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Identification of yeast *MAS17* encoding the functional counterpart of the mitochondrial receptor complex protein MOM22 of *Neurospora crassa*

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Abstract MOM22 of *Neurospora crassa* was suggested to be required for the transfer of mitochondrial precursor proteins from the receptors to the protein translocation machinery. We isolated a yeast mutant the viability of which depended on the expression of the introduced *N. crassa MOM22* gene. The mutant cells showed defects in protein import into mitochondria when the cells were depleted of MOM22. By screening for suppressor genes for the mutant, we could identify the yeast gene *MAS17* encoding the functional counterpart of MOM22. The *MAS17* gene was found to be essential for the viability of yeast cells.

Key words: Protein import; Mitochondrial outer membrane; MOM22; MAS17

1. Introduction

Most mitochondrial proteins are encoded by the nuclear genome, synthesized in the cytosol as a precursor protein, and transported to mitochondria. The mitochondrial outer membrane contains a protein complex mediating the specific recognition, membrane insertion and translocation of precursor proteins across the outer membrane. This receptor complex probably consists of several different proteins having distinct functions [1,2]. MAS20 and MAS70 in yeast *Saccharomyces serevisiae* or MOM19 and MOM72 in *Neurospora crassa* apparently function as import receptors with overlapping specificities [3–6]. ISP42 in yeast or MOM38 in *N. crassa* appears to form a machinery that mediates the insertion of precursor proteins into the outer membrane and their translocation across the membrane [7,8]. MOM22 in *N. crassa* was suggested to be required for the transfer of precursor proteins from the receptors to the protein translocation machinery involving MOM38 since the antibodies against MOM22 inhibited the import of many mitochondrial precursor proteins into mitochondria but not their binding to the receptors in vitro [9]. However, the yeast equivalent of MOM22 has not been identified yet and the function of MOM22 in vivo remains unknown.

In order to analyze the roles of MOM22 in mitochondrial protein import in vivo, we started identifying the yeast equivalent of *N. crassa* MOM22 by the 'cloning-by-function' approach [10]. We obtained a yeast mutant the viability of which depended on the expression of the introduced *N. crassa MOM22* gene. The mutant cells showed defects in protein import into mitochondria when the cells were depleted of *N. crassa* MOM22. By screening for suppressor genes for the mutant strain, we could identify the yeast functional counterpart of MOM22, which is now termed MAS17 (for mitochondrial assembly). Haploid cells lacking a functional copy of *MAS17* were shown to be inviable, suggesting that MAS17 is an essential protein of yeast.

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Abbreviations: MAS17, MAS20, MAS70, yeast mitochondrial outer membrane proteins (receptor complex proteins) of about 17 kDa, 20 kDa and 70 kDa; ISP42, yeast mitochondrial outer membrane protein (import site protein) of about 42 kDa; MOM19, MOM22, MOM38, MOM72, *N. crassa* mitochondrial outer membrane proteins (receptor complex proteins) of about 19 kDa, 22 kDa, 38 kDa and 72 kDa; PCR, polymerase chain reaction; $F_1\beta$, β-subunit of the F_1-ATPase; Cox IV, cytochrome oxidase subunit IV.

2. Materials and methods

2.1. Strains and plasmids

The following yeast strains were used: CH1305 (MAT**a** *ade2 ade3 leu2 ura3 lys2*) [10] and W303 (MAT**a**/MATα *ade2-1/ade2-1 his3-11, 15/his3-11, 15 ura3-1/ura3-1 leu2-3, 112/leu2-3, 112 trp1-1/trp1-1 can1-100/can1-100*). Yeast–*Escherichia coli* shuttle vector pCH1153 bearing *ADE3* and *URA3* as selectable markers, provided by Dr. C. Holm (Harvard University), and pYE-Ura3 (Clontech) bearing the yeast *GAL1-GAL10* promoter were used for the expression of the *N. crassa MOM22* gene in yeast. Plasmid pJJ217 [11] containing the *HIS3* gene was used for the disruption of *MAS17*.

2.2. Isolation of MOM22 cDNA and its expression in yeast

The *MOM22* cDNA was cloned from the *N. crassa* cDNA library, which was obtained from Fungal Genetics Stock Center (The University of Kansas Medical Center), by polymerase chain reaction (PCR) using primers based on the published sequence [9]. The amplified DNA fragment was inserted into pCH1153, placing the gene under control of the *GAL1* promoter (derived from pYE-Ura3). The resulting plasmid, pGAL:*MOM22*, was transformed into yeast strain CH1305. Expression of the *N. crassa MOM22* gene in yeast was induced by cultivating the transformant in rich medium (1% yeast extracts and 2% polypeptone) containing 2% galactose, or repressed by cultivating in the same medium containing 2% glucose instead of galactose.

2.3. Isolation of non-sectoring mutants

CH1305 cells containing pGAL:*MOM22* were mutagenized by UV-irradiation, and non-sectoring mutants were isolated according to the published procedure [10]. One of the non-sectoring mutants, which exhibited galactose-dependent viability and was inviable when grown on glucose medium, was designated as MN/M1-2, and used for further experiments.

2.4. Isolation of the MAS17 gene from yeast

MN/M1-2 cells were transformed with a yeast genomic DNA library which had been constructed on vector YEpM4 (provided by Dr. J. Nikawa, Kyusyu Institute of Technology). The transformants were screened for the regained ability to grow on glucose medium and to produce white sectors, and 16 colonies were isolated. Library plasmid DNA was purified from each of the colonies and restriction mapped. Comparison of the restriction digests revealed that each plasmid contained a common 1.7 kb fragment. The DNA fragment was sequenced with Taq Dye Primer Cycle Sequencing Kit (Applied Biosystems).

2.5. Disruption of the MAS17 gene

The 5′-upstream 770-bp and 3′-downstream 450-bp regions flanking the *MAS17* gene were PCR-amplified and inserted at both sides of the

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HIS3 gene of pJJ217. The resulting fragment containing the *MAS17* gene flanking regions and the *HIS3* gene was used to transform the diploid yeast strain W303. The heterozygous *MAS17* diploid strain was subjected to tetrad analysis.

2.6. Production of the anti-MOM22 antiserum
The *N. crassa* MOM22 cDNA was ligated into pGEM-EX1 expression vector (Promega), and the fusion protein between T7 gene *10* protein and MOM22 was expressed in *E. coli* cells and purified. The fusion protein was used to raise antibodies against MOM22.

2.7. Miscellaneous
The following procedures were performed essentially according to the published methods: DNA manipulations [12], yeast genetics [13], immunoblotting [14], and computer analyses of DNA sequences [14,15].

3. Results and discussion

We raised antibodies against MOM22 of *N. crassa* and used them to look for a possible yeast homologue of MOM22 by immunoblotting. However, the anti-MOM22 antibodies did not specifically recognize any yeast mitochondrial protein with MW of ≈20 kDa (data not shown).

The cloning-by-function strategy is useful for cloning essential yeast genes that are functionally equivalent to genes from other organisms, even in the case where antibody cross-reaction or DNA hybridization does not work [10]. This approach involves first identifying a yeast mutant that depends on a plasmid expressing a foreign gene of interest. The corresponding yeast gene is then cloned by complementation of the mutant defect. The plasmid dependence of the cells can be easily detected by using a red/white colony sectoring assay [16].

In order to apply the cloning-by-function strategy to identify a yeast homologue of *N. crassa* MOM22, we first constructed a plasmid-borne *N. crassa MOM22* gene the expression of which was controlled by the galactose-inducible *GAL1* promoter, and introduced it into the *ade2 ade3* yeast strain CH1305. The transformed cells exhibited normal cell growth on galactose medium (Fig. 1), and the expression of *MOM22* in

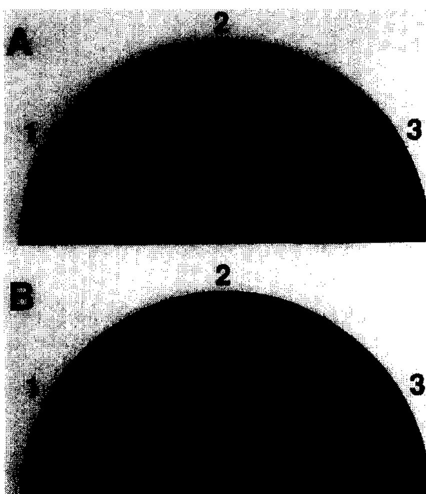

Fig. 1. Galactose-dependent growth of the mutant yeast strain, MN/M1-2. CH1305 cells (1), CH1305 cells harboring pGAL:*MOM22* (2) and MN/M1-2 cells (3) were streaked onto galactose-containing medium (A) or glucose-containing medium (B), and incubated for 5 days at 30°C.

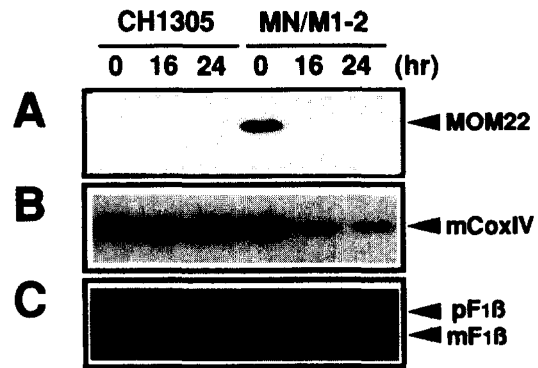

Fig. 2. Depletion of *N. crassa* MOM22 causes impaired mitochondrial protein import in MN/M1-2 mutant cells. Strains CH1305 (control) and MN/M1-2 (mutant) were grown in rich medium containing 2% galactose to late-log phase and then diluted into fresh rich medium containing 2% glucose instead of galactose. At various times after transfer to glucose medium, cell extracts were prepared and aliquots were analyzed for MOM22 (A), Cox IV (B), and $F_1\beta$ (C) by immunoblotting. mCoxIV, mature form of Cox IV; $pF_1\beta$ and $mF_1\beta$, precursor or mature form of $F_1\beta$, respectively.

galactose-containing medium was confirmed by immunoblotting (Fig. 2). Since the introduced plasmid contained the $ADE3^+$ gene, the *ade2* $ADE3^+$ transformant formed red colonies with many white *ade2 ade3* sectors which resulted from frequent plasmid loss events under the non-selectable conditions [10].

The transformed cells were then mutagenized, and the colonies they produced were examined for sectoring morphology on galactose-containing medium. If mutant cells require plasmid function for survival, they will form red colonies without white sectors. In a screen of 100,000 colonies, several reproducibly non-sectoring mutants were isolated. The obtained mutant strains were further analyzed as to whether or not they could grow on glucose-containing medium, where the expression of the introduced *N. crassa MOM22* was repressed. One of the mutants, named MN/M1-2, was inviable on glucose-containing medium, but viable on galactose-containing medium (Fig. 1), suggesting that the growth of the mutant cells depended on the expression of *N. crassa MOM22*.

If MN/M1-2 carries a mutation in a putative yeast homologue of the *N. crassa MOM22* gene, depletion of *N. crassa* MOM22 in the cells may impair mitochondrial protein import. As shown in Fig. 2, by 16 h after transfer to glucose-containing medium, *N. crassa* MOM22 became immunologically non-detectable in the MN/M1-2 cells. To determine the effect of reduced levels of *N. crassa* MOM22 on mitochondrial protein import in the MN/M1-2 cells, we analyzed the amounts of the mature and/or precursor forms of mitochondrial proteins by immunoblotting. The MN/M1-2 cells started to accumulate the precursor form of mitochondrial F_1-ATPase β subunit ($F_1\beta$) at 24 h after a shift to glucose-containing medium (Fig. 2), suggesting a defect in the import of the $F_1\beta$ precursor into mitochondria. Although accumulation of the precursor of another mitochondrial protein, cytochrome oxidase subunit IV (Cox IV), was not observed, the amount of mature Cox IV was already reduced significantly at 16 h after the shift to glucose-containing medium (Fig. 2). This may mean that the import of

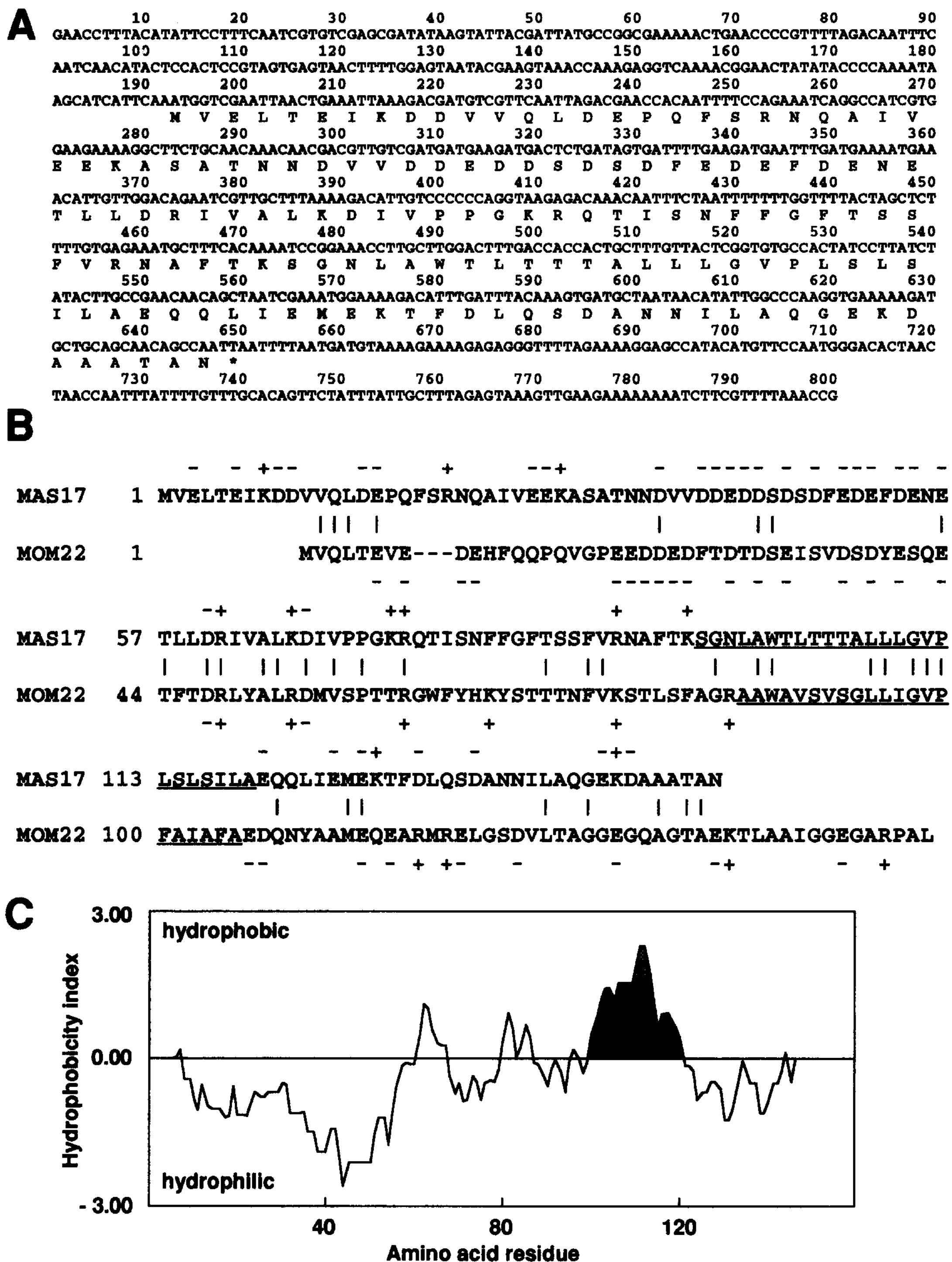

Fig. 3. Nucleotide sequence of the yeast *MAS17* gene (A), comparison of the deduced amino acid sequences of yeast MAS17 and *N. crassa* MOM22 [9] (single letter code) (B), and a hydropathy profile of MAS17 (C). In the sequence alignment (B), identical amino acid residues are marked by vertical bars and basic and acidic residues by + and −, respectively. Average hydropathicity was calculated by the methods of Kyte and Doolittle [15] with a window of 13 amino acids. Putative membrane spanning regions are underlined in B and shaded in C. The sequence data have been submitted to the EMBL/GenBank/DDBJ Data Libraries under the accession number X82405.

the Cox IV precursor into mitochondria was also impaired but that the precursor form was quickly degraded in the cells. These results suggest that the MN/M1-2 mutant has a defect in mitochondrial protein import which could be suppressed by the expression of *N. crassa MOM22*. The MN/M1–2 mutant most likely carries a mutation in the yeast homologue of the *N. crassa MOM22* gene.

Now the yeast gene homologous in function to the *N. crassa MOM22* gene can be cloned by transforming the MN/M1–2 mutant with a yeast library and looking for the regained ability

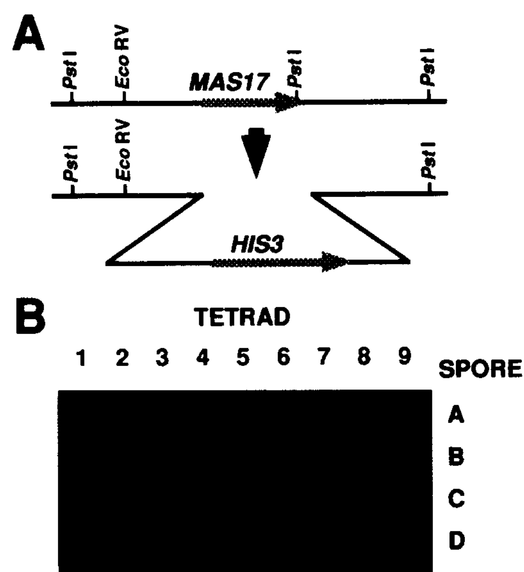

Fig. 4. *MAS17* is essential for cell viability. Strategy for disruption of the *MAS17* gene on the yeast chromosome (A) and the results of the tetrad analysis of the heterozygous *MAS17*+/Δmas17 diploid strain on glucose-containing medium (B).

to grow on gluocse medium and to lose the *N. crassa MOM22* gene on the plasmid [10]. The MN/M1–2 strain was thus transformed with the yeast library, and ≈40,000 transformants were screened directly on glucose-containing medium for reversal of the phenotype. 16 colonies that grew on glucose-containing medium were isolated and were found to exhibit the white sectoring morphology on both glucose-containing medium and galactose-containing medium. The library plasmid was purified from each clone, and then restriction mapped. All the plasmids contained a common DNA fragment of ≈1.7 kb. The nucleotide sequence of this fragment revealed an open reading frame encoding a putative protein of 152 amino acids with a calculated molecular weight of 16,790 (Fig. 3A). As shown in Fig. 3B, the deduced amino acid sequence shows distinct homology to that of *N. crassa* MOM22 [9] (25% identity). Although the sequence homology is somewhat low compared with those for MAS20/MOM19 (38% identity), MAS70/MOM72 (29% identity) and ISP42/MOM38 (40% identity), the deduced amino acid sequence exhibits characteristics common to *N. crassa* MOM22, as described below, and we have therefore concluded that this open reading frame corresponds to the yeast homologue of the *N. crassa MOM22* gene. This gene was named *MAS17*, and its gene product MAS17 (a protein of 17 kDa for mitochondrial assembly).

Like *N. crassa* MOM22 [9], MAS17 has a 25-residue uncharged hydrophobic stretch (residues 95–119) in the middle of the sequence (Fig. 3C), suggesting the same membrane insertion topology as *N. crassa* MOM22. Another interesting feature shared by MAS17 and MOM22 is that the N-terminal hydrophilic regions are highly acidic in both proteins. Since this acidic region was found to be exposed to the cytosol in *N. crassa* MOM22 [9] it may provide a binding site for the positively charged presequence of mitochondrial precursor proteins.

The success in identifying the yeast homologue of the *N. crassa MOM22* gene by the cloning-by-function approach in turn suggests that *N. crassa* MOM22 is functional in yeast and that the yeast MOM22 homologue, MAS17, is an essential protein of yeast. In order to confirm directly that MAS17 is an essential protein of yeast, we disrupted one of the two chromosomal copies of the *MAS17* gene with the *HIS3* gene in a homozygous *his3* diploid yeast strain. The diploid cells were then sporulated, the resulting tetrads were dissected, and spores were allowed to germinate. Only two spores from each tetrad formed visible colonies (Fig. 4), and all viable spores were His$^-$, indicating that spores carrying the null allele of *MAS17* were inviable. We concluded that *MAS17* is essential for the viability of yeast cells. This is in contrast to the other receptor complex proteins, MAS20 and MAS70, neither of which is essential for protein import or cell viability [3,17,18], but is similar to the other proteins (including ISP42, ISP45/MIM44, MAS6/MIM23, Sms1p/MIM17, mitochondrial hsp70 and hsp60, Yge1p, mitochondrial chaperonin 10, and the two subunits of the matrix processing protease) that mediate import, processing and folding of nuclear-encoded mitochondrial proteins and are essential for cell viability [19–21].

We report here the identification of the yeast gene *MAS17*, which encodes a functional equivalent to *N. crassa* MOM22. *N. crassa* MOM22 was functional in yeast, and the function of *N. crassa* MOM22 or that of yeast MAS17 appears essential for mitochondrial protein import and for the viability of yeast cells. The availability of the yeast *MAS17* gene now paves the way for a genetic analysis of the function of MAS17 (MOM22) in mitochondrial protein import.

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