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MAS5, a Yeast Homolog of DnaJ Involved in Mitochondrial Protein Import

DAVID P. ATENCIO AND MICHAEL P. YAFFE*

Department of Biology, B-022, University of California, San Diego, La Jolla, California 92093

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The nuclear *mas5* mutation causes temperature-sensitive growth and defects in mitochondrial protein import at the nonpermissive temperature in the yeast *Saccharomyces cerevisiae*. The *MAS5* gene was isolated by complementation of the mutant phenotypes, and integrative transformation demonstrated that the complementing fragment encoded the authentic *MAS5* gene. The deduced protein sequence of the cloned gene revealed a polypeptide of 410 amino acids which is homologous to *Escherichia coli* DnaJ and the yeast DnaJ homolog SCJ1. Northern (RNA blot) analysis revealed that *MAS5* is a heat shock gene whose expression increases moderately at elevated temperatures. Cells with a deletion mutation in *MAS5* grew slowly at 23°C and were inviable at 37°C, demonstrating that *MAS5* is essential for growth at increased temperatures. The deletion mutant also displayed a modest import defect at 23°C and a substantial import defect at 37°C. These results indicate a role for a DnaJ cognate protein in mitochondrial protein import.

Intracellular protein traffic depends on the function of heat shock proteins (HSPs) and their cognates (11, 41). HSPs are involved in the import of proteins into mitochondria (5, 9, 12, 26, 36) and chloroplasts (25), protein secretion into the lumen of the endoplasmic reticulum (8, 12, 40, 47), and the transport of certain cytosolic proteins into lysosomes as part of a protein-degradative pathway (7). In all of these processes, HSPs are thought to mediate protein conformational changes, including folding and unfolding of polypeptide chains and alterations in protein oligomerization states (14, 41). Many questions remain about the specific roles these conformational changes play in protein transport processes and about the details of HSP function.

Mitochondrial protein import depends on HSPs and other components to facilitate the import of precursor proteins (28). The 70-kDa HSPs (HSP70) of the cytoplasm have been implicated in an early step in the import process (12, 26) and may help to unfold precursors or maintain them in an import-competent conformation. Ssc1p, an HSP70 homolog located in the mitochondrial matrix (9), appears to play a role in both the translocation of proteins across the mitochondrial membranes and the refolding of precursors as they are transported into the matrix space (21). Additionally, HSP60 in the matrix mediates the assembly of newly imported polypeptides into functional enzyme complexes (5). Non-HSPs also have been identified as components of the import apparatus and include several polypeptides of the outer membrane (1, 35) and the two subunits of the matrix-localized processing protease (20, 49, 53). Other proteins that catalyze key steps in the import process have yet to be identified.

We have described previously the isolation and analysis of several *mas* mutants of *Saccharomyces cerevisiae* which display defects in mitochondrial protein import (52). Characterization of two of these mutations, *mas1* and *mas2*, led to the identification of products of the *MAS1* and *MAS2* genes as subunits of the protease that processes precursors upon their import into mitochondria (20, 49, 53). A third mutation, *mas3*, proved to be an allele of heat shock transcription factor and provided additional evidence for the role of HSPs in the import process (44). Here we describe another mutation, *mas5*, which causes temperature-sensitive growth and an import defect at the nonpermissive temperature. We report the isolation and analysis of the *MAS5* gene and demonstrate that this gene encodes a yeast homolog of the bacterial heat shock protein DnaJ.

MATERIALS AND METHODS

Strains and genetic techniques. The parent *S. cerevisiae* strain AH216 (*MATa leu2 his3 phoC phoE*) and isolation of the *mas* mutants were described previously (52). Strains MYY290 (*MATa leu2 his3 ura3*) and MYY291 (*MATα leu2 his3 ura3*) were derived from strain AH216 and were described previously (44). Strain MYY297 is a diploid strain obtained from a cross of MYY290 with MYY291. Strain MYY392 (*MATa leu2 his3 ura3 mas5*) and strain MYY392 (*MATα leu2 his3 ura3 mas5*) were derived as haploid spores from a cross of the original *mas5* mutant MYY244 (*MATα leu2 his3 mas5*) to strain MYY290. A yeast strain containing a deletion mutation in the *MAS5* gene, MYY406 (*MATα leu2 his3 mas5::URA3*), was created as described below. Strain MS177 (*MATα kar2-159 ura3 ade2*) was the gift of M. Rose (Princeton University). Media were prepared and standard genetic manipulations were performed as described by Sherman et al. (42). Yeast cells were transformed by the lithium acetate method (19).

Analysis of mitochondrial protein import. Cells were grown to an OD_{600} of between 0.5 and 2.0 in semisynthetic medium (10) containing 2% glucose. Cells were resuspended in 20 mM KPO_4 (pH 6)–1% glucose to an OD_{600} of 10 and incubated for 10 min at 37 or 23°C. Cells were then labeled with [^{35}S]Translabel (ICN) for 5 min. Labeling was stopped by addition of cycloheximide to 0.1 mg/ml and unlabeled methionine to 2 mM. Incubations were then continued at the same temperature as that used for preincubation and labeling. One-milliliter aliquots were removed periodically, and proteins were extracted as described previously (50). The labeling in 1% glucose–20 mM KPO_4, pH 6.0, was previously described by Reid and Schatz (37). These conditions produced a pulse-chase pattern identical with that obtained after

* Corresponding author.

labeling in the growth medium, but the overall level of incorporation was higher (data not shown). Raising of antisera against $F_1\beta$ and citrate synthase, immunoprecipitation, and analysis by polyacrylamide gel electrophoresis and fluorography were done as described previously (44).

Analysis of secretion. Secretion of α-factor was analyzed as previously described (44). Antiserum against α-factor was the gift of R. Schekman (University of California, Berkeley).

Isolation of the *MAS5* gene. The *MAS5* gene was isolated by genetic complementation of the *mas5* temperature-sensitive growth phenotype. The *mas5* mutant strain MYY391 was transformed with a yeast genomic plasmid library in the yeast centromere vector p366 (obtained from Meryl Hoekstra, Salk Institute, La Jolla, Calif.). Leu^+ transformants were selected at 23°C and screened for growth at the nonpermissive temperature by two successive replica platings at 37°C. Of 7,000 Leu^+ transformants analyzed, 1 complementing plasmid, p366M5, was isolated. This plasmid contained a 10.5-kbp insert of yeast DNA.

To further localize the *MAS5* gene, portions of the p366M5 plasmid were deleted, and other portions of the 10.5-kbp fragment were subcloned into the multiple cloning site of yeast shuttle vectors. Plasmid p366M5 was digested with *Hin*dIII and religated, removing 4.1 kbp, to create plasmid p366M5-1. The 2.3-kbp *Eco*RI fragment was isolated from plasmid p366M5 and ligated into the unique *Eco*RI site of plasmid YCp50 (39) to create plasmid YCp50M5. The 3.4-kbp *Sac*I-*Apa*I fragment isolated from plasmid p366M5 was ligated into the *Sac*I and *Apa*I sites of plasmid pRS315 (43) to create plasmid pRSM5. The ability of these constructs to complement the temperature-sensitive defect in *mas5* mutant cells was analyzed after transformation of strain MYY391.

Integrative transformation. A 2.3-kbp *Eco*RI fragment isolated from p366M5 was inserted into the *URA3*-containing vector YIp5 (46), and the resulting plasmid was linearized at the unique *Sac*I site prior to transformation of strain MYY290. Stable Ura^+ transformants were crossed to strain MYY392, and the diploids were sporulated at 23°C. The meiotic products were tested for growth at 37°C on YPD medium and for growth on medium lacking uracil. In this cross, all of the 38 tetrads analyzed were of the parental ditype (2 Ura^+, 2 ts^-:2 Ura^-, 2 ts^+). This lack of recombination between *mas5* and *URA3* in 38 tetrads placed the integration event less than 1.3 centimorgans (cM) from the *MAS5* locus.

Sequence analysis of the *MAS5* gene. The 3.4-kbp *Sac*I-*Apa*I fragment was isolated from plasmid pRSM5, the 3′ single-stranded ends were removed by using T4 DNA polymerase, and the resulting fragment was ligated into the *Sma*I site of BluescriptSK (Stratagene) to create plasmid ASA3-1. Templates for sequencing were obtained by using exonuclease III to create a series of nested deletions (18) from either end of the yeast insert. The nucleotide sequences of these fragments were determined by using the Sequenase 2.0 DNA sequencing kit (U.S. Biochemical Corp.).

The predicted amino acid sequence of the MAS5 protein, derived from the nucleotide sequence of the cloned gene, was compared with sequences in the GenBank data base with the FASTA program (24). Alignment of the protein sequences of MAS5, SCJ1, and DnaJ was performed as described by Feng and Doolittle (15).

Chromosomal mapping. The 2.3-kbp *Eco*RI fragment from p366M5 was labeled by random priming, using a kit from Bethesda Research Laboratories, and used to probe a blot of *S. cerevisiae* chromosomes separated by orthogonal-field-alternation gel electrophoresis (OFAGE; Clontech). Hybridizations were performed at 42°C, and radioactive bands were detected by autoradiography.

Analysis of gene expression. Yeast strain MYY290 was grown and RNA was extracted and characterized by Northern (RNA blot) analysis as described previously (44). Probes for hybridizations were made from portions of the *MAS5*, *SSA1*, and *ACT1* (actin) genes. These included a 0.5-kbp *Eco*RI-*Pvu*II fragment isolated from the *MAS5*-carrying plasmid ASA3-1, a 1-kbp *Pst*I-*Cla*I fragment isolated from the plasmid cen30-pADH-SSA1 (obtained from E. Craig), and a 0.9-kbp *Bgl*II-*Eco*RV fragment isolated from the *ACT1*-carrying plasmid pRP37 (obtained from R. Parker). DNA fragments were labeled by random priming with a kit from Bethesda Research Laboratories. Hybridizations were performed at 42°C, and radioactive bands were detected by autoradiography and quantified by laser densitometry.

Gene disruption of *MAS5*. A mutant lacking most of the *MAS5* coding region was created as follows. A 2.6-kbp *Sna*BI-*Apa*I fragment from plasmid pRSM5 was inserted into plasmid BluescriptSK (Stratagene) at the *Sma*I and *Apa*I sites. This plasmid was then cut with *Hin*dIII and *Nde*I, the single-stranded ends were filled in with Klenow fragment, and the ends were ligated, creating a new plasmid with most of the coding region of *MAS5* deleted and with a single *Hin*dIII site. The plasmid was cut with *Hin*dIII, and a 1.2-kbp fragment containing the *URA3* gene (isolated from the plasmid pFL1 [6]) was inserted into the site. This plasmid was then cut with *Bam*HI and *Apa*I. The resulting linear fragment was transformed into the diploid strain MYY297. Ura^+ integrants were isolated on selective medium lacking uracil. Transformants containing the expected disruption of one of two copies of the *MAS5* gene were identified by Southern analysis. Two independently isolated transformants were analyzed.

RESULTS

***mas5* mutants display a defect in mitochondrial protein import.** The *mas5* mutant was isolated in a screen of temperature-sensitive yeast cells for strains which accumulated the precursor of the $F_1\beta$ subunit of the mitochondrial ATPase during incubation at the nonpermissive temperature (52). In contrast to results obtained with several other mutations affecting mitochondrial protein import (52), the accumulation of large amounts of precursor (detectable by immunoblotting) disappeared after several backcrosses of *mas5* mutants to the wild-type parent (51). However, the temperature-sensitive growth phenotype in *mas5* mutants persisted after the backcrosses. Additionally, precursor of the $F_1\beta$ subunit was detected by immunoprecipitation of proteins from mutant cells labeled with [^{35}S]methionine for 5 min at 37°C (Fig. 1) but was not found for wild-type cells under the same conditions. This observation indicated the persistence of an import defect in the *mas5* mutants.

The inheritance of the import defect and temperature-sensitive growth phenotypes was analyzed in meiotic progeny resulting from a cross of *mas5* mutants to the wild-type parent. The increased precursor levels (import defect) cosegregated with the temperature-sensitive growth phenotype (Fig. 1) in 10 tetrads analyzed, indicating that a single nuclear lesion was responsible for both mutant traits. Additionally, heterozygous diploid cells displayed neither of the mutant phenotypes, indicating a recessive character for the mutation.

In order to characterize the import defect further, the fate

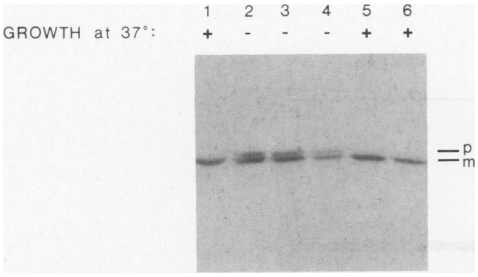

FIG. 1. Cosegregation of the *mas5* import defect with temperature-sensitive growth. Cells containing the *mas5* mutation were crossed to the wild-type parental strain, and the resulting diploid was sporulated. Colonies grown from individual spores of a tetrad were tested for growth at 37°C and for the appearance of precursor after 5 min of labeling at 37°C. Cells were grown and labeled and samples were analyzed as described in Materials and Methods. Precursor (p) and mature (m) forms of $F_1\beta$ subunit and growth (+) or no growth (−) at 37°C are indicated. Lane 1, wild-type strain (AH216); lane 2, original *mas5* mutant (MYY244); lanes 3 to 6, cells grown from individual spores from a single tetrad.

of labeled precursor was followed during a chase at the nonpermissive temperature under nonlabeling conditions. At the end of the pulse (0 min), little or no precursor to $F_1\beta$ was detected in the wild-type cells (Fig. 2). In contrast, in the *mas5* mutant, ~30% of the $F_1\beta$ was found in the precursor form at the end of the pulse, and this precursor disappeared only slowly during the subsequent chase (Fig. 2). Labeled precursor failed to disappear when the chase was carried out in the presence of the uncoupler carbonyl cyanide *m*-chlorophenylhydrazone (data not shown), suggesting that the *mas5* lesion affects an import step prior to the complete translocation of precursor into the mitochondrial matrix. The import of a second mitochondrial protein, citrate synthase, also was affected by the *mas5* mutation; a small but significant amount of precursor was present in the cells after a 5-min labeling at 37°C, and this precursor was subsequently chased slowly into the mature form (Fig. 2). No precursor to citrate synthase was detected in wild-type cells, reflecting the very rapid import of this protein. The import defects in the *mas5* mutants were detected within 10 min after a shift to the nonpermissive temperature and were not apparent at 23°C. These results demonstrate that *mas5* mutant cells display a defect in the import of at least two mitochondrial proteins and that this trait cosegregates genetically with the temperature-sensitive growth phenotype.

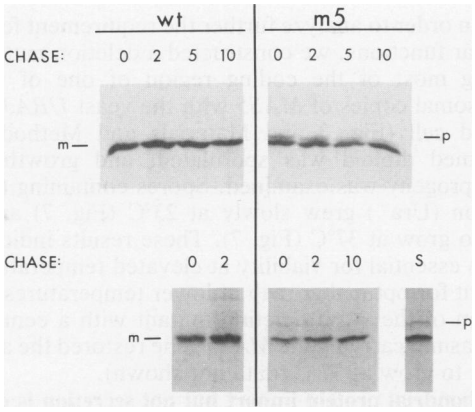

FIG. 2. Defective mitochondrial protein import in *mas5* mutant cells. Wild-type (wt) and *mas5* mutant (m5) cells were grown at 23°C, incubated at 37°C for 10 min, and labeled with [^{35}S]Translabel for 5 min. Labeling was stopped by addition of cycloheximide, and incubation was continued at 37°C. Proteins were extracted, and precursor (p) and mature (m) forms of $F_1\beta$ (upper panels) and citrate synthase (lower panels) were immunoprecipitated. Immunoprecipitates were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and fluorography. Chase indicates minutes after addition of cycloheximide, with the 0-min time point being the sample removed at the beginning of the chase. S, standard lane containing proteins immunoprecipitated from *mas2* mutant cells incubated at 37°C.

The *mas5* mutant import defect was only evident in the in vivo studies described above. Mitochondria isolated from the *mas5* mutant cells imported both pre-$F_1\beta$ and pre-citrate synthase in an in vitro import assay as efficiently as did wild-type mitochondria (data not shown). Additionally, deficiencies in other cellular processes were not apparent: cells stopped growing at a variety of stages in the cell cycle after several generations at the nonpermissive temperature, no defect in the secretion of the mating pheromone α-factor was detected in pulse-labeling experiments, and, similar to other import mutants (52), *mas5* mutant cells displayed normal respiration and growth on nonfermentable carbon sources.

Isolation of the *MAS5* gene. The wild-type *MAS5* gene was isolated by complementation of the temperature-sensitive growth phenotype with plasmids containing yeast genomic DNA. Of ca. ~7,000 transformants, one strain was isolated that contained a plasmid which allowed cells to grow at 37°C. Restriction analysis revealed that the isolated plasmid contained a 10.5-kbp insert of yeast genomic DNA (Fig. 3A). Subcloning portions of this insert and retesting these subclones for complementation in *mas5* mutant cells revealed a minimum complementing 3.4-kbp *Sac*I-*Apa*I fragment (Fig. 3). This fragment was found to complement both temperature-sensitive growth and the import defect at 37°C when present in a centromere-based plasmid in *mas5* mutant cells (not shown).

The isolated DNA was shown to correspond to DNA from the *MAS5* locus (rather than encoding an extragenic suppressor) by integrative mapping. A 1.7-kbp *Eco*RI fragment isolated from the larger complementing DNA integrated to a chromosomal position within 1.3 cM of the *MAS5* locus (see Materials and Methods). Additionally, the *MAS5* gene was mapped to chromosome XIV by hybridization of a 2.3-kbp *Eco*RI probe to an OFAGE blot of yeast chromosomes (data not shown).

***MAS5* gene encodes a homolog of DnaJ.** The nucleotide sequence of the complementing 3.4-kbp *Sac*I-*Apa*I fragment was determined by dideoxy sequencing with templates generated by exonuclease III digestion. A single long open reading frame (ORF) of 1,227 nucleotides, encoding a predicted protein product of 44.6 kDa, was identified (Fig. 4), and the location of this ORF in the *Sac*I-*Apa*I fragment corresponded to the complementation data (Fig. 3). Additionally, the size of the single ORF correlated with a 1.56-kbp mRNA detected by Northern analysis.

Comparison of the predicted protein sequence with sequences in the GenBank data base revealed homology to the heat shock protein DnaJ from *E. coli* (2, 29) (Fig. 5) and from *Mycobacterium tuberculosis* (22) (data not shown). The protein was also found to be homologous to the recently described SCJ1 (3) (Fig. 5), another yeast homolog of bacterial DnaJ. MAS5 is 35% identical to both *E. coli* DnaJ and SCJ1, with identical or conserved residues extending throughout almost the entire sequence. Two other features

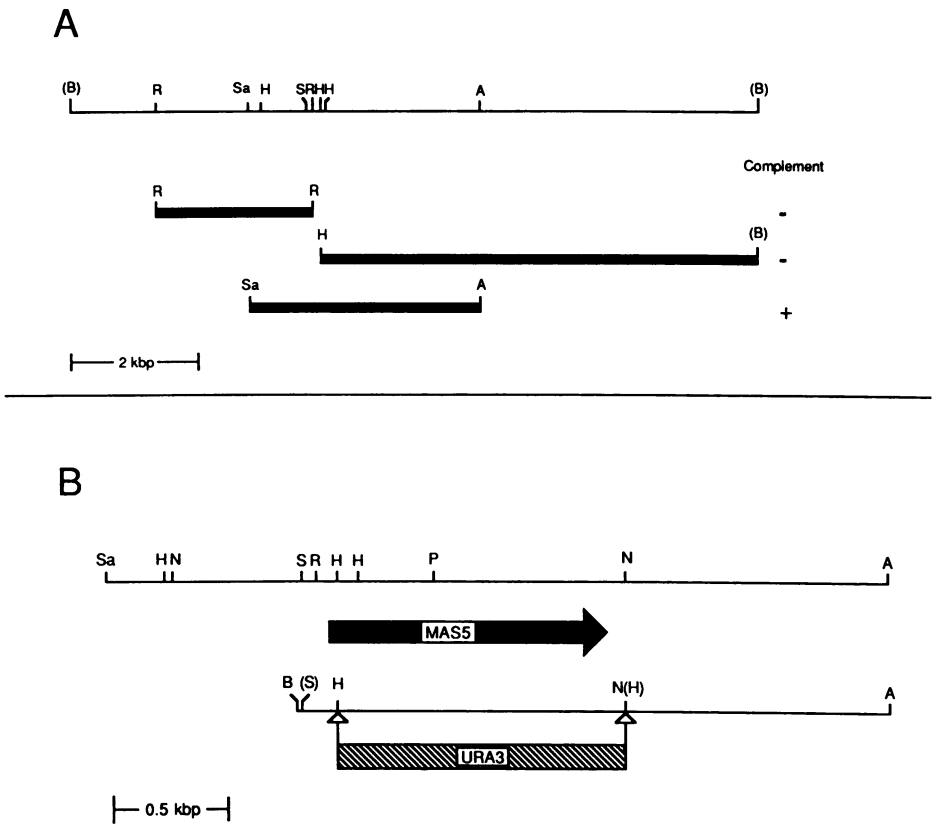

FIG. 3. Localization of the *MAS5* gene. (A) Map of some restriction endonuclease sites in the 10.5-kbp fragment which complemented the *mas5* lesion. The solid bars indicate DNA fragments tested for complementation of *mas5*. The results of complementation tests are shown to the right of the bars. (B) Map of the minimum complementing 3.4-kbp *Sac*I-*Apa*I fragment. The solid arrow indicates the position of the ORF encoding MAS5. The DNA fragment used to construct a null allele of *MAS5* is indicated at the bottom. In this fragment, most of the *MAS5* ORF was replaced by a fragment containing the yeast *URA3* gene. Restriction sites: A, *Apa*I; B, *Bam*HI; H, *Hin*dIII; N, *Nde*I; P, *Pst*I; R, *Eco*RI; S, *Sal*I; Sa, *Sac*I.

conserved among the three proteins are a glycine-rich region (residues 76 to 103 of MAS5) and four sets of paired cysteine residues (Fig. 5, arrowheads). The C terminus of MAS5 is unique from that of the other two homologs and ends with the amino acid sequence CASQ, a sequence resembling the recognition site for protein isoprenylation (38, 45).

MAS5 is a heat shock protein. Since bacterial *dnaJ* is a heat shock gene whose expression increases at elevated, sublethal temperatures, we examined whether *MAS5* was also a heat shock gene. The expression of *MAS5* was evaluated by Northern analysis with RNA isolated from wild-type cells grown at 23°C or shifted for various periods to 37°C. *MAS5* mRNA amounts increased approximately twofold after 15 min at 37°C and decreased slightly with longer incubation (Fig. 6). Although the increase was lower in magnitude, the pattern of *MAS5* expression was similar to that of the well-characterized HSP70 encoded by *SSA1* (Fig. 6) (48). Consistent with heat shock induction of *MAS5*, the DNA region upstream of the *MAS5* ORF (between nucleotides −390 and −320) contains multiple sites related to the heat shock element consensus sequence (Fig. 4). Additionally, adjacent to the heat shock element-containing region is the sequence TCGGCAGGTA (nucleotides −308 to −298), which is similar to the sequence (TCGGCGGCA) of a putative upstream repression site located in the promoter region of the HSP70 gene *SSA1* (34).

***MAS5* is essential for yeast cell viability at elevated temperatures.** In order to analyze further the requirement for *MAS5* in cellular functions, we constructed a deletion mutation by replacing most of the coding region of one of the two chromosomal copies of *MAS5* with the yeast *URA3* gene in a diploid cell (Fig. 3 and Materials and Methods). The transformed diploid was sporulated, and growth of the meiotic progeny was examined. Spores containing the gene disruption (Ura^+) grew slowly at 23°C (Fig. 7) and were unable to grow at 37°C (Fig. 7). These results indicate that *MAS5* is essential for viability at elevated temperatures and important for optimal growth at lower temperatures. Transformation of the *mas5* deletion mutant with a centromere-based plasmid carrying the *MAS5* gene restored the ability of the cells to grow at 37°C (data not shown).

Mitochondrial protein import but not secretion is defective in the deletion mutant. To evaluate further the cellular role of the MAS5 protein, mitochondrial protein import and protein secretion were analyzed in the deletion (null) mutant. The precursor to $F_1\beta$ was detected at the end of the labeling pulse, indicating an import defect at 23°C (Fig. 8). At 37°C, a substantial amount of precursor was present at the end of the pulse, and this material did not appear to chase significantly into the mature form during a subsequent 10-min incubation (Fig. 8). A modest import defect was also evident at 37°C for a second mitochondrial protein, citrate synthase (Fig. 9). These import patterns in the *mas5* deletion mutant are very similar to those previously described for another

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-450 TATAAAATAA GATGTGAAGT CGCTGGTTTG AGCACGTGAT ATACACCTGA CCTATAATAT TTCGTACAAA ATTATAGAAG GCCATCGAAA
-360 AAATAGAAAA TTTTTTCATT TCTTTTTCAA GAAATGAAAA GGCAATAGAG CATCGGCAGG TATGGAAAGT ATTTAACTTG CAGATCAATC
-270 CACGTACTTA TAAAACGTGT AAAAACTTGC TCTACGTTTA TATGTTGGTT AGGTAGTTCT TGTTTGATAA GGCGTGTAGT TCGTTTTTAT
-180 AAATCAAAGT CACAAAAAGT CCTTTTCCCC ATATATATTA GGCCCGAACA AGCGTCTTAT TTGATAACTG TTACGTATTT ATTTTTTTGT
-90  TATTTGTTAC CATATCTTTT GATAGAACAT AATTAAAAAT TATCCAAACT GAATTCTACA TCTTCCAACA ACAATAATAA ACGTCCAAAG

1   ATG GTT AAA GAA ACT AAG TTT TAC GAT ATT CTA GGT GTT CCA GTA ACT GCC ACT GAT GTC GAA ATT AAG AAA GCT
1   MET Val Lys Glu Thr Lys Phe Tyr Asp Ile Leu Gly Val Pro Val Thr Ala Thr Asp Val Glu Ile Lys Lys Ala

76  TAT AGA AAA TGC GCC TTA AAA TAC CAT CCA GAT AAG AAT CCA AGT GAG GAA GCT GCA GAA AAG TTC AAA GAA GCT
26  Tyr Arg Lys Cys Ala Leu Lys Tyr His Pro Asp Lys Asn Pro Ser Glu Glu Ala Ala Glu Lys Phe Lys Glu Ala

151 TCA GCA GCC TAT GAA ATT TTA TCA GAT CCT GAA AAG AGA GAT ATA TAT GAC CAA TTT GGT GAA GAT GGT CTA AGT
51  Ser Ala Ala Tyr Glu Ile Leu Ser Asp Pro Glu Lys Arg Asp Ile Tyr Asp Gln Phe Gly Glu Asp Gly Leu Ser

226 GGT GCT GGT GGC GCT GGC GGA TTC CCA GGT GGT GGA TTC GGT TTT GGT GAC GAT ATC TTT TCC CAA TTC TTT GGT
76  Gly Ala Gly Gly Ala Gly Gly Phe Pro Gly Gly Gly Phe Gly Phe Gly Asp Asp Ile Phe Ser Gln Phe Phe Gly

301 GCT GGT GGC GCA CAA AGA CCA AGA GGT CCC CAA AGA GGT AAA GAT ATC AAG CAT GAA ATT TCT GCC TCA CTT GAA
101 Ala Gly Gly Ala Gln Arg Pro Arg Gly Pro Gln Arg Gly Lys Asp Ile Lys His Glu Ile Ser Ala Ser Leu Glu

376 GAA TTA TAT AAG GGT AGG ACA GCT AAG TTA GCC CTT AAC AAA CAG ATC CTA TGT AAA GAA TGT GAA GGT CGT GGT
126 Glu Leu Tyr Lys Gly Arg Thr Ala Lys Leu Ala Leu Asn Lys Gln Ile Leu Cys Lys Glu Cys Glu Gly Arg Gly

451 GGT AAG AAA GGC GCC GTC AAG AAG TGT ACC AGC TGT AAT GGT CAA GGT ATT AAA TTT GTA ACA AGA CAA ATG GGT
151 Gly Lys Lys Gly Ala Val Lys Lys Cys Thr Ser Cys Asn Gly Gln Gly Ile Lys Phe Val Thr Arg Gln MET Gly

526 CCA ATG ATC CAA AGA TTC CAA ACA GAG TGT GAT GTC TGT CAC GGT ACT GGT GAT ATC ATT GAT CCT AAG GAT CGT
176 Pro MET Ile Gln Arg Phe Gln Thr Glu Cys Asp Val Cys His Gly Thr Gly Asp Ile Ile Asp Pro Lys Asp Arg

601 TGT AAA TCT TGT AAC GGT AAG AAA GTT GAA AAC GAA AGG AAG ATC CTA GAA GTC CAT GTC GAA CCA GGT ATG AAA
201 Cys Lys Ser Cys Asn Gly Lys Lys Val Glu Asn Glu Arg Lys Ile Leu Glu Val His Val Glu Pro Gly MET Lys

676 GAT GGT CAA AGA ATC GTT TTC AAA GGT GAA GCT GAC CAA GCC CCA GAT GTC ATT CCA GGT GAT GTT GTC TTC ATA
226 Asp Gly Gln Arg Ile Val Phe Lys Gly Glu Ala Asp Gln Ala Pro Asp Val Ile Pro Gly Asp Val Val Phe Ile

751 GTT TCT GAG AGA CCA CAC AAG AGC TTC AAG AGA GAT GGT GAT GAT TTA GTA TAT GAG GCT GAA ATT GAT CTA TTG
251 Val Ser Glu Arg Pro His Lys Ser Phe Lys Arg Asp Gly Asp Asp Leu Val Tyr Glu Ala Glu Ile Asp Leu Leu

826 ACT GCT ATC GCT GGT GGT GAA TTT GCA TTG GAA CAT GTT TCT GGT GAT TGG TTA AAG GTC GGT ATT GTT CCA GGT
276 Thr Ala Ile Ala Gly Gly Glu Phe Ala Leu Glu His Val Ser Gly Asp Trp Leu Lys Val Gly Ile Val Pro Gly

901 GAA GTT ATT GCC CCA GGT ATG CGT AAG GTC ATC GAA GGT AAA GGT ATG CCA ATT CCA AAA TAC GGT GGC TAT GGT
301 Glu Val Ile Ala Pro Gly MET Arg Lys Val Ile Glu Gly Lys Gly MET Pro Ile Pro Lys Tyr Gly Gly Tyr Gly

976 AAT TTA ATC ATC AAA TTT ACT ATC AAG TTC CCA GAA AAC CAT TTC ACA TCA GAA GAA AAC TTG AAG AAG TTA GAA
326 Asn Leu Ile Ile Lys Phe Thr Ile Lys Phe Pro Glu Asn His Phe Thr Ser Glu Glu Asn Leu Lys Lys Leu Glu

1051 GAA ATT TTG CCT CCA AGA ATT GTC CCA GCC ATT CCA AAG AAA GCT ACT GTG GAC GAA TGT GTA CTC GCA GAC TTT
351 Glu Ile Leu Pro Pro Arg Ile Val Pro Ala Ile Pro Lys Lys Ala Thr Val Asp Glu Cys Val Leu Ala Asp Phe

1126 GAC CCA GCC AAA TAC AAC AGA ACA CGG GCC TCC AGG GGT GGT GCA AAC TAT GAT TCC GAT GAA GAA GAA CAA GGT
376 Asp Pro Ala Lys Tyr Asn Arg Thr Arg Ala Ser Arg Gly Gly Ala Asn Tyr Asp Ser Asp Glu Glu Glu Gln Gly

1201 GGC GAA GGT GTT CAA TGT GCA TCT CAA TGA
401 Gly Glu Gly Val Gln Cys Ala Ser Gln ***

1231 TTTTCTTGAT AAAAAAAGAT CAACTTATTC ACGATTCATT TATTCATCAT ACTTTTCACT TCAAAATACA GTTTTGTAGC TATACATATG
1321 TAAAGTAATA ATGGTAGAAA ATGCAGTTTA CCAAAAAAAG TTCTAATTAT GACGAAATCT TAGGAAAGAG TAAAGCAGGG ATTATATGCT
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FIG. 4. Sequence of *MAS5* gene and its predicted protein product. Upstream sequences which are identical (double underline) or very similar (single underline) to the heat shock element consensus sequence are indicated.

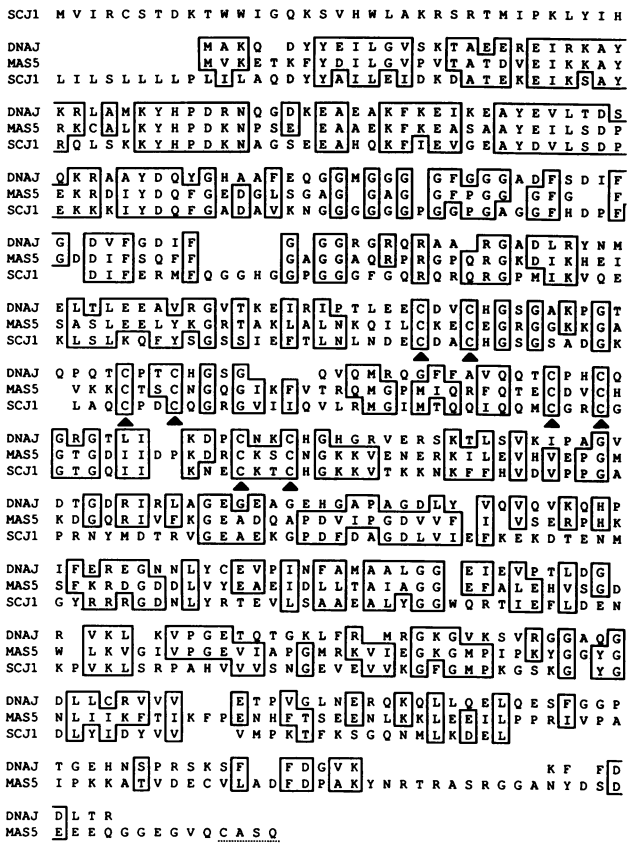

FIG. 5. Sequence comparison of MAS5 with SCJ1 and DnaJ. Protein sequences were aligned as described by Feng and Doolittle (15). Amino acids that are identical or similar between MAS5 and SCJ1 and between MAS5 and DnaJ are boxed. In this analysis, similar amino acids include E and D; K and R; T and S; and A, V, I, L, and F. Arrowheads indicate conserved cysteine residues. Dashed underline indicates the unique MAS5 C-terminal sequence CASQ.

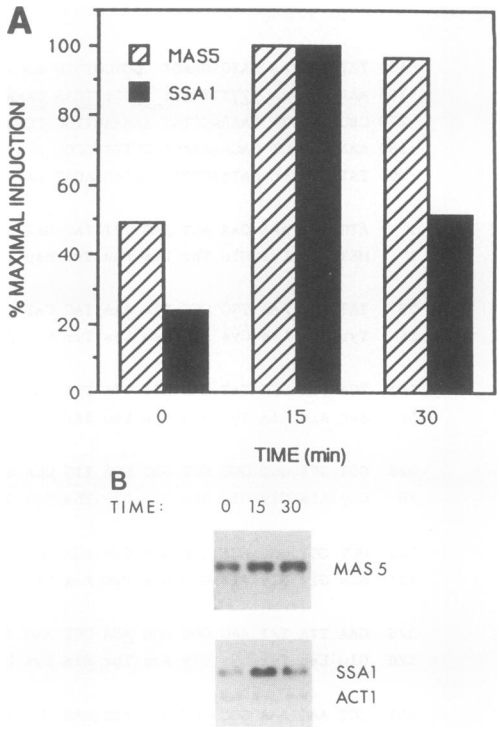

FIG. 6. Heat shock induction of *MAS5*. Yeast strain MYY290 (wild type) was grown at 23°C in SD medium. Immediately prior to and periodically following a shift to 37°C, aliquots were removed, and RNA was extracted from the cells. RNA was separated by electrophoresis and blotted as described in Materials and Methods. The blot was first probed with ^{32}P-labeled fragments of the *MAS5* gene. Following autoradiography, the probe was eluted from the blot, which was then reprobed with labeled fragments of the *SSA1* and *ACT1* (actin) genes. Bands on autoradiograms were quantified by laser densitometry. The deduced levels of *MAS5* and *SSA1* mRNA were corrected for differences in recovery by dividing by the level of *ACT1* message determined for the same sample. Percent maximal induction was calculated as described in Materials and Methods. (A) Percent maximal induction of *MAS5* and *SSA1* after a shift for various times to 37°C. (B) Autoradiograms of blots of total cellular RNA probed with *MAS5*, *SSA1*, and *ACT1* DNA. Time indicates minutes of incubation of cells at 37°C.

import mutation, *mas3* (44), and demonstrate a significant import defect at elevated temperatures. The deletion mutant transformed with a centromere-based plasmid containing the *MAS5* gene showed a wild-type pattern of mitochondrial protein import (data not shown).

Heat shock proteins or cognates have been implicated previously as important components for protein secretion (8, 11, 12). To assess the possible role of MAS5 in the secretory process, the secretion of the mating pheromone α-factor was compared in wild-type cells and cells with the null allele of *mas5* or the *kar2-159* mutation at 37°C by pulse-labeling analysis. While, as reported previously (47), the secretory defect in *kar2-159* mutant cells caused the accumulation of a substantial amount of prepro-α-factor, no accumulation of prepro-α-factor was detected in the *mas5* mutant (Fig. 10, lane 2). Consistent with the lack of a defect in secretion, cells with the deletion mutation in *mas5* continued to divide for several hours following a shift to 37°C and then ceased growing (data not shown). A similar delayed temperature-sensitive growth phenotype was described previously for other mutations affecting mitochondrial protein import, *mas1* and *mas2* (52). These results suggest that MAS5 is not required for the secretory process.

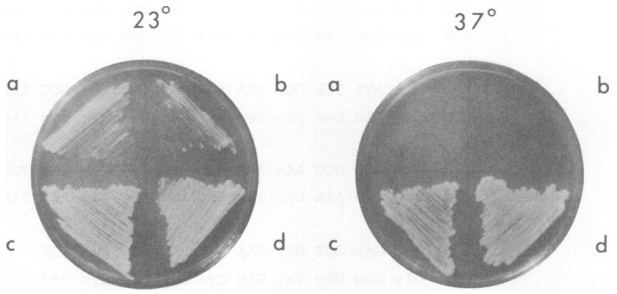

FIG. 7. Defective growth of *mas5* null allele mutant. One of two wild-type copies of *MAS5* was deleted in the diploid yeast strain MYY297 as described in Materials and Methods. The diploid was sporulated, and colonies grown from four spores (a through d) of a tetrad were tested for growth on YPD medium at 23 and 37°C. Spores a and b were Ura$^+$, indicating that they contained the disrupted *MAS5* gene. Plates were photographed after incubation for 3 days.

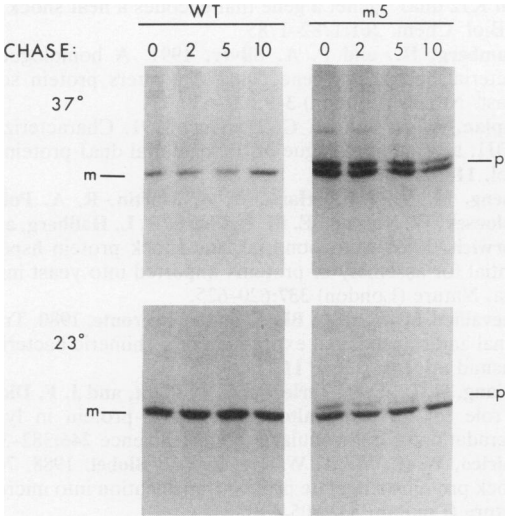

FIG. 8. Defective mitochondrial import of $F_1\beta$ in the *mas5* deletion mutant. Wild-type (wt) and *mas5* deletion mutant MYY406 (m5) cells were grown at 23°C, preincubated for 10 min at 23 or 37°C, and labeled with [^{35}S]Translabel for 5 min at the preincubation temperature. Following the addition of cycloheximide, incubations were continued at the same temperature, and aliquots were removed periodically. Samples were analyzed as described in the legend to Fig. 2. Immunoprecipitated precursor (p) and mature (m) forms of $F_1\beta$ subunit are shown.

DISCUSSION

We have demonstrated that the *mas5* mutation causes defects in mitochondrial protein import and cell viability at 37°C (Fig. 1 and 2). Isolation and analysis of the *MAS5* gene revealed its product to be a heat shock protein (Fig. 6) homologous to the bacterial DnaJ (Fig. 5). The phenotypes of both the original *mas5* mutant and cells containing a deletion allele of *mas5* indicate a role for the MAS5 protein in mitochondrial protein import and, perhaps, other cellular processes at elevated temperatures.

MAS5 is homologous to bacterial *dnaJ*. *dnaJ* was originally identified because mutations in the gene block bacteriophage λ DNA replication (16). In the replication process, DnaJ functions together with the HSP70 homolog DnaK to disassemble a protein complex at the phage replication origin (54). Further studies have revealed that *dnaJ* and *dnak* are also required for growth of *E. coli* cells at elevated temperatures, and mutations in these genes result in pleiotrophic effects (17), including defective DNA and RNA synthesis, blocks in the cell cycle, and filamentation of the bacterial cells.

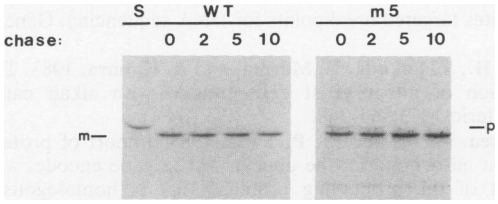

FIG. 9. Defective mitochondrial import of citrate synthase in the *mas5* deletion mutant. Import of citrate synthase at 37°C was analyzed in wild-type (wt) and mas5 deletion mutant MYY406 (m5) cells as described in the legend to Fig. 8. Precursor (p) and mature (m) forms of citrate synthase are indicated. S, standard of precursor to citrate synthase immunoprecipitated from cells labeled in the presence of carbonyl cyanide *m*-chlorophenylhydrazone as described previously (50).

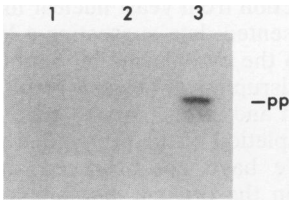

FIG. 10. Absence of a defect in α-factor secretion in the *mas5* deletion mutant. Cells were grown at 23°C, incubated at 37°C for 30 min, and labeled with [^{35}S]Translabel for 30 min at 37°C. Proteins were extracted following the addition of 3 A_{600} units of unlabeled *MAT***a** cells. Immunoprecipitates of α-factor were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and fluorography. Lanes: 1, wild-type (MYY291); 2, *mas5* mutant (MYY406); 3, *kar2-159* mutant (MS177). pp, prepro-α-factor.

MAS5 appears to be one of a family of yeast DnaJ homologs. Blumberg and Silver (3) identified the first such cognate, SCJ1, as a component whose overexpression could cause the missorting of a chimeric protein which contained both nuclear and mitochondrial targeting signals. The SCJ1 protein appears to be located both in the mitochondrial matrix and in the lumen of the endoplasmic reticulum, and it is postulated to play a role in sorting or refolding of precursor proteins following membrane translocation (3). A second protein, the product of the *SEC63/NPL1* gene, contains a domain homologous to a portion of the DnaJ protein, and the yeast protein is thought to play a role in the translocation of polypeptides into the endoplasmic reticulum (40). Southern blot analysis of yeast genomic DNA at lowered stringency revealed several other homologs of *SCJ1* (3), and *MAS5* appears to be one of these. Additionally, several other genomic DNA fragments which could complement the *mas5* lesion when present in multiple copies were isolated during the cloning of *MAS5* (data not shown). These fragments failed to map to the *MAS5* locus and may encode other DnaJ homologs.

A number of DnaK homologs, members of the HSP70 protein family, in *S. cerevisiae* have been described (23), and these proteins are thought to mediate conformational changes in protein structure for a variety of processes (41). Among these processes, mitochondrial protein import requires HSP70s of the SSA family functioning in the cytoplasm at an early step in the import pathway (12, 26). These HSPs may unfold precursor proteins or maintain them in an "import-competent" conformation (13, 14, 28). Another HSP70 homolog, SSC1, resides in the mitochondrial matrix (9) and is thought to assist both the translocation of precursor proteins across the membranes and precursor refolding (21). Blumberg and Silver (3) proposed that, by analogy to DnaJ activity in bacterial cells, the yeast DnaJ homologs may function in concert with HSP70s. SCJ1, located within the mitochondria, may act in a complex with SSC1 in the matrix. The MAS5 protein may be a functional partner of the cytoplasmic HSP70s.

A report describing the identification of another yeast DnaJ homolog, YDJ1, appeared during the preparation of this manuscript (4). Sequence comparison has revealed that YDJ1 is identical to MAS5. The *YDJ1* gene was isolated from a yeast expression library by using antiserum prepared

against a subfraction from yeast nuclear matrix. Caplan and Douglas (4) presented data suggesting a localization of the YDJ1 protein to the cytoplasm and perinuclear region and reported that a disruption of the gene resulted in slow growth on solid medium and an inability to grow in liquid culture. Additionally, depletion of the protein led to changes in cell morphology. We have not observed any morphological changes either in the original *mas5* mutant or in the null mutant at either 23 or 37°C. We have observed slower growth of cells with the null allele; however, the cells grow equally well on solid and liquid media. Caplan and Douglas (4) did not report defects in mitochondrial protein import or growth at 37°C, nor did they detect heat shock induction of gene expression. The sources of discrepancies between our observations and those described by Caplan and Douglas are unclear, but some phenotypic differences may be due to the varied effects of a rapid shift to nonpermissive conditions (our study) versus the dilution of preexisting protein via termination of gene expression.

MAS5 is likely to function in the cytoplasm or on the mitochondrial surface, since it lacks both obvious mitochondrial import signals and putative membrane-spanning regions. Additionally, Caplan and Douglas found a partially cytoplasmic distribution of the protein (4). Previous studies have provided evidence of cytoplasmic import components, including the cytoplasmic HSP70s (12, 26) and several other cytoplasmic proteins (26, 27, 30–33). The MAS5 protein may be one of these other polypeptides, and a cytoplasmic location would suggest a role for MAS5 early in the import pathway. By analogy to the bacterial system, MAS5 may act together with the cytoplasmic HSP70s to help unfold precursor proteins or to maintain them in a loosely folded state.

Our previous characterization of *mas3* (*hsf1-m3*), a mutant allele of heat shock transcription factor, revealed that mitochondrial protein import at elevated temperatures requires the induction of certain HSPs (44). One of these HSPs may be MAS5, since the protein is essential for cell growth at 37°C, its expression increases upon a shift of cells from 23 to 37°C, and loss of MAS5 substantially affects mitochondrial protein import at 37°C. These properties suggest a unique role for the MAS5 protein or for increased levels of the MAS5 protein at elevated temperatures. It is possible that precursor proteins are less stable or misfold more readily at 37°C, and MAS5 might function to maintain the import competence of these precursors. Alternatively, MAS5 could contribute to the stability or activity of some structure on the mitochondrial surface which is a component of the import apparatus. Future studies will assess the interaction of MAS5 with precursor proteins and with mitochondria and should lead to a better understanding of the role of this protein in the import process.

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