Regulators of mitochondrial translation in *Saccharomyces cerevisiae*

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*Citation for published version (APA):*
Mss51p, a putative translational activator of cytochrome c oxidase subunit 1 (COX1) mRNA, is required for synthesis of Cox1p in Saccharomyces cerevisiae

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Summary

Mutants of Saccharomyces cerevisiae that lack a functional MSS51 gene are respiratory-deficient due to the absence of cytochrome c oxidase subunit 1 (Cox1p). It has been previously suggested, but not formally proven, that Mss51p is required for translational activation of COX1 mRNA, rather than being involved in a subsequent step in the synthesis of Cox1p, or its assembly into cytochrome c oxidase. Pulse-chase labelling experiments now show that the absence of detectable levels of Cox1p in mss51-null strains is indeed due to the lack of synthesis of Cox1p, and is not caused by reduced stability of the protein. To get more insight into the exact function of Mss51p, we determined the subcellular localization of the protein. We were able to show that an epitope tagged version of Mss51p (Mss51HA) complements the mutation and can be localized in mitochondria where it is firmly associated with the mitochondrial inner membrane. In addition, we characterized the previously identified mutant allele mss51-3. Sequence analysis revealed the presence of a short open reading frame upstream of MSS51 resulting from the creation of an extra ATG startcodon.

Introduction

Mitochondria possess their own genome together with the machinery necessary to express the information contained within it. In yeast, the mitochondrial genome encodes seven protein subunits of the inner membrane, involved in respiration and oxidative phosphorylation (Pel et al., 1994). Although most of the genes encoding components of the mitochondrial translational apparatus have been identified in Saccharomyces cerevisiae, the process of translational initiation in yeast mitochondria is still poorly understood. Mitochondrial mRNAs in S. cerevisiae are unusual in that specific nuclear gene products are required for their translational activation (Grivell, 1995; Fox, 1996). Indirect evidence suggests that these proteins function by interacting with specific regions of the 5'-untranslated leaders (5'-UTL), with the mitochondrial ribosome and with the mitochondrial inner membrane. The nuclear genes PET54, PET122 and PET494, which specifically activate translation of cytochrome c oxidase subunit 3 (COX3) mRNA, are associated with the mitochondrial inner membrane. A complex of these proteins mediates the interaction of the COX3 mRNA with mitochondrial ribosomes (Brown et al., 1994; Wiesenberger et al., 1995). Translation of cytochrome b (COB) mRNA requires the action of CBS1 and CBS2. Cbs1p is tightly associated with the membrane, and Cbs2p has a peripheral association with the membrane and might be associated with mitochondrial ribosomes (Michaelis et al., 1991). These findings have been interpreted in terms of a model in which translational initiation occurs on the matrix face of the inner membrane, allowing the encoded proteins to be synthesized near the site of their assembly into multisubunit respiratory complexes (Michaelis et al., 1991; Dekker, 1993).

As part of a search for mutants specifically disturbed in the splicing of precursors of the mRNA for cytochrome c oxidase subunit 1 (COX1), Decoster identified a nuclear gene, MSS51, the product of which is specifically required for accumulation of normal levels of Cox1p (DeCoster et al., 1990). In an mss51-null strain devoid of mitochondrial introns, levels of COX1 mRNA were found to be normal, but Cox1p was reduced to almost undetectable levels, implying control at the level of translation or a subsequent step in maturation or assembly of the Cox1 protein. A potentially informative mutant allele is mss51-3, reported by DeCoster, mss51-3 mutants display a partial deficiency in respiratory growth (DeCoster et al., 1990). The effect of mss51-3 is enhanced in a strain carrying the PR454 (Li et al., 1982) mutation. This mutation is located in a conserved hairpin in the mitochondrial 15S rRNA and results in resistance to the antibiotic paromomycin. Paromomycin is known to interfere with the decoding process at the ribosomal A site. These findings are suggestive of, but do not prove a role for Mss51p as an initiation factor.
specific for the translation of the COX1 mRNA. As initial step in the elucidation of the function of Mss51p in Cox1p synthesis/assembly, we demonstrate here that (i) absence of detectable levels of Cox1p in a mss51-null strain is indeed due to lack of Cox1p synthesis (ii) Mss51p is a mitochondrial protein tightly associated with the inner membrane, and (iii) impaired respiratory growth of strains carrying the mss51-3 allele is caused by drastically reduced synthesis of Mss51p. Implications of these findings for the mode of action of Mss51p are discussed.

Material & Methods

Strains and media
To test complementation of an mss51-null strain by the HA-tagged MSS51 construct, yeast cells were grown at 30 °C on MM+AHW, YPGlyc or YPD plates. MM+AHW plates: 0.67% YNB, 2% glucose, 2.2% agar and 20 mg/l Adenine, Histidine and Tryptophan. Rich media: 1% bacto-peptone, 1% yeast-extract, 2% glycerol (YPGlyc) or 2% glucose (YPD) or 2% galactose (YPGal), 2.2% agar. To isolate mitochondria yeast were grown at 30 °C in YPGlyc or YPGal medium. The mutant gene mss51-3 was isolated from strain A278 (met6, lys2, can^R, mss51-3, [M12,54]), a kind gift of prof. dr. G. Faye (DeCoster ef al., 1990). Strain W303(167)/Δmss51 has the nuclear genotype MATα, ade2-1, his3-11,-15, trp1-1, leu2-3,-112, Δmss51::URA3, the mitochondrial genome is derived from KAR167. The Mss51HA fusion protein was expressed in this strain by transforming the strain with the desired plasmid. E. coli strain DH5α was used in all DNA manipulations requiring E. coli. E. coli cells were grown in 2YT (1.6% Bacto tryptone, 1% yeast extract, 0.5% NaCl), supplemented with ampicillin (100 mg/l) as necessary. Yeast was transformed with plasmid DNA by the one step method as described by Chen (Chen ef al., 1992). E. coli was made competent and transformed with plasmid DNA by electroporation (Sambrook ef al., 1989). Plasmid DNA was isolated from E. coli according to the CTAB-method (Del Sal ef al., 1988).

Cloning of the wild type and the mutant MSS51 gene
To clone the wild type MSS51 gene and the mutant gene mss51-3 mini libraries were constructed. Chromosomal DNA of wild type W303(167)alpha and mutant strain A278 was digested with EcoRV, leaving the MSS51 gene on a 3611 bp fragment. Fragments between 3 kb and 4 kb were isolated from gel (Qiagen extraction kit) and cloned into the Smal site in the multiple cloning site of YCplad 11 (Gietz ef al., 1988). The mini library was transformed to W303(167)/Δmss51 to isolate the genes by complementation on YPGlyc. From transformants that complemented the disruption strain to wild type level or to mutant level, plasmid DNA was isolated. The presence of MSS51 was checked by digestion with ORF specific restriction enzymes. This resulted in the constructs pMS1 (wild type gene in YCplad 11) and pMS10 (mutant gene in YCplad 11). Both the wild-type and the mutant gene were sequenced according to the dideoxy-method of Sanger (Sanger ef al., 1977).

Addition of C-terminal tags to MSS51
To be able to detect the Mss51 protein in whole cells, the protein was tagged with the green fluorescent protein (GFP) at its C-terminus. By means of PCR on chromosomal DNA (S. cerevisiae FY1679-28) the MSS51 gene was amplified, using primers: 5'-gggggttataataATGACCGTGCTATAT-CTCC-3' and 5'-cccgccgcccggcggcgccgGT-GCGT-CTCTTGTGATTGG-3'. In this PCR Pstl and NotI sites (underlined) were introduced at the 5'-end and 3'-end of the gene, respectively. The PCR product was digested with Pstl and NotI and ligated into pGH8, a modified version of p425TEF (Mumberg ef al.,...
Construct pMS1 was used to tag MSS51 at its C-terminus with the HA-tag (YPYDVDPYA), an epitope derived from the human influenza hemagglutinin protein (Wilson et al., 1984).

To create the C-terminal fusion a primer was designed that contains the coding information for the last 8 amino acids of the gene, changes the stop codon in a sense codon, introduces an in-frame HA-tag and a new stop codon. This primer, 5'-AAGGTACCATACAATCAAGAGAÇAGCTGTACCACTACGACGTCCCAGACTACGCTTG ACTAGTTATCGGGAGGCCATGCC-3', adds a PvuII restriction site directly upstream of the tag and a SpeI restriction site directly downstream of the tag (both are underlined in the primer sequence). Together with the universal M13 primer and under standard PCR conditions this yields a PCR-product of 172 bp. This PCR-product was digested with KpnI, in the 3' part of the gene (indicated in bold in the primer sequence), and in the multiple cloning site of YCplac111, and cloned in KpnI digested pMS1. Resulting clones were analyzed on restriction pattern by checking insertion of an extra PvuII restriction site. Positive clones were sequenced to confirm the in-frame fusion between the 3' end of the MSS51 ORF and the HA-tag. This construct (pMS3) was transformed to W303(167)Δmss51 to confirm the ability to complement the disruption. To look at higher expression levels of the fusion protein, the MSS51HA fusion was cloned into the multicopy vector YEplac181 (Gietz et al., 1988), resulting in construct pMS7. To confirm the changed expression level caused by the mutation in mss51-3, the C-terminal HA-tag was added to the mutant gene. A 970 bp Hpal/Xbal restriction fragment from pMS3 was cloned in pMS10, this resulted in an in-frame fusion of the mss51-3 gene and the HA-epitope (pMS11). All constructs used in this study are summarized in Table 1.

Table I: Constructs used in this study

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<td>pGH8</td>
<td>MSS51GFP</td>
<td>This study; Mumberg et al., 1995; Kruckeberg et al., 1999.</td>
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Detection of GFP

Independent yeast colonies from Δmss51::pGJ1 were picked from a fresh MM+AHW plate. After o/n growth in 3 ml of MM+AHW, these cultures were diluted into 3 ml fresh MM+AHW to an OD600 of about 0.3. Dapi was added to the culture (final concentration: 0.2 µg/ml). After growth for about one generation cells were harvested, and analysed by fluorescence microscopy.

Isolation of mitochondria

Strain Δmss51::pMS3 was grown on YPGlyc, after 2 days of growth cells were harvested and washed in water. Cells were resuspended in 2 ml/g wet weight DTT-buffer (100 mM...
TrisSO4 pH 9.4, 10 mM DTT) and shaken at 30 °C for 20 min. After washing in 1.2 M sorbitol, cells were treated with zymolyase in 7 ml zymolyase-buffer (1.2 M sorbitol, 20 mM KPi, pH 7.4) and 2 mg zymolyase per gram wet weight, gently shaking for 30-60 min. at 30 °C. After washing in 1.2 M sorbitol, cells were resuspended in 7 ml/g breaking buffer (0.6 M sorbitol, 10 mM Tris pH 7.4, 1 mM EDTA, 0.2% BSA (fatty-acid free), 1mM PMSF) and potted 10-20 times to break the cells. Mitochondria were isolated from this lysate by differential centrifugation. The mitochondrial pellet was resuspended in SEM (250 mM saccharose, 1 mM EDTA, 10 mM MOPS pH 7.2 (with KOH)). Protein concentration was determined by the method of Bradford (Bradford et al., 1976). Mitochondria were stored at -70 °C.

Fractionation of mitochondria.
Mitochondria of Δmss51::pMS3 were thawed on ice. The mitochondrial fraction (Mit) was prepared by addition of 10% SDS and 50% TCA, incubation on ice for 20 min. and centrifugation at 15,000 rpm for 10 min. Mitoplasts were prepared by osmotic shock, after 10 min. on ice and 15 min. 15,000 rpm the supernatant contains the intermembrane space (ImS) fraction and the pellet consists of mitoplasts. The pellet (mitoplasts) was resuspended in TE/PMSF (10 mM Tris pH 7.5, 1 mM EDTA, 1 mM PMSF). To break the mitoplasts this suspension was sonicated: 6 times 5 sec. After sonication the matrix (Mat) and membrane (Mem) fractions were separated by ultracentrifugation for 1 hour at 4 °C in an SW50.1 rotor. In the salt extraction experiment sonication was performed with mitoplasts in TE/PMSF with 0, 100, 200, 300, 400 and 500 mM NaCl. To extract soluble proteins and peripheral membrane proteins sodium carbonate extraction was carried out. Mitochondria were thawed on ice and harvested by centrifugation for 10 min. 10,000 rpm. The pellet was resuspended in 100 mM Na2CO3 pH 11.5 (0.2-1 mg protein/ml). After 45 min. on ice and 1 hour centrifugation at 40,000 rpm in an SW50.1 rotor the supernatant contains soluble proteins and peripheral membrane proteins and the pellet contains integral membrane protein.

Analysis of mitochondrial fractions by SDS-PAGE and Western blotting
Proteins were resolved in 15% SDS PAGE gels and transferred to nitrocellulose (0.45 μm, Schleicher & Schuell) or Immobilon-P (0.45 μm, Millipore). Gels were electrotransferred at 90 V for 1 hour. Prior to incubation with antibodies, the membranes were blocked by incubation in blocking buffer (Phosphate buffered saline (PBS), 0.05 % Tween-20, 0.5 % gelatin) for 1.5 hrs to overnight. After blocking the membranes were incubated with antibodies for 1.5 hrs to overnight. Mss51HAp was detected with the monoclonal antibody 12CA5 (Boehringer Manheim) at a 1: 1,000 dilution in PBS/Tween. Polyclonals anti-AAC and anti-TIM44 were used at a 1: 1,000 dilution. The goat anti-rabbit and the goat anti-mouse secondary antibodies, conjugated to horse radish peroxidase (Bio-Rad Laboratories), were used at 1: 10,000 dilution. Western blots were developed with HRP-color development (Bio-Rad Laboratories).

Labelling of mitochondrial translation products (Pulse-Chase)
Strains were grown in YPGal (1% bacto-peptone, 1% yeast extract, 2% galactose). Mitochondria were isolated as described previously. In the labelling experiment mitochondria were resuspended in translation buffer (750 mM mannitol, 187.5 mM KCl, 18.75 mM KH2PO4, 15.675 mM MgSO4, 5 mM ATP, 0.625 mM GTP, 6.25 mM ketoglutarate, 6.25 mM Phosphoenolpyruvate, 0.125 mM amino acids, 25 mM Tris-base, 6.25 μg/ml cycloheximide, 3.75 mg/ml BSA, pH 7.2). After addition of TRAN35S-LABEL (1175 Ci/mmol, 10.5 mCi/ml, containing 70% L-(35S)methionine and 15% L-(35S)cysteine; ICN Biomedicals, Inc.) and pyruvate kinase this reaction was incubated at 28 °C,
vigorously shaking. Pulse (protein synthesis) samples were taken after 10 and 30 min. At 30 min, 1/10 volume 0.2 M methionine was added and chase samples (protein degradation) were taken at 40, 60 and 90 min. Samples were pelleted, the pellet was washed once in 0.6 M mannitol/1 mM EDTA, pH 6.7. After electrophoresis on 11% SDS-PAGE, bands were visualized using a phosphorimager (Molecular Dynamics).

Miscellaneous
All DNA manipulations were done according to standard techniques as described by Sambrook (Sambrook et al., 1989). Sequencing was performed according to the dideoxy-method of Sanger (Sanger et al., 1977). Protein samples were separated by SDS-PAGE according to the method of Laemmli (Laemmli et al. 1970).

Results

Cloning and tagging of the MSS51 gene
The wild type MSS51 gene was cloned by functional complementation of a mss51-null strain (W303(167)Amss51 on YPGIyc using a mini-library (see Materials and Methods) of DNA from the strain W303(167)alpha constructed in the vector YCplac111 (Gietz et al., 1988). To allow detection of Mss51p, coding sequences for the hemagglutinin epitope (HA) or the green fluorescent protein (GFP) were fused in-frame to the 3'-end of the PCR amplified MSS51 gene (see Materials and Methods). Single- and multicopy plasmids containing either the MSS51HA- or the MSS51GFP fusions were introduced in a mss51-null yeast strain to verify the ability of these constructs to complement the disruption. Growth of these strains was indistinguishable compared to the wild-type strain under all conditions tested (data not shown).

Mss51p is necessary for translation of COX1 mRNA
DeCoster et al. have previously shown by labelling of mitochondrial translation products that lack of a functional MSS51 gene leads to a specific loss of Cox1p, without significantly affecting the level of the corresponding mRNA (DeCoster et al., 1990). They suggested that Mss51p is a factor specifically required for the translation of cytochrome c oxidase subunit 1 (COX1) mRNA. However, because the labelling times employed were long (60 minutes), they were unable to distinguish between lack of mRNA translation or destabilization of the resulting translation product with consequent enhanced turnover. To discriminate between these options we performed short-term pulse-chase labelling experiments to be able to look at the balance between initial rates of protein synthesis and turnover. Mitochondrial proteins were labelled for 5 and 30 min using 35S-methionine (pulse), followed by the addition of an excess of unlabelled methionine (chase; see Figure 1). The results clearly indicate that the absence of detectable levels of Cox1p in the disruptant strain is due to a lack of synthesis, and not the result of increased turnover (compare Fig. 1 panels A and B). A pulse-chase labelling experiment with the partial respiratory deficient mutant mss51-3 is consistent with this. Steady state levels of Cox1p were specifically decreased, while the stability of Cox1p was not changed (compare Fig. 1 panels A and C). These and previous results show that Mss51p is involved in the translation initiation of COX1 mRNA. Interestingly, in the disruptant strain Var1p, Cox2p/Cobp and Cox3p/Atp6p show a reduced level of synthesis as compared to wild type, while in mutant mss51-3 all other proteins were synthesized at levels comparable to wild type.
Figure 1: Mss51p is involved in translation initiation of COX1 mRNA. Mitochondria of W303(167) (panel A), Δmss51 (panel B) and Δmss51::pMS11 (panel C) were isolated from YPGal grown cultures. Equal amounts of mitochondria (500 μg) were incubated with 35S labelled methionine, after 10 and 30 minutes pulse samples were taken, at 30 minutes the labelling reaction was stopped by addition of excess of methionine, to follow the turnover of the synthesized proteins. During the chase, samples were taken after 10, 30 and 60 minutes. Samples (100 μg) were taken at the indicated time points and analyzed on 11% SDS-PAGE. Bands were visualized using a phosphorimager (Molecular Dynamics) and quantified using ImageQuant.

Mss51p is a mitochondrial protein, associated with the inner membrane
Based on the putative role of Mss51p as a translational activator, it seems reasonable to assume that the protein is located in mitochondria. Fluorescent microscopic examination of cells expressing Mss51p tagged at its C-terminus with GFP (pGJ1, Table 1) indeed showed that the Mss51GFP-fusion is found in particulate structures (Fig. 2A, right panel) that co-stain with dapi, which stains mtDNA and nuclear DNA (Fig. 2A, left panel).

Independent confirmation of a mitochondrial location was obtained by analysis of cells expressing HA-epitope tagged Mss51p. Mitochondrial and cytoplasmic fractions were isolated from the disruptant strain transformed with YCplac111 or the HA-tagged constructs (pMS3 and pMS7, Table 1), grown in YPGal and analysed by Western blotting. As shown in Fig 2B, a polypeptide of approximate Mr 44 kDa is observable in mitochondria of strain Δmss51::pMS3. This band is absent in the cytoplasmic fractions (data not shown) and in strains without tagged Mss51p (Fig. 2B, lane 1). Multicopy expression of Mss51HA (pMS7, Table 1) results in an increased signal (Fig. 2B, lane 3), providing additional confirmation of specificity of detection. From both fluorescent microscopic analysis and subcellular fractionation it is concluded that Mss51p is localized in the mitochondria.
Figure 2: Detection of Mss51p in mitochondria. A: a GFP-tagged version of the MSS51 gene was expressed in W303(167)Δmss51, in the right panel the fluorescent microscopic analysis of these cells is shown. The left panel shows dapi-staining of one of these cells, which stained the nuclear and mitochondrial DNA. B: Isolated mitochondria of strain W303(167)Δmss51 transformed with YCplac111, pMS3 or pMS7 (Table 1) were analyzed by Western blot analysis. Equal amounts (20 μg) of mitochondrial protein were resolved on a 15% SDS-PAGE gel. After transfer to nitrocellulose membrane, the HA-signal was detected with the monoclonal antibody 12CA5.
Figure 3: Mss51p is associated with the mitochondrial inner membrane. Mitochondria (Mit) of strain Δmss51::pMS3 were fractionated into three different fractions: the intermembrane space (Ims), matrix (Mat) and membrane (Mem), respectively. Mitochondrial protein fractions were resolved on 15% SDS-PAGE gels. Equal amounts (100 μg) of the respective fractions were analyzed. The HA-signal was detected with the monoclonal antibody 12CA5.

Figure 4: Mss51p is a peripheral membrane protein. Mitochondria (M) of strain Δmss51::pMS3 were subjected to Na2CO3-extraction. After centrifugation the supernatant (S) contained soluble proteins and peripheral membrane proteins and the pellet (P) consisted of integral membrane proteins. Equal amounts of the fractions (100 μg) were resolved on 15% SDS-PAGE gels. The HA-signal was detected with the monoclonal antibody 12CA5.

To determine in which mitochondrial compartment Mss51p is localized, mitochondria of strain Δmss51::pMS3 were fractionated into mitoplast and IMS fractions by osmotic shock. The mitoplast fraction was subsequently divided in matrix and membrane fractions by sonication. The quality of the fractionation was monitored by using antibodies for the ATP-ADP Carrier (Aac1p; integral inner membrane protein), and Tim44p (peripheral inner membrane protein). As observed previously (Blom et al., 1993), part of Tim44p appears in
the matrix fraction (Fig. 3, lane 3). Fig. 3 shows that Mss51HAp is firmly associated with the membrane fraction and that it co-fractionates with both Aac1p and Tim44p. In a separate experiment, the strength of the membrane association of Mss51p was assessed by sonication of mitoplasts in the presence of increasing NaCl concentration. Mss51HAp remains associated with the membrane in the presence of 500 mM NaCl (data not shown), indicating that Mss51p is either an integral membrane protein or a tightly bound peripheral membrane protein. To distinguish between these possibilities, mitoplasts were extracted with sodium carbonate. After Na$_2$CO$_3$ extraction Mss51HAp was detected in the soluble fraction (S, Fig. 4). Controls for Na$_2$CO$_3$ extraction are the integral membrane protein Aac1p (insoluble fraction P, Fig. 4), and the peripheral inner membrane protein Tim44p (soluble fraction S, Fig. 4). Co-localization of Mss51p with Tim44p indicates that Mss51p is a peripheral inner membrane protein. Taken together, these results clearly show that Mss51p is a mitochondrial protein, that is tightly associated with the mitochondrial inner membrane.

Characterization of mutant mss51-3

Decoster et al. described three mutant alleles of the MSS51 gene. mss51-1 is a nonsense mutation resulting in loss of function, while mss51-2 is simply a disruption produced by insertion of the LEU2 gene (DeCoster et al., 1990). A mutant strain containing the third allele, mss51-3, was identified in a screen for nuclear genes involved in mitochondrial splicing. In this screen, a strain lacking mitochondrial introns displayed strongly reduced respiratory growth. This suggests that at least a part of the effect on RNA splicing might be indirect, resulting from impaired translation. Respiratory deficiency of the mss51-3 mutation is enhanced in a strain additionally carrying the PR454 (Li et al., 1982) mutation, suggestive of an interaction between Mss51p and ribosomes.

To investigate the nature of the mutation, the mss51-3 gene was cloned in YCplac111 by complementation of an MSS51 disruption strain. mss51-3 complements the null mutant only partially, resulting in extremely slow growth (complementation was assessed after 5 days on YPGlyc, data not shown). Sequence determination revealed only one difference compared to the wild-type MSS51 gene (Fig. 5A). This is a G to A substitution 41 bp upstream of the translational start codon. This mutation creates an ATG, which could potentially serve as an initiation codon for an orf of 4 aa. The context of the ATG determines whether translation initiation might occur at this position. The presence of adenine (A) at position -3 and guanidine (G) at position +4, relative to the new ATG (Fig. 5A), is consistent with the preferences for efficient translation (Kozak, 1997). This makes it very likely that this new ATG is indeed used to initiate translation. Translation initiation at this ATG is expected to severely inhibit translation (Kozak, 1995; Pain, 1996) of the downstream Mss51p orf. In order to determine the expression levels of Mss51p in the mutant mss51-3, expression levels of Mss51p were compared in the disruptive strain transformed with either the HA-tagged wild-type gene (pMS3, Table 1) or the HA-tagged mutant gene (pMS11, Table 1). For the wild type gene we found a clear signal in the lanes containing 100 and 500 µg mitochondrial protein (Fig. 5B), while only a faint signal could be seen in the lane containing 10 µg mitochondrial protein. The expression level of the mutant gene was indeed strongly reduced since only the lane containing 500 µg mitochondrial protein showed a faint signal on the original blot, which could not be reproduced photographically. On the basis of this difference (several experiments, data not shown) it is estimated that the expression level of Mss51p in mutant mss51-3 is at least 100 fold lower than in wild-type. These results strongly suggest that in mutant mss51-3 translation of the MSS51 mRNA is reduced by the introduction of a small open reading frame upstream of the translational start codon.
Figure 5: Characterization of mutant *mss51-3*. **A**: Sequence comparison of the 5' part of the wild-type (*MSS51*) sequence and the mutant (*mss51-3*) sequence. In bold the G to A substitution at position -41, relative to the normal start site (in italics), is indicated. This substitution introduces an upstream ATG (in bold) and a four amino acid upstream open reading frame (underlined). *: G to A substitution, $\Rightarrow$: ATG of the Mss51p orf. **B**: Increasing amounts (as indicated) of mitochondrial proteins of $\Delta$mss51::pMS3 and $\Delta$mss51::pMS11 were resolved on 15% SDS-PAGE gels. The Mss51HA and mss51-3HA fusion proteins were detected with the 12CA5 monoclonal antibody.

**Discussion**

Genetic studies have led to the identification of an unexpectedly large number of nuclear genes, whose products are required for the translation of yeast mitochondrial mRNAs. The translational activators of COX2, COX3 and COB have been extensively studied (Mulero *et al.*, 1993; Brown *et al.*, 1994; Michaelis *et al.*, 1991). These mRNAs require one to three nuclear proteins which act as initiation factors. Less is known about factors required for ATP synthase subunits ATP6 and ATP9 and for COX1. Synthesis of Atp9p is under control of ATP13 (Finnegan *et al.*, 1991) and AEP1 (Payne *et al.*, 1993). Synthesis of Cox1p is controlled by PET309 and MSS51. Both emerged from screens for mRNA splicing defects. Mutations in both have variable effects, dependent on the presence or absence of introns in the COX1 gene. It has been described for the *MSS51* gene product that it is implicated in the translation of the COX1 mRNA. The most plausible function is that of an initiation factor specific for the translation of COX1 mRNA (DeCoster *et al.*, 1990). In this report we show that Mss51p is tightly associated with the mitochondrial inner membrane. A C-
terminal fusion of the Mss51 protein with the HA-tag can only be extracted from the membrane under stringent conditions and co-localizes with Tim44p (Blom et al., 1993), indicating a tight association with the inner membrane. DeCoster et al. (DeCoster et al., 1990) previously showed that Mss51p is involved in Coxlp synthesis. However, a role in stabilization of Cox1p could not be ruled out. Using pulse-chase experiments we have been able to show that Mss51p is required to initiate translation of COX1 mRNA, since during the pulse no Cox1p synthesis can be detected.

The mutation in mss51-3 lowers the expression level of Mss51p by at least 100 fold, due to the presence of a short upstream ORF in the mutated allele. The low expression level of Mss51p results in reduced synthesis of Cox1p, again indicating that Mss51p is required for translation and is not involved in stabilization of Cox1p. Since other mitochondrial translation products are present at normal levels, the effect on the synthesis of these products in the disruptant strain may be suggestive of secondary effects on translation. The mss51-3 mutation is apparently specific in its effect on Cox1p synthesis (Figure 1).

The effect of the mss51-3 mutation is enhanced when this allele is combined with the pR454 mutation which confers resistance to the aminoglycoside paromomycin. This mutation is located in a conserved hairpin in the 15S mitochondrial rRNA (Li et al., 1982). Paromomycin is known to interfere with the decoding process at the ribosomal A site. The observation that this mutation enhances the translational effect of the mss51-3 on Cox1p, gives further support to the idea that Mss51p acts together with other factors to initiate translation of COX1 mRNA. This may indicate a possible physical interaction of Mss51p with the mitochondrial ribosome, analogous to the situation for the COB mRNA translational activator Cbs2p (Michaelis et al., 1991).

We were able to detect the HA-tagged Mss51 protein expressed from a single copy vector (Fig. 2). This indicates that the steady state level of Mss51p is high enough to make it easily detectable, comparable to results obtained for the COX3 translational activator Pet54p (Fox, 1996). In contrast, to be able to detect the COX1 translational activator Pet309p (Manthey et al., 1998), the COX2 translational activator Pet111p and the COX3 translational activators Pet494p and Pet122p, multicopy expression is required (Fox, 1996). In this respect it may be significant that both Mss51p and Pet54p are peripheral membrane associated translational activators, while the others are integral membrane proteins. These observations suggest distinct roles for the integral membrane translational activators and the peripheral membrane translational activators.

Mss51p is not the only factor involved in translation of COX1 mRNA, the gene product of the nuclear gene PET309 is also necessary for Cox1p synthesis. Pet309p is an integral inner mitochondrial membrane protein (Manthey et al., 1998). Unlike mss51 disruptants, pet309 mutants can be suppressed by 5'-UTR exchanges within the COX1 mRNA which place Cox1p synthesis under control of other translational activators (Manthey et al., 1995). Similar revertants have been found for most other mitochondrial translational activators and this has been interpreted as evidence for direct interaction of the various activators with the 5'-UTRs of the respective mRNAs. The fact that such suppressors have not yet been found for mss51 mutants could mean either that Mss51p does not interact with mRNA, or that its site of interaction is not easily separable from the COX1 coding sequence.

The finding that the translation factors of the COX1 mRNA resemble other translational activators in being associated with the inner membrane is consistent with the idea that translation in yeast mitochondria takes place on the inner membrane in close proximity to sites of assembly of translation products into respiratory complexes (Sanchirico et al., 1998).
Until now very few nuclear genes required for translational activation have been identified in other organisms, with sequences of activators displaying high divergence even between yeast species (Costanzo et al., 1998). This may not be surprising for those factors that interact with mRNA, since *S. cerevisiae* mitochondrial mRNAs are unusual in possessing extremely long 5'-UTR's. For other factors that primarily interact with the ribosome, or other proteins at a membrane-associated assembly site, stronger sequence conservation of orthologous proteins might reasonably be expected. For the *MSS51* gene, however, no obvious homologs have thusfar been found.

How Mss51p and Pet309p function in the coupling of translation and insertion into the membrane remains to be determined. Further study will be required to identify possible interactions with other factors, such as Pet309p or the mitochondrial ribosome, to get more insight in the exact function of Mss51p in translation initiation of *COX1* mRNA.

**Acknowledgements**

We would like to thank Dr. G.A.J. Hakkaart for construction of the GFP-fusion and the fluorescent microscopy analysis and Dr. L.G.J. Nijtmans and Drs. R.F. Lascaris for critical reading of the manuscript. This work was supported in part by grants from the Chemical Sciences section of the Netherlands Organization for the Advancement of Pure Research (CW-NOW) and by the EU within the framework of the Human Capital and Mobility Program (contract no. CHRX-CT04-0520).
The mutation in mas5tf lowers the expression level of the 5UTR of the mRNA, leading to reduced translation. The presence of a short upstream CTRF in the mutated allele further exacerbates this effect, resulting in a translational block. This finding suggests that the mutation affects the efficiency of translation initiation factors, leading to a significant decrease in protein production.

The mas5tf mutation is apparently essential for its effect on Ctr5p synthesis. The effect of the mas5tf mutation is only observed in cells that are unable to maintain translation, indicating that a specific translational activator, Ctr5p, is required for efficient translation of the mRNA.

We were able to detect the NA-tagged Mas5tf protein expressed from a single copy vector. This indicates that the steady state level of Mas5tf is low enough to ensure rapid depletion, comparable to results obtained for the COX75 translational activator. In contrast, to be able to detect the COX75 translational activator, Pet115p, the COX75 translational activator Pet117p, and the CTRF translational activator Pet496p and Pet122p, multicopy expression is required (Pet117p). In this respect, it may be significant that both Mas5tf and Pet115p are membrane-associated translational activators, while the others are integral membrane proteins. These observations suggest distinct roles for the integral membrane translational activators and the peripheral membrane translational activators.

Mas5tf is not the only factor involved in translation of COX75 mRNA, as the gene product of the structural gene, PET117, is also necessary for Ctr5p synthesis. Pet117p is an integral membrane protein, and mutants can be suppressed by 5'-UTR exchanges within the COX75 mRNA, indicating that CTRF synthesis occurs under control of other translational activators (Manthey et al., 1996). Several regulatory elements have been found for most other mitochondrial translational activators, and they have been interpreted as evidence for direct interaction of the various factors with the 5'-UTR of the respective mRNA. The fact that such suppressors have not been found for mas5tf mutants could mean either that mas5tf is not involved in translation or that its 5'-UTR is not properly translated from the COX75 mRNA.