Import of proteins into yeast mitochondria: the nuclear *MAS2* gene encodes a component of the processing protease that is homologous to the *MAS1*-encoded subunit

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The *mas2* mutant of *Saccharomyces cerevisiae* is temperature sensitive for import of proteins into mitochondria. To identify the lesion in this mutant, we have cloned and sequenced the wild-type *MAS2* gene and determined the intracellular location of its protein product. *MAS2* encodes an essential 53-kd protein that is located in the mitochondrial matrix and is homologous to the *MAS1* protein, a previously identified subunit of the protease that cleaves presequences from mitochondrial precursor proteins. The activity of this enzyme is temperature sensitive in *mas2* cells. Together with the results of the accompanying study these results show that *MAS2* and *MAS1* encode the two subunits of the processing protease.

Key words: *mas* mutants/mitochondria/processing protease/protein import

Introduction

Mitochondrial protein import presents fundamental problems in cellular protein targeting. To be imported, proteins destined for the mitochondria must find the organelle, cross one or both of the mitochondrial membranes, and be sorted into the correct submitochondrial compartment. Mitochondrial protein import bears many similarities to the translocation of proteins across the endoplasmic reticulum and the secretion of proteins from bacteria (Schatz, 1986). To understand in molecular terms how proteins are translocated across cell membranes, it is necessary to identify the components required for these processes.

Most mitochondrial proteins are encoded by nuclear genes, synthesized in the cytoplasm, and imported into the mitochondria (reviewed by Attardi and Schatz, 1988). Many of these imported mitochondrial proteins are synthesized as precursors which carry 'presequences' at their amino (N)-termini. These presequences contain the information necessary to target these proteins to and into the mitochondria (Hurt *et al.*, 1984, 1985; Horwich *et al.*, 1985). Biochemical studies suggest that precursors are bound to specific receptors on the mitochondrial surface and then translocated across the mitochondrial membranes by a process that requires an energized inner membrane, ATP, and at least the partial unfolding of the precursor protein (Attardi and Schatz, 1988). Finally, the presequences of the imported proteins are removed by a processing protease

located in the matrix space. This metalloprotease specifically cleaves a variety of mitochondrial precursor proteins (Böhni *et al.*, 1980, 1983; McAda and Douglas, 1982). Since the processing protease does not recognize a specific amino acid sequence and fails to cleave denatured precursors, the protease must recognize a specific three-dimensional structure common to mitochondrial presequences.

To identify the proteins that mediate the various steps of the import pathway, *Saccharomyces cerevisiae* mutants defective in mitochondrial import have been isolated (Yaffe and Schatz, 1984). In particular, a collection of temperature-sensitive lethal yeast strains were screened for those that accumulated mitochondrial precursor proteins at the non-permissive temperature, 37°C. Two mutants, *mas1* and *mas2* (for mitochondrial assembly), were isolated, each of which carried a single temperature-sensitive mutation in a nuclear gene. Both mutants accumulated uncleaved precursors outside the mitochondrial inner membrane and failed to import these precursors at normal rates. Mitochondria isolated from *mas1* mutants contained 20-fold lower activity of the matrix-localized processing protease (Yaffe *et al.*, 1985). The *MAS1* gene was recently shown to encode a component of this protease (Witte *et al.*, 1988): antiserum to the *MAS1* protein cross-reacted with one of the two proteins in a purified preparation of the corresponding protease from *Neurospora crassa* mitochondria (Hawltischek *et al.*, 1988).

The ability to examine import *in vitro* with mitochondria isolated from the *mas1* mutant was critical for determining the function of the *MAS1* protein. Mitochondria isolated from the originally isolated *mas2* mutant, however, showed no apparent defect in the import of precursor proteins. As an alternative approach to understand the function of *MAS2* in the import pathway, we isolated the *MAS2* gene, determined its nucleotide sequence, and identified the intracellular location of the *MAS2* protein. Since the *MAS2* protein proved to be in the mitochondrial matrix, we tested the possibility that *MAS2* is required for processing imported mitochondrial precursors. By analyzing a *mas2* mutant which was more severely defective in *MAS2* function than the original *mas2* strain, we found that processing activity was temperature sensitive in mitochondrial extracts. In addition, sequence comparisons indicated that the *MAS2* protein is homologous to the *MAS1* protein, a subunit of the processing protease. Together with the results from the accompanying paper (Yang *et al.*, 1988), these results indicate that *MAS2* encodes the second subunit of the matrix-localized processing protease.

Results

Isolation of the *MAS2* gene

mas2 mutants are temperature sensitive for both mitochondrial protein import and cell growth (Yaffe and Schatz, 1984). To clone the wild-type *MAS2* gene, we first screened

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1      16      31      46      61      76
AAG TAC ACT TGT TTT TAT CAC CAT ATC TTT TCG AGA TGA TGA CTC TTG CAC AAA TTT TTC ATC CGG CGT GAG GGC GTA TCG AGA ATG AAC

91     106     121     136     151     166
AAT TCA GGT GAA AAA GCC TAC ACA GGT GGA CTA GAA GAA AGG TGT TTC AGG GCA GGT GGT CAA ATT AAT GCA AAG CAG AGA GCT GAT TAA

181    196    211    226    241    256
      Met Leu Arg Asn Gly Val Gln Arg Leu Tyr Ser Asn Ile Ala Arg Thr Asp Asn Phe Lys Leu Ser Ser Leu Ala Asn Gly
TAA CAA AAG ATG CTA AGG AAC GGC GTT CAG AGG TTG TAC TCT AAC ATC GCT AGA ACC GAT AAT TTT AAG CTT TCG TCA CTA GCG AAT GGA

271    286    301    316    331    346
Leu Lys Val Ala Thr Ser Asn Thr Pro Gly His Phe Ser Ala Leu Gly Leu Tyr Ile Asp Ala Gly Ser Arg Phe Glu Gly Arg Asn Leu
CTG AAA GTG GCG ACA TCA AAT ACT CCT GGA CAC TTT AGT GCC TTA GGC CTA TAC ATC GAC GCA GGA TCA CGG TTT GAA GGT AGG AAT TTG

361    376    391    406    421    436
Lys Gly Cys Thr His Ile Leu Asp Arg Leu Ala Phe Lys Ser Thr Glu His Val Glu Gly Arg Ala Met Ala Glu Thr Leu Glu Leu Leu
AAG GGT TGT ACA CAT ATC TTG GAT AGA CTA GCA TTC AAA TCA ACC GAA CAC GTC GAG GGC CGA GCA ATG GCA GAG ACG TTG GAG TTA CTG

451    466    481    496    511    526
Gly Gly Asn Tyr Gln Cys Thr Ser Ser Arg Glu Asn Leu Met Tyr Gln Ala Ser Val Phe Asn Gln Asp Val Gly Lys Met Leu Gln Leu
GGT GGG AAT TAC CAG TGT ACC TCG TCT AGA GAA AAT TTG ATG TAT CAG GCT TCT GTG TTC AAC CAG GAT GTG GGT AAA ATG CTT CAA TTA

541    556    571    586    601    616
Met Ser Glu Thr Val Arg Phe Pro Lys Ile Thr Glu Gln Glu Leu Gln Glu Gln Lys Leu Ser Ala Glu Tyr Glu Ile Asp Glu Val Trp
ATG TCA GAA ACT GTG AGG TTC CCT AAG ATT ACT GAG CAA GAA CTG CAA GAA CAG AAG CTG TCG GCA GAG TAT GAA ATC GAT GAA GTT TGG

631    646    661    676    691    706
Met Lys Pro Glu Leu Val Leu Pro Glu Leu Leu His Thr Ala Ala Tyr Ser Gly Glu Thr Leu Gly Ser Pro Leu Ile Cys Pro Arg Glu
ATG AAG CCA GAA TTG GTA TTA CCT GAG CTG TTG CAC ACA GCA GCA TAC TCC GGA GAA ACT TTG GGG TCC CCT TTA ATC TGC CCT AGA GAG

721    736    751    766    781    796
Leu Ile Pro Ser Ile Ser Lys Tyr Tyr Leu Leu Asp Tyr Arg Asn Lys Phe Tyr Thr Pro Glu Asn Thr Val Ala Ala Phe Val Gly Val
CTC ATT CCT TCT ATT TCT AAA TAC TAC TTA TTG GAC TAC AGA AAT AAA TTC TAC ACC CCA GAA AAT ACT GTT GCG GCA TTT GTT GGT GTT

811    826    841    856    871    886
Pro His Glu Lys Ala Leu Glu Leu Thr Glu Lys Tyr Leu Gly Asp Trp Gln Ser Thr His Pro Pro Ile Thr Lys Lys Val Pro Gln Tyr
CCT CAC GAA AAG GCA TTA GAG CTT ACA GAA AAG TAT CTC GGT GAC TGG CAA TCT ACA CAC CCT CCG ATC ACC AAA AAA GTT CCC CAA TAT

901    916    931    946    961    976
Thr Gly Gly Glu Ser Cys Ile Pro Pro Ala Pro Val Phe Gly Asn Leu Pro Glu Leu Phe His Ile Gln Ile Gly Phe Glu Gly Leu Pro
ACG GGC GGG GAA AGT TGT ATT CCA CCA GCA CCA GTG TTT GGG AAT TTA CCA GAA TTA TTC CAC ATC CAG ATC GGA TTT GAA GGT CTC CCT

991    1006   1021   1036   1051   1066
Ile Asp His Pro Asp Ile Tyr Ala Leu Ala Thr Leu Gln Thr Leu Leu Gly Gly Gly Gly Ser Phe Ser Ala Gly Gly Pro Gly Lys Gly
ATA GAT CAT CCA GAC ATT TAT GCT TTA GCA ACA TTA CAA ACT TTA CTT GGA GGT GGT GGT TCT TTC AGT GCT GGT GGG CCA GGA AAG GGG

1081   1096   1111   1126   1141   1156
Met Tyr Ser Arg Leu Tyr Thr His Val Leu Asn Gln Tyr Tyr Phe Val Glu Asn Cys Val Ala Phe Asn His Ser Tyr Ser Asp Ser Gly
ATG TAT TCT CGT TTG TAT ACC CAC GTT TTG AAC CAG TAT TAC TTT GTT GAA AAT TGT GTT GCG TTC AAC CAT TCT TAT TCG GAT TCT GGT

1171   1186   1201   1216   1231   1246
Ile Phe Gly Ile Ser Leu Ser Cys Ile Pro Gln Ala Ala Pro Gln Ala Val Glu Val Ile Ala Gln Gln Met Tyr Asn Thr Phe Ala Asn
ATT TTC GGT ATT TCA TTA TCT TGT ATA CCG CAG GCA GCT CCT CAA GCG GTT GAG GTA ATT GCA CAG CAA ATG TAT AAT ACA TTT GCT AAT

1261   1276   1291   1306   1321   1336
Lys Asp Leu Arg Leu Thr Glu Asp Glu Val Ser Arg Ala Lys Asn Gln Leu Lys Ser Ser Leu Leu Met Asn Leu Glu Ser Lys Leu Val
AAG GAT TTG AGG TTA ACT GAA GAC GAA GTC TCC AGG GCC AAG AAT CAG TTG AAG TCT TCA CTA CTA ATG AAC CTA GAG TCT AAA CTG GTA

1351   1366   1381   1396   1411   1426
Glu Leu Glu Asp Met Gly Arg Gln Val Leu Met His Gly Arg Lys Ile Pro Val Asn Glu Met Ile Ser Lys Ile Glu Asp Leu Lys Pro
GAA TTG GAA GAT ATG GGC AGA CAA GTT CTC ATG CAT GGT CCG AAA ATC CCA GTC AAC GAA ATG ATA AGT AAG ATC GAA GAT TTG AAG CCA

1441   1456   1471   1486   1501   1516
Asp Asp Ile Ser Arg Val Ala Glu Met Ile Phe Thr Gly Asn Val Asn Asn Ala Gly Asn Gly Lys Gly Arg Ala Thr Val Val Met Gln
GAT GAT ATC TCA CGT GTT GCC GAA ATG ATA TTT ACG GGA AAT GTA AAT AAT GCT GGC AAT GGT AAG GGT AGA GCT ACT GTT GTA ATG CAA

1531   1546   1561   1576   1591   1606
Gly Asp Arg Gly Ser Phe Gly Asp Val Glu Asn Val Leu Lys Ala Tyr Gly Leu Gly Asn Ser Ser Ser Lys Asn Asp Ser Pro Lys
GGA GAC AGA GGA TCC TTT GGA GAT GTG GAA AAT GTA CTG AAG GCA TAT GGA TTG GGT AAC AGC TCC TCC TCC AAA AAC GAT AAT CCC AAG

1621   1636   1651   1666   1681   1696
Lys Lys Gly Trp Phe ***
AAG AAG GGT TGG TTT TGA ACA ACT TTA GCA GTA CTT TAT TAC TTT TAT TTT GGG AGA CTT TCA AGA TAG GCA AAG ACT TTA TTT CCG CTG

1711
CTT ATC TAT

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Fig. 1. Sequence of the *MAS2* gene and its predicted protein product. The *MAS2* ORF is from nucleotide 190 to 1635.

Fig. 2. Antiserum against a peptide corresponding to the C-terminal 10 residues of the *MAS2* open reading frame recognizes the *MAS2* protein. Total proteins extracted from yeast cells were dissociated in SDS, subjected to SDS-PAGE, transferred to nitrocellulose, and decorated with antiserum. **Panel A** Protein (150 µg) isolated from strain AH216 was decorated with antiserum raised against the C-terminal *MAS2* peptide (112) or with preimmune serum (PRE). Mol. wts were determined from standards run in adjacent lanes; these are indicated on the left, with sizes given in kilodaltons. **Panel B** Total protein (150 µg) from strain DL1 carrying either the *ADHI-MAS2* fusion (pAC1-MAS2, which overexpresses *MAS2*) or the pAC1 vector without *MAS2* (WT), was analyzed by immune blotting with the anti-peptide antiserum.

a collection of yeast plasmids containing random genomic DNA fragments for plasmids that allow *mas2* cells to grow at 37°C, the restrictive temperature. Two plasmids were isolated and a 3.6-kbp DNA fragment common to both isolates was shown to have complementing activity. To verify that the complementing activity of the isolated DNA reflected the presence of the wild-type *MAS2* gene (rather than that of another gene that can substitute for *MAS2*), a DNA fragment from the cloned DNA was inserted into the plasmid YIp5, integrated into the yeast chromosome by homologous recombination, and the site of integration was mapped. This plasmid integrated close to, or at, the *MAS2* locus, indicating that the plasmid contained the *MAS2* gene (see Materials and methods). In additional experiments, the *MAS2* gene was mapped to the right arm of chromosome VIII (see Materials and methods, Table I).

Nucleotide sequence of the *MAS2* gene

The nucleotide sequence of a 1719-bp region shown to contain the *MAS2* gene was determined. Analysis of the DNA sequence revealed an open reading frame of 1445 bp which would encode a 53.3-kd protein (Figure 1). Two observations indicated that this open reading frame encodes the *MAS2* gene product (see Materials and methods). First, a frameshift mutation in the predicted open reading frame eliminated the ability of this DNA fragment to complement *mas2* mutations. Second, the 5' end of the putative *MAS2* RNA initiated ~20 bp upstream of the predicted initiation codon.

The predicted amino acid sequence of the *MAS2* protein was that of a typical soluble protein without any obvious membrane-spanning domains (Kyte and Doolittle, 1982). The N-terminal 15–30 residues resembled a 'matrix-targeting' signal since they had a strongly positive net charge

Fig. 3. *MAS2* is a mitochondrial protein. AH216 cells were grown to the mid-logarithmic stage in semisynthetic media containing 2% lactate, converted to spheroplasts and homogenized. The homogenate (HOM) was fractionated into a mitochondrial pellet (MITO) and a post-mitochondrial supernatant (CYTO). Crude mitochondria were banded on 20% Percoll gradients (P.MITO). Aliquots of homogenate (100 µg protein), cytoplasm (75 µg) and mitochondria (20 µg) corresponding to an equal amount of cells were analyzed by immune blotting with (**panel A**) antiserum to *MAS2* or (**panel B**) antisera to glyceraldehyde-3-phosphate dehydrogenase (G3PDH) and the F_1 -ATPase β -subunit ($F_1\beta$).

and several hydroxylated amino acids, but no extended hydrophobic stretches. This raised the possibility that *MAS2* encoded an imported mitochondrial protein.

MAS2 is an essential gene

mas2 is a temperature-sensitive lethal mutation, suggesting that *MAS2* encodes an essential gene product which is inactive at the non-permissive temperature in *mas2* mutants. However, some temperature-sensitive lethal mutations affect components that are essential only at the restrictive temperature (Hurt *et al.*, 1987). To determine whether *MAS2* encodes a gene product essential for growth at all temperatures, we constructed a null mutation in *MAS2* by inserting the yeast *LEU2* gene into one of the two copies of *MAS2* in a diploid cell (see Materials and methods). When these diploid cells were sporulated and the haploid progeny allowed to grow at 22°C, only *Leu*⁺ spores were found to be viable. All viable spores carried an intact *MAS2* gene. Thus, the *MAS2* gene is essential even at 22°C.

To determine how quickly haploid cells lacking an intact *MAS2* gene died, the four spores from several asci were allowed to germinate at 22°C and observed microscopically. Whereas two spores germinated and continued to grow into *Leu*⁺ colonies, the other two spores (inferred to carry the disrupted *MAS2* gene, *mas2-LEU2*) germinated, but stopped growth after four or five cell divisions. As was observed in *mas2* cells shifted to 37°C (Yaffe and Schatz, 1984), spores carrying the disrupted *MAS2* gene arrested at all stages of the cell cycle.

The *MAS2* protein is located in the mitochondrial matrix

To determine the intracellular location of the *MAS2* protein, we raised antibodies against a chemically synthesized peptide corresponding to the C-terminal 10 residues of the *MAS2*

protein. When this antiserum was tested against total yeast proteins by immune blotting, it specifically recognized a 51-kD protein (Figure 2A). Two observations suggest that the antiserum reacts with the MAS2 protein. First, overproduction of the *MAS2* gene product resulted in overproduction of the 51-kD antigen (Figure 2B). Second, the antiserum precipitated the protein produced by *in vitro* transcription/translation of the cloned gene (not shown). The 51-kD protein recognized by the antiserum was smaller than the 53-kD protein predicted from the DNA sequence, raising the possibility that the MAS2 protein is processed (see Discussion).

Immune blotting experiments to localize the *MAS2*-encoded protein revealed that it was associated with the mitochondrial fraction (Figure 3): *MAS2* cofractionated with the F_1 -ATPase β -subunit (a mitochondrial marker), but not with glyceraldehyde-3-P-dehydrogenase (a cytosolic marker). When mitochondria were further purified by banding in a Percoll density gradient, the *MAS2* protein still cofractionated with the F_1 -ATPase β -subunit.

The *MAS2* protein is located inside the inner membrane (Figure 4): *MAS2* was inaccessible to externally added trypsin in intact mitochondria (lane 2) and in mitoplasts (mitochondria whose outer membrane has been broken; lane 3), but was readily digested by trypsin if the mitochondrial membranes were solubilized by Triton X-100 (lane 4). Marker proteins representing specific submitochondrial compartments responded in the expected manner.

When mitochondria were disrupted by sonication and separated by ultracentrifugation into a membrane pellet and a soluble fraction, the *MAS2* protein cofractionated with the soluble proteins citrate synthase and cytochrome b_2 (Figure 5, lane S). In contrast, all of the 45- and 70-kD outer membrane proteins as well as the majority of the F_1 -ATPase β -subunit were recovered with the membrane pellet (Figure 5, lane P), suggesting that *MAS2* was soluble in the matrix. However, experiments in which the mitochondrial membrane was disrupted with a detergent, saponin, rather than sonication indicated that *MAS2* was associated with the mitochondrial inner membrane (not shown). In either case, *MAS2* resides within the mitochondrial matrix. Our conclusion is supported by the observation that the *MAS2* protein synthesized by transcription/translation of the cloned *MAS2* gene is imported into the matrix of mitochondria isolated from wild-type yeast cells (not shown).

Cells carrying the *mas2-10* mutation are temperature sensitive for processing protease activity

Because *MAS2* is a matrix protein, we directly tested the possibility that *MAS2* is required for processing imported precursors. However, mitochondria isolated from the original *mas2-1* mutant imported and processed precursors at the same rate as wild-type mitochondria, even though the mutant cells were import deficient. Because the lack of an *in vitro* phenotype may be a property of the *mas2-1* allele, we isolated additional temperature sensitive *mas2* mutants (see Materials and methods). One of these mutants, *mas2-10*, was at least 10-fold more defective in processing *in vivo* than *mas2-1* cells (Figure 6 and unpublished data).

Processing activity in mitochondrial extracts derived from *mas2-10* cells was temperature sensitive when assayed *in vitro*. Specifically, mitochondria were isolated from either *mas2-10* or wild-type cells, solubilized with Triton X-100,

Fig. 4. *MAS2* is located inside the mitochondrial inner membrane. Isolated mitochondria and mitochondrial subfractions (10 mg protein/ml) were digested with 1 mg/ml TPCK-treated trypsin for 20 min on ice, followed by the addition of a 5-fold weight-excess of soybean trypsin inhibitor. Aliquots corresponding to 100 μ g mitochondria were immune blotted with antisera to: the *MAS2* protein (panel A); the 45-kD outer membrane protein (panel B); cytochrome b_2 (cyt. b_2) and citrate synthase (cit. syn.) (panel C). Lane 1, Mitochondria, no trypsin; lane 2, trypsin-treated mitochondria; lane 3, trypsin-treated mitoplasts (mitochondria whose outer membrane was disrupted by osmotic shock); lane 4, mitoplasts solubilized in 1% Triton X-100 and digested with trypsin.

Fig. 5. *MAS2* is a soluble protein of the mitochondrial matrix. Mitochondria were converted to mitoplasts by osmotic shock and then sonicated. The membrane pellet (P) was separated from the soluble fraction (S) by ultracentrifugation. Aliquots corresponding to 100 μ g mitochondrial protein were analyzed by immune blotting with antisera against: the *MAS2* protein and the 70-kD outer membrane protein (panel A) the F_1 -ATPase β -subunit ($F_1\beta$) and the 45-kD outer membrane protein (panel B) and cytochrome b_2 (cyt. b_2) and citrate synthase (cit. syn.) (panel C).

and incubated with 35 S-labeled precursor proteins. As shown in Figure 7A, extracts from wild-type mitochondria efficiently cleaved the citrate synthase precursor to the mature form at both 22 (lane 3) and 37°C (lane 4). In contrast, *mas2-10* extracts processed the citrate synthase precursor at the permissive temperature, 22°C (lane 5); but little or no processing activity was observed at 37°C (lane 6), the restrictive temperature. (A variable fraction of the

Fig. 6. Cells that carry the *mas2-10* mutation are more defective in the processing of F_1 -ATPase β -subunit than are *mas2-1* cells. Wild-type strain AH216, *mas2-1* and *mas2-10* cells were grown at 22°C to an OD_{600} of 2–3 on semisynthetic medium containing 2% glucose, resuspended in fresh medium to an OD_{600} of 10, and preincubated for 10 min at 37°C. Cells were then labeled with [35 S]Translabel (ICN, 0.1 mCi/ml) for 5 min. Proteins were extracted (Yaffe and Schatz, 1984) from 1-ml aliquots and the F_1 -ATPase β subunit was immunoprecipitated (Maccacchini *et al.*, 1979) and analyzed by SDS–PAGE and fluorography (Chamberlain, 1979). Precursor (p) and mature (m) forms of the F_1 -ATPase β -subunit are indicated.

citrate synthase precursor was resistant to cleavage and may represent denatured molecules.) *mas2-10* mitochondrial extracts also exhibited temperature sensitive processing of two other precursor proteins, F_1 -ATPase β -subunit (Figure 7B) and a fusion protein containing the presequence of cytochrome oxidase subunit IV fused to mouse dihydrofolate reductase (Figure 7C). The defect in processing activity was observed within 2 min after the *mas2-10* extracts were shifted to 37°C. At 37°C, processing activity was at least 30-fold reduced in *mas2-10* extracts compared to wild-type extracts (not shown).

The processing activity of solubilized mitochondria appeared to represent authentic activity because processing was sensitive to the chelator 1,10-phenanthroline (Figure 7, lanes 2), and because processing generated a 'mature' cleavage product whose size was identical to that produced by import into isolated mitochondria (not shown). Surprisingly, although processing activity in *mas2-10* extracts was temperature sensitive, import and processing by isolated *mas2-10* mitochondria was not defective (not shown).

The thermolability of processing activity in *mas2-10* mitochondrial extracts was reversible. Extracts from *mas2-10* cells failed to process the citrate synthase precursor when incubated at 37°C but regained activity upon incubation at 22°C (Figure 8). Activity of a heat-treated extract returned to 22°C was identical to that of an extract held constantly at 22°C, suggesting that *mas2-10* encodes a protein required for protease activity which is reversibly inactivated at the restrictive temperature. Although the *mas2-10* protein was reversibly temperature sensitive for function, the lethality in *mas2-10* cells was not reversible; after several hours at 37°C, the *mas2-10* cells did not recover after they were returned to 22°C (not shown).

The MAS2 protein is homologous to the MAS1-encoded subunit of the matrix-localized processing protease

MAS2 is located in the mitochondrial matrix and is required for the processing of mitochondrial precursor proteins. MAS1 was recently shown to encode a subunit of the matrix-

Fig. 7. Processing activity is temperature sensitive in mitochondrial extract isolated from *mas2-10* cells. Mitochondrial extracts were prepared from wild-type and *mas2-10* cells (see Materials and methods). Three proteins were synthesized *in vitro* in the presence of [35 S]methionine: **panel A**, the citrate synthase precursor; **panel B**, the F_1 -ATPase β -subunit precursor; **panel C**, a fusion protein consisting of the 53 N-terminal residues of the cytochrome *c* oxidase subunit IV precursor, fused to the N-terminus of mouse dihydrofolate reductase. Each labeled precursor (5 μ l) was incubated for 15 min with either wild-type or *mas2-10* mitochondrial extracts (10 μ l; 10 μ g protein) at 22 or 37°C. (Extracts were preincubated at the indicated temperature for 2 min prior to addition of the labeled precursor.) Processing was stopped by the addition of 5 mM 1,10-phenanthroline, and proteins were analyzed by SDS–PAGE and fluorography (Hurt *et al.*, 1984). **Lane 1**, the precursor only; **lane 2**, precursor incubated with wild-type mitochondrial extract at 22°C in the presence of 5 mM 1,10-phenanthroline; **lane 3**, precursor incubated with wild-type mitochondrial extract at 22°C; **lane 4**, precursor incubated with wild-type mitochondrial extract at 37°C; **lane 5**, precursor incubated with *mas2-10* mitochondrial extract at 22°C; **lane 6**, precursor incubated with *mas2-10* mitochondrial extract at 37°C. Precursor (p) and mature (m) forms of the proteins are indicated.

localized processing protease (Witte *et al.*, 1988). Because MAS2 and MAS1 are both required for proteolytic processing, we investigated whether the two proteins were related. Figure 9 shows that more than one-quarter of the amino acids in the MAS2 and MAS1 proteins are identical. Only a few gaps were needed to maximize this homology, which was further emphasized by numerous matches between functionally analogous amino acids (e.g. leucine/isoleucine). The conserved residues were not confined to a single region, but were spread throughout the length of the two proteins. The homology between MAS2 and MAS1 was also seen at the level of the nucleotide sequences of the two genes (not shown). These results indicate that the two genes arose from a single ancestral gene via gene duplication. The homology also suggests that the MAS2 and MAS1 proteins perform similar functions. Indeed, the accompanying study shows that MAS2 encodes the larger of the two subunits in a purified preparation of the matrix-localized processing protease from yeast mitochondria (Yang *et al.*, 1988).

Discussion

The results presented in this and the accompanying paper strongly suggest that MAS2 encodes a component of the protease that processes imported mitochondrial precursor proteins. First, the MAS2 protein resides in the mitochondrial matrix, the location of the processing protease activity.

Fig. 8. Processing activity in *mas2-10* mitochondria is reversibly thermolabile. The citrate synthase precursor was incubated for 15 min with mitochondrial extracts from *mas2-10* cells at 22 or 37°C as described in Figure 7. 37→22 indicates that the *mas2-10* extract was first incubated at 37°C for 15 min, then returned to 22°C for 15 min prior to incubation with the citrate synthase precursor for 15 min at 22°C. Precursor (p) and mature (m) forms of citrate synthase are indicated.

MAS1	MFSRTASKFRNTRRLSTISSQIPCTRTSKPLNGLTIAETEYIPNTSSATVGFVDAGSRAENVKNGNTAHFLE
MAS2	MLR NGVQRLYSNI ARTDNFKLSSLANGLKATVSTNTPGHFA LGLYIDAGSRFEGRNKLGCTHILD
MAS1	HLAFKGTQNRSSQGEIELEINIGSHNAYTSRENTVYAKSLQEDIPKAVDILSDILTKSVLDNS
MAS2	RLAFKSTEHVEGRAMAETLELLGNGYQCTSSRENTVYAKSLQEDIPKAVDILSDILTKSVLDNS
MAS1	AIERERDVIIRESSEVDKMYDEVFDFHLEITYKQDPLGRITLGPINKIKSITRTDLKDYITKNY
MAS2	ELQEQKLSAEYEDDEVMPKELVLPPELLHTAAYSGETLGSPLICPRELIPSKYLLDYRNKFY
MAS1	KGDRMVLACAGADHEKLVQYQYFGHVPKSESVPPLGSPRGPLPVFCRVERFIKENTLPTTHI
MAS2	TPENTVAAPVFG VPHEKALELTERKLDGQSTHPPITKVPQYTGEGSCIPAPVFGNLPFLFHI
MAS1	AIALEGVSASDPYFVALATQAIQVND RAIGCTNSPPLAVASQNSLANSYMSFSTSYA
MAS2	QIGFEGLPIDHFDIYALATLQTLGCGGSFSAAGPGKMYSLYTHVLNQYFVFNCAVFNHSYS
MAS1	DSGLWGYI VTDSEHNVQLVNEILKEWKRIKSGKISDAEVNRAKAQKAAALLSLDGSTAI
MAS2	DSGIFGSLSCIPQAAQAEVIAQMYNTFAN KDLRLTEDEVSRAKQKSSLLMNLKSLVE
MAS1	VEDIGRQVVTGKRLSPFEEFQVDKIKDDIIMWANYRLQNKPVSMVALGITSTVNPVSYIEEK
MAS2	LEDMGKQVLMHGRKIPVNEIMISKIEDLKDDISRVAEMIFTGN VNNAGNGKGRATVVMQGDGRS
MAS1	LNQ
MAS2	FGDVENVLKAYGLNSSSSKNDSPKKKGWF

Fig. 9. The MAS2 protein is homologous to the MAS1 protein. The predicted amino acid sequence of the MAS2 protein was compared to that of the MAS1 protein (Witte *et al.*, 1988) using a program based on the Needleman–Wunsch algorithm (Needleman and Wunsch, 1970). Amino acids identical between MAS1 (upper sequence) and MAS2 (lower sequence) are connected by a line, and conservative amino acid substitutions (I/L, V/L, V/I, D/E and K/R) are connected by two dots. Gaps in either protein sequence were inserted by the program to maximize the alignment.

Second, extracts from *mas2* mitochondria are temperature sensitive for processing protease activity. Third, MAS2 is homologous to the MAS1 protein, which was previously shown to be a subunit of the protease (Witte *et al.*, 1988). Finally, the MAS2 protein is one of two subunits of the purified processing protease (Yang *et al.*, 1988).

The first 15 amino acids of the predicted MAS2 protein (MLRNGVQRLYSNIAR) appear to be a typical mitochondrial 'matrix-targeting' signal since they contain several basic amino acids, no acidic residues, and several hydroxylated amino acids interspersed among uncharged residues (Allison and Schatz, 1986; Roise *et al.*, 1986). Since most matrix-targeting signals are removed after import of the precursor into mitochondria, MAS2 itself may be initially synthesized as a precursor protein which is processed to a mature

protein of lower mol. wt. In support of this possibility, we find the mol. wt of the protein decorated by the MAS2 antiserum to be ~51 kd, 2 kd smaller than the 53-kd MAS2 protein predicted from the DNA sequence. On the other hand, the 53-kd MAS2 protein produced by transcription/translation of the cloned gene was imported, but not processed, by isolated wild-type mitochondria under our assay conditions (unpublished observations). Furthermore, the *in vitro* synthesized MAS2 protein was not processed by isolated wild-type matrix fractions. It therefore remains an open question whether the MAS2 protein is processed during import.

The homology between the MAS2 and MAS1 proteins is approximately the same as that between hemoglobin and myoglobin, suggesting that MAS2 and MAS1 perform similar functions. Our finding that both proteins are subunits of the same enzyme is consistent with this possibility (Witte *et al.*, 1988; Yang *et al.*, 1988). Indeed, nonidentical subunits of oligomeric complexes (such as F₁-ATPase or the acetylcholine receptor) are often homologous, probably because they originated by gene duplication (Anholt *et al.*, 1984; Walker *et al.*, 1985). However, some homology relationships are difficult to rationalize at present. For example, MAS1 and MAS2 are also homologous to the largest subunit (COR1) of the yeast cytochrome *bc*₁ complex (Tzagoloff *et al.*, 1986).

Like the yeast processing protease, the protease from *N. crassa* mitochondria also contains two subunits (Hawliitschek *et al.*, 1988). The MAS1 protein was found to be ~60% homologous to the smaller of the two *Neurospora* subunits (Witte *et al.*, 1988; Hawliitschek *et al.*, 1988). Since the sequence of the larger *Neurospora* subunit has not been determined, its relationship to the MAS1 and MAS2 proteins is not known. However, we propose that MAS2 encodes the yeast equivalent to the larger *Neurospora* protease subunit. Furthermore, we predict that a similar homology will be found between the two *Neurospora* processing protease subunits as that found between MAS1 and MAS2.

The identification of the MAS2 protein as a subunit of the processing protease fails to answer the question of why mutations in this polypeptide interfere with the translocation of precursors into mitochondria. In particular, *mas2* mutants (and *mas1* mutants) accumulate precursors outside the mitochondrial inner membrane (Yaffe and Schatz, 1984). This apparent translocation defect is seen *in vivo* within 10 min after *mas2* or *mas1* cells have been shifted to the restrictive temperature (E.Meier, R.Jensen and M.Yaffe, unpublished data). In contrast, processing but not translocation was deficient *in vitro* when precursors were imported into mitochondria isolated from *mas1* mutants (Yaffe *et al.*, 1985). The *in vivo* observations suggest that translocation and processing are more tightly coupled than was apparent from experiments with isolated mitochondria.

Yeast cells that carry a disrupted copy of the MAS2 gene are unable to grow at any temperature or on any carbon source. Why do cells lacking the processing activity die? One explanation for the lethality of *mas2* mutants is that the accumulation of mitochondrial precursor proteins *per se* may be toxic to the cell. Since mitochondrial presequences exhibit detergent-like properties (Roise *et al.*, 1986; von Heijne, 1986), large amounts of these precursors may perturb membrane functions. Similarly, the accumulation of precursors that retain their enzymatic activity outside the mitochon-

dria may lead to futile biochemical cycles. Alternatively, there may be one or more mitochondrial proteins essential for cell growth that are not imported or not assembled in *mas2* mutants. Our results support earlier observations indicating that mitochondria are essential for the yeast cell, regardless of the carbon source on which the cells are grown (Kovačová *et al.*, 1968).

Since mitochondrial presequences share no common amino acid sequence, the processing protease recognizes some three-dimensional structure common to all imported mitochondrial precursors. One might have predicted that *MAS1* and *MAS2* each encoded active proteases which cleaved different sets of precursor proteins. However, our present work and earlier studies, show that *mas1* and *mas2* mutants are each defective for processing the same precursors (e.g. F₁-ATPase β -subunit and cytochrome *c* oxidase subunit IV) (Yaffe and Schatz, 1984; Yaffe *et al.*, 1985). Our results indicate that, in yeast, there is only one major matrix-protease that cleaves all precursors, and that this protease requires both the *MAS1* and *MAS2* proteins for activity. Future investigations will provide insight into how the protease recognizes and cleaves the hundreds of different precursor proteins, and the relationship between processing and translocation.

Materials and methods

Strains and relevant genotypes

The parent strain AH216 (*MATa leu2-3 leu2-112*), and the *mas2* mutants MY11-4-2C (*MATa mas2-1 leu2-3 leu2-112*) and MY11-4-2B (*MATa mas2-1 leu2-3 leu2-112*) have been described (Yaffe and Schatz, 1984). Strain RJ25 (*MATa mas2-1 ura3-LEU2*) was constructed as follows. A 3-kbp *Bgl*III fragment carrying the *LEU2* gene was isolated from the plasmid YEp13 (Broach *et al.*, 1979), the DNA ends were filled in with DNA polymerase, and the fragment was inserted into the *EcoRV* site in the *URA3* gene of plasmid pFL1 (Chevalier *et al.*, 1980). The *URA3* gene carrying the *LEU2* insertion, *ura3-LEU2*, was isolated on a 4-kbp *Hind*III fragment and transformed into strain MY11-4-2C. A *Leu*⁺, *Ura*[−] transformant was isolated and shown by Southern analysis to carry the *ura3-LEU2* disruption. Strain RJ12-6b (*MATa mas2-1 ura3-52*) was derived from crossing strain MY11-4-2C to *MATa ura3-52* strain 1369 (Russell *et al.*, 1986). *MATa/MATa leu2/leu2* strain DHR125 (Russell *et al.*, 1986) and *MATa ura3* strain DL1 (van Loon *et al.*, 1983) were described previously. Mapping strains X3382-3A (*MATa arg4 pet17 ade1 gal1 trp1 his2 his6 asp5 aro7 cdc14*) and 1176 (*MATa leu2 arg4 arg1 cdc6 his2 his6 ade1 ade2 ilv3 lys7 his9 asp5 leu1 ura3 trp1 met2 met14 aro7*) were obtained from the Yeast Genetic Stock Center and I.Herskowitz (University of California, San Francisco) respectively. A collection of 1200 temperature sensitive lethals in the strain A364A (*MATa ade1 ade2 ura1 his7 lys2 tyr1 gal1*) was obtained from C.McGlaughlin (University of California, Irvine). Standard yeast genetic techniques and media were as described (Sherman *et al.*, 1979).

Isolation of the *MAS2* gene

The temperature-sensitive lethal *mas2* strain MY11-4-2C was transformed with the YEp13 library (provided by K.Nasmyth) as described (Beggs, 1978). *Leu*⁺ transformants were selected at 22°C and screened for growth at the nonpermissive temperature by two successive replica platings to 37°C. Of 8000 *Leu*⁺ transformants, two plasmids were isolated that allowed *mas2* strains to grow at 37°C. With both transformants, complementation of the *mas2* mutation was shown to be plasmid dependent. To localize the *MAS2* sequences, restriction endonuclease fragments from the original isolates in YEp13 were inserted into the *URA3*, *CEN4* vector YCp50 (obtained from C.Mann), transformed into strain RJ25 (Ito *et al.*, 1983), and tested for the ability to complement the *mas2* mutation. YCp50-R1 was constructed by inserting a 4.9-kbp *EcoRI* fragment common to both YEpMAS2-12 and YEpMAS2-15 into the vector YCp50. YCp50- Δ BR1 was constructed by partially digesting YCp50-R1 with *Bam*HI, ligating, and screening bacterial transformants for plasmids that lost the small *Bam*HI fragment. YCp50-XR1 and YCp50-XR2 were constructed by digesting YCp50-R1 completely with *Xba*I and partially with *EcoRI*, filling in the DNA ends with Klenow polymerase, and blunt-end ligation. Plasmids that retained the 1.5-kbp *Xba*I-*EcoRI* fragment (YCp50-XR1) or the 3.4-kbp *Xba*I-*EcoRI* insert

The *MAS2* protein

Fig. 10. Localization of the *MAS2* gene. Restriction endonuclease fragments, Δ BR1, B1, XR1 and XR2, were inserted into plasmid YCp50 and assayed for *mas2* complementation as described in Materials and methods (+, complements *mas2*; −, does not complement *mas2*). Thick upper line depicts a 4.9-kbp *EcoRI* fragment containing the *MAS2* gene. The zig-zag line at left indicates vector sequences. The bold arrow at the bottom right indicates the approximate position of the *MAS2* gene. The insertion of the *LEU2* gene into the *Xba*I site of *MAS2* resulted in the null mutation, *mas2-LEU2* (see text). Restriction endonuclease recognition sites: B, *Bam*HI; R, *EcoRI*; X, *Xba*I.

Table I. *MAS2* maps to the right arm of chromosome VIII

YEpMAS2-12 integrant crossed to	Total colonies examined	<i>Leu</i> [−]	<i>Arg</i> [−]	<i>Pet</i> [−]
(1) 1176 (<i>leu2 arg4</i> [VIII] <i>arg1</i> [XV])	946	127	64 ^a	n.d.
(2) X3382-3A (<i>arg4</i> [VIII] <i>pet17</i> [XV])	1020	n.d.	65	4

n.d., not determined.

^aOf the 64 *Arg*[−], 57 were also *Leu*[−].

(YCp50-XR2) were subsequently identified. Both YCp50-R1 and YCp50- Δ BR1 complemented the *mas2* mutation when transformed into strain RJ25. None of the three other fragments tested (B1, XR1 or XR2) complemented *mas2* when present on YCp50. Taken together, these results indicate that *MAS2* extends to the left of the *Xba*I site and to the right of the rightmost *Bam*HI site as shown in Figure 10.

Integrative transformation

A 3.2-kbp *Bam*HI fragment, B1 (see Figure 10), was inserted into the *URA3* vector YIp5 (Struhl *et al.*, 1979) and this plasmid was transformed into the strain DL1 (*MATa MAS2 ura3*). Stable *Ura*⁺ transformants were crossed to strain RJ12-6b (*MATa mas2 ura3*) and the diploids were sporulated at 22°C. The meiotic products were tested for growth at 37°C and for growth on media lacking uracil. In crosses to two independently isolated integrants, no recombinants between *mas2* and *URA3* were observed in 27 tetrads: all were parental ditype (2 *Ura*⁺ *Mas*⁺:2 *Ura*[−] *Mas*[−]). Thus YIp5-B2 integrated within 1.85 cM of *MAS2*.

MAS2 gene disruption

A 2.2-kbp *Sal*I-*Xho*I fragment containing the *LEU2* gene was isolated from plasmid YEp13, the DNA ends were filled in with DNA polymerase, and the fragment was blunt-end ligated into the *Xba*I site in the *MAS2* gene carried on plasmid YCp50-R1. This insertion interrupts the *MAS2* protein at amino acid 195 (out of 482 total amino acids). The *MAS2* gene carrying the *LEU2* insertion, *mas2-LEU2*, was isolated on a 5.5-kbp *Bam*HI fragment and used to replace one copy of *MAS2* in the *MATa/MATa* diploid strain DHR125 as described (Rothstein, 1983). *Leu*⁺ transformants were isolated and the meiotic products from three independently isolated diploid colonies analyzed at 22°C. Colonies resulting from viable spores were tested for growth on media lacking leucine. Of 36 tetrads, 32 gave rise to two viable spores, both of which were *Leu*[−]. Four tetrads gave rise to only one viable spore which was *Leu*[−]. Southern analysis of the *Leu*⁺ diploid strains confirmed

that one of the two copies of *MAS2* had been replaced by *mas2-LEU2* (not shown).

Chromosomal mapping

To map the *MAS2* gene to a specific chromosome, we used YEp13 plasmids carrying *MAS2* to induce chromosome loss as described (Falco and Botstein, 1983). Specifically, plasmid YEpMAS2-12, which carries *MAS2* on plasmid YEp13, was digested at the unique *Xba*I site in *MAS2* and used to transform strain DL1 (*MAT α leu2*). Stable *Leu*⁺ integrants were mated to a *MAT α* strain (strain 1176) which carries at least one recessive mutation on each chromosome. In a diploid cell, integrated copies of YEp13 plasmids are unstable and induce either chromosome breakage or chromosome loss. These events can be detected by the unmasking of recessive markers carried on the homologous chromosome. Four independently isolated integrants of YEpMAS2-12 were crossed to strain 1176 and the phenotypes of diploid colonies tested. As summarized in Table I, 127 out of 946 colonies lost the ability to grow on media lacking leucine due to the loss of YEpMAS2-12 sequences; ~45% (64/127) of these *Leu*[−] colonies were also phenotypically *Arg*[−]. The appearance of no other marker was correlated with the loss of *LEU2*, suggesting that *MAS2* is located on either chromosome VIII (*arg4*) or on chromosome XV (*arg1*). To distinguish these two chromosomes, *Leu*⁺ integrants in strain DL1 were mated to strain X3382-3A which carries *arg4* on chromosome VIII and *pet17* on chromosome XV. Of 1020 diploid colonies screened, 65 were *Arg*[−] while only four colonies were unable to grow on glycerol as the carbon source (*Pet*[−]). Taken together, these results indicate that *MAS2* is located on chromosome VIII. Furthermore, because loss of chromosomal sequences distal to the site of YEp13-plasmid integrations are more frequent than the loss of the entire chromosome, our results (loss of *Leu*⁺ > loss of *Arg*⁺, see above) suggest that *MAS2* is centromere-distal to *arg4*, on the right arm of chromosome VIII.

DNA sequence of MAS2

The nucleotide sequence of a 1719-bp region of the cloned *MAS2* gene was determined as described (Sanger *et al.*, 1977). Fragments of *MAS2* DNA to be sequenced were isolated in three ways. First, a series of overlapping deletions were constructed from the *Xba*I site through the 3' end of the gene. In particular, a 1.5-kbp *Xba*I–*Eco*RI fragment (XR1, see Figure 10) was inserted into the M13 vector, mp10 (Norlander *et al.*, 1983) and the resulting construct digested with *Xba*I and *Pst*I. *MAS2* sequences were removed (initiating from the *Xba*I site) by digestion with exonuclease III for various amounts of time as described (Henikoff, 1984). Second, clones from the opposite strand of *MAS2* to that sequenced above, as well as regions not represented in the deletions, were isolated by inserting specific restriction fragments into mp18 or mp19 (Yanisch-Perron *et al.*, 1985). Third, one strand of the 3' end of the *MAS2* gene was sequenced using an oligonucleotide primer (5'-GTCTCCCAAAATAAAG-3', UCSF Nucleotide Synthesis Facility) located at position 1678 of the *MAS2* gene (see Figure 1).

Construction of the MAS2 frameshift mutation

Plasmid YCp50-R1, which carries the 4.9-kbp *Eco*RI fragment inserted into the vector YCp50, was digested at the unique *Xba*I site in the *MAS2* gene (position 475, Figure 2), the DNA ends filled with Klenow polymerase, and ligated. The resulting plasmid, which contains an insert of 4 bp at the *Xba*I site, failed to complement *mas2* when transformed into strain RJ25.

Construction of the ADH1-MAS2 fusion (pAC1-MAS2)

A 1.8-kbp *Sna*FI–*Eco*RI fragment from *MAS2* was isolated, the DNA ends were filled, and the fragment was inserted into the *Sna*I site of the Bluescript KS vector (Stratagene). *Sna*FI cuts *MAS2* DNA at a position located 9 bp upstream of the initiation codon (position 184, Figure 2). One orientation of the *MAS2* fragment in Bluescript KS generates a 1.8-kbp *Eco*RI fragment containing the *MAS2* coding sequences (the *Eco*RI site within the Bluescript polylinker was located 19 bp upstream of the *MAS2* ATG). This *Eco*RI fragment was inserted into the *Eco*RI site of plasmid pAC1 (Hurt *et al.*, 1985), such that the 5' end of the *MAS2* gene was immediately adjacent to the *ADH1* regulatory sequences, to produce pAC1-MAS2. pAC1-MAS2 was transformed into strain RJ25 and found to complement the *mas2* mutation.

Mapping the 5' end of the MAS2 RNA

An oligonucleotide based on the *MAS2* sequence at position 516 (5'-GGATGTGGGTAAAGT-3', UCSF Nucleotide Synthesis Facility) was end labeled, annealed to total RNA isolated from strain AH216, and extended with reverse transcriptase as described (Domdey *et al.*, 1984). The length of the extended primer was ~360 bp, suggesting that the 5' end of the *MAS2* RNA is ~20 bp upstream of the predicted coding sequences.

Production of antiserum to the MAS2 protein

A peptide based on the 10 C-terminal amino acids of *MAS2* (peptide 112, CDSPKKKGWF; UCSF Peptide Synthesis Facility) was coupled to rabbit serum albumin (RSA, Sigma) using *m*-maleimidobenzoic acid-*N*-hydroxy-succinimide (Pierce) as the cross-linking agent (Doolittle, 1986). The asparagine residue of the *MAS2* protein was replaced by cysteine to facilitate coupling. For the primary injection, 5 mg peptide–RSA conjugate was mixed 1:1 with Freund's Complete Adjuvant (Calbiochem) and the emulsion was injected into rabbits (into the posterior lymph nodes and intradermally). For the boost injections, 5 mg peptide–RSA was emulsified in an equal volume of adjuvant (1:8 Freund's Complete:Incomplete), and injections were given s.c. and i.m. at 2-week intervals. Blood samples were taken and tested every 2 weeks, alternating weekly with the boosts. After the second boost, serum against peptide 112 reacted specifically with a 51-kD protein from whole yeast lysates (see Figure 2). Isolation of proteins from yeast cells was as described (Yaffe and Schatz, 1984).

Subcellular and submitochondrial fractionation

Subcellular fractionation, isolation of mitochondria, and submitochondrial fractionation were as described (Daum *et al.*, 1982) except that 1 mM aprotinin (Sigma) and 0.5 μ g/ml leupeptin (Sigma) were included in homogenization buffers. To purify the crude mitochondrial fraction, a Percoll gradient was first formed by centrifuging 20% Percoll (Pharmacia) in buffer (0.6 M mannitol, 20 mM Hepes–KOH, pH 7.5) at 15 000 r.p.m. for 12 min in a Sorvall SS34 rotor. The freshly formed gradient was then overlaid with 0.5 ml of mitochondrial suspension (10 mg protein/ml) and centrifuged for 10 min at 12 000 r.p.m. in a Sorvall HB4 rotor. To prepare mitoplasts, mitochondria were osmotically shocked by resuspension in 0.1 M mannitol, 20 mM Hepes–KOH (pH 7.5) at 10 mg protein per ml. After sonication with four 15-s pulses at maximum power, mitoplasts were separated into a membrane pellet and a soluble fraction by centrifugation at 100 000 g for 30 min. Proteins were separated by SDS–PAGE (Haid and Suissa, 1983), transferred to nitrocellulose (Towbin *et al.*, 1979), immune decorated with antiserum followed by [¹²⁵I] protein A, and autoradiographed (Haid and Suissa, 1983). Protein concentrations were determined with the BCA reagent (Pierce) with bovine serum albumin as the standard.

Antisera to mitochondrial marker proteins

Antibodies against the *F*₁-ATPase β -subunit were raised against a β -galactosidase–*F*₁ β fusion protein. Specifically, a *Pvu*II–*Hind*III DNA fragment containing most of the *ATP2* gene (encoding *F*₁ β) (Takeda *et al.*, 1985) was inserted into the multiple cloning site of the β -galactosidase vector pTRBO (Bürglin and DeRobertis, 1987). The fusion protein was expressed in the *Escherichia coli* strain 71-18, purified by SDS–PAGE and electroelution, and used to immunize rabbits (Carroll and Laughon, 1987). Antiserum to the 45-kD outer membrane protein was prepared as described (Riezman *et al.*, 1983a). Antisera to the 70-kD outer membrane protein (Riezman *et al.*, 1983a), citrate synthase (Riezman *et al.*, 1983b) and cytochrome *b*₂ (Daum *et al.*, 1982) were a gift from G.Schatz (University of Basel).

Isolation of additional mas2 alleles

A collection of temperature sensitive yeast mutants in the strain A364A was screened for strains that failed to complement the *mas2-1* mutant. *MAT α* colonies, each carrying an uncharacterized temperature sensitive lethal mutation, were replica-plated to lawns of *MAT α mas2-1* cells on synthetic media lacking amino acids. Diploids were selected at 22°C by their ability to grow on the unsupplemented medium. Diploid colonies were screened for growth at the restrictive temperature by two successive replica-platings to 37°C. Of 1200 diploids, four failed to grow at 37°C, indicating that the temperature sensitive lesion in the *MAT α* strain could not complement *mas2-1*. Each of the four *MAT α* strains was shown to carry a *mas2* allele. The *MAT α* strain carrying the putative *mas2* mutation was crossed to a *MAT α mas2-1* strain, the diploids were sporulated, and the meiotic products tested for growth at 37°C. As was the case for all four diploids, all spores were temperature sensitive for growth. Hence the mutations were allelic to *mas2*. The new *mas2* mutants were crossed to strain AH216 four successive times. One of these mutants, which carried the *mas2-10* mutation, showed a severe defect in processing (Figure 6). This defect was shown to cosegregate with the temperature sensitive growth phenotype (not shown), indicating that the import defect and the inability to grow at 37°C were due to the same mutation.

In vitro processing assays

Mitochondria were isolated from either *mas2-10* or wild-type cells (strain AH216) as described (Daum *et al.*, 1982) with the following modifications: (i) incubation with dithiothreitol was for 30 min at 22°C; (ii) treatment with

zymolyase 20 000 (3 mg/g wet weight of cells) was for 90 min at 22°C. Extracts to be tested for processing activity were isolated by resuspending mitochondria in 1% Triton X-100, 50 μ M ZnCl₂, 50 μ M MnCl₂, 1 mM phenylmethylsulfonylfluoride (Sigma), 1 mM aprotinin, 1 μ g/ml leupeptin, 100 μ g/ml α 2-macroglobulin (Boehringer, Mannheim), 20 mM Hepes-KOH, pH 7.5, at a mitochondrial protein concentration of 10 mg/ml.

Mitochondrial precursor proteins were synthesized by the *in vitro* transcription/translation of cloned genes. The precursor to citrate synthase was produced as follows. A 1.7-kbp *Asu*II-*Eco*RV DNA fragment containing the citrate synthase gene (Suissa *et al.*, 1984) was isolated, the DNA ends filled in with Klenow polymerase, and this fragment was inserted into the *Hinc*II site of plasmid pUC18 (Yannisch-Perron *et al.*, 1985). Citrate synthase sequences were isolated from the pUC18 construction by digestion with *Bam*HI and *Pst*I (the *Bam*HI site is located 14 bp upstream of the citrate synthase ATG), and inserted downstream of the SP6 bacteriophage promoter in the vector pSP65 (Melton *et al.*, 1984) and transcribed *in vitro*. RNA was translated in rabbit reticulocyte lysates (Promega Biotech) in the presence of 10 mCi/ml [³⁵S]methionine (New England Nuclear). The F₁-ATPase β -subunit precursor was produced by transcription/translation of the cloned *ATP2* gene (Takeda *et al.*, 1985). The *in vitro* synthesis of a fusion protein consisting of the first 53 amino acids of the cytochrome *c* oxidase subunit IV precursor fused to the mouse dihydrofolate reductase protein, was as described (Hurt *et al.*, 1985).

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