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MPI1, an essential gene encoding a mitochondrial membrane protein, is possibly involved in protein import into yeast mitochondria

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To identify components of the mitochondrial protein import pathway in yeast, we have adopted a positive selection procedure for isolating mutants disturbed in protein import. We have cloned and sequenced a gene, termed *MPI1*, that can rescue the genetic defect of one group of these mutants. *MPI1* encodes a hydrophilic 48.8 kDa protein that is essential for cell viability. Mpi1p is a low abundance and constitutively expressed mitochondrial protein. Mpi1p is synthesized with a characteristic mitochondrial targeting sequence at its amino-terminus, which is most probably proteolytically removed during import. It is a membrane protein, oriented with its carboxy-terminus facing the intermembrane space. In cells depleted of Mpi1p activity, import of the precursor proteins that we tested thus far, is arrested. We speculate that the Mpi1 protein is a component of a proteinaceous import channel for translocation of precursor proteins across the mitochondrial inner membrane.

Key words: gene cloning/membrane proteins/mitochondria/protein import/yeast mutants

Introduction

Most mitochondrial proteins are synthesized on cytoplasmic ribosomes and post-translationally imported into mitochondria. Targeting information in these proteins is typically specified by a stretch of amino acids at their amino-terminus. This targeting signal, which carries a net positive charge and often has the ability to form an amphiphilic structure at the surface of the mitochondrial outer membrane, is usually removed by metalloprotease upon entry into the mitochondrial matrix (reviewed by Hartl *et al.*, 1989; Pfanner and Neupert, 1990). A rapidly increasing number of protein factors appears to mediate the specific import of proteins by mitochondria and routing to submitochondrial compartments. Components that participate in this import pathway are located both in the cytosol and inside mitochondria. In the cytosol, 70 kDa heat shock proteins accelerate import of precursor proteins by preventing misfolding of the polypeptide chains (Deshaies *et al.*, 1988; Murakami *et al.*, 1988). Import-competent precursor proteins are bound to receptors on the mitochondrial surface [MAS70 in yeast (Hines *et al.*, 1990; Steger *et al.*, 1990); MOM72 and MOM19 in *Neurospora crassa* (Söllner *et al.*, 1989, 1990)] and subsequently transferred to a common insertion site, GIP, in the outer membrane (Pfaller *et al.*, 1988). ISP42 in yeast (Vestweber *et al.*, 1989; Baker *et al.*, 1990) and its *N.crassa* homologue MOM38 (Kiebler *et al.*, 1990) are part of GIP. Recent crosslinking studies showed that MOM7, MOM8 and MOM30 are also components of GIP (Söllner *et al.*, 1992). Translocation of proteins across the inner membrane is dependent on a membrane potential and also involves the matrix-localized heat shock protein mhsp70 (Kang *et al.*, 1990; Scherer *et al.*, 1990; Manning-Krieg *et al.*, 1991). Both mhsp70 and the mitochondrial heat shock protein hsp60 (Cheng *et al.*, 1989; Ostermann *et al.*, 1989; Reading *et al.*, 1989) participate in the refolding of translocated proteins in the matrix, where proteolytic maturation is accomplished by the mitochondrial processing peptidase MPP and the processing enhancing protein PEP (Hawlitschek *et al.*, 1988; Jensen and Yaffe, 1988; Pollock *et al.*, 1988; Witte *et al.*, 1988; Yang *et al.*, 1988, 1991; Schneider *et al.*, 1990). Other proteases, including IMP1, which removes the sorting sequence of cytochrome b2 (Behrens *et al.*, 1991; Schneider *et al.*, 1991), are required to deliver some imported proteins to their correct mitochondrial location. While some proteins are functionally redundant, others seem to participate in key steps of mitochondrial protein import. ISP42, MPP, PEP and the stress proteins hsp60 and mhsp70, which are essential for viability of yeast, belong to the latter category. As yet, no essential proteins have been identified that constitute a protein transport channel through the inner membrane.

Here we report the isolation and characterization of *MPI1*, encoding another mitochondrial protein involved in import of proteins into mitochondria. Mpi1p is a mitochondrial membrane protein and is probably another essential component of the mitochondrial import machinery.

Results

Isolation of mutants disturbed in mitochondrial protein import

To identify components of the mitochondrial protein import machinery of *Saccharomyces cerevisiae* we used a positive screening procedure for mutants with (partial) defects in mitochondrial protein import. This genetic approach has been successfully applied to isolate mutants in the yeast secretory pathway (Deshaies and Schekman, 1987) and is based on mislocalization of a chimeric protein that is efficiently imported into a cellular organelle of a wild-type strain and that has enzyme activity normally residing in the cytosol (Figure 1). To isolate mitochondrial import mutants, we constructed a centromeric test plasmid that carries a hybrid gene, encoding an SOD-URA fusion protein. This chimeric protein contains the first 100 amino acids of the precursor form of manganese superoxide dismutase (SOD; a mitochondrial matrix enzyme) at its amino-terminus, the *URA3* gene product (a cytosolic enzyme with OMP decarboxylase activity) at its carboxy-terminus with a HIS4C bridge in

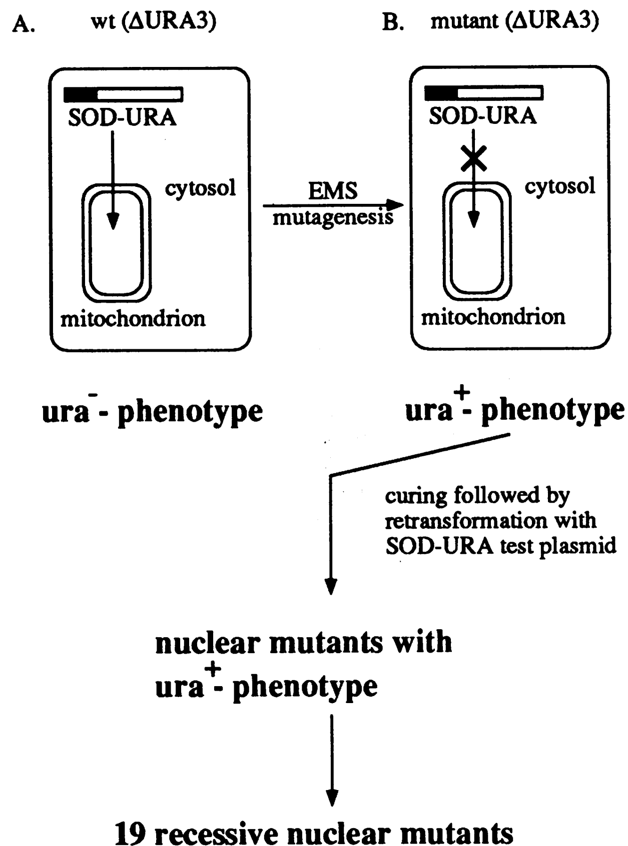

Fig. 1. Selection of recessive nuclear yeast mutants with defects in mitochondrial protein import. (**A**) Strain MB3 harbouring a test plasmid encoding an SOD–URA fusion protein displays a Ura^- phenotype because of efficient import of the chimeric protein into mitochondria. (**B**) After ethylmethanesulfonate (EMS) mutagenesis mutants with a Ura^+ phenotype were selected. Mutants carrying nuclear mutations were selected by curing the test plasmid from Ura^+ cells and re-transformation of plasmid-free cells with the original test plasmid. Nuclear mutants bearing recessive mutations were selected by testing the Ura-phenotype after a cross to MB5.

between. After transformation of the test plasmid into the yeast strain MB3, in which the complete chromosomal *URA3* coding region has been deleted, OMP decarboxylase activity was determined in detergent treated cellular extracts. As considerable enzyme activity was detected in lysates of transformed cells, we concluded that the fusion protein is synthesized and carries enzymically active OMP decarboxylase (data not shown). However, transformed MB3 cells still required exogenously supplied uracil for growth (Figure 2). The most likely explanation for these observations is that the matrix targeting signal of superoxide dismutase efficiently targets this enzymically active fusion protein into mitochondria, preventing it from participation in cytosolic pyrimidine biosynthesis. As a control, MB3 cells were transformed with a similar construct in which SOD coding sequences were replaced by an ATG initiation codon. Thus, this plasmid-encoded ATG–URA fusion protein has no mitochondrial targeting information. As expected, OMP decarboxylase activity was also present in extracts of transformed cells and cells could grow on selective medium lacking uracil (Figure 2).

Mutations that block import of the SOD–URA chimeric protein into mitochondria should allow cells to grow in the absence of uracil. Therefore MB3 cells harbouring the

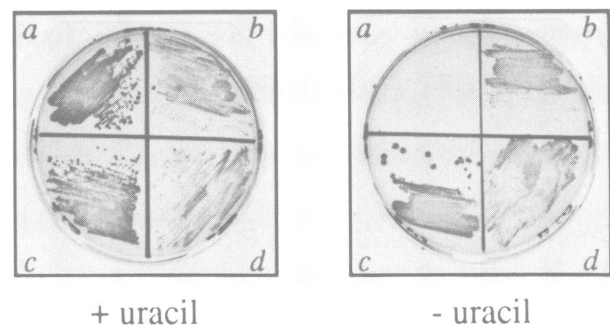

Fig. 2. Import of an SOD–URA fusion protein into mitochondria of mutant MB3-52 is impaired. The wild-type strain MB3 (panels a and c) and the mutant MB3-52 (panels b and d), harbouring either the *SOD–URA* test plasmid (panels a and b) or the ATG–*URA* construct (panels c and d) were plated onto selective medium supplemented with adenine, leucine and uracil (left panel) or with adenine and leucine (right panel).

SOD–URA test plasmid were subjected to ethylmethanesulfonate mutagenesis and mutants with a Ura^+ phenotype were selected on minimal medium containing glucose as a carbon source. Potentially, this screening procedure selects for mutations in the SOD targeting signal of the fusion protein and mutations in components of the mitochondrial protein import machinery. Both classes of mutations can be distinguished by curing the test plasmid from mutant cells and re-transforming plasmid-free cells with the original test plasmid. Twenty-seven mutants were found to carry nuclear mutations. Genetic crosses revealed that 19 of these mutants harbour recessive mutations that fall into minimally three and maximally 12 different complementation groups. Figure 2 illustrates the phenotype of MB3-52, the mutant on which we report in this paper. The cytosolic OMP decarboxylase deficiency of MB3-52 can be complemented with both the ATG–*URA* construct (Figure 2, panel d) and the *SOD–URA* test plasmid (Figure 2, panel b). This restoration of the pyrimidine biosynthetic pathway by the SOD–URA fusion protein is most simply explained by an impaired import of the fusion protein into mitochondria.

Isolation and structural analysis of the MPI1 gene

To clone the wild-type copy of the gene responsible for the protein import defect in MB3-52, mutant cells harbouring the *SOD–URA* test plasmid were transformed with a library of yeast genomic DNA fragments in the plasmid vector YEp13 (Nasmyth and Tatchell, 1980). Double transformants were selected by plating cells on minimal medium supplemented with uracil and 5-fluoro-orotic acid (Boeke *et al.*, 1984). This permitted the identification of a complemented clone that had again acquired uracil auxotrophy. We obtained one YEp13 derivative that complements the Ura^+ phenotype of MB3-52 cells containing the test plasmid (Figure 3, construct 1). We found that this clone could also rescue the genetic defect of mutants MB3-68 and MB3-75, but not that of mutant MB3-67. Several subclones of this primary clone were tested for their complementing ability. The 2.7 kb *Hin*dIII fragment, either cloned into a high copy number vector (Figure 3, construct 4) or into a low copy number vector (Figure 3, construct 8), contains all information for full complementation. Partial complementation of the Ura^+ phenotype was achieved with the *Hin*dIII–*Xba*I

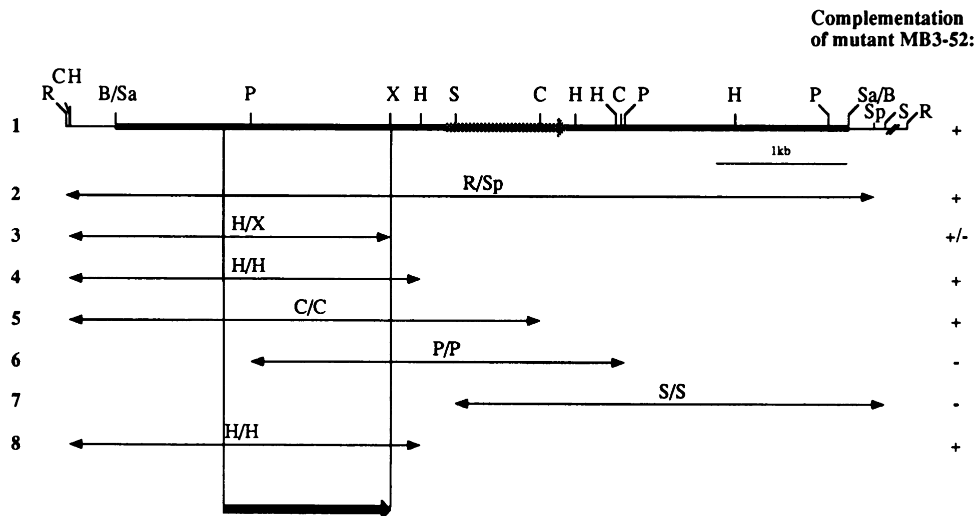

Fig. 3. Demarcation of complementing activity by deletion analysis. The indicated fragments from the YEp13 derivative (1) were subcloned either into the high copy number vector YEplac181 (2–7) or into the low copy number vector YCplac111 (8). Full, partial or no complementation of the Ura^+ phenotype of mutant MB3-52 harbouring the test plasmid is indicated respectively by +, +/– and –. The single headed black arrow indicates the *MPI1* reading frame determined by DNA sequencing whereas the grey arrow indicates the *RPB3* reading frame. B, *Bam*HI; C, *Cla*I; H, *Hin*dIII; P, *Pst*I; R, *Eco*RI; S, *Sal*I; Sa, *Sau*3A; Sp, *Sph*I; X, *Xba*I.

subclone (Figure 3, construct 3), whereas no complementation was observed with the *Pst*I subclone (Figure 3, construct 6). This indicates that the *Pst*I–*Xba*I segment within the *Hin*dIII fragment is essential but insufficient for full complementation. DNA sequence analysis of the complete *Hin*dIII fragment revealed that both the *Pst*I and *Xba*I sites are located inside a reading frame, corresponding to a protein of 431 amino acids and a molecular weight of 48.8 kDa (black arrow in Figure 3). The nucleotide sequence of the gene, termed *MPI1* (*M*itochondrial *P*rotein *I*mport 1), and the derived amino sequence are shown in Figure 4. As nearly half of the amino acids of Mpi1p are either acidic, basic or polar, the protein is rather hydrophilic. Moreover, Mpi1p has no obvious membrane spanning domains. The amino-terminal 55 amino acids are completely devoid of acidic residues, whereas 10 basic residues (including nine arginines) and 17 polar residues (serine and threonine) are contained within this area. These features are characteristic for mitochondrial targeting sequences and suggest that Mpi1p is an imported mitochondrial protein. The rather low codon adaptation index of MPI1 (0.14; Sharp and Li, 1987) suggests that *in vivo* the expression of the gene is low.

A computer search revealed no homology or similarity of Mpi1p to protein sequences in the EMBL data base (release 28.0). However, it did show a presence of the gene encoding the RPB3 subunit of RNA polymerase II, downstream of *MPI1* and transcribed from the same strand. This physical linkage means that *MPI1* is located on chromosome IX, as determined for *RPB3* (Kolodziej and Young, 1989).

Mpi1p is a mitochondrial membrane protein

To determine the location of Mpi1p in the yeast cell, we have made a DNA construct encoding a fusion protein in which the last four carboxy-terminal amino acid residues of Mpi1p were replaced by a human c-myc epitope tag (Munro and Pelham, 1987). This plasmid encoded an Mpi1–c-myc fusion protein that could be detected in yeast cells with commercially available antibodies recognizing the c-myc epitope. When transformed cells were fractionated into a cytosolic fraction (marked by hexokinase) and a mitochondrial fraction (marked by the β-subunit of F_1-ATPase), Mpi1p exclusively cofractionates with mitochondria (Figure 5A, lane 2). This result was confirmed by immunoelectron microscopy, which also supports a mitochondrial localization of Mpi1p (not shown).

Subfractionation of mitochondria revealed that Mpi1p cofractionates with ISP42 (an outer membrane protein) and the F_1-ATPase β-subunit (an inner membrane protein), but not with superoxide dismutase (a soluble matrix protein) (Figure 5B, lanes 3 and 4). The rather loose association of the $F_1\beta$ subunit with the inner face of the inner membrane explains why some of this protein is also found in the matrix fraction (Figure 5B, lane 3). However, Mpi1p was found almost exclusively in the membrane fraction (Figure 5B, cf. lanes 3 and 4), which indicates a rather tight association with mitochondrial membranes. With intact mitochondria the protein was completely resistant to proteinase K (Figure 5B, lane 5). In mitoplasts, however, the c-myc tail of the fusion protein was degraded (Figure 5B, compare lanes 2 and 6) even though the matrix enzyme superoxide dismutase and the $F_1\beta$ subunit in the inner membrane (Figure 5B, lane 6) remained intact. Mpi1p is thus a mitochondrial membrane protein with its carboxy-terminus facing the intermembrane space.

The mitochondrial location of Mpi1p was determined with a fusion protein of Mpi1 and c-myc. The MPI1–c-myc construct encoding this fusion protein contains identical *MPI1* coding sequences as the *Hin*dIII–*Xba*I subclone shown in Figure 3 (construct 3). We found that the MPI1–c-myc construct can also partially complement the Ura^+ phenotype of MB3-52 cells harbouring of *SOD–URA* test plasmid (not shown). In addition, we found that introduction of low copy number plasmids carrying the MPI1–c-myc construct into a strain with a disrupted nuclear *MPI1* gene, could rescue the lethal effect of this gene inactivation (see later in the Results section). These two experimental results prove that this fusion protein is functional, implying that the presence of the c-myc epitope in the chimeric protein does not interfere with targeting to the authentic submitochondrial location of Mpi1p.

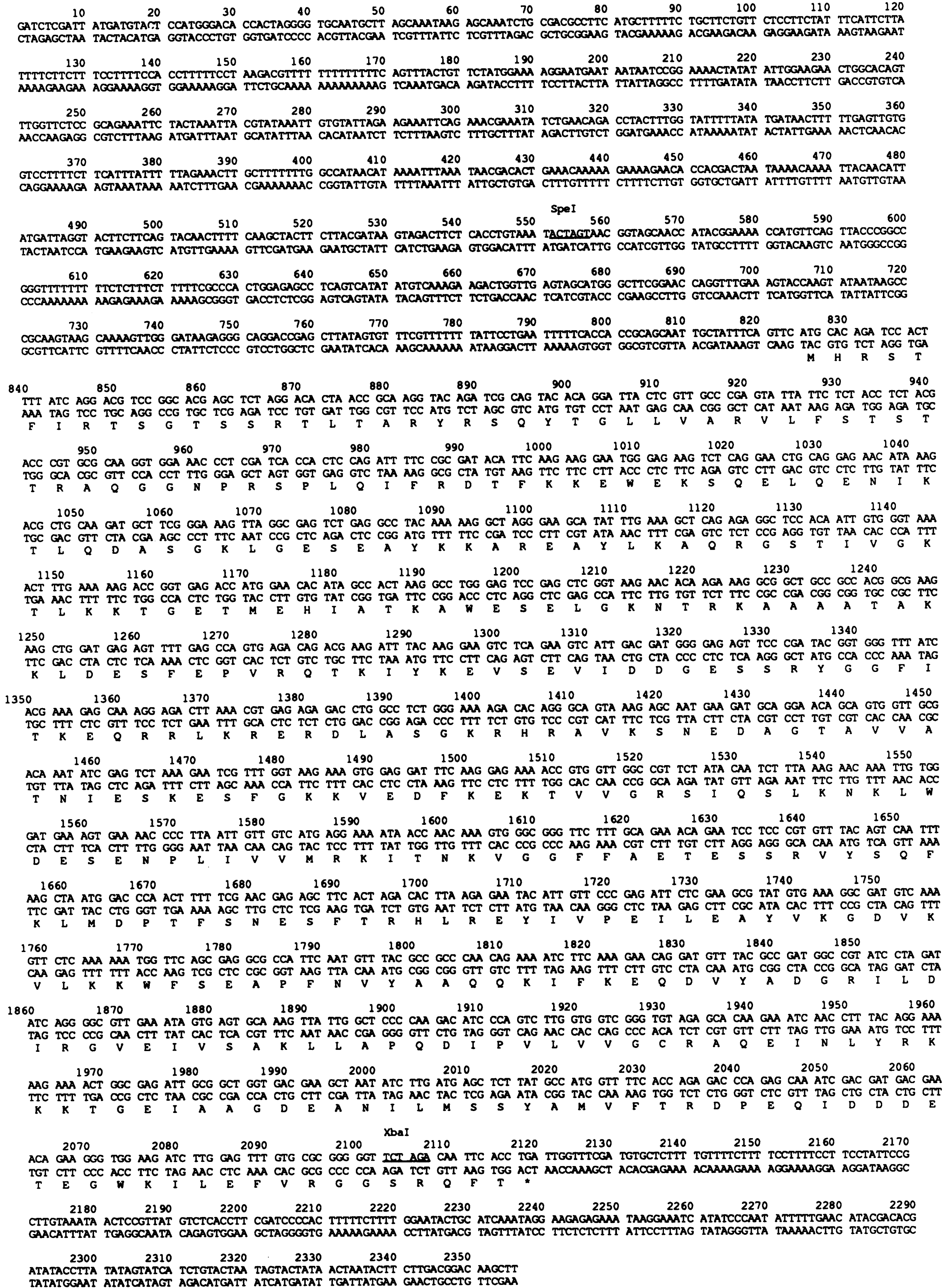

Fig. 4. Nucleotide sequence of *MPI1* and deduced amino acid sequence. The unique restriction enzyme recognition sites for *Spe*I and *Xba*I are underlined.

Mpi1 protein is proteolytically processed in vivo

Imported mitochondrial proteins contain targeting sequences that in most cases are located at the amino-terminus as cleavable presequences. Mpi1p also carries a characteristic mitochondrial targeting signal at its amino-terminus. We have explored the possibility that this targeting signal is proteolytically removed *in vivo*. Labelled Mpi1−c-myc fusion protein was synthesized *in vitro*, mixed with a protein

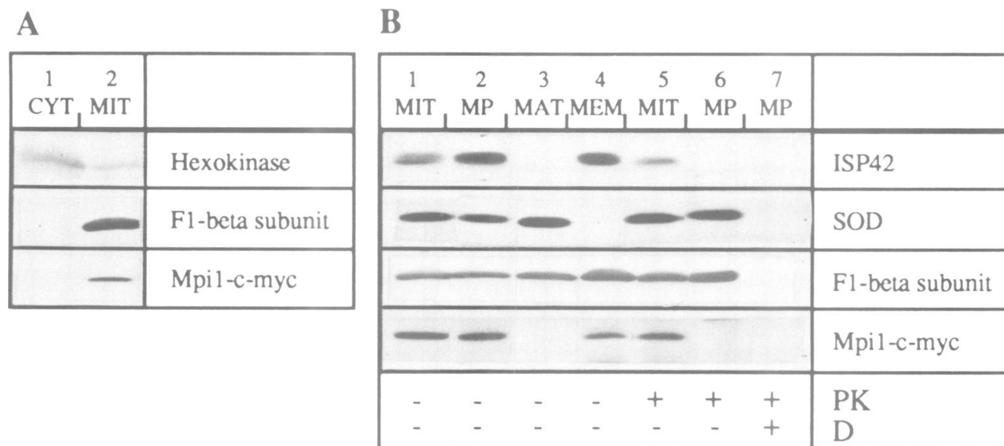

Fig. 5. Mpi1p is a mitochondrial membrane protein. (**A**) YP102 cells harbouring the *MPI1*−c-*myc* DNA construct, were fractionated into a cytosolic fraction and a mitochondrial fraction, which were analysed by SDS−PAGE, Western blotting and subsequent immunodecoration with antibodies raised against hexokinase, the β-subunit of F_1-ATPase or c-myc. Lane 1, cytosolic fraction; lane 2, mitochondrial fraction. (**B**) Mitoplasts, obtained by subjecting mitochondria to an osmotic shock, were subsequently fractionated into a membrane fraction and a matrix fraction. Intact mitochondria and mitoplasts were treated with proteinase K (0.2 mg/ml, 30 min on ice) as indicated. Samples, equivalent to 100 μg of mitochondrial protein were analyzed as described above. In addition to the β-subunit of F_1-ATPase and Mpi1-c-myc also superoxide dismutase (SOD) and ISP42 were immunodecorated. Lane 1, mitochondria (MIT); lane 2, mitoplasts (MP); lane 3, matrix fraction (MAT); lane 4, membrane fraction (MEM); lane 5, mitochondria treated with proteinase K (PK); lane 6, mitoplasts treated with proteinase K; lane 7, mitoplasts treated with proteinase K in the presence of 0.2% SDS (D).

lysate of yeast cells harbouring the MPI1−c-myc DNA construct and run on an SDS−polyacrylamide gel. After blotting proteins onto nitrocellulose, the *in vivo* synthesized fusion protein was decorated with antibodies raised against the c-myc epitope, whereas the *in vitro* synthesized protein was detected by autoradiography. As shown in Figure 6, the *in vivo* synthesized fusion protein migrated significantly faster than the *in vitro* synthesized protein. We conclude that the Mpi1 protein is proteolytically processed *in vivo*. We assume that this proteolytic cleavage occurs by the action of a specific processing peptidase inside mitochondria.

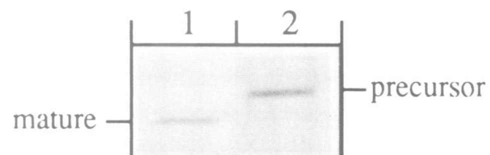

Fig. 6. *In vivo* processing of Mpi1 protein. *In vitro* synthesized Mpi1−c-myc fusion, labelled with [^{35}S]methionine, was mixed with a protein lysate of yeast cells containing the *MPI1*−*c-myc* DNA construct; proteins were separated on an SDS−polyacrylamide gel and subsequently blotted onto nitrocellulose. Lane 1, immunodecoration of *in vivo* synthesized fusion protein with antibodies raised against the c-myc epitope; lane 2, autoradiographic detection of the *in vitro* synthesized chimeric protein after blotting.

MPI1 is essential for viability of yeast

To determine whether *MPI1* encodes an essential mitochondrial protein, we constructed a null mutation in *MPI1* by inserting the yeast *LYS2* gene into one of the two *MPI1* alleles of the homozygous *lys2* diploid MB2. The presence of a wild-type and a disrupted *MPI1* allele in the resulting strain MB2-22 was verified by Southern blot analysis with probes complementary to either *MPI1* sequences or *LYS2* sequences. When strain MB2-22 was sporulated and ascospores were dissected, each tetrad maximally yielded two viable spores, even on medium containing a fermentable carbon source (data not shown). All viable spores were Lys^- and therefore did not carry the null allele of *MPI1*. When MB2-22, harbouring a plasmid containing the intact *MPI1* gene, was sporulated, viable spores that do not require lysine were also obtained. All Lys^+ spores carried the plasmid marker. These results prove that *MPI1* is an essential gene in yeast.

The involvement of Mpi1 protein in import of proteins into mitochondria in vivo

To explore the function of Mpi1p, expression of *MPI1* was placed under control of the *GAL1* promoter. This promoter is induced by galactose and repressed when glucose is used as the carbon source. A haploid *mpi1* null mutant harbouring this plasmid (pGAL-MPI1) was obtained by transformation and subsequent sporulation of the diploid strain MB2-22. To our surprise, these cells not only survived on medium supplemented with galactose but also on medium supplemented with glucose. Apparently, even a residual amount of Mpi1p, synthesized under glucose-repressed growth conditions, was sufficient for viability of yeast cells. This observation suggests that Mpi1p is a rather low abundance mitochondrial protein, a feature in agreement with the low codon adaptation index of the gene, indicating a poor translational efficiency of *MPI1* mRNA. Moreover, Northern blot analysis revealed a low steady state level and a constitutive expression of endogenous *MPI1* mRNA in wild-type cells, either grown on glucose- or galactose-containing medium (Figure 7, lanes 1 and 2). The transcription level of *MPI1* was comparable to the residual level of transcription of *QCR8* (encoding the 11 kDa subunit of ubiquinol cytochrome *c* oxidoreductase; Maarse and Grivell, 1987; Figure 7, lane 1) and *HIS3* (Hill *et al.*, 1986; data not shown) in cells where expression of these genes is repressed.

To be able to test the effect of depletion of Mpi1p activity on import of proteins into mitochondria, the *GAL1* promoter was therefore placed in front of a modified gene encoding a crippled Mpi1 protein. Deletion analysis of *MPI1* (Figure 3) has shown that *MPI1* coding sequences running until the *Xba*I site only partially complement the Ura^+

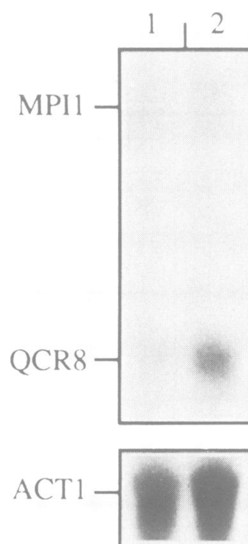

Fig. 7. Steady state *MPI1* mRNA level is constitutively low in yeast cells. RNA was isolated from YP102 cells, grown to early stationary phase in rich medium containing 2% glucose or 3% galactose, size-fractionated on a 1.2% agarose gel, blotted onto Hybond-N and hybridized to probes specific for: *MPI1* (1325 bp *Pst*I−*Hin*dIII fragment), *QCR8* (840 bp *Hin*dIII−*Sal*I fragment; Maarse and Grivell, 1987) and *ACT1* (1600 bp *Bam*HI−*Kpn*I fragment; Ng and Abelson, 1980). The actin probe was used to assess whether comparable amounts of RNA were loaded in each slot. Lane 1, RNA from cells grown in the presence of glucose; lane 2, RNA from cells grown in the presence of galactose.

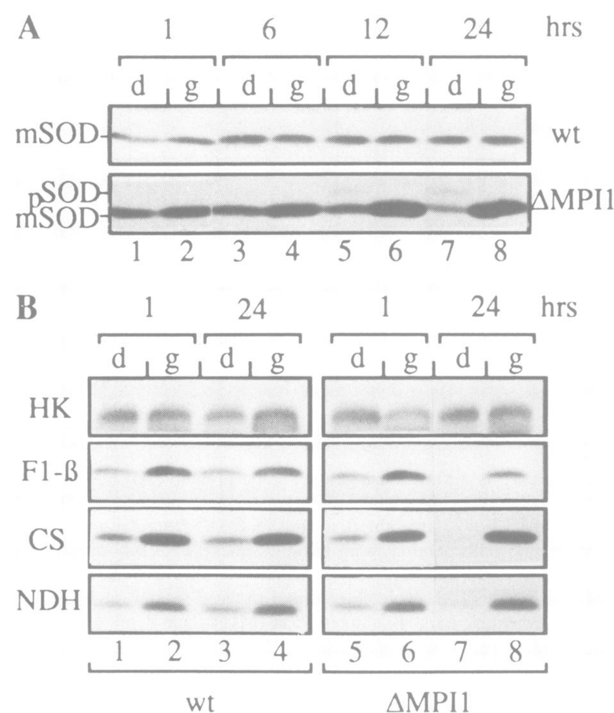

Fig. 8. The effect of Mpi1p depletion on steady state levels of nuclear-encoded mitochondrial proteins. Wild-type cells and cells from the *mpi1* null mutant, both containing a plasmid-borne gene fusion of the *GAL1* promoter and a truncated *MPI1*, were grown overnight on medium containing 3% galactose, collected and divided into two equal portions. One portion was shifted to medium containing 2% glucose (d) whereas the other half was resuspended in fresh galactose-containing medium (g). At different time points the steady state levels of hexokinase (HK) and the mitochondrial proteins superoxide dismutase (SOD), subunit β of F_1-ATPase (F1-β), citrate synthetase (CS) and internal NADH dehydrogenase (NDH) were determined by size-fractionation, Western blotting and immunodecoration. (**A**) Steady state levels of mature superoxide dismutase (mSOD) and the precursor of superoxide dismutase (pSOD) in wild-type cells (wt) and cells of the mpi1 null mutant (ΔMPI1) both harbouring the fusion gene. The levels were determined 1, 6, 12 and 24 h after the nutrient shift. (**B**) Steady state levels of hexokinase and the other mitochondrial proteins, visualized 1 and 24 h after the nutrient shift.

phenotype of mutant MB3-52. Indeed, the growth of a haploid *mpi1* null mutant containing a plasmid-borne gene fusion of the *GAL1* promoter and this truncated *MPI1* coding sequence was severely impaired on glucose-containing medium compared with growth on galactose-containing medium. A nutrient shift experiment was performed with wild-type cells and cells of an *mpi1* null mutant, both harbouring this DNA construct (Figure 8). After preculturing in galactose-containing medium, cells were collected and divided into two equal parts. One half was shifted to glucose-containing medium, whereas the other half was resuspended in fresh galactose-containing medium. At different time points samples were taken and the steady state levels of the cytosolic protein hexokinase and the mitochondrial proteins superoxide dismutase, citrate synthetase, subunit β of F_1-ATPase and internal NADH dehydrogenase were determined. Figure 8A shows the steady state levels of superoxide dismutase in this time course experiment. After 6 h of incubation in glucose-containing medium, the level of mature superoxide dismutase in the *mpi1* null mutant diminished and after 24 h most of the protein had disappeared (Figure 8A, bottom panel, cf. lanes 3, 5 and 7). This decrease was accompanied by a gradual increase of the amount of precursor superoxide dismutase. Turning off expression of the *GAL1−MPI1* fusion gene was responsible for this effect since the level of superoxide dismutase remained constant in mutant cells, grown in galactose-containing medium (Figure 8A, bottom panel, cf. lanes 2, 4, 6 and 8). In addition, the level of superoxide dismutase remained unchanged in wild-type cells, containing also the authentic chromosomal copy of *MPI1*, either grown in galactose- or glucose-containing medium (Figure 8A, top panel). Figure 8B shows that comparable results were obtained for the imported mitochondrial proteins citrate synthetase, subunit β of F_1-ATPase and internal NADH dehydrogenase. After 24 h of incubation in glucose-containing medium, these proteins were virtually absent in *mpi1* null mutant cells harbouring the *GAL1−MPI1* DNA construct (Figure 8B, lane 7). Part of the reduction of these protein levels can be explained by glucose-repressed expression of the nuclear genes (Figure 8B, cf. lanes 3 and 4), whereas the superimposed reduction (Figure 8B, cf. lanes 3 and 7) is caused by depletion of the Mpi1p activity. This effect is specific for mitochondrial proteins, as the level of the cytosolic protein hexokinase was not significantly affected after the nutrient shift from galactose to glucose (Figure 8B, top panel, cf. lanes 3 and 7). These results therefore strongly imply an involvement of Mpi1p in mitochondrial protein import.

After depletion of Mpi1p activity, the decrease of the mature forms of the imported mitochondrial proteins was not compensated by a comparable increase of their precursors. This suggests that these proteins probably accumulate in an unfolded state at an import stage that makes them very sensitive to proteolytic degradation.

To provide additional experimental evidence for a role of Mpi1p in mitochondrial protein import, a pulse−chase

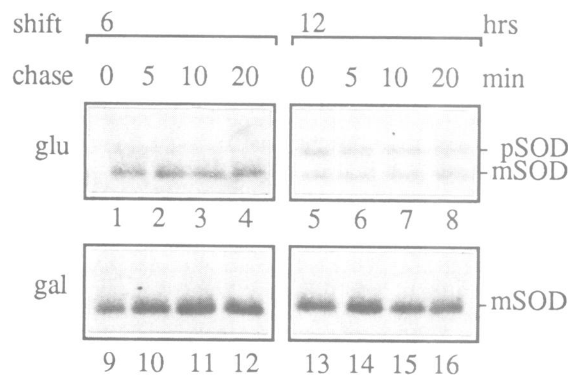

Fig. 9. Rate of maturation of precursor superoxide dismutase in Mpi1p-depleted cells. A nutrient shift experiment was performed with the *mpi1* null mutant containing the *GAL–MPI1* construct (see legend of Figure 8). Cells were grown for 6 or 12 h in the presence of either glucose (glu) or galactose (gal) to an OD_{600nm} of 1.5, collected by centrifugation, resuspended in 40 mM KP_i pH 6.0, 2% glucose or galactose to an OD_{600nm} of 20 and pulse-labelled with [^{35}S]methionine (75 μCi/ml) for 2.5 min at 28°C. Labelling was stopped by adding 10 mM unlabelled L-methionine and 0.1 mg/ml cycloheximide. The cells were chased at 28°C and at the times indicated, aliquots were removed, cells were spun down and the cell pellet was frozen in liquid nitrogen. Cellular proteins were extracted and subjected to immune precipitation with rabbit antisera against superoxide dismutase. Immunoprecipitates were collected on protein A–sepharose beads and analysed by SDS–PAGE and autoradiography.

experiment was performed with the *mpi1* null mutant containing the *GAL–MPI1* DNA construct. After 6 and 12 h growth in glucose- or galactose-containing medium, cells were pulse-labelled for 2.5 min with [^{35}S]methionine and subsequently chased for the indicated time periods. In cells grown in the presence of galactose, the labelled superoxide dismutase was exclusively of mature size (Figure 9, lanes 9–16). However, after 6 h growth in glucose-containing medium, a significant amount of labelled precursor superoxide dismutase was present, which was slowly converted into the mature form (Figure 9, lanes 1–9). Twelve hours after the nutrient shift to glucose, even more precursor superoxide dismutase was present and the rate of maturation was further reduced (Figure 9, lanes 5–8).

Discussion

To identify components of the mitochondrial protein import pathway in yeast we have performed a new screening for mutants disturbed in protein import. Positive selection of such mutants was based on mislocalization of a plasmid-encoded fusion protein that is efficiently imported into mitochondria and that has OMP decarboxylase activity, normally residing in the cytoplasm. Mutants were isolated with *trans*-acting recessive nuclear mutations that belong to at least three complementation groups. By transformation with a yeast nuclear DNA bank we have identified a new gene, *MPI1*, which can rescue the genetic defect of mutant MB3-52. *MPI1* encodes a mitochondrial protein that is essential for cell viability and import of precursor proteins into mitochondria.

The intramitochondrial location of Mpi1p was determined with a functional Mpi1p derivative, furnished with a c-myc epitope tag at its extreme carboxy-terminus. The fractionation behaviour of Mpi1p is consistent with being a protein of the inner or outer mitochondrial membrane. The sensitivity of the MPI1–c-myc fusion protein to proteinase K suggests that the carboxy-terminus of Mpi1p faces the intermembrane space. The fact that Mpi1p is proteolytically processed *in vivo* suggests that it is an inner membrane protein, since imported outer membrane proteins do not usually carry a cleavable presequence (Hartl *et al.*, 1989). Although the amino acid sequence of Mpi1p predicts a hydrophilic protein without obvious transmembrane domains, Mpi1p still behaves like a membrane protein. In this respect Mpi1p resembles the import site protein ISP42, which is located in the yeast mitochondrial outer membrane (Baker *et al.*, 1990). Stabilization of such hydrophilic proteins in a lipid environment can be achieved by interactions with other membrane proteins. Crosslinking studies revealed that MOM38, the *N.crassa* homologue of ISP42, is associated not only with the import receptors MOM19 and MOM72, but also with the outer membrane proteins MOM7, MOM8 and MOM30 (Kiebler *et al.*, 1990; Söllner *et al.*, 1992). Whereas these proteins seem to constitute a multi-subunit hydrophilic transmembrane channel for translocation of precursor proteins across the outer membrane, Mpi1p may be a component of a proteinaceous import channel through the inner membrane.

So far five components of the mitochondrial protein import apparatus in yeast are essential for cell viability. One of them is the outer membrane protein ISP42 (Baker *et al.*, 1990). The others are four matrix proteins: the two subunits of the matrix-processing protease (encoded by *MAS1* and *MAS2*; (Jensen and Yaffe, 1988; Witte *et al.*, 1988; Yang *et al.*, 1988), mitochondrial hsp60 (encoded by *MIF4*; Cheng *et al.*, 1989) and mitochondrial mhsp70 (encoded by *SSC1*; Craig *et al.*, 1989; Scherer *et al.*, 1990).

In contrast to these essential components of the import machinery, others are dispensable. These include the mitochondrial import receptor MAS70 (Hines *et al.*, 1990; Steger *et al.*, 1990), cytochrome *c* heme lyase, which is involved in import of apocytochrome *c* (Dumont *et al.*, 1991) and the inner membrane protease I, which removes the cytochrome *b2* sorting sequence (Behrens *et al.*, 1991; Schneider *et al.*, 1991). Mpi1p is the sixth essential component and the first that is localized in the inner membrane. This fits in a picture that is emerging for the import route of proteins translocated into the mitochondrial matrix (Pfanner and Neupert, 1990). After recognition by functionally redundant receptors on the surface of the outer membrane, precursor proteins enter a common import pathway in the outer membrane in which essential components such as ISP42 participate. Subsequently, proteins are transferred via a unique transport channel across the inner membrane to essential import catalysts within the matrix space. It is tempting to speculate that Mpi1p is part of the protein import system in the inner membrane and functions either before, during or after the membrane potential dependent translocation of targeting sequences across the inner membrane. According to this hypothesis, we found that mitochondrial protein import is specifically inhibited in Mpi1p-depleted cells as illustrated by: (i) a strongly reduced rate of maturation of the precursor of superoxide dismutase and (ii) a virtually unaffected level of the cytosolic enzyme hexokinase and drastically reduced steady state levels of the mature forms of all imported proteins that we have tested so far. As the latter decrease is not compensated by a parallel increase of the corresponding precursor proteins, imported proteins arrested at an

Mpi1p dependent translocation step are probably very susceptible to proteolytic degradation.

All mitochondrial proteins known to be essential for viability of yeast cells are key components of the mitochondrial protein import machinery (Baker and Schatz, 1991). The results presented in this paper show that Mpi1p is a new essential mitochondrial protein, which also participates in protein import. Future experiments are necessary to establish whether Mpi1p plays a direct or indirect role in mitochondrial protein import.

Materials and methods

Strains and growth media

S.cerevisiae strains used in this study are listed in Table I. MB3 is a derivative of YP102 (kindly provided by P.Philippsen) in which all coding sequences of the mutant *ura3-52* allele of YP102 are replaced by *LYS2* sequences. MB3 was constructed by transformation of YP102 with the 5.1 kb *Hin*dIII fragment from pUC18ura3::LYS2 and selection of a Lys$^+$ transformant with the predicted *URA3* deletion by Southern blot analysis. MB4, a spontaneous Lys2$^-$ derivative of S150-2B (Cold Spring Harbor Laboratory collection), was selected according to a method described by Chattoo *et al.* (1979). MB5 was derived from MB4 in a similar way as MB3 from YP102. MB1 was selected by random tetrad analysis of a sporulated diploid obtained by crossing YP102 and S150-2B. The diploid strain MB2 was constructed by crossing MB1 and YP102. MB2-22, harbouring one inactivated MPI1 allele, was obtained by transforming MB2 with a 5.5 kb *Bal*I–*Sph*I fragment from pUC18mpi1::LYS2 and selecting a Lys-positive transformant with one disrupted *MPI1* allele by Southern blot analysis.

Selective minimal medium of growth of yeast contained 0.67% yeast nitrogen base, 2% glucose or 3% galactose and was supplemented with appropriate amino acids, uracil and adenine (each at 20–50 μg/ml). Rich broth contained 2% bactopeptone, 1% yeast extract and 2% glucose or 3% galactose.

Recombinant DNA constructs in M13 vectors were propagated in *Escherichia coli* JM101 (Yanisch-Perron *et al.*, 1985) grown on 2YT medium (1.6% bactotryptone, 1% yeast extract, 0.5% NaCl). *E.coli* HB101 (Boyer and Dussoix, 1969) or JF1754 (*lac*, *gal*, *metB*, *leuB*, *hisB*, *hsdR*) was used for propagation of DNA constructs in plasmid vectors. *E.coli* transformants were selected on 2YT medium containing 100 μg/ml ampicillin, JF1754 was used for transformation of plasmid DNA isolated from double transformed yeast cells. This permitted a direct selection of transformants carrying plasmids containing the yeast *LEU2* gene that can complement the *leuB* mutation.

DNA manipulations

To generate pUC18ura3::LYS2, pUC18 (Sambrook *et al.*, 1989; Yanisch-Perron *et al.*, 1985) was restricted with *Pst*I, treated with T4 DNA polymerase, digested with *Sma*I and ligated. This plasmid, pUC18ΔPS, was linearized with *Hin*dIII and ligated with the 1.1 kb *Hin*dIII *URA3* fragment to construct pUC18URA3. Finally, the 0.9 kb *Pst*I–*Sma*I *URA3* fragment was replaced with the 4.9 kb *Pst*I–*Hin*dIII (blunt) *LYS2* fragment liberated from pDP6 (Fleig *et al.*, 1986).

pBPHura3::HIS3 was obtained from pBPH1 (Van Loon *et al.*, 1987) by replacing the *Eco*RV fragment containing part of the *URA3* coding region by a 1.8 kb blunt-ended *Bam*HI *HIS3* fragment, isolated from YEp6 (Struhl *et al.*, 1979). The *SOD–URA* test plasmid was generated in two steps. Firstly, a 369 bp *Eco*RI–*Bam*HI fragment, liberated from an *SOD* deletion construct and encoding the first 100 amino acids of the precursor of superoxide dismutase, was cloned into pBPH1ura3::HIS3. Secondly, this construct was linearized with *Bam*HI, treated with Klenow fragment and ligated with a 1.2 kb blunt-ended *Bam*HI fragment isolated from pNKY48 (Alani and Kleckner, 1987). The ATG–*URA* plasmid was constructed by cloning successively a synthetic 10 bp *Eco*RI–*Bam*HI fragment containing an ATG codon and the same *Bam*HI fragment from pNKY48 into pBPH1ura3::HIS3. The nucleotide sequences of the in frame fusions at the *SOD–HIS4* and ATG–*HIS4* junctions in the constructs are indicated in Table II.

pUC18mpi1::LYS2 was constructed by cloning a 3.0 kb *Eco*RI–*Sal*I *MPI1* fragment from the original YEp13 derivative into a pUC18 derivative lacking the *Pst*I site and replacing the 1.6 kb *Spe*I–*Xba*I *MPI1* fragment with the 4.9 kb *Xba*I *LYS2* fragment from pDP6. In this construct *LYS2* is transcribed from the same strand as *MPI1* before replacement.

To construct the *MPI1*–c-*myc* fusion gene, a synthetic *Sma*I–*Eco*RI fragment encoding a c-myc epitope was cloned into YEplac181 and YCplac111 (Geitz and Sugino, 1988). A stop codon was created in both constructs by filling in the *Eco*RI site downstream of c-*myc* coding sequences with the Klenow fragment of *E.coli* DNA polymerase, generating the plasmids YEpmyc181 and YCpmyc111. The almost complete *MPI1* coding sequence, liberated from the *Hin*dIII–*Xba*I subclone (Figure 3, construct 3) by restriction with *Xba*I, treatment with Klenow fragment and restriction with *Hin*dIII, was cloned into YEpmyc181 and YCpmyc111, both digested with *Xma*I at the *Sma*I site, treated with Klenow fragment and restricted with *Hin*dIII, creating YEpmycMPI1 and YCpmycMPI1. The nucleotide sequence running from the *MPI1*–c-*myc* junction to the filled in *Eco*RI site in these plasmids is shown in Table II.

To construct pGAL-MPI1 we introduced by PCR techniques a *Bam*HI site immediately in front of the ATG start codon of *MPI1* in one of the low copy number *Hin*dIII subclones. A 1.2 kb *Eco*RI–*Bam*HI fragment containing the *MPI1* promoter was then replaced with a 0.8 kb *Eco*RI–*Bam*HI *GAL1* promoter fragment isolated from pNN265 (kindly provided by M.Johnston). The nucleotide sequence at the *GAL1–MPI1* junction is indicated in Table II. The truncated *MPI1* gene regulated by

Table I. Yeast strains used in this study

Strain	Genotype
YP102	*MATα, ade2-101*^{ochre}*, his3-Δ200, leu2-Δ1, lys2-801*^{amber}*, ura3-52*
MB3	*MATα, ade2-101*^{ochre}*, his3-Δ200, leu2-Δ1, lys2-801*^{amber}*, ura3::LYS2*
S150-2B	*MAT***a**, his3-Δ*1*, *leu2,3,112, trp1-289, ura3-52*
MB4	*MAT***a**, *his3-Δ1, leu2-3, 112, lys2, trp1-289, ura3-52*
MB5	*MAT***a**, *his3-Δ1, leu2-3, 112, lys2, trp1-289, ura3::LYS2*
MB1	*MAT***a**, *his3, leu2, lys2-801*^{amber}*, trp1-289, ura3-52*
MB2	*MAT***a***/α, ADE2/ade2-101*^{ochre}*, his3/his3-Δ200, leu2/leu2*-Δ1, lys2-801^{amber}/ *lys2-801*^{amber}*, trp1-289/TRP1, ura3-52/ura3-52*
MB2-22	*MAT***a***/α, ADE2/ade2-101*^{ochre}*, his3/his3-Δ200, leu2/leu2*-Δ1, lys2-801^{amber}/ *lys2-801*^{amber}*, trp1-289/TRP1, ura3-52/ura3-52, MPI1/mpi1::LYS2*

Table II. Nucleotide sequence of junctions in hybrid genes

SOD–HIS4	C CAT Ggg gga tcg atc c*AA GCT*
ATG–*HIS4*	GAATTC ATG ggg atc c*AA GCT*
MPI1–c-*myc*	TCT *AGC CGG GAA CAA AAA CTT ATT TCT GAA GAA GAT CTG AAT TAA TTC*
GAL1–MPI1	GGAGAAAAAACcccggatcc *ATG CAC*

Sequences introduced by constructing the hybrid genes are shown in lower case. *HIS4*, c-*myc* and *MPI1* coding sequences flanking the junctions are shown in upper case italics. The CAT codon in the *SOD* sequence corresponds to the histidine residue at position 100 of the wild-type precursor of superoxide dismutase (Marres *et al.*, 1985). Initiation and termination codons are underlined.

the *GAL1* promoter was derived from pGAL-MPI1 by digestion with *Xba*I and *Hind*III and recircularization of the plasmid after filling in both ends with Klenow fragment.

To synthesize MPI1−c-myc fusion protein *in vitro*, a fusion gene was cloned into pEP30 (Laird, 1988) in a two-step procedure. A 0.2 kb *Bam*HI−*Pst*I fragment from pGAL-MPI1, encoding the amino-terminus of *MPI1*, was cloned into pEP30. The resulting construct was linearized with *Hind*III, treated with Klenow, digested with *Pst*I and ligated with a 1.6 kb *Pst*I−*Rsa*I fragment from YCpmycMPI1 encoding the carboxyl-terminus of the fusion protein.

Miscellaneous

The following procedures were performed essentially according to published methods: manipulation of nucleic acids (Sambrook *et al.*, 1989); isolation of RNA from yeast (Losson and Lacroute, 1979); isolation of total yeast cell extracts (Needleman and Tzagoloff, 1975); pulse-labelling and immune precipitation (Brandt, 1991); protein electrophoresis (Laemmli, 1970); immunoblotting using HRP-conjugated secondary antibodies as described in the Promega Protocols and Applications Guide, second edition, 1991; DNA sequence analysis with chain elongation inhibitors (Sanger *et al.*, 1977); EMS mutagenesis and sporulation of diploid yeast cells (Rose *et al.*, 1988); transformation of yeast cells (Klebe *et al.*, 1983).

For *in vitro* synthesis of the MPI1−c-myc fusion protein, the *MPI1*−*c-myc* DNA construct in pEP30 was linearized with *Pvu*II, a run-off transcript was made with T7 RNA polymerase according to standard methods (Melton *et al.*, 1984) and the transcript was translated *in vitro* in the presence of [^{35}S]methionine as recommended by the supplier of rabbit reticulocyte lysate (GIBCO BRL).

Fractionation of yeast cells was performed essentially as described by Daum *et al.* (1982) except that isolated mitochondria were resuspended in 0.65 M sorbitol, 20 mM KP_i, 1 mM $MgCl_2$, 1 mM EDTA. Mitoplasts were obtained by diluting the mitochondrial suspension 3.25-fold in 20 mM Tris−HCl pH 7.4, incubating the suspension 10 min on ice, sedimenting the mitoplasts at 165 000 *g* for 20 min in a Beckman Type SW50.1 rotor and finally resuspending them in 10 mM Tris−HCl pH 7.4, 1 mM EDTA. After sonicating mitoplasts 6 × for 5 s at 0°C and sedimentation at 165 000 *g* during 60 min in a Beckman Type SW50.1 rotor, a pellet and supernatant were obtained containing respectively mitochondrial membranes and matrix proteins.

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