; the other two, DMR3 and GMS4, were from different cultures. The spontaneously derived mutant was designated ASR3. A non-Ts variant of an *ssa1-3 ssa2-2 ssa3-1* strain which arose following transformation was shown to contain a single nuclear mutation which caused suppression and thus was retained for further study and designated XSS6.

Dominance: To test for dominance at least two diploids that were homozygous for *ssa1-3* and *ssa2-2* and heterozygous for each suppressor mutation were isolated and tested for the ability to grow at 37.5°. The suppressor phenotype was still apparent in diploids heterozygous for the mutations contained in isolates AMR5, AMS4 and XSS6. Suppression was not apparent in diploids heterozygous for the mutations contained in isolates DMR3, GMS4 and ASR3 (data not shown). This analysis indicates that the suppressor mutations in isolates AMR5, AMS4 and XSS6 are dominant while the suppressor mutations in isolates DMR3, GMS4 and ASR3 are recessive.

Linkage of suppressor mutations: Reciprocal crosses between each of the different revertants were performed to determine how many different loci were represented by these six mutations (Table 2). The mutations in AMR5, AMS4, DMR3 and GMS4 appear to be very tightly linked and are likely to be in the same gene. The gene identified by this linkage group is designated *EXA1*. Also, suppression was apparent in diploids containing both of the recessive suppressor mutations DMR3 and GMS4, providing further support for allelism between these two mutations. Tetratype and the occasional nonparental ditype (DT) te-

TABLE 2

Linkage of suppressor mutations

Cross	PD	TT	NPD
AMR5/AMS4	47	5	0
AMR5/DMR3	8	0	0
AMR5/GMS4	30	0	0
AMS4/DMR3	38	0	0
AMS4/GMS4	46	1	1
DMR3/GMS4	40	0	1
ASR3/AMR5	8	16	7
ASR3/GMS4	5	11	4
ASR3/DMR3	2	6	3
ASR3/GMS4	8	10	8
XSS1/AMR5	3	4	1
XSS1/ASR3	0	2	4

PD = parental ditype, TT = tetratype, NPD = nonparental ditype.

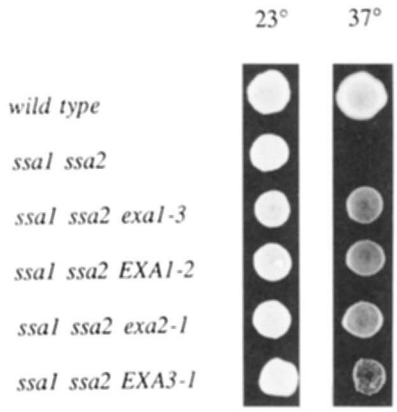

FIGURE 1.—Growth test of suppressor containing strains. Strains of the indicated genotypes were grown into log phase in liquid rich media, diluted to equal cell density, then 5 μl of each were placed onto rich media at the indicated temperature and incubated for 72 hr.

trad seen in the crosses between these four revertants is most likely due to the effects of background genetic variation which was observed when out crossing the original revertants. The other two mutations ASR3 and XSS6 do not appear to be linked to each other or *EXA1*. The ASR3 mutation was designated *exa2-1* and the XSS6 mutation was designated *EXA3-1*. Thus, suppression of the Ts phenotype in these six revertants is the result of a mutation at one of three loci.

Growth characteristics of revertant strains: Growth tests were performed to determine the relative strength of Ts suppression exhibited by both the dominant and recessive alleles of *EXA1*, *exa2-1* and *EXA3-1* (Figure 1). The strength of suppression by *exa2-1*, *exa1-3*, and *EXA1-2* and *EXA3-1* is approximately equal but only partial; in no case is suppression back to wild type. The other two alleles of *EXA1*, *EXA1-1* and *exa1-4* showed similar properties (not shown).

Suppressor mutations are not linked to *SSA1* or *SSA2*: The ability of the revertants to grow on histidine and uracil omission media suggested that they still contained the *ssa1-3* and *ssa2-3* alleles, yet it is possible that the genetic events causing suppression could be at one of these two loci. This possibility was tested by crossing the revertants by strains JN54 and JN55 which are wild type for *SSA1* and *SSA2*. If the genetic event causing suppression was intragenic it would be unlikely that Ts *ssa1-3 ssa2-2* segregants would be recovered. In contrast, an extragenic suppressor could segregate away from *ssa1-3* and *ssa2-2* causing some of the *ssa1-3 ssa2-2* segregants to be Ts. Approximately one half of the *ssa1-3 ssa2-2* segregants in crosses with all the *EXA1* mutations and *EXA3-1* were Ts (data not shown), indicating that the reversion event in these two linkage groups strains is not at *SSA1* or *SSA2*.

The *exa2-1* mutation seems to be highly sensitive to background genetic variation. Eleven out of 12 *ssa1-3 ssa2-2* segregants from these crosses were Ts. Thus by this test it cannot be determined if *exa2-1* is allelic to *SSA1* or *SSA2*. However, this seems unlikely because isolates are still Ura$^+$ and His$^+$ and no Ssa1p or Ssa2p or novel hsp70 species were observed by either 1D or 2D gel electrophoretic analysis of *exa2-1* lysates (data not shown). Also, *exa2-1* showed stronger centromere linkage than would be expected for *SSA2* and weaker centromere linkage than expected for *SSA1*. Centromere linkage of a closely CEN-linked marker of unknown location can be detected when scoring against the *MAT* locus, which is approximately 25 cM from its centromere. The combined effects of centromere linkage of the unknown mutation and *MAT* can result in an elevation of the proportion of tetrads which are either parental DT or nonparental DT. When *exa2-1* was scored against *MAT* in 78 tetrads 41 were DT (53%), either parental DT or nonparental DT. In contrast, when *SSA1* was scored against *MAT* of 64 tetrads, 45 were DT (70%), and when *SSA2* was scored against *MAT* of 71 tetrads 24 were DT (34%). This indicates that *exa2-1* is not at these loci. Therefore it seems unlikely that *exa2-1* is a result of intragenic reversion of *ssa1-3* or *ssa2-2*. In summary, none of the suppressor mutations appears to be at the *SSA1* or *SSA2* loci and thus these mutations are extragenic suppressors.

Linkage to SSA3: To determine if *EXA1* was allelic to *SSA3*, *ssa1-3 ssa2-2 EXA1-2* strain JN29 was crossed to *ssa1-3 ssa2-2 ssa3-1* strain JN131. If *EXA1* was allelic to *SSA3* then it would not be possible to recover Ts$^+$ *ssa3-1* segregants. In the eight complete tetrads from this cross the suppressor mutation segregated 2:2. Of the 16 Ts$^+$ segregants 10 were Trp$^+$, indicating the presence of *ssa3-1* and the remaining six segregants were Trp$^-$ (*SSA3*). This indicates that *EXA1-1* is not allelic to *SSA3* and also that Ssa3p is not necessary for suppression.

TABLE 3

Suppression of *ssa1 ssa2 ssa4* lethality

Cross	Viable:nonviable spores[a] 4:0	3:1	2:2	1:3	0:4
ssa1 ssa2 ssa4 [GAL1p-SSA1] ×					
ssa1 ssa2	0	0	22	11	15
ssa1 ssa2 exa1[b]	7	86	80	22	45
ssa1 ssa2 exa2-1	0	0	72	15	17
ssa1 ssa2 EXA3-1	0	1	31	5	12

[a] Scored as the ability to germinate and form colony at 23° on YPD.

[b] This is the sum of crosses with all of the *EXA1* alleles.

The results and interpretation are essentially the same for *EXA3-1*. *ssa1-3 ssa2-2 ssa3-1 EXA3-1* strain JN62 was crossed to *ssa1-3 ssa2-2* strain JN14. In 17 complete tetrads the suppressor mutation segregated 2:2. Eighteen of the Ts^+ segregants were *ssa3-1* while the remaining 16 were *SSA3*.

No centromere linkage is detected for *SSA3* when scored against *MAT* [23 DT out of 90 tetrads (26%)], while as mentioned above when *EXA2* was scored against *MAT* 41 out of 78 tetrads were DT (53%), indicating that *EXA2* and *SSA3* are probably not allelic.

Suppression of *ssa1ssa2ssa4* lethality: Strains carrying null alleles of *SSA1*, *SSA2* and *SSA4* are inviable. This strain can be rescued by the plasmid p*GAL1-SSA1* that has the $SSA1^+$ structural gene coupled to a galactose-inducible promoter. To determine if any of the suppressor mutations could suppress this phenotype, strain MW141 of genotype *ssa1-3 ssa2-1 ssa4-1 pGAL1-SSA1* was crossed to *ssa1-3 ssa2-2* strains which contained each of the suppressor alleles of *EXA1*, *EXA2* and *EXA3*. Diploids were induced to sporulate on potassium acetate media containing galactose and meiotic segregants were dissected onto glucose-based media (Table 3). If the suppressor was allelic to *SSA4* two spores of each tetrad would be viable and two would be inviable. The same result would be expected if the suppressor mutation is not allelic to *SSA4* and cannot suppress the triple mutant phenotype. A preponderance of tetrads that have more than two viable spores indicates that the suppressor is not allelic to *SSA4* and that the mutation can suppress the lethal combination *ssa1 ssa2 ssa4*.

More than two viable spores from each tetrad were recovered from all of the crosses with the suppressor alleles of *EXA1*, indicating that *SSA4* and *EXA1* are not allelic and that these mutations can suppress *ssa1 ssa2 ssa4* lethality. Departure from the 1:4:1 proportions of 4:0, 3:1 and 2:2 type segregation of spore viability (which should be observed for independent segregation of two markers) could be due to random inviability, variable strength of suppression, and/or linkage of *EXA1* and *SSA4*. Although the *EXA1* mutations rescue the viability of *ssa1 ssa2 ssa4* mutants the *ssa1 ssa2 ssa4 exa1* strains exhibit a severe growth defect at 23°, with a doubling time of 6.75 hr, and cannot grow at 37°.

No more than two viable spores were recovered from the above crosses involving *exa2-1* indicating that this mutation cannot suppress the lethal phenotype or that it is allelic to *SSA4*. Since centromere linkage of *EXA2* can be detected when scored against *MAT* but no centromere linkage can be detected for *SSA4 vs. MAT* (12 DT out of 60 tetrads, 20%), it is unlikely that *EXA2* and *SSA4* are allelic.

Only one out of 49 tetrads from crosses of MW141 with *ssa1-3 ssa2-2 EXA3-1* had more than two viable spores. One of the segregants from this tetrad had wild-type growth properties indicating that it was probably a product of a recombination event that produced a fully functional *SSA1* or *SSA2* gene. As will be described below *EXA3-1* is tightly linked to *HSF1* thus the above results indicate that *EXA3-1* is unable to suppress the lethality caused by the triple inactivation of *SSA1*, *SSA2* and *SSA4*.

***EXA1* and *EXA2* mutations are not allelic to *SSC1* or *KAR2*:** It is likely that cytosolic hsp70 and organelle-localized hsp70 both play a role in translocation of nascent proteins across organellar membranes (Kang *et al.* 1990; Vogel, Misra and Rose 1990). An alteration in the activity of an organellar hsp70 could perhaps compensate for a defect in cytosolic hsp70 function. The possibility that *EXA1* or *EXA2* were mutations in either *SSC1*, which encodes mitochondrial hsp70, or *KAR2*, which encodes ER-resident hsp70, was tested by transforming a wild-type copy of these genes into *ssa1-3 ssa2-2 exa1-4* strain JN37 and *ssa1-3 ssa2-2 exa2-1* strain JN9. Since *exa1-4* and *exa2-1* are recessive, these strains should become Ts if either of these genes were allelic to *EXA1* or *EXA2*. The presence of the plasmid borne *SSC1* gene on pKT12 or the *KAR2* gene on pMR713 in these strains had no effect on the growth characteristics of JN37 and JN9 (not shown) indicating that *EXA1* and *EXA2* are not allelic to *SSC1* or *KAR2*.

Hsp70 profile of suppressor containing strains: Since overexpression of Ssa3p and Ssa4p can at least partially compensate for the loss of Ssa1/2p (R. J. Nelson and E. A. Craig, unpublished) we compared the levels of these and other hsp70s in *ssa1-3 ssa2-2* strains with and without each of the suppressor mutations to determine if increased expression of a related protein could be causing suppression.

Proteins were harvested from cells in logarithmic growth at 23°, and 6 hr after shift to 37°. Six hours after shift to 37° was chosen as a relevant point to assess the effects of the suppressors because this is approximately the time at which viability of *ssa1-3*

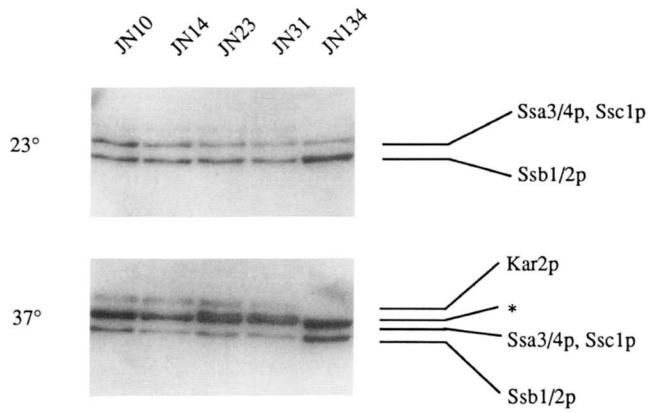

FIGURE 2.—Hsp70 profile of suppressor containing strains at 23° and 37°. Strains JN10, JN14, JN23, JN31 and JN134 are all *ssa1-3 ssa2-2* and are *exa2-1*, *EXA*$^+$, *exa1-3*, *EXA1-2* and *EXA3-1*, respectively. These strains were grown into log phase at 23° in complete synthetic media then a portion of each culture was harvested by filtration and the rest of the culture was placed in a 37° water bath. After 6 hr at 37° cells were harvested again. Proteins were prepared and immunoblotted with sera 535 and then incubated with ^{125}I-conjugated protein A. The hsp70 species are indicated to the right "*" denotes the novel form.

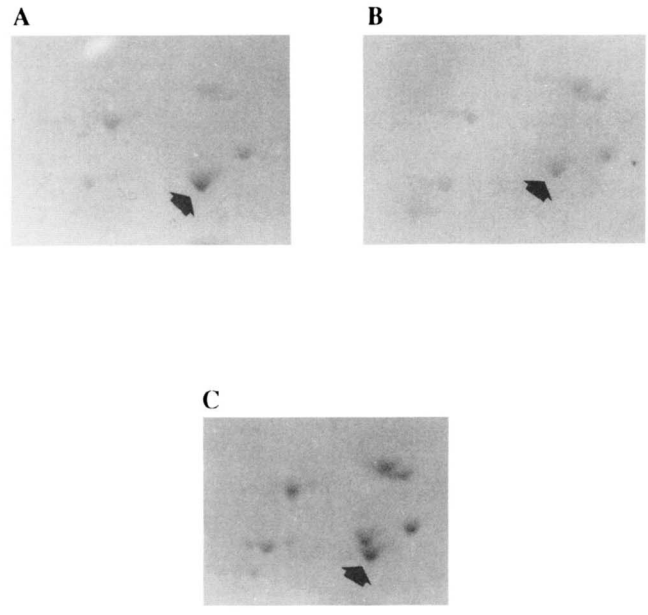

FIGURE 3.—Two-dimensional gel electrophoresis of protein lysate from *ssa1 ssa2 ssa4 exa1* strain. Lysate was prepared from *ssa1 ssa2* strain JN16 which contains Ssa3/4p as indicated with arrow (A) and *ssa1 ssa2 ssa4 exa1* strain JN41 which contains protein with anomalous migration as indicated with arrow (B). Panel (C) is a superimposition of the gels from (A) and (B). Two-dimensional gel electrophoresis was performed as decribed previously in WERNER-WASHBURNE, STONE and CRAIG (1987).

ssa2-2 cells begins to decline but the number of viable cells of all three suppressor containing strains continues to increase (not shown). Equal amounts of total protein of each lysate were run on a one-dimensional SDS polyacrylamide gel. In these gels Kar2p, Ssa3/4p and Ssb1/2p can be clearly resolved but Ssc1p and Ssa3/4p comigrate. The levels of hsp70s were determined by western blotting using an antibody raised against the highly conserved amino terminus of hsp70 which reacts with all the hsp70s listed above (Figure 2). At 23°, only *EXA3-1* had a striking affect on hsp70 levels. The *EXA3-1 ssa1-3 ssa2-2* strain JN134 had elevated levels of Ssb1/2p.

At 37° different effects were observed for each suppressor mutation. *EXA1* mutations resulted in accumulation of a novel form of hsp70. This species was seen in strains containing both dominant and recessive *EXA1* mutations. This band migrated slightly above the Ssa3/4p-Ssc1p band in one-dimensional SDS gels. In two-dimensional gels of these lysates, a protein that migrates just above and with a slightly more basic isoelectric point than Ssa3/4p was seen. Lysate from *ssa1 ssa2 ssa4 exa1-3* strain JN41 contains no Ssa3p that migrated at the position that it would be normally found, only the novel form was seen (compare Figure 3, A with B). This novel form was not observed to accumulate in *exa2-1* or *EXA3-1* strains.

Lysates from *exa2-1* containing strain JN10 (*ssa1-3 ssa2-2 exa2-1*) contained slightly more material in the Ssa3/4p-Ssc1p band than *ssa1-3 ssa2-2* strain JN14. Two-dimensional gels which clearly resolve Ssa3/4p from Ssc1p were run on these lysates and indicated that the increase in this band was due to higher levels of Ssa3/4p (not shown). Thus *exa2-1* may be causing suppression by increasing the level of these two related proteins.

EXA3-1 had striking effects at 37°. Lysates from JN134 an *ssa1-3 ssa2-2 EXA3-1* strain contained an increased levels of Ssb1/2p and Ssa3/4p. Also, the level of the ER-localized hsp70, Kar2p was dramatically reduced. Identical results were observed for another *ssa1-3 ssa2-2 EXA3-1* strain JN130. As mentioned above, *EXA3-1* also had a strong affect in the levels of Ssb1/2p at 23°. This mutation seems to affect either the regulation or stability of two different classes of hsp70.

Linkage of *EXA3-1* to *HSF1*: The dramatic affects of *EXA3-1* on the levels of the stress proteins suggested that this mutation may affect the regulation of the stress response. A central transcriptional regulatory factor in this response is the heat shock factor "HSF." We checked the possibility that *EXA3-1* and *HSF1* might be allelic by integrating the *LEU2* gene next to *HSF1* in an *ssa1-3 ssa2-2* strain and crossing the resultant strain with a *ssa1-3 ssa2-2 EXA3-1* strain. In this way the suppressor phenotype conferred by *EXA3-1* could be scored against the Leu$^+$ phenotype conferred by *LEU2* integrated near the *HSF1* locus. In 12 tetrads complete repulsion was observed between the Leu$^+$ and Ts$^+$ traits both which segregated 2:2. Complete segregation of Leu$^+$ away from the suppressor phenotype indicates that *EXA3* is closely linked or allelic to *HSF1*.

Protein translocation: The members of the *SSA*

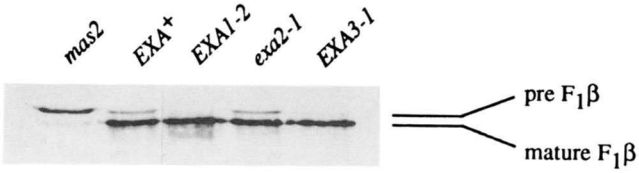

FIGURE 4.—Effect of the suppressor mutations on the accumulation of $F_1\beta$ at 37° in *ssa1 ssa2* strains. Strain RSY130 (mas2-10) which accumulates precursor to $F_1\beta$ at 37° and strains JN14, JN31, JN10 and JN134 which are all *ssa1-3 ssa2-2* and either *EXA*$^+$, *EXA1-2*, *exa2-1* or *EXA3-1*, respectively, and as indicated were grown at 23° into log phase in complete synthetic media minus methionine, shifted to 37° for 6 hr then labeled for 30 min by the addition of [^{35}S]methionine. In the immunoprecipitation reaction equal counts were used. No accumulation was detected in *ssa1-3 ssa2-2* strains at 23°.

hsp70 subfamily, which are cytoplasmic, seem to be involved in translocation of proteins into the ER and mitochondrium. Yeast cells from which these essential proteins have been depleted accumulate unimported precursor of the mitochondrial F_1 ATPase β-subunit ($F_1\beta$) and the secretory protein, prepro-α-factor (ppαF) (DESHAIES *et al.* 1988). Also, the *SSA* hsp70s (Ssaps) act in a ATP-dependent manner, along with an undefined *N*-ethylmaleimide-sensitive activity, to stimulate *in vitro* translocation into mitochondria (MURAKAMI, PAIN and BLOBEL 1988) and ER (CHIRICO, WATERS and BLOBEL 1988).

As mentioned above, it was shown that as *ssa1 ssa2 ssa4* strains die they accumulate unimported $F_1\beta$ precursor and ppαF (DESHAIES *et al.* 1988). We wanted to determine if processing of ppαF or $F_1\beta$ was defective in *ssa1 ssa2* strains. Cells were labeled with [^{35}S] methionine at 23° or 6 hr after shift to 37°. PpαF and $F_1\beta$ were then immunoprecipitated from lysate prepared from these cells (Figure 4). Using this method no $F_1\beta$ precursor was detected in *ssa1 ssa2* or wild type strains at 23°. However, at 37° $F_1\beta$ precursor, as judged by comigration with $F_1\beta$ that accumulates in a *mas2-10* strain, was detected in *ssa1-3 ssa2-2* strains. This accumulation was suppressed by *exa1-3* (not shown), *EXA1-2* and *EXA3-1* but not by *exa2-1*. No accumulation of ppαF was detected in *ssa1-3 ssa2-2* mutants (not shown).

DISCUSSION

We report the isolation and characterization of mutations at three different loci that can suppress the temperature sensitive growth caused by the double inactivation of *SSA1* and *SSA2*, two members of the *SSA* subfamily of yeast hsp70. These mutations are at loci designated *EXA1*, *EXA2*, and *EXA3*. All of the suppressors affect either expression of *SSA3* and *SSA4* or the properties of their protein products. Although their mechanism of suppression appears to be through utilization of the two remaining *SSA* hsp70 subfamily members, they are genetically and functionally distinct.

The mutations at *EXA1* result in the accumulation of a protein that migrates in SDS gels with a molecular mass of approximately 70 kD and reacts with a sera raised against the conserved amino terminus of hsp70. This protein is present at extremely low levels in *ssa1-3 ssa2-2* strains that contain no suppressor mutations suggesting that *EXA1* mutations do not cause its production *de novo*. Cellular fractionation experiments indicate that, like Ssa3p and Ssa4p, this form which has an isoelectric point that is very close to those of Ssa3/4p, is cytoplasmically localized. Lysate from *ssa1 ssa2 ssa4 exa1-3* strain JN41 contains no Ssa3p that migrated at the position that it would be normally found, only the novel form was seen. Taken together these results suggest this protein is most likely an isoform of Ssa3/4p. Consistent with the possibility that the *EXA1* mutations act through Ssa3/4p, we have not been able to isolate the quintuple mutant, *ssa1 ssa2 ssa3 ssa4 exa1*. This isoform may be better able to substitute for Ssa1/2p than the Ssa3/4p found in *EXA1*$^+$ strains. It is also possible that accumulation of this protein is a byproduct of the mechanism of suppression. Since both dominant and recessive *EXA1* mutations cause the accumulation of this isoform it does not seem to represent undermodified Ssa3/4p that results from a reduction in activity of a modifying enzyme.

EXA3-1 caused a large increase in the accumulation of Ssb1/2p at 23° and 37° and a large increase in the accumulation of Ssa3/4p at 37°. This mutation, like *exa2-1*, may be suppressing the *ssa1-3 ssa2-2* Ts phenotype by increasing the amounts of these related proteins. Interestingly, the level of Kar2p is lower in *ssa1 ssa2 EXA3-1* strains as compared to the level found in *ssa1 ssa2* strains. Northern blot analysis of the *SSA4*, *SSB1/2* and *KAR2* transcripts revealed a direct correspondence between the message levels and the protein levels. Genetic evidence indicates that *EXA3* is either allelic or closely linked to *HSF1*. We propose that *EXA3-1* is an allele of *HSF1*. The observation that all stress proteins are not effected in the same way by the *EXA3-1* mutation suggests that it is an allele of *HSF1* which has complex and interesting effects on regulation of the stress response. Experiments are in progress to test this hypothesis.

Since the *exa2-1* mutation causes an increase in the accumulation of Ssa3/4p at 37° suppression may result from an increase in the levels of these related proteins. This is consistent with our observation that when present on high copy number plasmids, the *SSA3* and *SSA4* genes can partially suppress the 37° growth defect of *ssa1 ssa2* strains (NELSON and CRAIG, unpublished results). Thus *exa2-1* causes either increased expression or stability of Ssa3/4p. Mutations that result in the induction of the heat-shock response but are not involved in the heat-shock regulatory pathway,

per se, have been described previously (PARKER-THORNBERG and BONNER 1987). This could be the manner in which *exa2-1* causes increased levels of Ssa3/4p. Alternatively *EXA2* may be involved directly in the regulation of *SSA3* and *SSA4*.

EXA3-1 caused a large increase in the accumulation of Ssb1/2p at 23° and 37° and a large increase in the accumulation of Ssa3/4p at 37°. This mutation, like *exa2-1*, may be suppressing the *ssa1-3 ssa2-2* Ts phenotype by increasing the amounts of these related proteins. Genetic evidence indicates that *EXA3* is either allelic or closely linked to *HSF1*. We propose that *EXA3-1* is an allele of *HSF1* and directly affects the regulation of the transcription of SSA3/4 and SSB1/2 and other stress proteins. Experiments are in progress to test this hypothesis.

Ssa1/2p have been implicated in translocation of proteins into mitochondria and ER. To determine whether a translocation defect could be the reason for inviability of *ssa1 ssa2* strains we investigated processing of the mitochondrial protein $F_1\beta$ and the secreted protein ppαF. In the *ssa1 ssa2* mutant at the restrictive temperature we found a weak defect in the processing of $F_1\beta$ but not for ppαF. Presumably this reduction in $F_1\beta$ processing is due to a decrease in its rate of import. Both dominant and recessive *EXA1* mutations strongly suppress this import defect suggesting that the hsp70 isoform produced in these strains may have a direct role on the function of the import pathway. Molecular cloning and analysis of the *EXA1* locus will be illuminating as to the mechanism of production as well as the significance of this hsp70 isoform.

exa2-1, which results in a slight increase in the levels of Ssa3/4p did not suppress the $F_1\beta$ import defect. This raises the question of whether Ts growth of *ssa1 ssa2* strains is due to a defect in translocation. The possibility that there may be other important roles for Ssa1/2p other than protein translocation is supported by the observation that no accumulation of ppαF was detected in *ssa1 ssa2* mutants even after prolonged incubation at the restrictive temperature.

Since the overall role of cytoplasmic hsp70 is not well understood, we undertook pseudoreversion analysis of the *ssa1 ssa2* double null mutant in the hopes of determining in which cellular processes Ssa1/2p were involved. Instead, all the suppressor mutations we isolated affect either the regulation or function of the remaining *SSA* hsp70 subfamily members.

Ssa1/2p are very abundant proteins and probably have multiple cellular functions. Thus, in order to suppress the growth defect caused by their absence, a suppressor mutation would have to simultaneously suppress multiple different defects. All of the suppressor mutations we isolated seemed to have accomplished this by recruiting Ssa3/4p to cover for the loss of Ssa1/2p. It may be impossible to determine what cellular processes Ssa1/2p are involved in by isolating and characterizing extragenic suppressors since the cell has many different defects to overcome. However, the mutations isolated in this study will provide a good starting point for further understanding of the regulation of hsp70 activity at both the level of transcription and protein function.

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