

The Cold Sensitivity of a Mutant of *Saccharomyces cerevisiae* Lacking a Mitochondrial Heat Shock Protein 70 Is Suppressed by Loss of Mitochondrial DNA

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Abstract. *SSH1*, a newly identified member of the heat shock protein (hsp70) multigene family of the budding yeast *Saccharomyces cerevisiae*, encodes a protein localized to the mitochondrial matrix. Deletion of the *SSH1* gene results in extremely slow growth at 23°C or 30°C, but nearly wild-type growth at 37°C. The matrix of the mitochondria contains another hsp70, Ssc1, which is essential for growth and required for translocation of proteins into mitochondria. Unlike *SSC1* mutants, an *SSH1* mutant showed no detectable defects in import of several proteins from the cytosol to the matrix compared to wild type. Increased expression of Ssc1 partially suppressed the cold-sensitive growth de-

fect of the *SSH1* mutant, suggesting that when present in increased amounts, Ssc1 can at least partially carry out the normal functions of Ssh1. Spontaneous suppressors of the cold-sensitive phenotype of an *SSH1* null mutant were obtained at a high frequency at 23°C, and were all found to be respiration deficient. 15 of 16 suppressors that were analyzed lacked mitochondrial DNA, while the 16th had reduced amounts. We suggest that Ssh1 is required for normal mitochondrial DNA replication, and that disruption of this process in *ssh1* cells results in a defect in mitochondrial function at low temperatures.

H EAT shock proteins of the hsp70 class are found in all organisms and play essential roles as molecular chaperones (for reviews see 7, 18, 20). Related hsp70s are found in cellular compartments such as the cytosol, ER, and mitochondria (5). The cytosol of yeast contains two functionally distinct classes of hsp70s, the *SSA* and *SSB* proteins (9). The *SSA* proteins, besides being involved in the regulation of expression of heat-inducible genes, are required for normal rates of translocation of some proteins into the ER or mitochondria. The *SSB* proteins associate with translating ribosomes, and they are required for normal protein translation. The presence of both *SSA* and *SSB* classes of hsp70s in the same cellular compartment illustrates the evolution of related but functionally distinct types of hsp70s.

To date, Ssc1 has been the only hsp70 identified in the mitochondria matrix, and Kar2 has been the sole family member identified in the ER (8, 30, 36). In the yeast *Saccharomyces cerevisiae*, Ssc1 is required for the translocation of proteins from the cytosol into the matrix and is involved in their subsequent folding, maturation, and proteolysis (16, 21, 22, 40, 49). Ssc1 is proposed to bind to

an incoming polypeptide, preventing backsliding into the cytosol. Cycles of binding and release provide a driving force for the vectorial movement of polypeptides across the two mitochondrial membranes into the matrix. Similarly, Kar2 plays an important role in translocation and folding of proteins into the ER (39, 48). The functions of these organellar hsp70s are critical; both *SSC1* and *KAR2* are essential genes.

Mitochondria are ubiquitous, complex, and essential organelles (32). Most of the several hundred proteins that comprise mitochondria are encoded by the nuclear genome and transported into the organelle from the cytosol. The mitochondrial genome of yeast encodes eight major proteins; seven polypeptides are components of the respiratory apparatus and one is a component of mitochondrial ribosomes. The remainder of the mitochondrial genome encodes ribosomal and transfer RNAs specific for the mitochondrial translation machinery and several minor proteins involved in intron splicing and gene conversion. The mitochondrial genome is required for respiration competence, but not for the integrity of the structure of mitochondria, since cells that lack mitochondrial DNA (ρ^0) are viable, but respiration deficient. Mitochondria are essential structures, even when cells are grown on fermentable carbon sources. Therefore, components of the translocation apparatus required for the import of cytosolically synthesized mitochondrial proteins are essential genes (2).

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Here, we describe the characterization of a gene encoding the second identified hsp70 of yeast mitochondria, *SSH1*. This hsp70 is required for growth of yeast cells in the lower temperature range, but not at 37°C. Surprisingly, loss of mitochondrial DNA (mtDNA)¹ suppresses this cold-sensitive phenotype.

Materials and Methods

Yeast Strains

PJ53 is a member of the W303 isogenic family with the following genotype: *trp1-1/trp1-1 ura3-1/ura3-1 leu2-3,112/leu2-3,112 his3-11,15/his3-11,15 ade2-1/ade2-1 can1-100/can1-100 GAL2⁺/GAL2⁺ met2-Δ1/met2-Δ1 lys2-Δ2/lys2-Δ2* (James, P., personal communication). PJ43-2B is an α -mating type haploid derivative of PJ53 containing a wild-type *SSH1* gene, and it is referred to as *SSH1* throughout the paper. PJ53 was transformed to yield PJ53ΔH1, which is heterozygous for the *ssh1-1* allele (this study). Haploid derivatives of PJ53ΔH1 containing the *ssh1-1* allele are referred to as *ssh1* throughout the paper. CMV1 is a *MATa* haploid of PJ53 containing the temperature-sensitive allele of *SSC1 ssc1-2::LEU2*. The wild-type *S. cerevisiae* strain D273-10B (ATCC 25657) was used for preparation of mitochondria. Other strains used in this study have the following genotypes: DUL2 is *ura3Δ lys2 rho⁺*, and DL2rho^o is *MATa lys2 rho^o* (28).

Identification, Isolation, and Manipulation of the *SSH1* Gene

Yeast sequence databases were searched for additional hsp70 genes using the yeast *Ssa1* protein as a query sequence in the BLASTp search algorithm. In addition to the known yeast hsp70s, this search identified an open reading frame on chromosome 12 (GenBank/EMBL/DDBJ accession No. YSCL8039), which is predicted to encode a 657-amino acid protein with a high degree of homology to hsp70 proteins. We have named this gene *SSH1*.

Based on the sequence in the GenBank/EMBL/DDBJ database, the following oligonucleotide primers were designed to amplify a fragment of the identified gene using the PCR: 5'-TTGGTTGGCGTTCACAAG-3' and 5'-TTGATGGGCAAGCGGGTG-3'. Approximately 4,400 bacterial colonies from a yeast genomic library (37) were grown on LB plates with 100 mg/ml ampicillin and transferred onto nylon filters. The 815-nucleotide double-stranded PCR product, which spans the region from +1396 to +2211, with +1 being the A of the initiating ATG, was used to screen the genomic library of yeast chromosomal DNA. The PCR product was labeled using the random priming method and colonies were probed using a standard colony hybridization protocol (1). Colonies generating positive signals were isolated and plasmid DNA was prepared. Plasmid restriction maps were compared to the *SSH1* GenBank/EMBL/DDBJ sequence to identify clones that contained the entire coding region.

To construct a strain with a deletion of *SSH1*, a 2.1-kb HindIII-MunI fragment containing all but the last 100 bp of the coding sequence for *SSH1* was replaced with a 4.6-kb HindIII-EcoRI fragment carrying the gene for *LYS2*. This deletion, which has been given the allele number *ssh1-1*, removed 256 bp of *SSH1* upstream of the initiating ATG in addition to the protein-coding region. A linear 6.0-kb ClaI-EcoRI fragment containing the *ssh1::LYS2* mutation was purified and used to transform the diploid strain PJ53 by the lithium acetate protocol (19). *Lys⁺* transformants were subjected to Southern analysis to verify that one of the copies of *SSH1* had been replaced with the deletion allele.

Fusions were constructed between *SSH1* and *SSC1* by using a KpnI site located in a highly conserved region in hsp70 genes at the extreme NH₂ terminus (see Fig. 5 A). DNA encoding the promoters and 37 amino acids for Ssh1 or 39 amino acids for Ssc1 is 5' of the KpnI site. The carboxyl-encoding end of each gene was isolated as a KpnI-Sac I fragment and cloned behind the promoter of the other gene contained on a derivative of pRS316 (pRS316K; 44), which had its KpnI site destroyed by S1 nuclease treatment (James, P., personal communication). The *SSC1-SSH1* fusion

was moved to the 2- μ m plasmid, pRS426 (44), as a Sall-SacII fragment. The *SSH1-SSC1* fusion was moved as an EcoRI-SacI fragment.

To construct an expression vector for in vitro transcription/translation of *SSH1*, a 2.6-kb *Bst1107I*-EcoRI fragment, containing the coding region for *SSH1* plus 124 bp of upstream DNA, was cloned into pSP64 (Promega, Madison, WI) restricted with HincII-EcoRI.

The promoters of *SSC1* and *SSH1* were translationally fused to the reporter gene, *lacZ*. The vector carrying the promoterless *lacZ* gene was derived from pHT102 (46), which had its EcoRI-SacI fragment replaced with the EcoRI-SacI fragment from pMC2010 (6) to generate pBS1-*lacZ* (this study). The *SSC1* promoter was isolated as a 350-bp EcoRI-HindIII (blunted) fragment from pRS316K-C1 (James, P., personal communication) and cloned into pRS316 (44) restricted with EcoRI and SmaI. From this plasmid, an EcoRI-BamHI (blunted) fragment was isolated and cloned into pBS1-*lacZ* restricted with EcoRI-SmaI. The fusion protein contains the first 12 amino acids of Ssc1. The *SSH1* promoter was isolated as a 1.4-kb EcoRI-PleI (blunted) fragment and cloned into pBS1-*lacZ* restricted with EcoRI-SmaI. The resulting fusion protein contains the first four amino acids of Ssh1.

A modified *SSH1* gene capable of expressing Ssh1 with a hemagglutinin (HA) tag on the very carboxyl end was constructed to localize the protein in the cell by immunological techniques. First, a NotI site was generated at the site in the *SSH1* gene encoding the extreme carboxyl end of the Ssh1 protein using standard PCR protocols. Then a 114-bp NotI fragment carrying three tandem copies of the HA tag was isolated from pGTEPI (35) and cloned into the generated NotI site in *SSH1*. The clone was sequenced to determine the correct orientation of the tag and the absence of any mutations caused by PCR. The tagged gene was cloned into pRS316 and transformed into the haploid *ssh1* deletion strain to test for function.

β -Galactosidase Assay

Transformants were grown in minimal media lacking tryptophan with either glucose or galactose as the carbon source (43) to an OD₆₀₀ of 0.5–0.8. Cells were collected in 1.5-ml Eppendorf tubes, and the pellets were rapidly frozen in a dry ice ethanol bath. The pellets were stored at –80°C until they were assayed. β -galactosidase activity in Miller units was measured as described previously (45). Miller units were calculated as $(OD_{420} \times 1,000) / (OD_{600} \times t \times v)$, where *t* is incubation time in min and *v* is volume of cell culture harvested in milliliters (25).

Translocation of Proteins into and Fractionation of Mitochondria

Mitochondria were isolated from the wild-type yeast strain D273-10B, as described previously (16). [³⁵S]Methionine-labeled precursor proteins were synthesized in vitro using a coupled rabbit reticulocyte lysate system (Promega). All methods for translocation experiments have been described previously (16). 50 μ g of mitochondria were preincubated for 5 min at 23°C, and then incubated at 23°C with lysate containing ³⁵S-labeled Ssh1 in the presence or absence of a membrane potential for 5 or 30 min before addition of VAO (final concentration = 0.25 μ M valinomycin, 4 μ M antimycin A, and 10 μ M oligomycin). Half of each sample was then treated with proteinase K (PK; 100 μ g/ml) for 25 min at 0°C. 25- μ g samples were analyzed by SDS-PAGE and autoradiography.

For fractionation of mitochondria, 50 μ g of isolated mitochondria were resuspended in 50 μ l of SEM (250 mM sucrose, 1 mM EDTA, 10 mM Mops, pH 7.2). Mitochondria were swollen to form mitoplasts by diluting 10-fold in 25 mM Hepes pH 7.4 and incubated at 0°C for 25 min with vortexing for 10 s every 5 min. Mitoplasts were disrupted by adding sodium deoxycholate (DOC) to 1% in the presence or absence of 75 μ g/ml PK and incubating 15 min at 0°C. Samples were centrifuged for 15 min at 14,000 rpm in an Eppendorf 5415 microfuge (Brinkman Instruments, Inc., Westbury, NY) to separate pellet and supernatant fractions. The supernatant of the sample not treated with DOC or PK (intermembrane space) was precipitated by the addition of 1/10 vol of 75% TCA. 25- μ g samples were analyzed by SDS-PAGE and autoradiography or Western analysis using the ECL detection kit from Amersham (Arlington Heights, IL).

Analysis of Chromosomal and mtDNA

Cellular DNA was isolated and analyzed as previously described (14). Briefly, 250-ml cultures were grown to an OD₆₀₀ of 2.0–3.0, and the cells harvested. After treatment with zymolyase, the resulting spheroplasts were washed with 10 ml of 1 M sorbitol and resuspended in 0.25 ml of 1 M

1. Abbreviations used in this paper: CAI, codon adaptation index; DOC, deoxycholate; HA, hemagglutinin; mtDNA, mitochondrial DNA; PK, proteinase K.

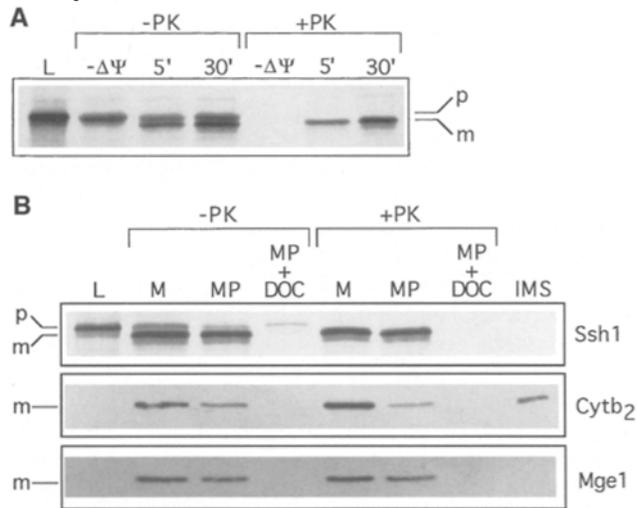


Figure 2. Ssh1 preprotein can be imported into the matrix of mitochondria and is cleaved in the process. (A) Ssh1 preprotein synthesized in a reticulocyte lysate was added to isolated mitochondria. Translocation was carried out in the presence of a membrane potential, $\Delta\psi$, for 5 or 30 min and in the absence of membrane potential ($-\Delta\psi$). PK, proteinase K; p, precursor; m, mature; L, reticulocyte lysate containing ^{35}S -labeled Ssh1 preprotein. (B) Fractionation of mitochondria (M) after a 30-min translocation reaction into mitoplasts (MP) and intermembrane space fractions (IMS). An equivalent portion of mitoplasts was disrupted with deoxycholate detergent (MP + DOC). Equivalent amounts of the fractions were either treated with (+PK) or without (-PK) proteinase K. Immunoblot analysis was carried out on fractions with antibodies specific for Cytb₂ and Mge1 after separation by electrophoresis.

tein, mitochondria were treated with a hypotonic solution that ruptures the outer, but not the inner, mitochondrial membrane to form mitoplasts. Under these conditions, proteins of the intermembrane space, such as cytochrome b₂, are released from the mitochondria and become sensitive to exogenously added protease, while matrix proteins, such as Mge1, remain insensitive to protease (Fig. 2 B). Ssh1 was not sensitive to protease treatment after mitoplast formation, becoming sensitive only after the mitoplasts were treated with detergent to disrupt the inner membrane. This result suggests that Ssh1 is localized to the matrix.

These in vitro translocation experiments demonstrated that Ssh1 can be imported into the matrix of mitochondria, but did not directly address the question of its normal cellular location. Therefore, we constructed a gene that encoded Ssh1 with a HA tag at its carboxyl terminus. The tagged protein was recognized by HA-specific antibodies (Fig. 3 A) and was found to be functional, since it rescued the cold-sensitive growth defect of the *ssh1* null mutant (data not shown). Mitochondria, purified from a strain containing the HA-tagged Ssh1 protein as the only Ssh1 protein, were subjected to hypotonic treatment and found to contain Ssh1. As in the in vitro import experiments, Ssh1 was completely resistant to protease digestion in mitoplasts, becoming susceptible only after disruption of the inner membrane with detergent (Fig. 3 B). These results confirm that Ssh1 is a protein of the mitochondrial matrix.

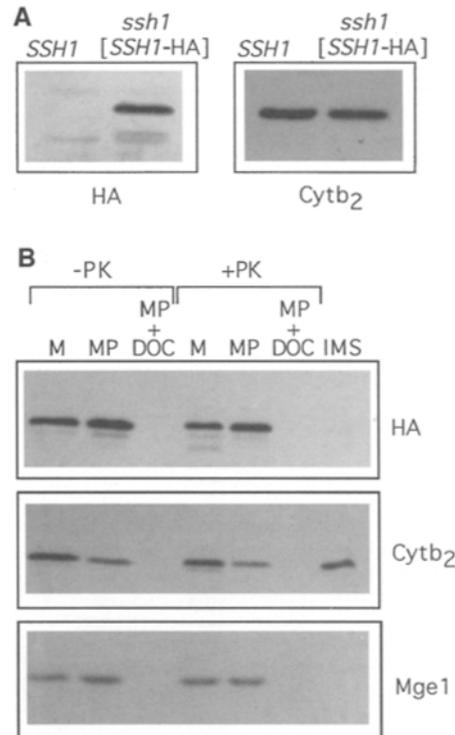


Figure 3. Ssh1 is localized to the matrix of mitochondria in vivo. (A) HA-tagged Ssh1 can be detected by HA-specific antibodies. Whole-cell lysates of cells containing wild-type *SSH1* or cells with a disrupted copy of *SSH1* and a plasmid carrying an *SSH1* gene with a HA tag at the carboxyl end (*ssh1* [*SSH1*-HA]) were separated by electrophoresis and subjected to immunoblot analysis using HA- and cytochrome b₂ (Cytb₂)-specific antibodies. (B) Mitochondria (M) isolated from cells carrying the HA-tagged form of Ssh1 were separated into mitoplast (MP) and intermembrane space (IMS) fractions. An equivalent portion of mitoplasts was disrupted by addition of deoxycholate detergent (MP + DOC). Equivalent amounts of the fractions were either treated (+PK) or not treated (-PK) with proteinase K. Immunoblot analysis was carried out on the fractions with antibodies specific for the HA tag (HA), cytochrome b₂ (Cytb₂), or Mge1 after separation by electrophoresis.

Deletion of *SSH1* Results in a Cold-sensitive Phenotype

To determine the phenotype of a strain lacking the *SSH1* gene, a deletion/insertion mutation was constructed. The 2.1-kb HindIII-MunI fragment (-256 to $+3347$, with $+1$ being the ATG of the initiating methionine) was replaced by the *LYS2* gene (Fig. 4 A), and a diploid wild-type strain (PJ53) was transformed as described in Materials and Methods. Transformants were selected based on their lysine prototrophy, and the presence of the mutant allele was confirmed by Southern blot analysis (data not shown). The heterozygous diploid was sporulated. After incubation of the dissection plates at 30°C, the tetrads showed a uniform 2:2 segregation of pinpoint and large colonies after 7 d (Fig. 4 B). The lysine prototrophy segregated 2:2 concomitantly with the small colonies, indicating that they carried the *ssh1::LYS2* allele. At temperatures of 23°C and below, *ssh1* cells did not form visible colonies. At the higher temperature of 37°C, the growth difference between *SSH1* and *ssh1* strains was minimal (Fig. 5 C). The

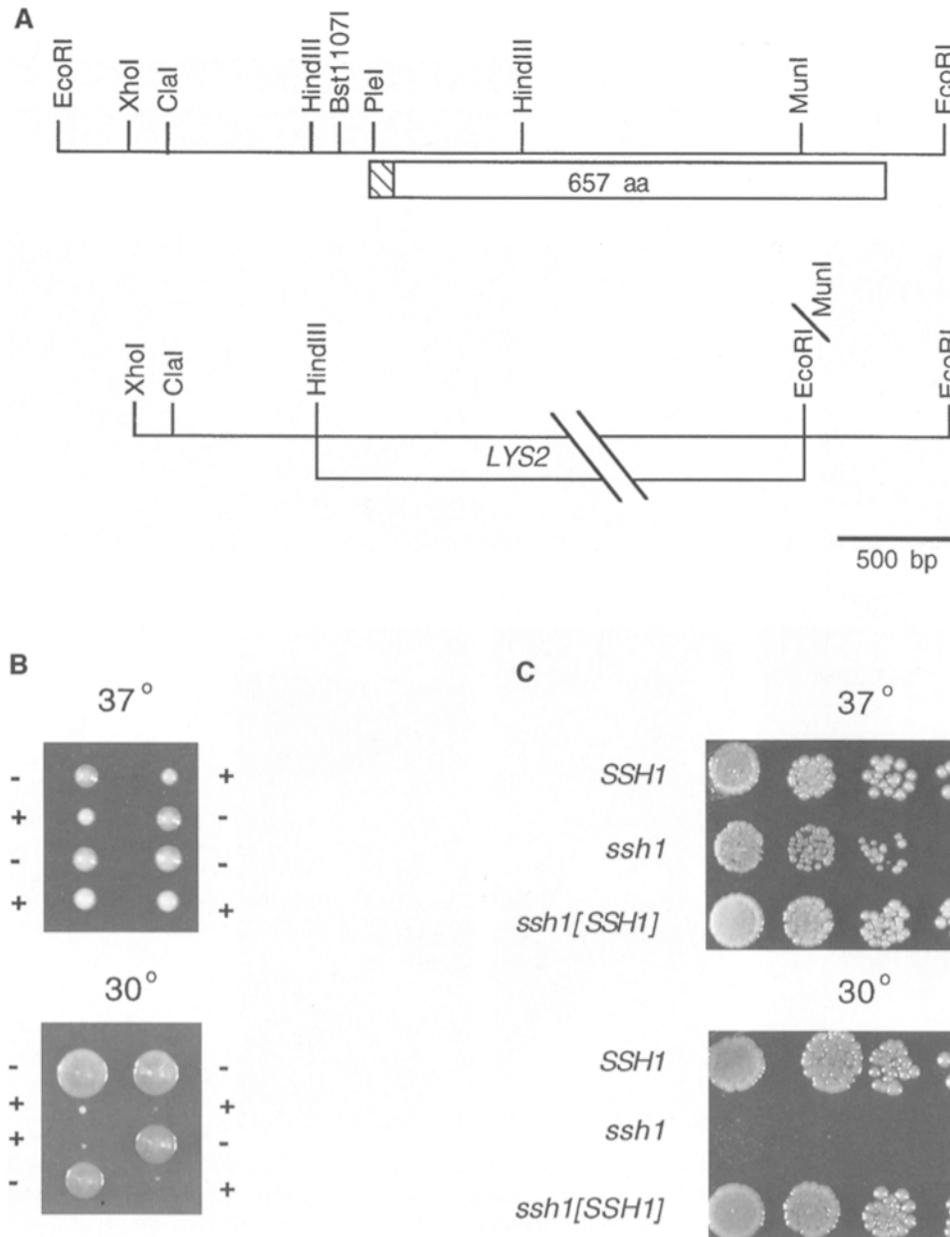


Figure 4. Deletion of *SSH1* results in a cold-sensitive growth phenotype. (A) Insertion/deletion mutation of *SSH1*. Transcription of the *SSH1* gene is from left to right. The box beneath the DNA represents the protein coding region of *SSH1* with the hatched portion indicating the putative presequence. The bottom part of the figure represents the mutant allele formed by insertion of the *LYS2* gene after deletion of the *SSH1* DNA between the HindIII and MunI sites (B) Asci of P153ΔH1 were dissected onto glucose-based media, and the plates were incubated at 30 or 37°C for 7 and 4 d, respectively. The plates were replica plated to lysine omission medium, and growth or lack of growth was indicated by the + and - symbols, respectively. (C) Rescue of the growth defect of the *ssh1* mutant by *SSH1* carried on a low copy plasmid (*SSH1*). Both the 37°C and 30°C plates were incubated for 5 d.

cold-sensitive phenotype was relieved by the presence of the wild-type *SSH1* gene on a centromeric plasmid (Fig. 4 C). A growth rate difference between *ssh1* and wild-type cells was also observed on medium containing carbon sources other than glucose. On the nonfermentable carbon sources, glycerol or lactate, mutant colonies formed slowly or not at all at 23°C, respectively; at 37°C, visible colonies formed in both cases, but at a reduced rate compared to wild type. On galactose-based medium, *ssh1* cells formed colonies, but grew more slowly than wild type at 23°C (data not shown).

Overexpression of *Ssc1* Partially Suppresses the Cold-sensitive Phenotype of *ssh1* Cells

Two hsp70s are present in the mitochondrial matrix, the newly identified Ssh1 protein and Ssc1 protein. As described above, cells lacking Ssh1 protein are cold-sensitive

for growth. The other hsp70 identified in mitochondria, Ssc1, is essential for growth at all temperatures. To better address the question of whether these related proteins are functionally distinct, we created fusions, such that the presequence coding region as well as the promoter was derived from one gene and the remainder of the fusion from the other gene (see Materials and Methods). Construction of these fusions was facilitated by the presence of a KpnI site in the regions of both genes that encode the extreme NH₂ terminus. Because the NH₂ termini of hsp70s are highly conserved, the proteins resulting from expression of the fusion constructs contain only a few alterations at the very NH₂ terminus (Fig. 5 A).

The *SSC1:SSH1* fusion was transformed into the *ssh1* null mutant. The presence of the *SSC1:SSH1* fusion resulted in suppression of the cold sensitivity of the *ssh1* strain (Fig. 5 B), consistent with the finding that both Ssc1 and Ssh1 are present in the mitochondrial matrix. The

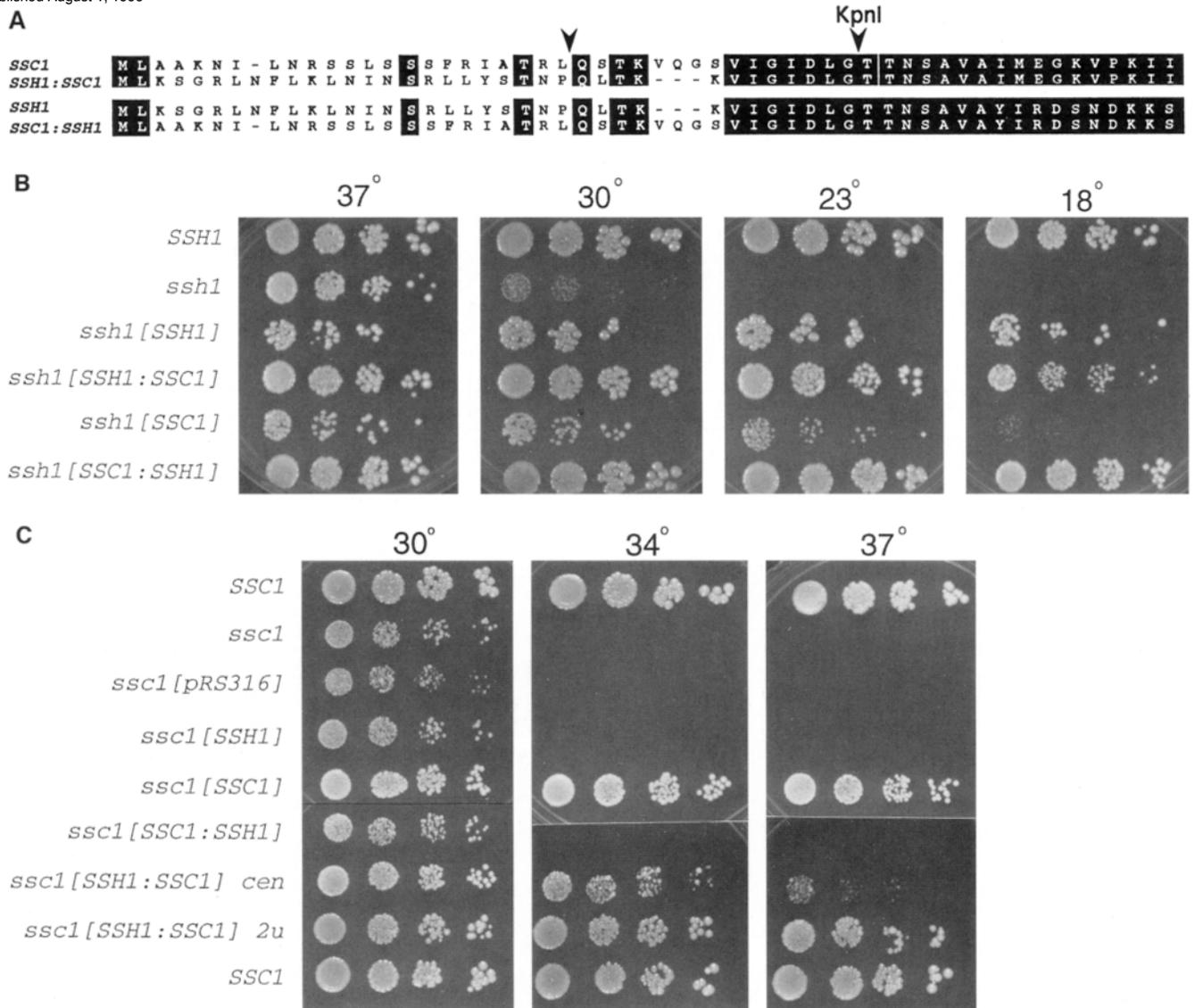


Figure 5. Suppression of the growth defects of *SSC1* and *SSH1* mutants by *SSH1* and *SSC1* fusion constructs. (A) The NH₂ regions of the predicted protein products of the *SSH1::SSC1* and *SSC1::SSH1* are shown in comparison with *SSC1* and *SSH1*. The known cleavage site of *SSC1* is indicated by the arrow. (B) Growth of *ssh1* cells in the presence of plasmids carrying wild-type genes or fusion constructs. Cells, plated on yeast extract/peptone/dextrose, were incubated at the indicated temperatures for 5 d. All plasmids were constructed from the centromeric vector pRS316. (C) Growth of *ssc1-2* cells in the presence of plasmids carrying wild-type genes or fusion constructs of *SSC1* and *SSH1*. Cells plated on yeast extract/peptone/dextrose were incubated for 3 d. All genes and fusions were in the centromeric vector pRS316 except the fusion *SSH1::SSC1* 2- μ m, which was cloned into the 2- μ m vector pRS426.

SSH1::SSC1 fusion was also transformed into the *ssh1* null mutant. Partial suppression of the cold-sensitive phenotype was conferred by the presence of this fusion, allowing substantial growth at 30°C, but no observable growth at 18°C (Fig. 5 B). A centromeric plasmid containing an *SSC1* gene under control of its own promoter was able to support faster, but not wild-type, growth rates of *ssh1* cells at 23° and 18°C. In addition, growth on galactose-based media, which results in increased Ssc1 expression (see below), partially suppressed the cold-sensitive phenotype of *ssh1* (data not shown). These results indicate that when present in increased amounts, Ssc1 is able to carry out, at least partially, some or all of the functions of Ssh1.

The fusions were also transformed into a temperature-

sensitive *SSC1* mutant, *ssc1-2* (22). No rescue of the temperature-sensitive growth defect was observed in *ssc1-2* cells carrying the *SSC1::SSH1* fusion (Fig. 5 C). The lack of suppression suggests that Ssh1 cannot compensate for the absence of functional Ssc1. As expected, a centromeric plasmid carrying the *SSC1* gene under control of its own promoter allowed complete rescue of the temperature-sensitive growth defect (Fig. 5 C). On the other hand, the *ssc1-2* cells carrying the *SSH1::SSC1* fusion on a low copy number plasmid containing a centromere showed only slightly better growth at the intermediate temperature of 34°C than *ssc1-2* cells with the vector alone and no colony formation at the more restrictive temperature of 37°C. However, the same fusion gene carried on a high copy, 2- μ m

plasmid allowed complete rescue of the temperature sensitive phenotype (Fig. 5 C).

The requirement that the *SSH1:SSC1* fusion be in high copy number in order to suppress the growth defect of the *ssc1-2* strain suggests that the *SSH1* gene might be expressed at a lower level than the *SSC1* gene. Analysis of the codon usage of *SSH1* and *SSC1* allowed us to make a comparison of the codon adaptation index (CAI), allowing a prediction of the relative levels of expression (42). Genes of *S. cerevisiae* that are highly expressed, such as ribosomal protein genes and histones, usually have a CAI between 0.5 and 1.0. Genes that are expressed at very low levels, such as *GAL4*, have CAIs of 0.098 to 0.12. Using the DNAstrider program, *SSC1* was calculated to have a CAI of .522 and *SSH1* a CAI of 0.148, suggesting that *SSH1* is expressed at relatively low levels while, as already known, *SSC1* is a relatively highly expressed gene (8).

To attempt to address the question of the level of expression more closely, we constructed fusions between the promoters of *SSC1* and *SSH1* and the reporter gene, *lacZ* (see Materials and Methods). Wild-type cells (PJ43-2B) carrying the *SSC1:lacZ* and *SSH1:lacZ* fusions on centromeric vectors were grown at two temperatures, 23 and 30°C, and assayed for β -galactosidase activity (Table I). The nearly 1,000-fold difference in activity suggests that *SSH1* is expressed at much lower levels than *SSC1*. Expression levels of both *SSC1* and *SSH1* are twofold higher when cells are grown in media with galactose as the carbon source instead of glucose as expected of genes encoding mitochondrial proteins.

ssh1 Mitochondria Show No Obvious Defect in Preprotein Translocation

Ssc1, the first mitochondrial hsp70 identified, is required for translocation of a variety of proteins from the cytosol into mitochondria (22). To investigate whether *Ssh1* was required for normal translocation into mitochondria, we tested the ability of mitochondria isolated from *ssh1* cells to import radiolabeled precursor proteins in vitro. No significant difference was observed in the import kinetics or efficiency of translocation of the Rieske Fe/S protein, cytochrome *b*₂ (Fig. 6), or cytochrome *c*₁ (data not shown), when import of these precursors into *ssh1* mitochondria was compared to import into mitochondria from wild-type cells (Fig. 6). Therefore, we were unable to find a requirement for *Ssh1* in the translocation of several proteins into mitochondria. This is not surprising, considering the deduced low abundance of *Ssh1* protein in the cell.

Table I. Activity of the *SSC1* and *SSH1* Promoters at Different Temperatures and on Different Carbon Sources

Plasmid [‡]	β -Galactosidase activity*		
	23°C	30°C	Galactose
pBS1- <i>lacZ</i>	0.14	0.15	0.16
<i>SSC1:lacZ</i>	477	469	1019
<i>SSH1:lacZ</i>	0.5	0.7	1.4

* β -Galactosidase activity was determined in PJ43-2B, as described in Materials and Methods.

[‡] Promoter regions included in fusions to *lacZ* are described in Materials and Methods.

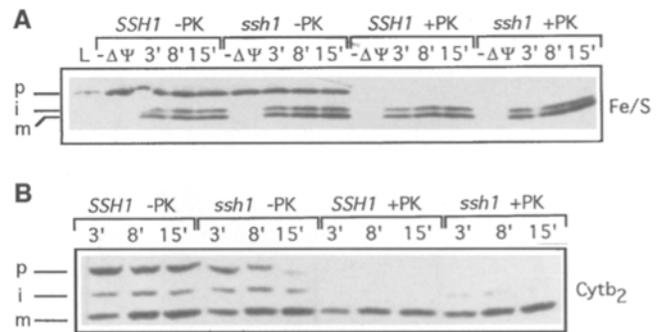


Figure 6. No defect in translocation of proteins into mitochondria was observed in *ssh1* mutants. (A) Import of pre-Fe/S protein and (B) precytochrome *b*₂ into isolated mitochondria from wild-type and *ssh1* cells with time was compared. After the import reaction was stopped, the reactions were divided; half were treated with proteinase K (+PK) and half were not (-PK). p, precursor, i, intermediate, m, mature.

Loss of Mitochondrial DNA Suppresses the *ssh1* Cold-sensitive Phenotype

During growth studies of the *SSH1* mutant, we observed fast-growing colonies arising at 23 and 30°C at a frequency of 0.6 and 2.5%, respectively. The high frequency of appearance of fast-growing colonies suggested that the growth suppression was not caused by a mutation in the nuclear genome, but to some other more frequent extragenomic event. Initially, four phenotypic suppressors were chosen for more detailed analysis. All four grew more slowly than the *ssh1* parent at 37°C, but were able to form colonies at lower temperatures (for example, see Fig. 7). Since the frequency of the appearance of suppressors was similar in magnitude to the appearance of respiration-deficient petites in the parent strain (5–10% at 30°C), we tested the growth of the suppressors on glycerol, a nonfermentable carbon source. All four suppressors were unable to grow on glycerol at any temperature, indicating that they were respiration-deficient petites (for example, see Fig. 7).

There are several types of mutations that can cause a petite phenotype: *pet*⁻, *mit*⁻, and *rho*⁻. A *pet*⁻ mutation is a nuclear mutation, a *mit*⁻ mutation is a point mutation or small deletion in the mitochondrial genome, and a *rho*⁻ mutation is a larger deletion mutation in the mitochondrial genome that results in the absence of mitochondrial protein synthesis. Cells most commonly become petite because of a *rho*⁻ mutation. Deletion of mtDNA segments is accompanied by amplification of the remaining DNA, such that the total DNA mass remains similar to that found in wild-type strains (10). Subsequently, *rho*⁻ cells may lose all their mtDNA to become *rho*^o.

To assess the cause of the defect in respiration, we crossed the suppressors with *rho*^o and *rho*⁺ tester strains (DL2*rho*^o and DUL2, respectively). The diploids constructed with the *rho*^o tester were unable to grow on nonfermentable carbon sources, while those formed with the *rho*⁺ strain were respiration competent (data not shown). These results indicated that a nuclear *pet*⁻ mutation was not responsible for the respiration deficiency, and suggested that the suppressors did not contain normal mtDNA.

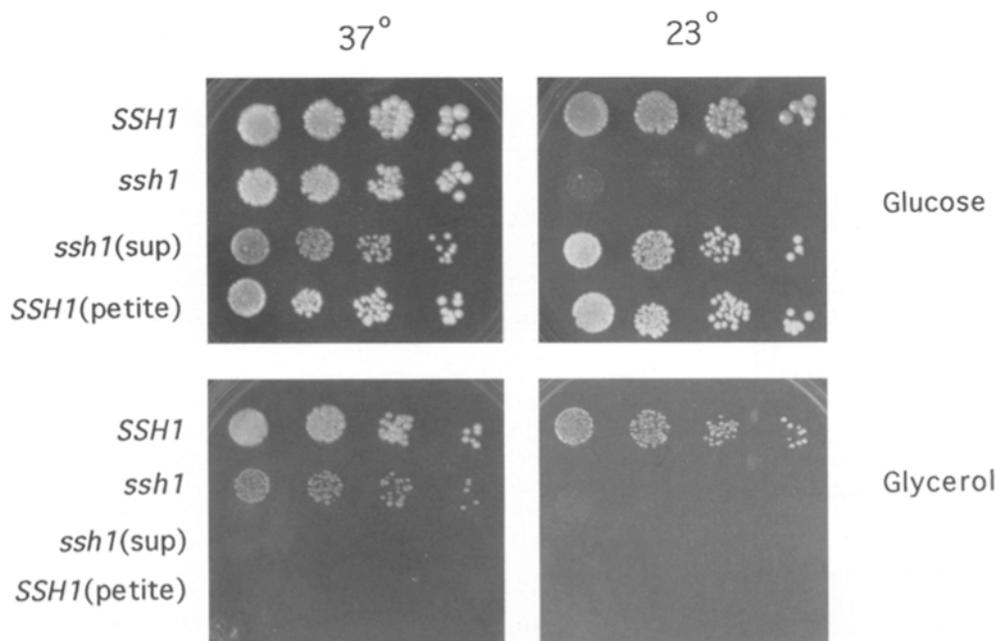


Figure 7. *ssh1* suppressors grow robustly at 23 and 30°C, but not 37°C, and are respiration deficient. *SSH1*, *ssh1*, *ssh1* suppressor, and a petite derivative of *SSH1* were spotted onto rich medium and incubated for 5 d at either 37 or 23°C.

To determine whether the suppressors lacked mtDNA or were of the more common ρ^- type, two tests were performed. First, mtDNA was visualized as bands in CsCl gradients containing the dye bisbenzimidazole, which preferentially binds (A + T)-rich DNA, and thus allows separation of mtDNA from genomic DNA (see Materials and Methods). While genomic and mtDNA from the ρ^+ controls, which included the *ssh1* parental strain, showed about equivalent staining with bisbenzimidazole, no band of mtDNA was observed in any of the four suppressors (Fig. 8 A). We also carried out fluorescence microscopy of DAPI-stained cells in order to visualize mtDNA. While mtDNA was easily visible as small dots in the cytosol of wild-type and *ssh1* mutant cells, no such fluorescence was observed in the suppressors (Fig. 8 B). Based on these two tests, we conclude that the four suppressors did not contain mtDNA and are ρ^0 petites.

The original four phenotypic suppressors of *ssh1* tested lacked mtDNA. However, these suppressors were stored for several weeks before the analysis, and it is known that ρ^- strains are unstable, resulting in larger deletions or total loss of DNA (14). We wished to test whether *ssh1* suppressors were ρ^0 immediately after isolation. Therefore, a fresh, respiration-competent *ssh1* isolate was plated on rich media at 23°C, and 11 new suppressors were selected. All were respiration deficient, as indicated by their inability to form colonies at 23°C on glycerol-based medium. 10 of the 11 *ssh1* suppressors lacked detectable mtDNA (data not shown); one had mtDNA, but the amount was reduced fourfold relative to wild type as determined by comparison of the staining intensity of the chromosomal and mitochondrial DNA bands. Since this analysis detected the average amount of mtDNA to genomic DNA in the cell population, we wondered if the suppressor that had reduced amounts of mtDNA was undergoing mtDNA loss. To test this, six colonies of this line were picked, grown up, and the DNA analysis was repeated on these newly established lines. Five of the lines

showed reduced amounts of mtDNA similar to the parent suppressor line; the sixth line showed no detectable mtDNA (data not shown).

The respiration-deficient strains that we isolated as suppressors of *ssh1* were predominantly ρ^0 , while petite strains arising from a wild-type population are predominantly ρ^- . Two alternate hypotheses could explain this observation: either ρ^0 deficiencies can better suppress the *ssh1* phenotype or *ssh1* strains have an unusually high tendency to become ρ^0 . To discern between these possibilities, we screened *ssh1* cells at 37°C to identify those which had become respiration deficient, and then tested those cells for mtDNA. This screen was aided by the fact that the yeast strain PJ53 carries an *ade2* mutation that causes the accumulation of a red pigment when respiratory competent, but not when respiration deficient. Small white colonies were picked and streaked on glycerol-containing media to confirm that they were respiration deficient. Only six isolates that were unable to grow on glycerol-based media were obtained from three separate plating experiments, screening a total of ~4,500 colonies. Five of the six isolates failed to form colonies at 23°C, indicating that the *ssh1* cold-sensitive phenotype was not suppressed. DNA isolated from the six isolates was banded in CsCl gradients containing bisbenzimidazole. The five isolates that failed to suppress the *ssh1* cold-sensitive phenotype contained normal amounts of mtDNA, while the isolate that could form colonies at 23°C was ρ^0 (data not shown). To discriminate between a mutation in nuclear or mitochondrial DNA being responsible for the respiratory deficiencies, the five nonsuppressing isolates were mated to a ρ^0 tester strain. The resulting zygotes were all able to grow on glycerol-based media, indicating that the respiration deficiency in these five isolates, which could be siblings, was caused by a nuclear mutation. Therefore, the *ssh1* mutation does not induce ρ^- or ρ^0 mutations at a permissive growth temperature. In conclusion, we have been unable to isolate a ρ^- mutation in the *ssh1* back-

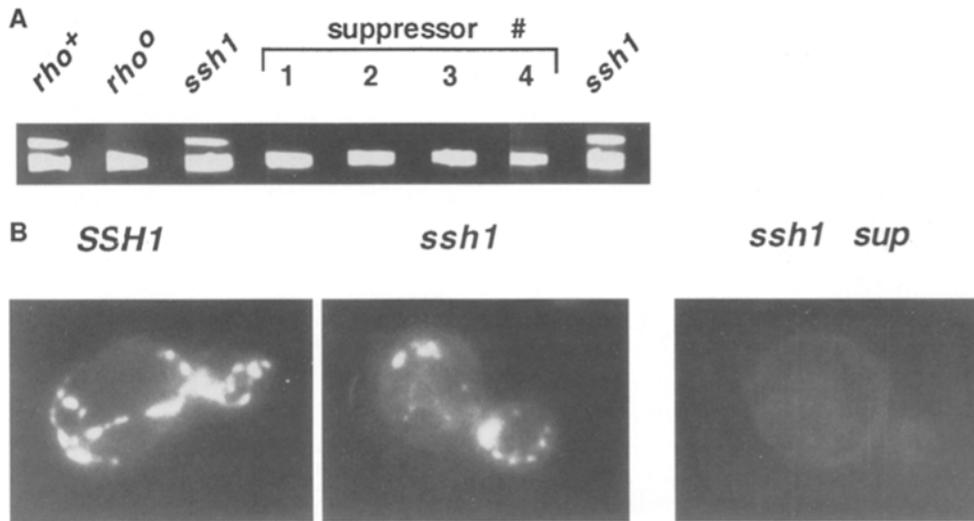


Figure 8. *ssh1* suppressors lack mitochondrial DNA. (A) Detection of mtDNA by staining with bisbenzamide. DNA isolated from cells grown at 37°C (*ssh1*) or 23°C (ρ^+ , ρ^0 , and *ssh1* suppressors [lanes 1–4]) was centrifuged in CsCl gradients containing bisbenzamide, and DNA was visualized under UV light. (B) DAPI staining of *SSH1*, *ssh1*, and an *ssh1* suppressor (*ssh1 sup*).

ground with normal amounts of mtDNA at any temperature.

Discussion

In this study, we have demonstrated the existence of a second hsp70-related protein (Ssh1) in the mitochondrial matrix. While Ssc1 protein is essential for growth at all temperatures, Ssh1 protein is only required at temperatures of 30°C and below, since *ssh1* cells grow nearly as well as wild type at 37°C. Given the vastly different expression levels between the two mitochondrial hsp70 genes, it was surprising to find that overexpression of Ssc1 can partially suppress the cold-sensitive phenotype of *ssh1* cells. These results suggest two possible explanations for the nature of the *ssh1* phenotype. Perhaps Ssh1 performs an essential function at lower temperatures that is not required at higher temperatures; Ssc1 can functionally replace Ssh1, but only when at higher than normal concentrations. On the other hand, Ssh1 activity may be required at all temperatures, but sufficient Ssc1 protein is available at the higher temperature of 37°C to perform the normal function of Ssh1 protein. While Ssc1 can compensate for the absence of Ssh1, the opposite does not appear to be the case as the *SSC1:SSH1* fusion was unable to complement a temperature-sensitive *SSC1* mutant. These results suggest that Ssh1 may play a very specialized role in mitochondrial function.

What role does Ssh1 play in mitochondria? Ssc1 has been shown to be required for the translocation of preproteins from the cytosol into the matrix (16, 22, 40). However, we observed no requirement for Ssh1 in translocation, consistent with the failure of overexpression of Ssh1 to rescue the growth defect of a temperature-sensitive *SSC1* mutant. However, we cannot exclude the possibility that a defect in translocation of a subset of proteins is responsible for the observed *ssh1* phenotypes. A clue to the function of Ssh1 comes from the characterization of the suppressors of the cold-sensitive growth defect of *ssh1* cells. 15 out of 16 suppressors examined had lost their mtDNA becoming ρ^0 , while the 16th had reduced amounts of mtDNA, suggesting having no or reduced

amounts of mtDNA is advantageous to *ssh1* cells. Normally, when cells become respiration deficient, mtDNA is not completely lost; rather, a deletion of mtDNA occurs, rendering cells incapable of synthesizing the mitochondrially encoded components of the respiratory chain, and the remaining DNA is amplified (13). Therefore, respiratory incompetent ρ^- cells have normal amounts of mtDNA. Since both ρ^- and ρ^0 cells fail to synthesize mitochondrially encoded proteins, two simple hypotheses would state that either the loss of mtDNA or the lack of mitochondrial protein synthesis is the cause of suppression. Since we were unable to isolate ρ^- *ssh1* cells with normal amounts of mtDNA, we were unable to determine if mitochondrial protein synthesis is directly responsible for the growth defect in mutant cells. Analysis of the *ssh1* mutant, however, does not point to detrimental effects of protein synthesis. The pattern of proteins synthesized in *ssh1* mutant and wild-type mitochondria was the same, and no increased aggregation of mitochondrially synthesized proteins was observed (Walter, W., and E. Craig, unpublished results). In addition, *ssh1* cells grew better at 23°C under some conditions, where mitochondrial protein synthesis is known to increase (i.e., galactose- or glycerol-based, compared to glucose-based media).

The idea that the presence of normal amounts of mtDNA is detrimental to *ssh1* cells is supported by the fact that we were unable to isolate the more common ρ^- mutation in the *ssh1* background. It is possible that Ssh1 functions in DNA replication, particularly at low temperatures. In the absence of Ssh1 at these temperatures, DNA replication may proceed in a manner that is deleterious to the cell. Mitochondria are critical for the synthesis of a number of important biological compounds such as lipids, heme, amino acids, and nucleotides (47). Therefore, defects in DNA replication that disrupt biogenesis or distribution of mitochondria would be lethal events. However, it is also known that loss of mitochondrial DNA can affect expression of nuclear genes (31). It is therefore possible that mtDNA loss is not directly the cause of suppression of the growth defect of *ssh1* cells.

Involvement of several hsp70s with DNA-related processes has been observed. A role for *E. coli* hsp70, DnaK,

in DNA replication has been known for many years (15). DnaK is required for the replication of phage λ DNA, playing an essential role in the disassembly of the initiation complex, thus allowing replication to proceed (17). One report has also suggested a role for DnaK in the initiation of *E. coli* DNA replication, based on phenotypic similarities between strains carrying mutations in *dnaA*, which encodes a protein required for the initiation of DNA replication (38), and a particular allele of *dnaK*.

For many years, it was thought that procaryotes, the theorized progenitors of mitochondria, have only a single hsp70, DnaK. Recently, however, a second hsp70, Hsc66 encoded by the *hscA* gene, which is only 35% identical to DnaK, has been identified in *E. coli* (23, 24, 41). Both mitochondrial proteins, Ssc1 and Ssh1, are similarly related to each of the two procaryotic proteins. Ssc1 and DnaK share 59% amino acid identity, Ssh1 and DnaK 49%, Ssc1 and Hsc66 39%, and Ssh1 and Hsc66 38%. A role for Hsc66 in DNA metabolism has also been suggested. Mutations in the gene encoding Hsc66, *HscA*, were initially identified as suppressors of mutations of *hns-1* (23). *hns-1* encodes a major component of the nucleoid (12). One phenotype of *hns-1* mutants is a dramatic increase in site-specific DNA inversions that normally occur at low levels. Mutations in *hscA* are able to suppress this high level of DNA inversion. However, the mechanism by which this suppression occurs is not understood, and the role of either procaryotic hsp70 in host DNA metabolism therefore remains unresolved. It is also intriguing to note that Ssc1 has been identified as an essential, noncatalytic subunit of the mitochondrial site-specific endonuclease, *Endo.SceI*, which is thought to be involved in genetic recombination (27, 29).

A mitochondrial hsp70 has been identified in trypanosomes. This hsp70 has been localized to the site of mtDNA replication within the unusually large mitochondrion called a kinetoplast (11). This location has been an enigma because of the known involvement of the mitochondrial hsp70 from *S. cerevisiae*, Ssc1, in protein translocation. We suggest that the mitochondria of trypanosomes, like those of yeast, are likely to have more than one hsp70, one involved in DNA replication and one involved in the translocation and folding of proteins synthesized in the cytosol.

The fact that loss of mtDNA results in suppression of the *ssh1* cold-sensitive phenotype is unusual, if not unique. This observation may have implications concerning mitochondrial metabolism in mammalian cells, since alterations in mtDNA are a frequent cause of maternally inherited genetic diseases characterized by defects in respiratory metabolism (for review see 33). Most of these mitochondrial myopathies are caused by deletions of portions of the mtDNA, but patients with one type have been shown to have reduced amounts of mtDNA (26). Additional research with this mutation has shown that over many generations the mitochondria ultimately lose all their mtDNA, becoming rho⁰ (3, 4). The reduction in mtDNA is caused by a nuclear-encoded mutation whose gene product has not yet been identified. We have shown that mutation of a yeast mitochondrial hsp70 gene is more detrimental in the presence of normal amounts of mtDNA than in its absence. It is intriguing to speculate that a similar mutation in humans would result in selection of cells with lower levels of mtDNA.

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