The EMBO Journal vol.12 no.11 pp.4115–4123, 1993

Functional cooperation of mitochondrial protein import receptors in yeast

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Communicated by G.Schatz

We have identified a 20 kDa yeast mitochondrial outer membrane protein (termed MAS20) which appears to function as a protein import receptor. We cloned, sequenced and physically mapped the *MAS20* gene and found that the protein is homologous to the MOM19 import receptor from *Neurospora crassa*. MAS20 and MOM19 contain the sequence motif F-X-K-A-L-X-V/L, which is repeated several times with minor variations in the MAS70/MOM72 receptors. To determine how MAS20 functions together with the previously identified yeast receptor MAS70, we constructed yeast mutants lacking either one or both of the receptors. Deletion of either receptor alone had little or no effect on fermentative growth and only partially inhibited mitochondrial protein import *in vivo*. Deletion of both receptors was lethal. Deleting only MAS70 did not affect respiration; deleting only MAS20 caused loss of respiration, but respiration could be restored by overexpressing MAS70. Import of the F_1-ATPase β-subunit into isolated mitochondria was only partly inhibited by IgGs against either MAS20 or MAS70, but both IgGs inhibited import completely. We conclude that the two receptors have overlapping specificities for mitochondrial precursor proteins and that neither receptor is by itself essential.

Key words: gene disruption/mitochondrial biogenesis/precursor proteins/protein translocation

Introduction

Import of proteins into isolated mitochondria is inhibited by treating the mitochondria with proteases under conditions that maintain the integrity of the outer membrane (Riezman *et al.*, 1983b; Zwizinski *et al.*, 1984). Antibodies or Fab fragments against total outer membranes also inhibit import (Ohba and Schatz, 1987). Efforts to identify the inhibited target molecules have so far led to the identification of two highly protease-sensitive outer membrane proteins of approximate molecular weights 70 and 20 kDa, respectively. The larger protein has been termed MAS70 (in *Saccharomyces cerevisiae*; Hines *et al.*, 1990) or MOM72 (in *Neurospora crassa*; Söllner *et al.*, 1990) and the smaller one MOM19 (in *N.crassa*; Söllner *et al.*, 1989). Both proteins are integral membrane components that appear to be concentrated at regions where the two mitochondrial membranes are in close contact. As MAS70/MOM72 and MOM19 accelerate the import of precursor proteins into isolated mitochondria, they are generally regarded as 'import receptors' that bind precursors at the mitochondrial surface and deliver them to the translocation apparatus (Hartl and Neupert, 1990; Schatz, 1993).

The relative importance of these two receptors has been controversial. Our studies in yeast indicated that MAS70 is a general import receptor that accelerates import of most precursor proteins. The only known exceptions are chimeric precursors containing mouse dihydrofolate reductase as the mature domain. Since deletion of MAS70 from yeast is not lethal and inhibits mitochondrial protein import *in vivo* only 2- to 3-fold, we proposed that the different import receptors of yeast mitochondria have overlapping precursor specificities (Hines *et al.*, 1990).

A more hierarchical model was suggested for *N.crassa*. The MAS70 homologue MOM72 was viewed as a specialized receptor whose function was mainly restricted to the adenine nucleotide translocator (Söllner *et al.*, 1990), whereas MOM19 was proposed to be the 'master receptor' required for import of the other precursors, including MOM72 itself (Schneider *et al.*, 1991; Keil and Pfanner, 1993).

In the present study we characterized a 20 kDa outer membrane protein of yeast mitochondria that appears to function as a protein import receptor. We have termed this protein MAS20, in line with our earlier nomenclature (for mitochondrial assembly; Yaffe and Schatz, 1984). The sequence of its nuclear gene identified MAS20 as the yeast homologue of MOM19 from *N.crassa*. Since the yeast *MAS70* gene was already available (Hase *et al.*, 1983; Riezman *et al.*, 1983c), we could now construct yeast mutants lacking either one or both receptors. A study of these mutants revealed that neither receptor is by itself essential for mitochondrial protein import *in vivo*. Strains lacking only one receptor grow somewhat more slowly and importprecursors several-fold more slowly than wild-type cells. Also, cells lacking only MAS20 cannot grow on nonfermentable carbon sources, but can be 'cured' of this defect by overproduction of MAS70. However, deletion of both receptors is lethal, presumably because it blocks or severely slows mitochondrial protein import. These results suggest that the two receptors have overlapping precursor specificities and can thus at least partly substitute for one another.

Results

Identification of MAS20

Antisera raised against total mitochondrial outer membranes inhibit protein import into mitochondria, but not into inner membrane vesicles (Hwang *et al.*, 1989). The inhibiting antibodies in these sera are therefore presumably directed against components of the protein import machinery in the outer membrane. When one of these complex antisera was

depleted of antibodies recognizing outer membrane proteins in the range between 16 and 28 kDa, its inhibitory effect on mitochondrial protein import was significantly diminished (V.Hines, unpublished). This finding prompted us to excise the 23 kDa protein band (Figure 1) from isolated outer membranes and to raise antisera against it. For the reasons outlined below, this 23 kDa protein will from now on be referred to as MAS20.

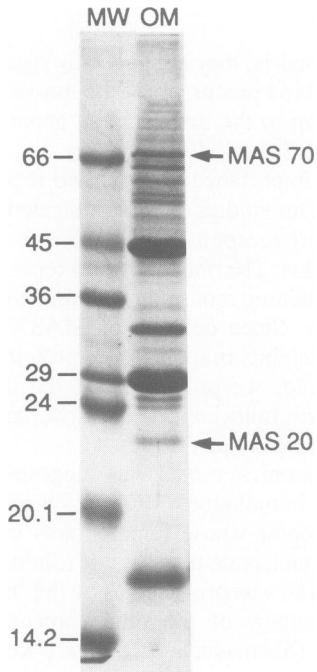

Fig. 1. Mitochondrial outer membrane proteins from *S.cerevisiae*. One hundred micrograms of purified yeast mitochondrial outer membrane (OM) proteins were resolved by SDS–13% PAGE. The gel was stained with Coomassie brilliant blue R-250. The positions of MAS70 and MAS20 are indicated by arrows. The band marked 'MAS20' was electroeluted from the gel and used for the production of antiserum. MW, molecular weight standards (sizes given in kDa on the left).

IgGs which had been affinity-purified on the MAS20 protein band inhibited import of the F_1-ATPase β-subunit precursor and of an artificial precursor (SU9-DHFR; Ostermann *et al.*, 1989) into isolated mitochondria. In contrast, inhibition of adenine nucleotide translocator import was only marginal and similar to that seen with IgGs affinity-purified against outer membrane porin (Figure 2). As porin is not directly involved in mitochondrial protein import (Dihanich *et al.*, 1987), the small inhibition by these IgGs serves as a control for nonspecific effects of contaminants which are often present in IgGs isolated by our affinity purification method.

MAS20 is readily removed from the mitochondrial surface by treating the mitochondria with protease under conditions in which the outer membrane barrier remains intact (Figure 3). As MAS20 is not extracted at pH 11.5 (not shown), it seems to be an integral protein exposed on the outer surface of the mitochondrial outer membrane. Immunoelectron micrographs confirm that the protein is located in the mitochondrial outer membrane and also suggest that MAS20 is distributed over the entire mitochondrial surface (not shown).

The combined evidence suggests that MAS20 functions during an early step of mitochondrial protein import, presumably as a receptor. Further evidence for this view will be given below.

Isolation and characterization of the MAS20 gene

IgGs affinity-purified on the MAS20 protein band (Materials and methods) were used to select immune-positive clones from a random library of yeast genomic DNA in the phage lambda gt11. A 650 bp DNA insert, isolated from one of these positive clones by PCR, then served as a probe for screening a library of large yeast genomic DNA fragments

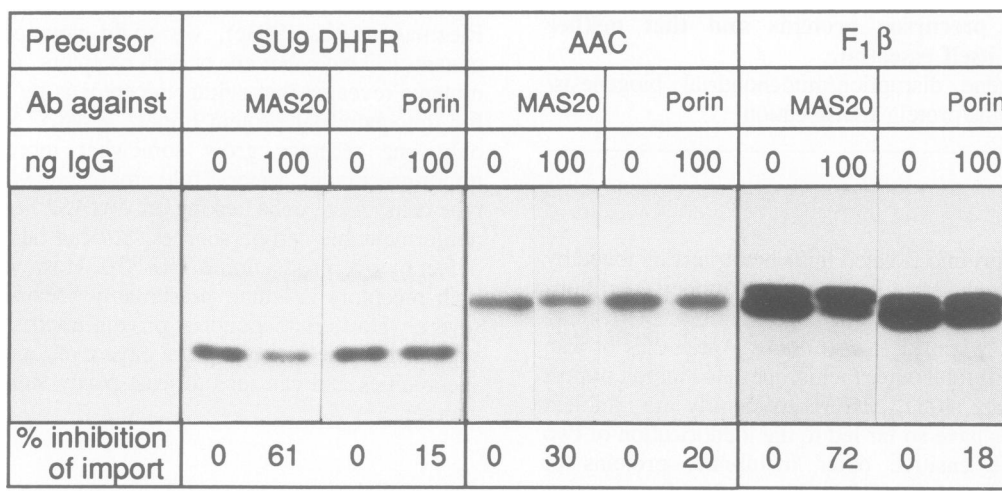

Fig. 2. Effect of anti-MAS20 IgGs on the import of different precursors into isolated yeast mitochondria. Ten micrograms of mitochondria were incubated for 60 min on ice in the absence or the presence of 100 ng IgGs (see horizontal top line) recognizing either MAS20 or porin. The IgGs had been affinity-purified against either porin or overexpressed MAS20 immobilized on nitrocellulose membranes. The incubation was in import buffer in a final volume of 100 μl. NADH and ATP were each added to final concentration of 2 mM. The reactions were prewarmed for 3 min to the temperature at which import was to take place and 5 μl of radiolabelled precursor were then added. Import was for 10 min at 30°C for SU9-DHFR (a fusion protein containing the presequence of *N.crassa* ATPase subunit 9 fused to mouse dihydrofolate reductase; Ostermann *et al.*, 1989) and the F_1-ATPase β-subunit, and 10 min at 15°C for the adenine nucleotide translocator. Import was stopped by adding valinomycin to 0.1 μg/ml and chilling to 0°C. In the case of the adenine nucleotide translocator, nonimported precursor was digested with proteinase K (100 μg/ml) for 30 min on ice. The protease was inactivated by adding PMSF to 1 mM. The samples were treated with trichloroacetic acid as described (Glick, 1991) and analysed by SDS–PAGE, fluorography and densitometric quantification of the bands.

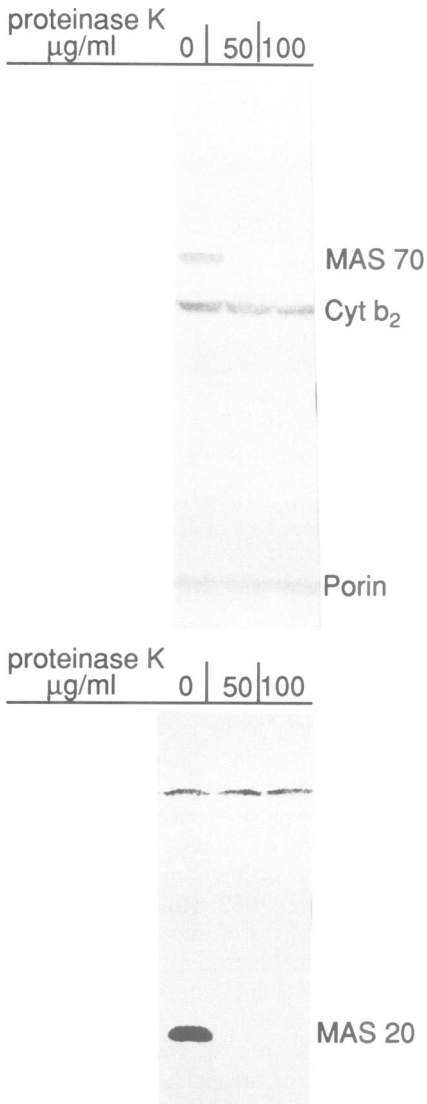

Fig. 3. MAS20 is exposed on the mitochondrial surface. Mitochondria (120 µg) were treated for 30 min on ice with the indicated concentrations of proteinase K. PMSF was then added to 1 mM and the mitochondria were pelleted by centrifugation, resuspended in 0.6 M sorbitol, 20 mM HEPES–KOH pH 7.4 and 1 mM PMSF, treated for 5 min at 60°C, then for 5 min at 0°C with trichloroacetic acid (Glick, 1991) and analysed by SDS–PAGE and immunoblotting with antisera to MAS70 (protease-sensitive outer membrane marker), cytochrome b_2 (intermembrane space marker) and MAS20. The blots were developed with anti-rabbit IgG antibody coupled to alkaline phosphatase and colour development as recommended by the manufacturer (Promega).

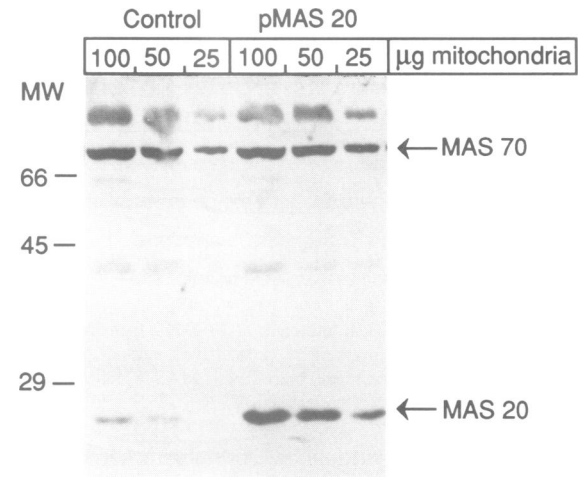

Fig. 4. A recombinant pFL1 clone causes overexpression of MAS20 in yeast. The yeast strain JKR101 was transformed with an insert-free pFL1 plasmid (Control) or with a recombinant pFL1 plasmid which had given a positive hybridization signal with the 650 bp DNA fragment from the lambda gt11 clone bank (pMAS20; see text). The transformants were grown on 0.67% yeast nitrogen base, 0.5% casamino acids, 1% galactose and 0.002% adenine, and different amounts of their mitochondria were analysed by SDS–PAGE and immunoblotting with antisera against MAS70 or MAS20. Molecular weight markers (MW) are indicated on the left.

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-60  TGCCTAGACCTACAAGAAACATTGCCTCAAGTGCCACCTTCATAAAGTTTATTTTCTATT

  1  ATGTCCCAGTCGAACCCTATCTTACGTGGCCTCGCTATTACAACAGCCATAGCTGCTCTA
     MetSerGlnSerAsnProIleLeuArgGlyLeuAlaIleThrThrAlaIleAlaAlaLeu

 61  TCAGCCACCGGTTATGCTATCTACTTTGACTATCAAAGAAGAAATAGCCCGCAATTCAGG
     SerAlaThrGlyTyrAlaIleTyrPheAspTyrGlnArgArgAsnSerProGlnPheArg

121  AAAGTGTTGAGACAAAGGGCCAAAGAGCAGGCCAAGATGGAAGAACAAGCTAAAACTCAT
     LysValLeuArgGlnArgAlaLysGluGlnAlaLysMetGluGluGlnAlaLysThrHis

181  GCTAAGGAAGTGAAGCTGCAAAAGGTTACCGAATTCTTATCCATGGAATTAGCCAAGGAC
     AlaLysGluValLysLeuGlnLysValThrGluPheLeuSerMetGluLeuAlaLysAsp

241  CCCATCCCTAGTGATCCCTCCGAAAGAGAAGCTACATTTACCACCAACGTAGAAAATGGT
     ProIleProSerAspProSerGluArgGluAlaThrPheThrThrAsnValGluAsnGly

301  GAAAGATTATCCATGCAACAAGGTAAGGAACTGGAAGCAGCCTCTAAGTTTTATAAAGCA
     GluArgLeuSerMetGlnGlnGlyLysGluLeuGluAlaAlaSerLysPheTyrLysAla

361  TTGACTGTATACCCTCAGCCAGCCGATTTATTGGGAATTTACCAAAGATCCATTCCTGAA
     LeuThrValTyrProGlnProAlaAspLeuLeuGlyIleTyrGlnArgSerIleProGlu

421  GCCATTTACGAATATATTATATTAATGATTGCCATCTTGCCTCCTGCTAATGTGGCTTCT
     AlaIleTyrGluTyrIleIleLeuMetIleAlaIleLeuProProAlaAsnValAlaSer

481  TTCGTTAAAGGAGTTGTTGGAAGCAAGGCCGAATCTGATGCGGTTGCTGAAGCTAACGAT
     PheValLysGlyValValGlySerLysAlaGluSerAspAlaValAlaGluAlaAsnAsp

541  ATCGATGACTGAGAGTAACATTTTGCTTCGCTTTTTTTCTCCGTTTTTGTTTCTTTTACT
     IleAspAspEnd
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Fig. 5. Nucleotide sequence of the *MAS20* gene and deduced amino acid sequence of the protein product. The numbers on the left indicate the nucleotide number in the open reading frame.

in the yeast–*Escherichia coli* shuttle vector pFL1 (Chevalier *et al.*, 1980). One of the recombinant pFL1 plasmids identified by this approach contained a 5.5 kb insert and appeared to carry the entire *MAS20* gene as this plasmid caused a pronounced overexpression of the protein in yeast (Figure 4).

The *MAS20* gene was further localized to a 2.5 kb *Bam*HI fragment by probing restriction fragments derived from the plasmid with the 650 bp fragment from the lambda gt11 library, using Southern blotting. Overexpression of the MAS20 protein in yeast was then used to confirm the presence of the intact gene. The complete nucleotide sequence of the *MAS20* gene revealed an open reading frame of 183 codons, corresponding to a 20 268 Da protein (Figure 5). This size is in reasonable agreement with the mobility of MAS20 on SDS–PAGE.

Chromosomal mapping of the MAS20 gene

When yeast chromosomes separated by pulse-field gel electrophoresis were tested with the *MAS20* probe, the gene proved to reside on chromosome VII. Physical submapping of the gene localized it to the right arm of chromosome VII, 140 kb from the centromere (not shown).

Topology of the MAS20 protein

The amino-terminus of the MAS20 open reading frame lacks a typical matrix targeting signal (Roise and Schatz, 1988). Starting at residue 9, there is an uninterrupted stretch of 20

```
MAS 20   1 M SQSNPIL RGLAITT AIAALSATGY   AIYFDYQRRNSFQFRKVLRQ   45
           | ||        :  || |:||: |||:   |:||||:||| |:||: ||
MOM 19   1 MPSQA       VTYTTAAVAAV AIGFLAYAVYFDYKRRNDPEFRRQLRR   43

          46 RAKEQAKMEEQAKTHAKEVKLQKVTEFLSMELAKDPI P  SDPSEREATFTT 95
             |: ||: | :      :   |:: : :  | ||:   |  ||  |:|| |
          44 SARRQARQEKEYAELSQQAQRQRIRQMVD.E AKEEGFPTTSD  EKEAYFLE 92

          96 NVENGERLSMQQGKELEAASKFYKALTVYPQPADLLGIYQRSIPEAIYEY   145
             :|: || |     | ::|   ||||| ||| |:||: || ::::  | :
          93 QVQAGEILGQDPIKAIDASLAFYKALKVYPTPGDLISIYDKTVAKPILDI   142

         146 IILMIAILPPANVASFVKGVVGSKAESD   AVAEANDIDD            183
             : :|||  |   :::   | |   ||     | | :  :|
         143 LAIMIAYDPSLKIGINYTGGVDV AELMRIMASAFGVGLD             181
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Fig. 6. MAS20 is the yeast homologue of MOM19 from *N.crassa*. Identical amino acids are marked by vertical bars, similar amino acids by two dots. The sequence motif shared with MAS70 and other proteins (discussed below) is underlined.

uncharged, mostly hydrophobic residues; a second hydrophobic stretch of 12 residues starts at amino acid 145. As only the amino-proximal stretch is long enough to form a typical transmembrane helix, MAS20 may be anchored to the outer membrane via this amino-terminal sequence. The mitochondrial import receptor MAS70 has a similar topology (Hase *et al.*, 1984). Indeed, both MAS20 and MAS70 are similarly sensitive to low levels (<10 μg/ml) of externally added proteinase K (not shown).

MAS20 is the yeast homologue of MOM19 from N.crassa

A computer search revealed strong similarity between MAS20 and MOM19, a protein import receptor in *N.crassa* (Schneider *et al.*, 1991). The degrees of identity and similarity are 38 and 58%, respectively (Figure 6). The hydropathy plots of the two proteins are also quite similar (not shown). MOM19, like MAS20, is an integral protein exposed on the outer face of the outer membrane. We conclude that MAS20 is the yeast homologue of MOM19. As there is good evidence that MOM19 functions as a protein import receptor (Söllner *et al.*, 1989; Schneider *et al.*, 1991), the high degree of identity between MAS20 and MOM19 further supports the view that MAS20 is a mitochondrial protein import receptor in yeast.

Yeast cells lacking MAS20 are viable and still import precursor into their mitochondria

Southern blot analysis using the *MAS20* gene as a probe suggested that *S.cerevisiae* contains a single *MAS20* gene. This result was also obtained upon lowering the stringency of the hybridization conditions (not shown). Disruption of this gene generated MAS20-deficient cells (Figure 7) which were viable. These cells grew nearly as fast as wild-type cells on a synthetic medium containing glucose as the major carbon source: the generation times were 180 min for the mutant and 120 min for the wild-type. However, the MAS20-deficient cells could not grow under nonfermentative conditions, suggesting that at least one protein essential for respiration-driven growth was no longer imported at sufficient rates. This respiratory deficiency could account for the small difference in growth rates between MAS20-deficient and wild-type cells.

As mitochondrial protein import is essential for viability of yeast (Baker and Schatz, 1991), deletion of an indispensible component of the protein import machinery

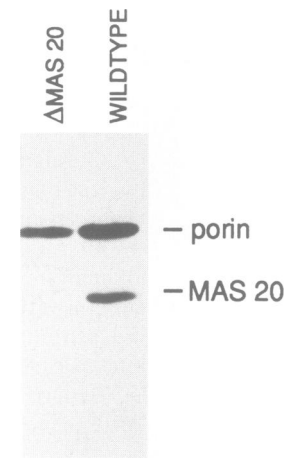

Fig. 7. Disruption of the single *MAS20* gene of yeast. The diploid strain carrying one disrupted copy of the *MAS20* gene was sporulated and the ascospores were dissected. Equal amounts of mitochondria from a *URA3*$^+$ spore (ΔMAS20) and from a *ura3*$^-$ spore (WILDTYPE) were analysed by SDS–PAGE and immunoblotting with antisera against MAS20 or outer membrane porin.

should be lethal. As a deletion of MAS20 is not lethal, the protein does not appear to be essential for mitochondrial protein import *in vivo*. This conclusion was corroborated by measuring the import rate of the F_1-ATPase β-subunit precursor *in vivo* by pulse–chase experiments. In these experiments, the rate of import was measured as the uncoupler-sensitive conversion of the precursor to the mature form (Reid and Schatz, 1982; Hines *et al.*, 1990). In two experiments, the half-lives of import of this precursor into MAS20-deficient mitochondria *in vivo* were 40 and 33 s. In the experiment shown in Figure 8, the wild-type cells had processed all of the β-subunit molecules after 1 min of chase. In other experiments (not shown), they still contained between 5 and 15% of the pulse-labelled subunit as the precursor. Thus *in vivo* import of the F_1-ATPase β-subunit into the mitochondria of MAS20-deficient cells was about five times slower than into mitochondria of wild-type cells; these rates are quite similar to those found for MAS70-deficient cells (see Figure 6 of Hines *et al.*, 1990).

Cells lacking both MAS20 and MAS70 are inviable

The results described so far suggested that most of the functions of MAS20 in mitochondrial protein import could be fulfilled by MAS70, or by some as yet undiscovered import receptor. However, it also seemed possible that mitochondrial protein import *in vivo* could occur without either MAS20 or MAS70. To address this question, we constructed a yeast strain lacking both proteins. In the experiment shown in Figure 9, a heterozygous diploid cell containing one disrupted *MAS20* gene and one disrupted *MAS70* gene was sporulated, each of the four spores was given a chance to grow on a rich glucose-containing medium and each viable clone was then analysed for its genotype. Because of the exponential nature of the growth process, the observed colony sizes accentuate small differences in growth rates. Cells lacking either MAS70 or MAS20 grew somewhat slower than the wild-type, but cells lacking both MAS20 and MAS70 were inviable. Although the doubly deficient spores germinated, they stopped growing after ~8

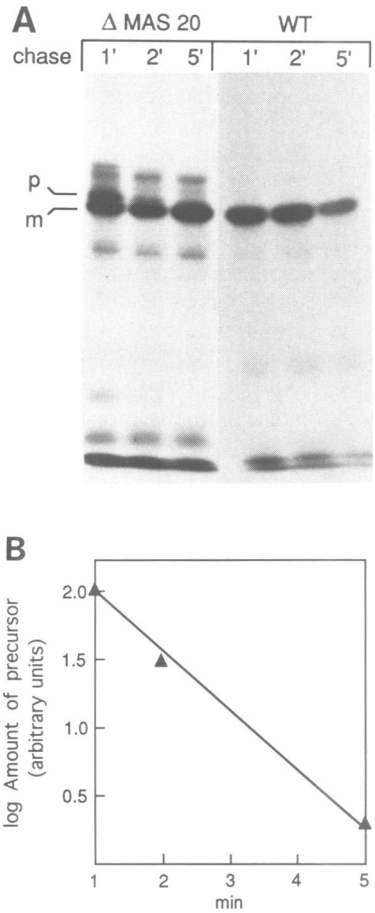

Fig. 8. Disruption of the *MAS20* gene retards, but does not block mitochondrial import of the F_1-ATPase β-subunit *in vivo*. Cells derived from the MAS20-deficient spore (ΔMAS20) and the wild-type spore (WT), both derived from the disruption experiment shown in Figure 7, were grown to the late logarithmic phase (OD_{600} = 0.50) on 0.67% yeast nitrogen base, 0.5% casamino acids, 2% glucose and 20 μg/ml each of uracil and adenine. The cells were pulse-labelled for 2 min at 30°C with [^{35}S]methionine, chased for the indicated times at 30°C (by the addition of cycloheximide to 100 μg/ml and unlabelled methionine to 2 mM) and analysed by immunoprecipitation with rabbit antiserum against the F_1-ATPase β-subunit as described by Brandt (1991). (**A**) Fluorogram of the immunoprecipitates. p, precursor; m, mature form. (**B**) Quantification of the β-subunit precursor band in the MAS20-deficient cells. Quantification was performed by densitometry of three different exposures of the fluorogram.

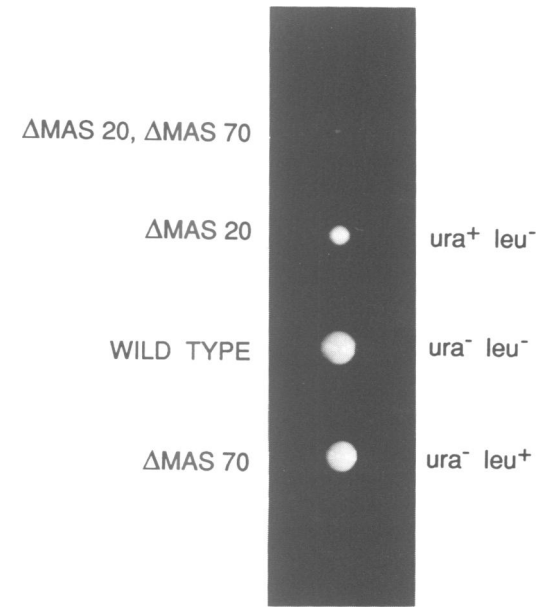

Fig. 9. Disrupting both the *MAS20* and the *MAS70* genes is lethal. A yeast strain carrying a disrupted *MAS20* gene (*MATa ura3 leu2 his4 ade2 lys, MAS20::URA3*) was mated with a yeast strain carrying a disrupted *MAS70* gene (*MATα ura3 leu2 his4 ade2 MAS70::LEU2*). The resulting heterozygous diploid was sporulated and each of the four spores derived from a single ascus were plated onto rich solid glucose medium (1% yeast extract, 2% peptone and 2% glucose). The plates were incubated for 3.5 days at 30°C and then photographed. The phenotypes of the three viable spores were scored on appropriate selective plates and are given on the right. The deduced genotypes for the *MAS20* and *MAS70* genes are indicated on the left.

generations. Thus mitochondrial protein biogenesis *in vivo* requires at least one of these two receptor proteins.

Import of the F1-ATPase β-subunit into isolated mitochondria is completely inhibited by a combination of anti-MAS20 IgGs and anti-MAS70 IgGs

Protein import into isolated mitochondria is only partly inhibited by IgGs monospecific for either MAS20 (Figure 2) or MAS70 (Hines *et al.*, 1990; Hines and Schatz, 1993). This result is indeed that predicted by our suggestion that both receptors contribute to the import process. In order to corroborate this model further and in order to test whether import is can also be mediated by additional as yet unknown receptors, we tested the effect of both types of IgGs together. Figure 10A and B confirm that either type of IgG alone inhibits import of the F_1-ATPase β-subunit precursor only partly; however, both IgGs together give complete inhibition (Figure 10B and C).

Overproduction of MAS70 suppresses the defect caused by deleting MAS20

Since deletion of MAS20, but not of MAS70 (Riezman *et al.*, 1983c) renders cells unable to grow on a nonfermentable carbon source, it remained possible that some mitochondrial precursors can only be imported via MAS20. This would imply a strict division of function between these two receptors. This model became unlikely when it was found that the respiration defect of the MAS20-deficient cells could be overcome by overexpression of MAS70. As shown in Figure 11A, the restored growth was nearly as fast as that of wild-type cells. Immunoblot analyses confirmed that MAS70 was overproduced ~3-fold, in agreement with earlier results (Riezman *et al.*, 1983c) and that the MAS70-overproducing cells still lacked MAS20 (Figure 11B). While MAS20 and MAS70 may thus have different affinities for particular precursors, the specificities of the two receptors overlap.

Discussion

The MAS20 protein

The results presented here strongly suggest that the outer membrane protein MAS20 is part of the machinery which mediates import of proteins from the cytosol into mitochondria: blocking of MAS20 function with specific IgGs partly inhibits protein import into isolated mitochondria and deletion of MAS20 from yeast cells retards mitochondrial protein import *in vivo*. The exact role of MAS20 in the import pathway is more difficult to define. The amino acid sequence of MAS20 suggests that only a

single transmembrane helix is buried in the outer membrane and that most of the protein protrudes into the cytoplasm. In contrast, putative subunits of the transport channels across the mitochondrial membranes (ISP42, ISP45/Mpi1p and MAS6) appear to be largely buried in the membrane, being only poorly accessible to proteases or inhibitory IgGs (Vestweber *et al.*, 1989; Maarse *et al.*, 1992; Scherer *et al.*, 1992). Furthermore, the sequence of MAS20 indicates that this protein is the yeast homologue of MOM19, a protein whose function as an import receptor in *Neurospora* is well-established (Söllner *et al.*, 1989; Schneider *et al.*, 1991). It is thus reasonable to assume that MAS20, as well, functions as a mitochondrial receptor for protein import from the cytoplasm. Interestingly, both known import receptors from yeast and *Neurospora* share the sequence motif F-X-K-A-L-X-(V/L) which is located near the carboxy-terminal part of these proteins (Figure 12). This motif, with minor variations, is repeated seven times in the MAS70 protein. Similar repeats of this motif are found as parts of a 34-residue repeat (termed tetratricopeptide repeat) in several other proteins which appear to function in cell cycle control and in mediating interactions with the cytoskeleton or the nuclear scaffold (Boguski *et al.*, 1990; Sikorski *et al.*, 1990). The functional significance of this consensus motif is unknown.

Interaction of MAS20 with MAS70

The function of MAS20, like that of MAS70, is not essential for protein import or cell viability. However, loss of MAS20 has more serious consequences than that of MAS70, since it causes loss of respiration-driven growth. The molecular lesion responsible for this effect is not known with certainty, but mitochondria from MAS20-deficient cells contain lower levels of cytochromes *b* and aa_3 (not shown), suggesting that loss of nonfermentative growth is a result of defective respiration. At least in the yeast strain used in this study, MAS20 seems to be rate-limiting for importing one or more

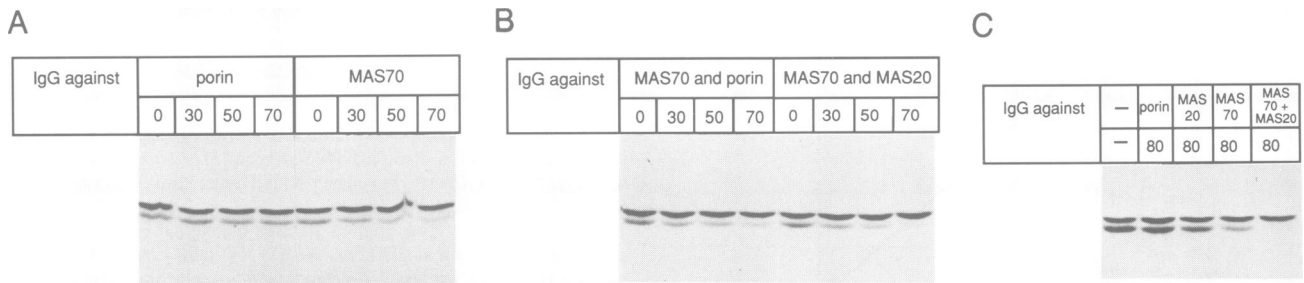

Fig. 10. Import of the F1-ATPase β-subunit precursor into isolated mitochondria is only partly inhibited by IgGs against either MAS20 or MAS70, but is completely inhibited by a combination of these IgGs. (**A**–**C**) Ten micrograms of yeast mitochondria were incubated for 60 min on ice with the amounts of IgGs(μg) indicated. In order to compensate for any nonspecific inhibitory effects of IgGs, the effect of anti-MAS70 IgGs was tested in the presence of an equal amount of IgG against outer membrane porin. In these instances, each of the two types of IgG was added in the amount shown. Anti-porin IgGs were also tested by themselves to show the absence of significant nonspecific inhibitory effects of these IgGs. IgGs were purified by chromatography on protein A–Sepharose and shown to be monospecific by immunoblotting against total mitochondrial proteins that had been subjected to SDS–PAGE and transfer of the resolved protein band onto a nitrocellulose sheet (see Figure 7 for the purity of the anti-MAS20 IgGs). The mitochondria were then assayed for import of the F_1-ATPase β-subunit precursor as described in Figure 2.

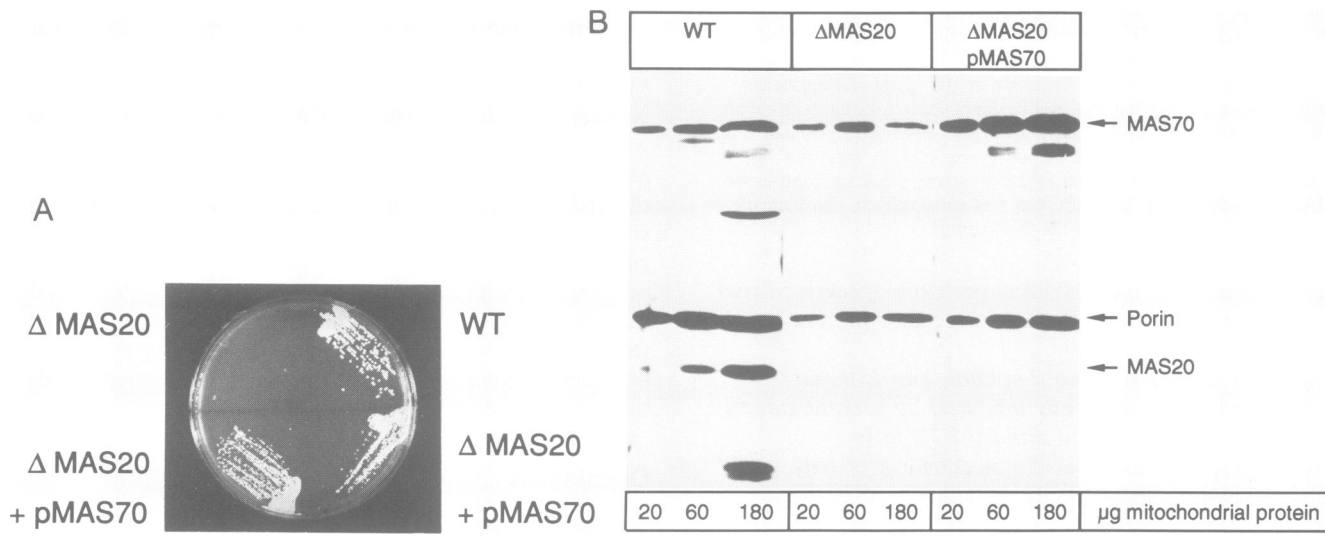

Fig. 11. Overexpression of MAS70 suppresses the respiratory defect of a MAS20-deficient strain. A 4 kb *Bam*HI fragment derived from plasmid pHR64 (Riezman *et al.*, 1983b) was cloned into the single *Bam*HI site of the 2 μm-based plasmid YEplac181 (Gietz and Sugino, 1988). The resulting recombinant plasmid was transformed into the MAS20-deficient haploid (*MATa ura3 leu2 his4 mas20::URA3*) derived from the disruption described in Figure 7 to generate a MAS20-deficient haploid strain overproducing MAS70. The same MAS20-deficient spore without the plasmid served as a control. (**A**) Growth on a nonfermentable carbon source. The wild-type spore (WT), the corresponding MAS20-deficient spore (ΔMAS20) or the MAS20-deficient spore transformed with the MAS70-bearing multicopy plasmid (ΔMAS20 + pMAS70; see above) were streaked onto a YPEG plate (1% yeast extract, 2% peptone, 3% glycerol and 3% ethanol) and allowed to grow for 4 days at 30°C. (**B**) Overproduction of MAS70 in MAS20-deficient cells. Increasing amounts of mitochondria isolated from the three types of cells shown in Figure 11A were subjected to SDS–PAGE on a 10–15% gradient gel and analysed by immunoblotting with antisera against MAS20, MAS70 and outer membrane porin. The blot was developed with radioiodinated protein A and autoradiography.

proteins of the respiratory system. However, this finding does not imply a strict hierarchy of receptor function. Most and perhaps all functions of MAS20 appear to be performed by MAS70 when that protein is overproduced. Such a receptor substitution is not easily reconciled with a model in which MOM19/MAS20 functions as the 'master receptor' for protein import into mitochondria (Figure 13B). Our data also argue against the proposal (Schneider *et al.*, 1991) that MAS20 is required for the import of MAS70: this possibility could not explain why MAS20-deficient cells (in contrast to the doubly deficient cells) are viable and why the respiratory defect caused by MAS20 deficiency is 'cured' by overexpression of MAS70.

The concept of a receptor hierarchy was based exclusively on studies with isolated mitochondria. We have recently shown that the standard import experiments with isolated mitochondria may fail to reveal the involvement of a particular import receptor, presumably because the rate-limiting steps for import *in vitro* are different from those in intact cells. For example, the initial interaction of a precursor with the mitochondrial import machinery may be much slower *in vitro* than *in vivo*, so that the effect of MAS70 is not apparent *in vitro* (Hines and Schatz, 1993).

Our results favour a model in which the precursor specificities of the two receptors are not identical, but overlap considerably. As a result, the entire range of precursors imported into mitochondria can be accomodated by only one of the two receptors, particularly if the remaining receptor is overproduced (Figure 13A). Since different 'wild-type' yeast strains may vary widely in the polypeptide composition of their mitochondria, it is possible that in some strains the effect of deleting MAS20 could be fully compensated for by the normal levels of MAS70.

MAS20	117	**Phe**	Tyr	**Lys**	**Ala**	**Leu**	Thr	Val	Tyr Pro	125
MOM19	114	**Phe**	Tyr	**Lys**	**Ala**	**Leu**	Tyr	Val	Tyr Pro	122
MAS70	386	**Phe**	Asp	**Lys**	**Ala**	**Leu**	Lys	Leu	Asp Ser	394
MOM72	321	**Phe**	Lys	**Lys**	**Ala**	**Leu**	Asp	Leu	Gly Glu	330

Fig. 12. A common sequence motif in the two major protein import receptors from *S.cerevisiae* and *N.crassa* mitochondria. The numbers on the left identify the position of the phenylalanine residue and the numbers on the right the position of the last amino acid shown, in each of the four proteins. The sequences of MAS70, MOM19 and MOM72 were taken from Hase *et al.* (1984), Schneider *et al.* (1991) and Söllner *et al.* (1990).

Other possible receptor models

In principle, MAS20 and MAS70 might not be distinct receptors that can function independently, but subunits of a hetero-oligomeric receptor (Hines *et al.*, 1990; Moczko *et al.*, 1992). We consider this possibility unlikely because it does not readily explain why loss of MAS20 can be compensated for by an excess of MAS70. The hetero-oligomeric model would have to postulate that all of the MAS20 sites in a complex can be filled by MAS70, even though MAS70 is much larger than MAS20, and has a very different amino acid sequence.

MAS20 and MAS70 may not be the only protein import receptors of yeast mitochondria. A 32 kDa protein has been suggested to function as a receptor for precursors carrying an amphiphilic matrix-targeting signal (Pain *et al.*, 1990), but the role of this protein in import of precursors is still unclear (Phelps and Wohlrab, 1991; Phelps *et al.*, 1991). A recently described 22 kDa outer membrane protein of *N.crassa* may be yet another protein import receptor (Moczko *et al.*, 1992). The results presented here show that any putative additional receptors could not, by themselves, sustain protein import at rates which could be detected in our *in vitro* assay system or which are compatible with viability of the yeast cells.

Materials and methods

Yeast strains

Mitochondria for *in vitro* import studies and for isolating outer membranes were isolated from the wild-type *S.cerevisiae* strain D273-10B (*MATα*; ATCC25657). Cells were grown to early stationary phase in semi-synthetic medium containing 2% lactate and 0.1% glucose (Daum *et al.*, 1982). Transformation and genetic experiments were performed with the following yeast strains: JKR101 (*MATa ura3 leu2 his4 ade2*) (Bibus *et al.*, 1988); YKB5 (*MATa*/α ura3 leu2 his4 ADE2/ade2 *LYS2/lys2*); YVH1 (*MATα ura3 leu2 his4 ade2 MAS70::LEU2*).

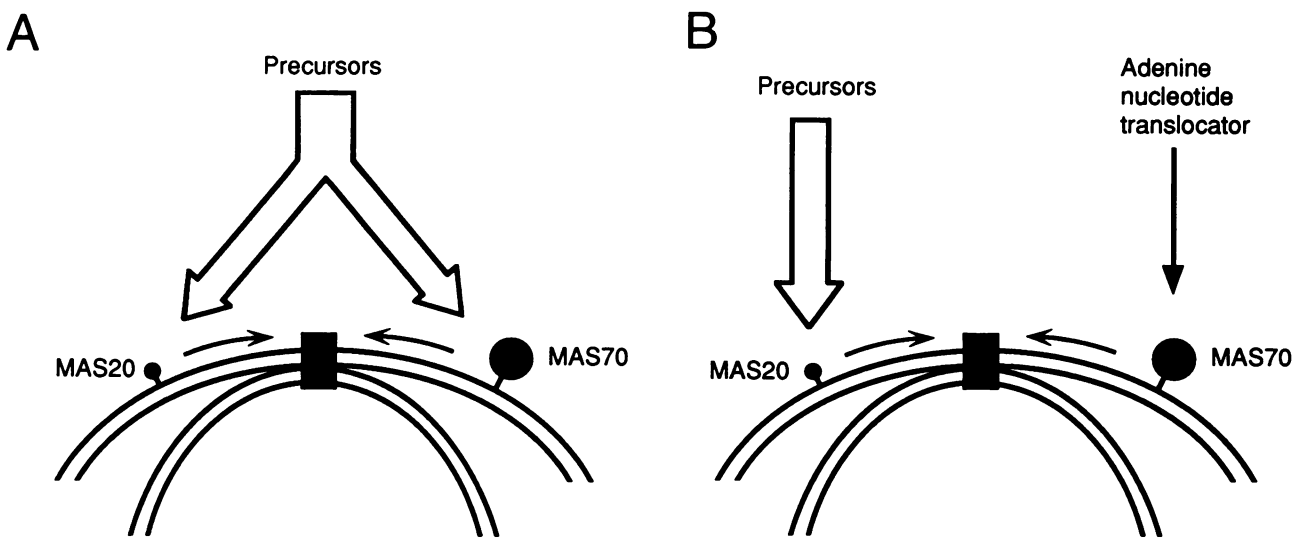

Fig. 13. Two contrasting models for the function of MAS20/MOM19 and MAS70/MOM72 as mitochondrial protein import receptors. (**A**) The two receptors have overlapping precursor specificities. Our data favour this model. (**B**) MAS20/MOM19 is the 'master receptor', whereas MAS70/MOM72 is specialized for the import of only a few precursors.

Isolation of mitochondrial outer membranes

Outer membranes were prepared by swelling mitochondria in 10 mM Tris−P_i pH 7.4 and 1 mM PMSF for 30 min on ice, then shrinking them by the addition of sucrose, $MgCl_2$ and ATP to final concentrations of 0.45 M, 5.4 mM and 1.2 mM, respectively, and exposing them to sonic oscillation (Riezman *et al.*, 1983a). The sonicated mitochondria were centrifuged at 20 000 *g*. The resulting supernatant was centrifuged at 235 000 *g* for 2 h, the pellet was resuspended in buffer B (5 mM HEPES−KOH pH 7.4, 10 mM KCl, 1 mM $MgCl_2$ and 1 mM dithiothreitol) and centrifuged for 16 h at 140 000 *g* in a sucrose step gradient (1.6/1.35/1.1/0.85 M sucrose in buffer B). The outer membranes collected at the 0.85/1.1 M interface; they were removed, diluted with buffer B, pelleted at 235 000 *g*, resuspended in buffer B and quick-frozen in liquid nitrogen as small aliquots.

Antiserum and IgGs

Mitochondrial outer membranes were subjected to SDS−13% PAGE (see Figure 1). The gel was stained with Coomassie brilliant blue, the MAS20 band was excised, and the protein was electroeluted. One hundred micrograms of the SDS-denatured protein were injected into multiple subcutaneous sites of a female New Zealand rabbit. The first injection was in 50% Freund's complete adjuvant and subsequent injections were at three-week intervals in 50% Freund's incomplete adjuvant. Antibody titres were monitored by immunoblotting, using mitochondrial outer membranes resolved on SDS−PAGE as antigen. IgGs were affinity-purified on proteins that had been immobilized on nitrocellulose sheets, visualized by transient staining with Ponceau Red and excised. Anti- MAS20 IgGs were affinity-purified on the prominent 23 kDa band from mitochondria isolated from a MAS20-overproducing yeast strain (see Figure 4). The anti-porin IgGs were purified against the porin band from outer membranes derived from normal yeast mitochondria. The IgGs were eluted with 4 mg/ml bovine serum albumin adjusted to pH 2.3 with acetic acid, quickly neutralized with NaOH, then further purified and concentrated on protein A−Sepharose beads (Ey *et al.*, 1978).

Isolation of the MAS20 gene

A library of random yeast genomic DNA fragments in the phage lamda gt11 (Davis, 1976) was screened with affinity-purified anti-MAS20 IgGs. The inserts of the immunoreactive clones were examined by PCR using primers specific for lambda gt11. The 650 bp fragment obtained from one of the positive lambda clones was labelled using ECL random primers according to the manufacturer's instructions (Amersham International) and used to screen a library of large random fragments of yeast genomic DNA in the yeast−*E.coli* shuttle vector pFL1 (Chevalier *et al.*, 1980). The positive pFL1 clones were tested for their ability to overexpress MAS20 by transforming the yeast strain JKR101 with the plasmids (see Figure 4).

Sequencing

The *MAS20* gene was sequenced by the dideoxy method (Sanger *et al.*, 1977) using a Sequenase kit (United States Biochemicals) following the manufacturer's instructions. A direct step-wise approach, employing nucleotide sequence information to design priming oligonucleotides, was used to sequence both DNA strands.

Gene disruption

A 1.2 kb genomic *Bam*HI−*Cla*I fragment containing the *MAS20* gene was subcloned into the *E.coli* vector pBluescript KS (Stratagene). The yeast *URA3* gene was inserted as a blunt-ended 1.2 kb fragment into the *Eco*RI site (rendered blunt-ended) of the *MAS20* gene. The resulting 2.4 kb *Bam*HI−*Cla*I fragment was excised and used to transform the diploid yeast strain YKB5 to uracil prototrophy. The result was verified by Southern blotting (Southern, 1975). Chromosomal DNA was prepared from the resulting transformants as described by Riezman *et al.* (1983c).

Chromosomal location of MAS20

S.cerevisiae chromosomes (Boehringer) were resolved on a 1% agarose gel on a Rotophor apparatus as recommended by the manufacturers. The chromosomes were transferred onto Genescreen Plus (New England Nuclear) and probed with the ECL-labelled *Bam*HI−*Cla*I fragment containing the *MAS20* gene. Subsequently, filter grids containing immobilized DNA from an ordered set of overlapping lambda clones carrying fragments of yeast genomic DNA (provided by Dr Maynard Olson, Washington University) were probed with the *MAS20* gene.

Miscellaneous

Published methods were used for the transformation of yeast cells (Ho *et al.*, 1983) and *E.coli* (Mandel and Higa, 1970), SDS−PAGE (Daum *et al.*, 1982), isolation of mitochondria (Daum *et al.*, 1982), *in vitro* transcription/translation of precursor proteins in the presence of [^{35}S]methionine (Hurt *et al.*, 1984), import into isolated mitochondria (Glick *et al.*, 1992), pulse−chase experiments followed by immunoprecipitation (Brandt, 1991) and immunoblotting (Haid and Suissa, 1983). Protein was measured by the BCA procedure according to a protocol distributed by Pierce Chemicals.

Acknowledgements

We thank Dr Maynard Olsen and Ms Linda Kiles (Washington University) for their invaluable advice and help in the chromosomal mapping experiments, Drs Walter Neupert (University of Munich) and Michael G.Douglas (University of North Carolina, Chapel Hill) for the genes encoding SU9-DHFR and the adenine nucleotide translocator, respectively, Hildegard Brütsch for excellent technical assistance, Clemens Wachter for his help with the *in vitro* import experiments and Kitaru Suda for his help in the computer-aided analysis of the *MAS20* gene. This study was supported by grants from the Swiss National Science Foundation (3-26189.89), the US Public Health Service (2-RO1-GM-37803), and the Human Frontiers Science Program Organization. Trevor Lithgow is supported by a long-term fellowship from the Human Frontiers Science Program Organization.

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Received on June 7, 1993; revised on July 29, 1993