**Abstract.** To identify new components that mediate mitochondrial protein import, we analyzed *mas6*, an import mutant in the yeast *Saccharomyces cerevisiae*. *mas6* mutants are temperature sensitive for viability, and accumulate mitochondrial precursor proteins at the restrictive temperature. We show that *mas6* does not correspond to any of the presently identified import mutants, and we find that mitochondria isolated from *mas6* mutants are defective at an early stage of the mitochondrial protein import pathway. *MAS6* encodes a 23-kD protein that contains several potential membrane spanning domains, and yeast strains disrupted for *MAS6* are inviable at all temperatures and on all carbon sources. The Mas6 protein is located in the mitochondrial inner membrane and cannot be extracted from the membrane by alkali treatment. Antibodies to the Mas6 protein inhibit import into isolated mitochondria, but only when the outer membrane has been disrupted by osmotic shock. Mas6p therefore represents an essential import component located in the mitochondrial inner membrane.

Most mitochondrial proteins are encoded in the nucleus, synthesized in the cytoplasm, and imported into mitochondria (Attardi and Schatz, 1988; Hartl and Neupert, 1990). Imported mitochondrial proteins must be recognized by mitochondria, cross one or both of the mitochondrial membranes, and sort themselves into one of four compartments: the outer membrane, the inner membrane, the intermembrane space, and the matrix. Many imported proteins destined for the inner mitochondrial compartments are synthesized in the form of precursors with cleavable amino-terminal presequences. These presequences contain all the information necessary to direct proteins to the mitochondria (Hurt et al., 1984, 1985; Horwich et al., 1985; van Loon et al., 1986).

Import of proteins into the mitochondrial matrix occurs via a multistep process that includes binding of precursors to receptors on the surface of the mitochondria (Pfänder and Neupert, 1987; Söllner et al., 1989, 1990; Hines et al., 1990), translocation of precursors across both membranes by a process requiring ATP and an inner membrane potential (Schleyer et al., 1982; Chen and Douglas, 1987; Pfänner et al., 1987; Eilers et al., 1987; Hwang and Schatz, 1989), and processing of precursors to their mature form (McAda and Douglas, 1982; Böhm et al., 1983). Translocation of precursor proteins has been shown to occur, at least initially, at contact sites between the inner and outer membranes (Schleyer and Neupert, 1985; Pon et al., 1989). In addition, some precursors require cytosolic factors, including the 70-kD heat shock proteins, for efficient import (Deshaies et al., 1988; Murakami et al., 1988). After import, many matrix proteins are proposed to be folded into their native conformation by matrix-localized chaperone proteins (Cheng et al., 1989; Ostermann et al., 1989; Kang et al., 1990).

Several components of the import pathway have been isolated using genetic approaches. Six mutants, *mas1-mas6*, were isolated as temperature-sensitive yeast mutants that accumulated precursors at the restrictive temperature (Yaffe and Schatz, 1984). We previously showed that *mas1* and *mas2* are defective in the activity of the matrix-localized processing protease, which removes the presequences from imported mitochondrial proteins (Yaffe et al., 1985; Jensen and Yaffe, 1988). We further showed that *MAS1* and *MAS2* encode the two subunits of this protease (Jensen and Yaffe, 1988; Witte et al., 1988; Yang et al., 1988). *mas3* mutants are defective in a transcription factor for the genes encoding many heat-shock proteins (Smith and Yaffe, 1991). *MAS5* encodes a non-essential, cytoplasmic dnaJ-like protein, that may play a chaperone role in import (Atencio and Yaffe, 1992). Subsequent genetic screens yielded new alleles of *mas1* and *mas2*, as well as a mutation in an hsp60-like protein (*mif4*) of the mitochondrial matrix (Cheng et al., 1989). *MIF4* is required for the ATP-dependent refolding and assembly of proteins imported into the matrix (Ostermann et al., 1989). A mutation in the matrix-localized hsp70 protein leads to a defect in translocation of proteins into the matrix, and in the folding of imported proteins to their native conformation (Kang et al., 1990). Recently, *MPI2*, encoding a membrane-bound protein required for import has been identified (Maarse et al., 1992).

Several potential import components located in the mitochondrial outer membrane have recently been identified. Antibodies to a 42-kD protein, ISP42, inhibit import of...
proteins into isolated mitochondria (Obha and Schatz, 1987a; Baker et al., 1990). In addition, a precursor protein “jammed” in the import machinery can be cross-linked to, or coimmunoprecipitated with, the ISP42 protein (Westeber et al., 1989; Scherer et al., 1990). Antibodies to two outer membrane proteins, MOM19 and MOM22, were shown to inhibit import into isolated Neurospora crassa mito-
chondria (Sölner et al., 1989, 1990). MOM19 appears to be the receptor for most mitochondrial proteins synthesized with amino-terminal presequences, whereas MOM22 is required for the import of the ATP/ADP carrier protein, an imported protein that does not carry a cleavable presequence. MOM38, which is homologous to the yeast ISP42 protein, is proposed to function as the general insertion protein (GIP), which interacts with all imported precursors at a step after the initial binding of precursors to the mitochondrial surface (Pfaller et al., 1988; Kiebler et al., 1990). The yeast MA670 gene encodes the functional homologue of Neurospora MOM22 (Hines et al., 1990).

Although a number of components of the mitochondrial protein import pathway have been identified, import components located in the inner membrane are conspicuously abs-
ent. For example, although precursors are thought to be translocated across the mitochondrial membranes through an aqueous channel (Pfanner et al., 1987), no inner membrane proteins of this putative channel have yet been identified. As described below, we find that a previously uncharacterized mutant, mas6Δ, is defective in mitochondrial protein import, and that MA6 encodes an essential protein located in the mitochondrial inner membrane.

Materials and Methods

Strains and Relevant Genotypes

Strain AH216 (MATα leu2-3 leu2-112) and the mas2 and mas6 mutants have been described previously (Yaffe and Schatz, 1984). ma6 strains JE4-3c (MATα mats6-1 leu2-3 leu2-112) and JE8-1b (MATα mats6-1 leu2-3 leu2-112) were obtained by backcrossing the mas6 mutant to AH216. JE4-3b (MATα mat6-1 trpl) was isolated by crossing JE8-1b to MATA trpl strain YPH250 (Sikorski and Hieter, 1989). MA6/MATα ura3-52/ura3-52 strains were used.

Cells were lysed and total proteins were precipitated with TCA as described (Michaelis et al., 1986), and strain D273-10B (Sherman, 1964) have been described. Standard yeast genetic techniques (Rose et al., 1988), and yeast media (Sherman et al., 1982) were used.

Cell Labeling and Immunoprecipitation

Yeast cells to be labeled were grown to an OD600 of 0.7 to 1.0 in SD medium (Sherman et al., 1982) supplemented with the appropriate amino acids. Cells were harvested and resuspended to an OD600 of 10 in fresh SD medium. 1 ml of cells were preincubated at 38°C for 30 min, then labeled for 4 min with 150 μCi of 35S-Translabel (1,000 Ci/mmol, ICN) at 38°C. Cells were lysed and total proteins were precipitated with TCA as described (Yaffe and Schatz, 1984). TCA pellets were resuspended in 100 μl of SDS-buffer (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1% SDS), heated at 95°C for 5 min, and diluted with 1.1 ml TNE (150 mM sodium chloride, 5 mM EDTA; 1% Triton X-100, 50 mM Tris-HCl, pH 8.0). Samples were con-
tinued at 12,000 g for 10 min, and specific proteins were immunoprecipit-
tated as described (Jensen et al., 1992). Labeled proteins were separated by SDS-PAGE (Laemmli, 1970; Haid and Suissa, 1983), and visualized by fluorography (Chamberlain, 1979).

Isolation of the MAS6 Gene

mas6 trpl strain JE14-5b was transformed with a library of random yeast genomic sequences in the TRPl-CEN6 vector pRS200 (Connelly, C., and P. Hieter, unpublished data) as described (Scheffii and Gietz, 1989). Trp" transforms were selected at 22°C, and then tested for growth at 37°C. From 5,000 total transformants, one plasmid, pJE1, was found that allowed growth of the temperature-sensitive mas6 strain at 37°C. Complementation of the mas6 mutation was shown to be plasmid dependent. To localize MAS6 sequences, restriction endonuclease fragments from the pJE1 plasmid were inserted into pRS200, transformed into the mas6 strain JE4-5b, and transformants were tested for growth at 37°C. The MAS6 complementing activity was localized to a 2.2-kbp SacI-BamHI fragment (see Fig. 2).

To show that pJE1 contained MAS6 sequences, a 4.2-kb XhoI-BamHI fragment was subcloned into the LEU2-containing vector pRS305 (Sikorski and Hieter, 1989) to form plasmid pJE4. pJE4 was cut with HindIII to target the site of integration (Rothstein, 1991), and transformed into the MAS6 leu2-3 strain AH216. Stable Leu+ integrants were crossed to mas6 strain JE8-1b, and the meiotic products of the diploid were analyzed. In 38 tetrads, no recombination between LEU2 and MAS6 was found indicating that the LEU2-containing plasmid, pJE4, had integrated into 1.3 map units of MAS6.

DNA Sequence of MAS6

pJE2 and pJE3 were constructed by inserting the 2.2-kbp SacI fragment of MAS6 into plasmid pRS200 (Sikorski, R., and P. Hieter, unpublished data) in both possible orientations. A series of overlapping deletions of the MAS6 fragment was made using exonuclease III digestion (Henikoff, 1984). To prevent exonuclease digestion of vector sequences, pJE2 and pJE3 were digested with XhoI, and the ends were filled in with α-phosphoroethanol nucleotides (Stratagene, La Jolla, CA) using DNA polymerase (Putney et al., 1981). MAS6 sequences carried on pJE2 and pJE3 were exposed to exonuclease III digestion by Clal digestion. Single-stranded DNA was isolated by S1 nuclease digestion, and plasmids were circularized by ligation. Using this collection of plasmids, both strands of the MAS6 gene were completely sequenced (Sanger et al., 1977; Jensen and Yaffe, 1988) using oligonucleotide primers specific to the pRS200 vector.

MAS6 Gene Disruptions

A precise deletion of MAS6 coding sequences was constructed as follows. First, a unique NotI site was engineered into the amino-terminus of MAS6. PCR fragment A, which contains the upstream region of MAS6, was isolated from plasmid pJE2 using oligonucleotide No. 21 (5'-ATACCGCTCATAAAACGAAG-3') and oligonucleotide No. 30 (5'-GGGGAGCGCGCTTGAATTTGTTGTTGATCTT-3'), and the polymerase chain reaction (Saiki et al., 1985). Similarly, PCR fragment B containing the MAS6 open reading frame (ORF) and downstream sequences was isolated using oligonucleotide No. 20 (5' -ATACCGCTCATAAG-3') and oligonucleotide No. 59 (5'-GGGGAGCGCGCTTGAATTTGTTGTTGATCTT-3'). PCR fragment A was digested with NotI and SacI, and PCR fragment B was digested with NotI and BamHI. Both fragments were ligated into SacI-BamHI digested pRS315 (Sikorski and Hieter, 1989) to form plasmid pJE5. pJE5 contains a unique NotI site immediately following the stop codon of MAS6.

A unique NotI site was engineered into the carboxy terminus of MAS6 as follows. PCR fragment C, which contains sequences downstream of MAS6, was isolated from plasmid pJE2 using oligonucleotide No. 20 (5'-ATACCGCTCATAAG-3') and oligonucleotide No. 30 (5'-GGGGAGCGCGCTTGAATTTGTTGTTGATCTT-3'). Similarly, PCR fragment D containing the MAS6 ORF and upstream sequences was isolated using oligonucleotide No. 21 (5'-ATACCGCTCATAAAACGAAG-3') and oligonucleotide No. 50 (5'-GGGGAGCGCGCTTGAATTTGTTGTTGATCTT-3'). Fragment C was digested with NotI and BamHI, and PCR fragment D was digested with NotI and SacI. Both fragments were ligated into SacI-BamHI digested pRS315 to form plasmid pJE7. pJE7 contains a unique NotI site immediately preceding the stop codon of MAS6.

Plasmid pJE9, which has the MAS6 coding sequences deleted, was constructed by removing a NotI–SacI fragment carrying the MAS6 ORF and upstream sequences from plasmid pJE7, and replacing them with a 5-kbp SacI fragment (lacking the MAS6 ORF) from PCR product A (see above). pJE10, which contains the MAS6 gene whose coding sequences were replaced by URA3, mas6Δ: URA3, was constructed as follows. A L-bkb HindIII fragment containing the yeast URA3 gene was isolated from plasmid YEp24 (Botstein et al., 1979). The DNA ends were filled in with DNA polymerase, and the fragment was blunt-end ligated into the NotI site of pJE10 to form plasmid pJE10:URA3. pJE10:URA3, carried on a 2.9-kb SacI–KpnI fragment, was used to replace one copy of MAS6 in the MATA/MATα diploid strains SM1060 or YPH501 (Rothstein, 1983). Stable UraΔ transformants were isolated and the meiotic products of two independently isolated diploids were analyzed at 22°C. Colonies resulting from viable spores were downloaded from jcb.rupress.org on November 18, 2017.
tested for growth on medium lacking uracil. Of 18 tetrads, all gave rise to no more than two viable spores, even after 2 wk of incubation at 22°C. The viable spores in every tetrad were Ura-. Southern analysis of the diploids no more than two viable spores, even after 2 wk of incubation at 22°C. The Isolation of Antiserum to the MAS6 Protein

transposon was found in the MAS6 open reading flame, near the SalI site. The plasmid containing this transposon was cut with KpnI and the DNA fragment in plasmid pRS200, was transformed into bacterial strain DB1329. Beverley, MA). Bacterial cells carrying this construct were induced to ex-
opJE5 into the EagI-BamI-II site of pMAL-c (New England Biolabs Inc., Mas6 protein was created by ligating a 1.5-kb NotI-BamHI fragment from strain SM1060. Ura + transformants (TUl0-LUK carries the URA3 gene) were selected, and the meiotic products analyzed at 22°C. Of 10 tetrads, all gave rise to two viable spores, both of which were Ura-.

Isolation of Antiserum to the MAS6 Protein

A fusion between the E. coli maltose binding protein (MBP) and the entire Mas6 protein was created by ligating a 1.5-kb NotI-BamHI fragment from pJE5 into the EagI-BamHI site of pMAL-c (New England Biolabs Inc., Beverly, MA). Bacterial cells carrying this construct were induced to ex-

Subcellular and Submitochondrial Fractionation

Subcellular fractionation, isolation of mitochondria, and submitochondrial fractionation were done as described (Daum et al., 1982; Jensen and Yaffe, 1988) except that the breaking buffer consisted of 0.6 M mannitol, 10 mM EDTA, and 20 mM Hepes-KOH, pH 7.4, when mitochondrial membranes were being fractionated. Proteins were separated by SDS-PAGE, stained with Coomassie blue, and the band containing the MBP-MAS6 fusion protein was excised with a razor blade. The gel slices were frozen in liquid nitrogen, ground in a mortar and pestle, and lyophilized. Samples were mixed with adjuvant and injected into rabbits as described (Carroll and Laughon, 1987).

Subcellular Fractionation

Subcellular fractionation, isolation of mitochondria, and submitochondrial fractionation were done as described (Daum et al., 1982; Jensen and Yaffe, 1988) except that the breaking buffer consisted of 0.6 M mannitol, 10 mM EDTA, and 20 mM Hepes-KOH, pH 7.4, when mitochondrial membranes were being fractionated. Proteins were separated by SDS-PAGE, transferred to nitrocellulose filters (Haid and Suissa, 1983), immune decorated with antisera and visualized with chemiluminescence (ECL, Amersham). To separate mitochondrial inner and outer membrane vesicles, 49 mg mitochondria isolated from strain D273-10B were converted to mitoplasts by osmotic shock, and membrane vesicles isolated as described (Pon et al., 1988) except that the breaking buffer consisted of 0.6 M mannitol, 15 % rabbit reticulocyte lysate, 50 mM potassium chloride, 1 mM magnesium chloride, 1.2 mM GTP, 1 mM ATP, 1 mM phosphoenol pyruvate, 1 mM methionine, 15 mM potassium succinate, 20 mM potassium malate, 20 mM Hepes-KOH, pH 7.4, and 0.1 mg/ml rabbit pyruvate kinase (Boehringer Mannheim Corp., Indianapolis, IN). Mitoplasts were prepared by diluting mitochondria with 9 vol of 20 mM Hepes-KOH, pH 7.4, followed by incubation on ice for 25 min. Mitoplasts were recovered by centrifugation (12,500 rpm for 10 min), and were resuspended in import buffer. Import reactions were terminated by cooling the reaction tubes on ice. After import, mitochondria and mitoplasts were reisolated by centrifugation through 1 ml 0.625 M sucrose, 20 mM Hepes, pH 7.4. Proteins were separated by SDS-PAGE, and visualized by fluorography.

Measurement of Mitochondrial Inner Membrane Potential

The inner membrane potential of mitochondria isolated from wild-type cells and mas6 mutants was measured as described (Elliott et al., 1987). Briefly, mitochondria were suspended in 0.6 M mannitol, 20 mM Hepes-KOH, pH 7.4, at 10 mg/ml total protein concentration. Assays using the fluorescent dye (3,3′-dipropylthiocarbocyanine iodide (Molecular Probes Inc., Eugene, OR) were carried out at 22°C in 0.6 M mannitol, 10 mM magnesium chloride, 0.5 mM EDTA, 20 mM potassium phosphate, pH 7.4, with 1 mg/ml BSA. The dye was diluted 1,000-fold from a 2-mM stock solution in DMSO. Measurements were performed in a fluorimeter (model 650-105; Perkin-Elmer Corp., Norwalk, CT) with excitation at 620 nm, emission at 670 nm, and slit widths of 6 nm. The final concentration of mitochondria in each reaction was 200 μg/ml.

Construction of MAS6 under the Control of the GAL1 Promoter

The MAS6 gene was placed under the control of the yeast GAL1 promoter as follows. A 939-bp MSel fragment, which contains the entire MAS6 open reading frame and 20 bp of upstream sequences, was isolated from plasmid pJE2. The DNA ends were filled in with DNA polymerase, and the fragment was blunt-end ligated into the HindIII site of the Bluescript II SK+ plasmid (Stratagene) to form pBf1. The MAS6 gene was excised from pBf1 by XhoI–BamHI digestion and inserted downstream of the GAL1 promoter in plasmid pRS314GU (Nigro et al., 1992) to form the plasmid pGAL-MAS6. To construct a strain dependent on pGAL-MAS6 expression, a SM1060 diploid heterozygous for the mas6::Tnl0-LUK disruption (see above) was transformed with a plasmid that carries MAS6 on the LEU2-containing plasmid pRS315 (Silkows and Hieter, 1989). The diploid was sporulated, and a haploid segregant was isolated that contained both the mas6::Tnl0-LUK disruption and the MAS6-LEU2 plasmid. This strain was transformed with the pGAL-MAS6 plasmid, and the transformants transferred to medium containing galactose. Mitotic segregants that contained only the pGAL-MAS6 plasmid, and not the MAS6-LEU2 plasmid were then isolated.

Inhibition of Import Using anti-Mas6p Antibodies

Immunoglobulin from antisera to Mas6p and preimmune serum were
purified as described (Ey et al., 1978; Harlow and Lane, 1988). Briefly, 1 ml serum was heated to 55°C for 20 min to inactivate complement, and then mixed with 10 ml 0.1 M Tris-HCl, pH 8.0. Serum was passed through a 2-ml column of Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ), and then through a 0.5 ml column of protein A-Sepharose CL-4B (Pharmacia). The Protein A column was washed with 10 vol 100 mM Tris-HCl, pH 8.0, followed by a wash with 10 vol 10 mM Tris-HCl, pH 8. IgGs were eluted from the protein A column by the addition of 100 mM glycine, pH 3.0. 0.5-ml fractions were collected and neutralized with 50 μl 1 M Tris-HCl, pH 8. IgG-containing fractions were pooled and dialyzed against PBS (140 mM sodium chloride, 10 mM sodium phosphate, pH 7.4). IgG was concentrated to ~10 mg/ml using Centricon 30 columns (Amicon Corp., Danvers, MA) according to manufacturer’s instructions.

Antibody Inhibition Using Mitochondria. Mitochondria were isolated from wild-type strain AH216, and aliquots representing 100 μg of mitochondrial proteins in 100 μl import buffer (see above) were preincubated with either 70 μg Mas6p IgG, or 120 μg IgG isolated from preimmune serum. After a 90-min incubation on ice, an [35S]-labeled precursor to the F1β protein was added, and the reaction tubes shifted to 25°C for 20 min. Mitochondria were reisolated by centrifugation, proteins subjected to SDS-PAGE, and the F1β protein identified by fluorography.

Antibody Inhibition Using Mitoplasts. Isolated mitochondria were incubated with 1 mg/ml trypsin for 30 min on ice to inactivate membrane import components (Ohba and Schatz, 1987a). After the addition of soybean trypsin inhibitor (STI) to 10 mg/ml, the mitochondria were isolated by centrifugation and resuspended in breaking buffer (0.6 M mannitol, 20 mM HEPES-KOH, pH 7.4) containing 1 mg/ml STI. The mitochondrial outer membrane was disrupted by osmotic shock and the mitoplast pellet recovered by centrifugation as described above. Aliquots representing 100 μg of mitoplast proteins in 100 μl import buffer were preincubated with the indicated amounts of Mas6p IgG, or 120 μg IgG isolated from preimmune serum. After a 90-min incubation on ice, the [35S]-labeled F1β protein was incubated with the mitoplasts for 20 min at 30°C, and analyzed by SDS-PAGE and fluorography.

Miscellaneous
pSP6-COX4, a plasmid for expressing the cytochrome oxidase subunit IV protein (Cox4p) by transcription/translation was obtained from D. Allison (University of Alabama, Birmingham, AL). The injection of MBS-MAS6 fusion protein into rabbits, and the isolation of antiserum was carried out by Hazleton Research Products, Inc. (Denver, PA). Standard methods were used for restriction endonuclease digestions, DNA ligations, bacterial transformations, and small-scale plasmid isolations from bacteria. The MAS6 DNA and protein sequences were compared with sequences contained in the GenBank (ver. 73.1), EMBL (ver. 32), PIR-Protein (ver. 34), PIR-Nucleic (ver. 36), Swiss-Prot (ver. 23), and VecBas3 (ver. 3) databases using the Fasta and tFasta algorithms (Lipman and Pearson, 1985).

Results

mas6 Mutants Are Defective in the Import of Mitochondrial Precursor Proteins

To identify additional components of the mitochondrial protein import pathway, we have analyzed mas6, a mutant isolated in a genetic screen that yielded the previously characterized mas1 and mas2 mutants (Yaffe and Schatz, 1984). As shown in Fig. 1, mas6 mutants are defective in the import of a mitochondrial matrix protein, subunit IV of cytochrome oxidase (Cox4p). Compared to wild-type cells (WT), mas6 mutants accumulated a significant amount of the Cox4p precursor, at least as much as the previously characterized mas2 mutant (Yaffe and Schatz, 1984; Jensen and Yaffe, 1988). In pulse-chase experiments, we showed that the rate of Cox4p import was reduced 10–20-fold in mas6 strains relative to wild-type strains (not shown). In similar labeling studies, we also found that mas6 strains were defective in the import of two other mitochondrial proteins, the β subunit of the F1-ATPase (F1β) and the citrate synthase protein (not shown).

We found that the temperature-sensitive growth defect in mas6 strains, and the defect in mitochondrial protein import cosegregated in genetic crosses (not shown). Hence the import defect in mas6 mutants is due to a single genetic lesion. mas6 mutants were found to be complemented by all previously identified import mutants: mas1, mas2, mif4 (Cheng et al., 1989), and sscl (Kang et al., 1990). Furthermore, a plasmid encoding the ISP42 protein failed to rescue the temperature-sensitive growth defect of our mas6 mutant (J. Entage and R. Jensen, unpublished data). mas6 therefore represents a new mutant defective in mitochondrial protein import.

Isolation of the MAS6 Gene

The MAS6 gene was cloned by genetic complementation of the temperature-sensitive mas6 mutant. Briefly, mutant cells were transformed with a genomic DNA library of wild-type DNA fragments in the shuttle vector pRS200 (Sikorski and Hieter, 1989), and P. Hieter, unpublished) as described in Materials and Methods. One plasmid was isolated that suppressed the temperature-sensitive growth defect of our mas6 mutant (J. Emtage and R. Jensen, unpublished data). mas6 therefore represents a new mutant defective in mitochondrial protein import.

Subcloning of the original 18-kbp DNA fragment located the MAS6 gene on a 2.2-kbp SacI–BamHI fragment (Fig. 2). This DNA fragment was completely sequenced (Fig. 3 A), revealing an open reading frame of 666 bp, encoding a protein of 222 amino acids with a molecular mass of 23.2 kDa. Two observations suggest that this open reading frame is derived from the original plasmid was subcloned into the plasmid pRS305 (Sikorski and Hieter, 1989), integrated into the yeast chromosome by homologous recombination, and the site of integration genetically mapped. The plasmid integrated at, or close to, the MAS6 locus, indicating that the original complementing plasmid carried the bona fide MAS6 gene.

Hydropathy analysis (Kyte and Doolittle, 1982) of the MAS6 coding sequence suggested that Mas6p is a membrane protein (Fig. 3 B). The carboxy-terminal half of Mas6p contains several potential membrane spanning domains. In con-
trast, the amino-terminal half of Mas6p is hydrophilic, with an unusual number of acidic residues. A comparison of MAS6 with sequences in the available databases failed to identify any significant homologies.

MAS6 Is an Essential Gene

mas6-1 is a temperature-sensitive lethal mutation, suggesting that MAS6 encodes an essential protein that is inactive at the nonpermissive temperature in mas6 mutants. However, some temperature-sensitive lethal mutations affect components that are only required at high temperatures (Atencio and Yaffe, 1992). To determine whether MAS6 encodes a gene product required at all temperatures, we constructed two disruptions of the MAS6 gene. First, the entire MAS6 open reading frame was replaced by the yeast URA3 gene, and the mas6::URA3 construction was used to replace one of the two copies of MAS6 in a ura3/ura3 diploid cell (see Materials and Methods). When these diploid cells were sporulated and the haploid progeny allowed to grow at 22°C on glucose-containing medium, only Ura- spores were found to be viable. Hence all viable spores carried an intact MAS6 gene, indicating that MAS6 is essential at 22°C. When germination of the spores inferred to carry the mas6::URA3 mutation was observed microscopically, all spores were seen to arrest in their growth after three to five divisions. Strikingly, >90% of the cells arrested as unbudded cells. Similar results were seen when the chromosomal MAS6 gene was replaced with a MAS6 gene disrupted by a Tn10-LUK insertion.

The MAS6 Protein is Located in the Mitochondrial Inner Membrane

Although mitochondria isolated from mas6 mutants are defective for import, the MAS6 gene product does not carry a typical amino-terminal mitochondrial presequence. To determine the intracellular location of Mas6p, we raised antibodies to a fusion protein consisting of the E. coli maltose binding protein and the entire Mas6 protein. When this antiserum was tested against total yeast proteins by immune

Figure 2. Restriction endonuclease map of the cloned MAS6 gene. The top solid line indicates the relevant restriction sites in the 18-kbp insert carried in plasmid pJE1. The lower solid line shows an enlarged view of the 2.2-kbp fragment shown by subcloning to carry the MAS6 gene. The striped lines indicate adjacent vector sequences. The shaded arrow at the bottom represents the approximate location of the MAS6 gene. Restriction endonuclease sites: B, BamHI; C, Clal; K, KpnI; S, SacI; Sa, SalI; and X, Xhol.

Figure 3. MAS6 encodes a 23.2-kD protein with several potential membrane-spanning domains. (A) DNA sequence of MAS6 and its predicted protein product. Underlined amino acids represent potential trans-membrane domains predicted from hydropathy analysis. (B) Hydrophathy plot of the predicted MAS6 protein. The Mas6 protein was analyzed using the algorithm of Kyte and Doolittle (1982) with a window size of 12. Hydrophobic stretches in Mas6p are indicated as shaded areas with negative values. These sequence data are available from EMBL/GenBank/DDBJ under accession number X71633.

Figure 4. Mas6p is a mitochondrial protein. AH216 cells were grown to the midlogarithmic stage in semisynthetic medium containing 2% lactate, converted to spheroplasts, and homogenized. The homogenate (cells) was centrifuged at 2,500 g for 5 min, and the supernatant fractionated into a mitochondrial pellet (mitos), and a postmitochondrial supernatant (cytosol) by centrifugation at 9,600 g for 10 min. Aliquots of homogenate, mitochondria, and cytosol representing equivalent numbers of cells were subjected to SDS-PAGE and analyzed by immune blotting with antisera to Mas6p, hexokinase, and the F1,ATPase β subunit (F1β).

Figure 5. The Mas6 protein fractionates with the inner membrane. Mitochondria were converted to mitoplasts and sonicated (see Materials and Methods). Mitochondrial membrane vesicles were loaded onto sucrose step gradients, and centrifuged at 100,000 g for 17 h. Fractions were collected, and an aliquot from each fraction analyzed by immune blotting with antisera to Mas6p, the outer membrane OM45 protein, and the inner membrane F1β protein. The top of the gradient is to the left.
Figure 6. Immunoelectron microscopy indicates that Mas6p is a mitochondrial inner membrane protein. Mitochondria were swelled, fixed and stained as described in Materials and Methods. After imbedding in LR White resin, ultrathin sections of mitochondria were taken and decorated with antiserum to Mas6p, OM45 (an outer membrane protein), and F1β (an inner membrane protein). Antibody-antigen interactions were detected using a gold-coupled secondary antibody and electron microscopy. Bars, 100 nm.

blotting, a single 23-kD protein was identified. Two observations suggest that this antiserum recognized Mas6p. First, overproduction of Mas6p (due to its expression from the GAL1 promoter region) resulted in overproduction of the 23-kD antigen (not shown). Second, the antiserum immunoprecipitated the protein produced by in vitro transcription/translation of the cloned MAS6 gene (not shown).

Immune decorations of yeast cell fractions indicate that Mas6p is a mitochondrial protein. When a yeast cell homogenate was separated into a mitochondrial pellet and crude cytosol, Mas6p cofractionated with the mitochondrial F1β protein (Fig. 4). No Mas6p was found in the supernatant fraction, which contains most of the cytosol, as indicated by the hexokinase enzyme. The mitochondrial location of Mas6p was also seen in immunofluorescent labeling of permeabilized yeast cells (not shown), and in immunofluorescent labeling of mammalian COS-7 cells transiently expressing Mas6p (Jensen, R., unpublished results).

Several observations demonstrate that Mas6p is an integral membrane protein located in the mitochondrial inner membrane. First, Mas6p could not be extracted from mitochondrial membranes with 0.1 M sodium carbonate, whereas F1β, a peripheral membrane protein, was readily extracted with carbonate (not shown). Second, when mitochondria were disrupted by sonication and the membrane vesicles separated on sucrose gradients, Mas6p cofractionated with the inner membrane-bound F1β protein (Fig. 5), as well as the inner membrane Cox4p and the ATP/ADP carrier protein (not shown). Little or no Mas6p was found in the sucrose gradient fractions that contained OM45p, a mitochondrial outer membrane protein (Yaffe et al., 1989). Third, immunoelectron microscopy of mitochondrial sections showed that Mas6p was located in the inner membrane, along with the F1β protein (Fig. 6). Essentially no Mas6p was found to colocalize with OM45p on the mitochondrial surface. Fourth, Mas6p synthesized by transcription/translation of the cloned gene was imported into the inner membrane of isolated mitochondria (not shown).

Mitochondria Isolated from mas6 Mutants Are Defective at an Early Stage in the Import Pathway

To determine the step in import that is defective in mas6 mutants, mitochondria were isolated from mas6 strains and tested for the ability to import 35S-labeled precursor proteins. The Cox4p precursor was efficiently imported and processed to the mature form in wild-type mitochondria at 23, 30, and 37°C (Fig. 7 A). In contrast, mas6 mitochondria were defective for import of Cox4p at all temperatures. Similarly, mas6 mitochondria were defective in the import of the F1β precursor protein, at 23°C (Fig. 7 B), and at 30 and 37°C (not shown). We also found that the import of two additional precursors, citrate synthase and cytochrome c1, was defective in mas6 mitochondria (not shown). Although mas6 strains are temperature-sensitive for viability, isolated mas6 mitochondria are defective in import even at room temperature. We suggest that the altered Mas6 protein is more labile after subcellular fractionation than in intact cells. Similarly, mitochondria isolated from temperature-sensitive mas1 strains are defective in import even at room temperature. We suggest that the altered Mas6 protein is more labile after subcellular fractionation than in intact cells. Similarly, mitochondria isolated from temperature-sensitive mas1 strains are defective in import at both the permissive and restrictive temperatures (Yaffe et al., 1985).

In contrast to wild-type mitochondria, very little Cox4p or F1β protein pellets with the mas6 mitochondria after the import reaction (compare total recovery of precursor and mature in wild-type and mas6 mitochondria in Fig. 7). Hence the import defect in mas6 mutants appears to be early in the import pathway. The mas6 import defect, however, does not seem to be due to a defect in the initial binding of precursors to the mitochondrial surface. In particular, previous studies have shown that precursor binding to mitochondrial outer membrane import components does not require
an inner membrane potential (Pfeller and Neupert, 1987; Söllner et al., 1989, 1990; Hines et al., 1990). We found that when mitochondria were deenergized with valinomycin (Fig. 7, -Δψ), similar amounts of Cox4p or the Fβ precursor protein pelleted with both wild-type and mas6 mitochondria.

mas6 Mutants Are Directly Blocked in the Import Pathway

We have shown that the import defect in mas6 mutants is not an indirect effect, for example due to defective mitochondrial energy metabolism. (a) The potential across the mitochondrial inner membrane, measured with a potential-sensitive fluorescent dye (Sims et al., 1974; Eilers et al., 1987), was not significantly different from that of wild-type mitochondria (Fig. 8). The addition of the respiratory substrates, succinate and malate, elicited a near maximal potential in both mas6 and wild-type mitochondria. (b) Coupling ratios measured with an oxygen electrode (Yaffe et al., 1985) showed no significant differences between wild-type, mas1, and mas6 mitochondria (not shown). (c) Respiration-driven protein synthesis (Yaffe and Schatz, 1984), and aliquots containing 80 μg protein were run on SDS–polyacrylamide gels. The Mas6p and the Fβ-ATPase α subunit were identified by immune blotting. Relative amounts of the Mas6p (●), and the precursor form of the Fβ protein (○) were determined by densitometry.

Depletion of MAS6 from Cells Results in the Accumulation of Mitochondrial Precursor Proteins

mas6 mutants are defective in import presumably due to the inactivation of Mas6p at the restrictive temperature, 37°C. To determine the effect of Mas6p inactivation at lower temperatures, we placed the MAS6 gene under the control of the galactose-inducible GAH promoter region. We introduced this construct into cells deleted for the chromosomal MAS6

Figure 7. Mitochondria isolated from mas6 mutants are defective at an early stage of the import pathway. Mitochondria were isolated from mas6 and wild-type AH216 cells and incubated with an 35S-labeled Cox4p precursor (A) or the precursor to the Fβ-ATPase β subunit, Fβ (B) at the indicated temperatures. After 20 min, mitochondria were reisolated by centrifugation, and proteins were solubilized in SDS-sample buffer. Proteins were separated on SDS–polyacrylamide gels, and the radiolabeled Cox4p and Fβ proteins were identified by fluorography. -Δψ indicates no inner membrane potential due to the addition of valinomycin to the import reaction. Precursor (p) and mature (m) forms of the imported proteins are indicated. 20% of the precursor added to each import reaction is also shown.

Figure 8. mas6 mitochondria are not defective in establishing or maintaining the potential across the inner membrane. Mitochondria isolated from mas6 or wild-type AH216 cells were incubated with the potential sensitive dye, diS-C3-(5), and the fluorescence was recorded. A downward deflection indicated an increase in the inner membrane potential. The following were added to the mitochondria as indicated: ATP (2 mM); potassium malate (20 mM), potassium succinate (15 mM); and the potassium ionophore, valinomycin (10 μg/ml).

Figure 9. Cells depleted of the Mas6p accumulate mitochondrial precursor proteins. Cells carrying the mas6::Tn10-LUK disruption and the pGAL-MAS6 plasmid were grown at 30°C on YEP-galactose medium to an OD600 of 1.0. Cells were centrifuged and pellets were resuspended in glucose medium (YEPrD) to an OD600 of 0.1. At the indicated times, total cell proteins were extracted (Yaffe and Schatz, 1984), and aliquots containing 80 μg protein were run on SDS–polyacrylamide gels. The Mas6p protein and the Fβ-ATPase α subunit were identified by immune blotting. Relative amounts of the Mas6p (●), and the precursor form of the Fβ protein (○) were determined by densitometry.
gen (see Materials and Methods), and examined mitochondrial protein import when the expression of \textit{MAS6} was inhibited. When cells that contain the \textit{GAL1-MAS6} construct were grown in galactose-containing medium, Mas6p was overproduced \textasciitilde10–20-fold (not shown). When these cells were shifted to glucose medium, which inhibits the expression of \textit{MAS6}, a striking correlation between the level of Mas6p and mitochondrial protein import was seen (Fig. 9). 7 h after shifting to glucose-containing medium, the amount of Mas6p was slightly below wild-type levels. By 12 h, Mas6p was no longer detectable by immune blotting. Concomitant with the loss of Mas6p, the precursor to the \( \alpha \) subunit of the \( F_1 \)-ATPase protein accumulated. This defect in mitochondrial protein import was not simply due to cell inviability since cells containing the \textit{GAL1-MAS6} construct continued to divide for at least 24 h after their shift to glucose-containing medium (not shown). In similar experiments, we found that import of the \( F_1 \beta \) and Cox4 proteins was blocked when expression of \textit{MAS6} was inhibited by glucose (not shown). Therefore, depletion of Mas6p from cells leads to a defect in mitochondrial protein import.

\textbf{Antibodies that Recognize the MAS6 Protein Inhibit Import into Mitoplasts}

Our studies with different \textit{mas6} mutants suggest that Mas6p is an essential import component. To test this conclusion, we asked whether antibodies directed against the Mas6p protein inhibit mitochondrial protein import in vitro. Since \textit{MAS6} encodes an inner membrane protein, we examined import of precursors into mitoplasts, mitochondria whose outer membranes have been disrupted. In mitoplasts, precursors can be translocated directly across the inner membrane, bypassing outer membrane import components (Ohba and Schatz, 1979b; Hwang et al., 1989). For our inhibition experiments, we inactivated outer membrane import components by digestion with trypsin, and mitoplasts were then formed by breaking open the outer membrane by osmotic shock (see Materials and Methods). When 7 \( \mu \)g Mas6p IgG was added to 100 \( \mu \)g of mitoplasts, a slight decrease in the import of \( F_1 \beta \) was seen as compared to mitoplasts not treated with IgG (Fig. 10). 35 \( \mu \)g of Mas6p IgG markedly inhibited import, and increasing the amount of Mas6p IgG to 70 \( \mu \)g led to a virtual block in import, with little or no mature-sized \( F_1 \beta \) protein produced. 120 \( \mu \)g of preimmune IgG did not inhibit the import of \( F_1 \beta \). We obtained similar results when we examined the import of another precursor, Cox4p (not shown). As an additional control, we showed that neither Mas6p IgG nor preimmune IgG inhibited the import of precursors into mitochondria whose outer membranes remained intact (Fig. 10). Therefore, the inhibition of import with Mas6p antibodies complements our studies with the \textit{mas6} mutants described above, and strongly suggests that the Mas6 protein is an inner membrane component acting directly in the import pathway.

\textbf{Discussion}

We have analyzed a new import mutant, \textit{mas6}, which carries a mutation that causes both temperature-sensitive growth and defective mitochondrial protein import. Several genetic observations show that \textit{MAS6} encodes an essential import component: (a) the \textit{mas6} mutant is a temperature-sensitive lethal and accumulates the precursor form of several imported mitochondrial proteins; (b) mitochondria isolated from \textit{mas6} cells are defective in the import of at least four different proteins; and (c) depletion of Mas6p from cells results in cell death and a defect in the import of mitochondrial precursor proteins.

Our results strongly suggest that \textit{MAS6} plays a direct role in import, and that the import defect in \textit{mas6} mutants is not due to a defect in some other mitochondrial function such as energy metabolism. First, we showed that \textit{mas6} strains, and mitochondria isolated from \textit{mas6} mutants, are not markedly defective in establishing or maintaining the potential across the inner membrane. Second, we find that wild-type Mas6p function can be inhibited in mitoplasts using IgG directed against Mas6p. Under these conditions we also find no detectable decrease in inner membrane potential (Emtage, J., unpublished data). Third, we find that \textit{MAS6} is essential for viability, even when cells are grown on glucose-containing medium. All previously characterized mitochondrial components that are required for electron transport and ATP synthesis are only necessary for growth on nonfermentable carbon sources (Tzagoloff and Dieckmann, 1990). Hence \textit{MAS6} joins a family of essential mitochondrial proteins (Baker and Schatz, 1991), all of which are components of the mitochondrial protein import pathway: \textit{MAS1} (Witte et al., 1988) and \textit{MAS2} (Jensen and Yaffe, 1988), subunits of the matrix-localized processing protease; \textit{MIF4} (Cheng et al., 1989), a groEL homologue located in the matrix; \textit{SSC1} (Kang et al., 1990), a matrix-localized chaperonin protein; \textit{ISP4} (Baker et al., 1990), an import component located in the outer membrane; and \textit{MP11} (Maarse et al., 1992), a membrane-bound protein required for import. Since we have localized Mas6p to the inner membrane, \textit{MAS6} represents the first essential inner membrane import component.

The Mas6 protein, predicted from its DNA sequence, contains two domains. The amino-terminal half of Mas6p is hydrophilic, and the carboxy-terminal half contains several potential membrane spanning regions. Preliminary experiments using "epitope-tagged" Mas6p constructs suggest that the extreme carboxy terminus and the hydrophilic amino-terminal domain of Mas6p face the intermembrane space.
tion, the Mas6 protein does not itself contain a typical amino-terminal presequence, and following import into the inner membrane, Mas6p is not processed to a lower molecular weight form. Therefore, the signal to target Mas6p to the mitochondria and to localize it to the inner membrane must be located within the mature protein. Preliminary experiments have shown that at least one targeting signal in Mas6p is carried within its first putative transmembrane domain (Ryan, K., unpublished observations).

When an in vitro synthesized precursor protein such as Cox4p or the F1β protein is incubated with mitochondria isolated from mas6 strains, very little precursor or mature-sized protein pellets with mas6 mitochondria whose inner membrane potential has been dissipated with valinomycin. These results suggest that mas6 mitochondria are defective at a step in the import pathway after the binding of precursors to outer membrane components. Apparently in mas6 mitochondria, the precursor binds to outer membrane components, but fails off the mitochondria when the precursor reaches the defective Mas6 protein. Consistent with this hypothesis, time course of import studies with mas6 mitochondria indicate that the imported precursor initially binds to the mitochondrial surface, but fails to pellet with the mitochondria at later times during import (Emtage, J., unpublished observations). Since MAS6 encodes an inner membrane protein, our results raise the exciting possibility that import may be reversible even after the precursor has penetrated the outer membrane import machinery. It is important to note, however, that we have not directly shown that the precursor initially bound to the surface of mas6 mitochondria is subsequently released. Consequently, we cannot exclude the possibility that the radiolabeled precursor is simply being degraded by mas6 mitochondria.

There are several possible roles for the Mas6 protein in import. For example, Mas6p may be required for the binding of precursors to the inner membrane translocation machinery. The amino terminus of MAS6 contains a large number of acidic amino acids (17%), with some of these aspartate and glutamate residues clustered. We speculate that one of the functions of Mas6p may be to interact with the arginine- and lysine-rich presequences of imported mitochondrial proteins as they come through the outer membrane translocation machinery. Another possibility is that Mas6p may translocate precursor proteins across the inner membrane. Mas6p is an integral membrane protein containing several potential membrane spanning domains. Hence Mas6p may form part of a protein-translocating channel in the inner membrane. Furthermore, since half of the Mas6 protein is predicted to reside in the membrane, while the other half is hydrophilic, Mas6p may have more than one function.

Since Mas6p is an essential inner membrane import component, it provides a valuable tool to learn more about the mechanism by which proteins are translocated into mitochondria. Experiments are in progress to determine the step in the import pathway in which precursors come into physical contact with the Mas6 protein. For example, we have recently shown that a precursor partially translocated across the inner membrane can be chemically cross-linked to Mas6p (Ryan, K., and R. Jensen, manuscript in preparation). Furthermore, since protein import through the mitochondrial outer membrane requires many different proteins (Klebl et al., 1990; Sölter et al., 1992), we anticipate that Mas6p does not act alone in the translocation of proteins through the inner membrane. Hence we are using both biochemical and genetic approaches to identify new inner membrane proteins with which Mas6p may interact.

We wish to thank Jeff Schatz, in whose lab this work was initiated. We also thank Michael Yaffe for the mas6 mutant, and Barbara Smith for communicating her preliminary results with mas6. We are grateful to Mike Delannoy for his special expertise with the immunoelectron microscopy, and to Dan Allison for the SP6-COX4 plasmid, David Bedwell for the SP6-F1β construct, Mike Douglas for the AAC2 antiseraum, and Carla Connolly and Phil Hieter for the TRP1-CENI6 library. We thank Bernard Khoo for help with experiments measuring the mitochondrial inner membrane potential of mas6 mutants. We thank Kathy Wilson and Carolyn Machamer for valuable discussions, and for critical reading of the manuscript. We also thank Kathleen Ryan, Jennifer Kalish, and Shawn Burgess for helpful comments on the manuscript.

This work was supported by an American Cancer Society grant, MV-454 to R. E. Jensen.

Received for publication 21 April 1993 and in revised form 4 June 1993.

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