Regulators of mitochondrial translation in Saccharomyces cerevisiae

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Chapter V

Functional analysis of Mss51p

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Summary

In this final experimental chapter, we describe our attempts to determine the function of Mss51p in the synthesis of Cox1p. One approach to get more insight in the function of a gene product is the study of mutants. However, for MSS51 there were no mutants available. To obtain useful mutants, we performed PCR mutagenesis to create a mutant library that could be screened for temperature sensitive (ts) mutants. Comparison of sequences might lead to clues on a proteins' function, therefore we carried out database searches with the Mss51p sequence. From earlier work an interaction of Mss51p and the translation machinery (the mitoribosome) was suggested. To investigate this possibility, we isolated mitochondrial ribosomes.

Introduction

We were able to localize Mss51p to the mitochondrial inner membrane and using pulse labeling experiments we showed that this protein functions in an early step of the cytochrome c oxidase subunit 1 (Cox1p) synthesis (Chapter III). However, the exact function of Mss51p is still unknown. Based on the evidence presented in Chapter IV, the mechanism whereby Mss51p activates translation appears to differ from that of other translational activators. For the functional analysis of proteins naturally occurring variants are often used. Despite extensive searching, no spontaneous mutants in MSS51 have been found (DeCoster et al., 1990; Siep and van der Spek, unpublished results). mss51-3, the only mutant described so far, is an expression mutant: introduction of an upstream ATG decreases translation of the MSS51 ORF to 1% of wild type (Chapter III). Mutations in the MSS51 ORF would facilitate the analysis of the protein function. Because of the lack of available mutations we decided to create point mutations by PCR mutagenesis. Mutant libraries were screened for presence of temperature-sensitive (cs/ts) mutants during growth on a non-fermentative carbon source (YPGlyc) at different temperatures (23 °C, 28 °C and 37 °C).

In chapter III we describe the characterization of mutant mss51-3. The phenotype of this mutation is enhanced by the P_R\textsuperscript{454} mutation (Li et al., 1982), this mutation is located in a conserved hairpin in the 15S mitochondrial ribosomal RNA and interferes with the decoding process at the ribosomal A site. These observations suggest an interaction of Mss51p with the translation machinery. Mitochondrial fractionation experiments (as described in Chapter III), showed that Mss51p protein is firmly associated with the mitochondrial inner membrane. This localization resembles that of other mitochondrial translational activators in S. cerevisiae. As suggested by others, it is thought that membrane-localized translation initiation is needed to synthesize the hydrophobic translation products near their site of assembly (Michaelis et al., 1991; Fox, 1996). Most mitochondrially encoded mRNAs are translated by membrane-associated ribosomes (Marzuki and Hibbs, 1986). The translational activators Cbs2p and Pet122p for COB and COX3 mRNA, respectively, display interaction with the mitochondrial inner membrane and the mitochondrial ribosome (Michaelis et al., 1991; Hafter et al., 1991). Based on the observation that the respiratory-deficient phenotype of the mss51-3 mutation is enhanced by a mutation in the mitochondrial 15S rRNA (P_R\textsuperscript{454}), an interaction with the mitochondrial ribosome has also been suggested for Mss51p. To investigate a possible interaction of Mss51p with ribosomes, we isolated mitochondrial ribosomes from a yeast strain expressing HA-tagged Mss51p. Using Western blot analysis with antibodies directed against a mitochondrial ribosomal subunit protein and the HA-epitope we determined whether Mss51p displays co-localization.
Material & Methods

Mutagenesis of MSS51
All constructs in this study were based on construct pMS3: a Spl-Xbal chromosomal DNA fragment containing the MSS51 gene in yeast-Escherichia coli shuttle vector YCplac111 (Gietz and Sugino, 1988).

Deletion mutants: All single copy mutants were constructed in YCplac111 and YEplac181. From pMS3 different internal fragments were deleted. pS51NHA was constructed by deleting a 324 bp Ncol fragment. Before ligating the vector, the sticky ends were made blunt with T4 DNA polymerase. Plasmid pS51EHA was constructed by deleting a 187 bp Espl-PmlI fragment, the sticky Espl end was made blunt with T4 DNA polymerase. For the construction of plasmid pS51BHA the Spl-Xbal fragment of pMS3 (containing the MSS51HA-fusion) was first cloned in vector pEP30 (Laird, 1988). Then a 126 bp BstBI fragment was deleted and the Spl-Xbal fragment with the BstBI deletion was cloned in YCplac111. Multi copy plasmids pMS7, pM51NHA, pM51EHA and pM51BHA were constructed by cloning a HindIII-Xbal fragments of respectively pMS3, pS51NHA, pS51EHA and pS51BHA in YEplac181.

PCR mutagenesis: Random mutagenesis experiments on the MSS51 gene were performed according to the method of Spee et al. (1993). PCR reactions were performed using pMS3 as template. Reactions were done in a final volume of 50 μl, containing: 0.2 mM of the universal M13 primers, 1 unit of Taq DNA Polymerase (Promega Madison, USA), Thermophilic Buffer (50 mM KCl, 10 mM Tris-HCl pH 9, 0.1% Triton X-100), 5 mM MgCl2, 20 mM of one dNTP and 200 mM of the three remaining dNTPs. PCR was performed in 30 cycles, each cycle consisting of a denaturing step at 94 °C for 1 min., a primer annealing step at 54 °C for 1.5 min. and an extension step at 72 °C for 2.5 min., using a Biometra T3-thermocycler. In a mutagenesis experiment four separate PCR reactions were performed, each of which contained limiting amounts of one of the four dNTPs and afterwards the products were pooled. Two different libraries were made. PCR-products digested with HindIII and XbaI were ligated in YCplac111, resulting in insertion of a MSS51 gene with possible mutations over the complete ORF (EWL). For the other library a vector was created that was suitable for inserting only part of the PCR-products: an internal 1148 bp BamHI-KpnI fragment. pMS3 was digested with SplI and BsaBI, a 1872 bp fragment was inserted in YCplac111 vector. First, the empty vector was digested with KpnI, treated with T4 DNA Polymerase and digested with SplI. The 1148 bp internal BamHI-KpnI fragment was exchanged with primers MWG#112 and MWG#113, introducing a linker containing a Pmel site. This vector, pMR2, contained the mitochondrial targeting signal of MSS51, a linker and the 3’-part of the gene fused with the HA-epitope. A library was constructed in this vector by exchanging the linker with the BamHI-KpnI fragment from the PCR-products. This library (MRL) contained inserts with wild type targeting signal and wild type HA-epitope.

Isolation of mitochondrial ribosomes
Mitochondrial ribosomes were isolated as described previously (Grivell et al., 1971), with minor modifications. In brief, cells from a late logarithmic phase culture were collected by centrifugation (MSE-rotor), and spheroplasts were prepared by zymolyase (Seikagaku) treatment at 30 °C for 1 hr. After resuspension in 5 ml Mannitol medium (0.6 mM mannitol, 0.1 mM EDTA, 10 mM Tris-HCl pH 6.7), cells were disrupted in a blender. To remove unbroken cells this suspension was centrifuged twice at 3,000 rpm for 5 min in a GSA-rotor. The supernatant was centrifuged at 10,000 rpm for 10 min in a SS34-rotor to collect the mitochondrial pellet. After washing in Mannitol medium, the mitochondria were resuspended in Medium A (60 mM mannitol, 150 mM KCl, 10 mM KH2PO4, 2 mM ATP...
(sodium salt), 5 mM 2-oxoglutaric acid, 2 mg/ml bovine serum albumin, 20 mM Tris pH 6.7 in a concentration of 5 mg/ml, and incubated at 30 °C for 10 min (gently shaking) in Erlenmeyer flasks holding 1/10th of their nominal volume. After 10 min, puromycin was added to a final concentration of 50 μg/ml and the incubation was continued for a further 3 min. The suspension was rapidly chilled to 0 °C by addition of an equal volume of Mannitol medium. To recover the mitochondria, this suspension was centrifuged at 13,000 rpm for 5 min at 4 °C (SS34-rotor). Mitochondria were resuspended in A50MT (50 mM NH₄Cl, 10 mM MgAc, 10 mM Tris-HCl pH 7.5, 6 mM 2-mercaptoethanol), and lysed by rapid addition of 1/10th volume 3% sodium deoxycholate. After clarification at 13,000 rpm for 20 min (SS34-rotor), the lysate was layered directly over 0.4 volume 1 M sucrose in A500MT (500 mM NH₄Cl, 10 mM MgAc, 10 mM Tris-HCl pH 7.5). This sucrose cushion was centrifuged at 40,000 rpm for 16 hr in a Ti65-rotor or a type 60 rotor. After centrifugation, the pellet was washed three times in A50MT, and resuspended in the same buffer and the suspension was clarified by centrifugation at 10,000 rpm for 10 min in an Eppendorf centrifuge at 4 °C. The supernatant was transferred to a new Eppendorf tube and the pellet was again extracted with the same buffer, centrifuged and the supernatants were combined. Fractions were frozen in liquid nitrogen and stored at -70 °C.

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).

Results

Mutagenesis of MSS51
The sequence of Mss51p does not display any obvious motifs or features that could give clues to a possible function of the protein or location of functional domains in the protein. Based on its hydropathy, Mss51p can be divided into two parts: a rather hydrophilic N-terminal part of about 160 amino acids, and a more neutral C-terminal part with occasional hydrophilic regions. The protein is tightly associated with the mitochondrial inner membrane (Siep et al., 2000). A database search using Psi-BLAST (Altschul et al., 1997) identified a protein in Schizosaccharomyces pombe that displays similarity with Mss51p over its whole length, showing 37% identity with S. cerevisiae Mss51p (Fig. 1). Alignment of S. cerevisiae Mss51p and the S. pombe orthologue revealed a number of highly conserved regions (Fig. 1). To search for additional conserved domains that might indicate the function of Mss51p, these candidate patterns together with the S. cerevisiae Mss51p sequence were used as input in a Phi-BLAST search against all non-redundant GenBank translations (Zhang et al., 1998). No other protein sequences that contain the input patterns were found.

In order to determine experimentally which domains are important for activity of Mss51p, deletion mutants were created. Through digestion and religation, deletions of 108, 64 and 42 amino acids were obtained. The deleted regions are shown schematically in Figure 2. All three deletions were checked by DNA sequencing and turned out to be in-frame. However, because all deletions led to loss of function (data not shown), we turned to creation of point mutations by random mutagenesis (Spee et al., 1993), followed by a screen for a mutant phenotype. Two different libraries were created: EWL, in this library the whole gene (including targeting signal and 3'-HA-tag) was mutagenized and MRL, for this library a vector was created that contained the wild type targeting signal and 3'-HA-tag and was suitable for insertion of an internal 1148 bp fragment of the mutagenized PCR-
products. Library EWL gave rise to about 2,000 E. coli clones, from which DNA was isolated and transformed to the mss51-null strain. Screening of this yeast library on YPGlyc at 23°C, 28°C and 37°C yielded seventeen thermosensitive (ts) mutants out of 4,000 transformants: eight ts mutants which did not grow at 37°C, and nine partial ts mutants which grew poorer at 37°C compared to growth at 28°C. For the MRL library 1,800 E. coli clones were pooled to isolate DNA. Transformation of the mss51-null strain resulted in a yeast library of 12,000 clones. Screening of this library yielded four ts mutants and 5 partially ts mutants at 37°C. Western blot analysis showed that at 28°C all mutant proteins were normally expressed and localized in the mitochondria (data not shown). In order to determine which alterations are responsible for the ts phenotypes a number of mutants with the most clear phenotype were chosen for sequence analysis: one ts mutant from the EWL library and four ts mutants from the MRL library.

Figure 1: Sequence alignment of S. cerevisiae Mss51p and a S. pombe homologue. Using DIALIGN 2.1 (Morgenstern, 1999) the protein sequence of the S. cerevisiae Mss51 protein was aligned with a protein sequence that was identified using Psi-BLAST (Altschul et al., 1997). This protein from S. pombe (GenBank Accession number SPAC25B8) shows similarity to S. cerevisiae mitochondrial protein Mss51p. The underlined sequences were used as candidate patterns to search for conserved domains using Phi-BLAST (Zhang et al., 1998). Amino acid substitutions created by PCR-mutagenesis are depicted in bold type.
Figure 2: Organization of the Mss51HA protein and positions of the deletions. Mss51p is represented as an open box of 436 amino acids. The Ha-tag is represented as a shaded box of 10 amino acids. The deletions are shown as black boxes in the open box. N, 108-aa *NcoI-NcoI* deletion. E, 64-aa *Esp1-Pml1* deletion. B, 42-aa *BstBI-BstBI* deletion. The square dotted line represents the N-terminal region containing the point mutations.

Table I: Nature of the point mutations in Mss51p. Point mutant ts1 from library EWL and point mutants ts3, ts8, ts13 and ts17 from library MRL were characterized using sequence analysis. This table depicts the nucleotide substitutions and the corresponding amino acid changes. Positions are relative to the initiator ATG of the *MSS51* orf.

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Nucleotide substitution</th>
<th>Amino acid change</th>
</tr>
</thead>
<tbody>
<tr>
<td>ts1</td>
<td>C227T, A499T</td>
<td>A76V, T167S</td>
</tr>
<tr>
<td>ts3</td>
<td>deletion (139-159), T494C</td>
<td>DPPPSSPE (47-53), F165S</td>
</tr>
<tr>
<td>ts8</td>
<td>A401G</td>
<td>D134G</td>
</tr>
<tr>
<td>ts13</td>
<td>G577A</td>
<td>G193R</td>
</tr>
<tr>
<td>ts17</td>
<td>C701T</td>
<td>P234L</td>
</tr>
</tbody>
</table>

Sequence analysis of the point mutants showed that the ts phenotype of three of the yeast transformants was caused by a single point mutation in the *MSS51* gene (Table I). Mutant ts1 is the only mutant that contained two nucleotide changes. The first being a C to T alteration at nucleotide position 227 (relative to the ATG) resulting in a change from valine to alanine at amino acid position 76 and the second substitution was an A to T alteration at nucleotide position 499 resulting in a threonine to serine change at amino acid position 167. In mutant ts3 sequence analysis showed a 21 bp deletion resulting in loss of amino acids DPPPSSPE from position 47 to 53, in combination with a T to C nucleotide substitution at position 494 resulting in a change from phenylalanine to serine at position 165. The amino acid changes in these mutants range from relatively neutral changes (alanine to valine, threonine to serine) to more drastic changes (neutral glycine to hydrophilic, positively charged arginine) (Fig. 1). Interestingly, all mutations (except A76V) that change conserved amino acids are in a 100-aa region in the N-terminal half of the protein (Fig. 2).

Does Mss51p interact with the mitochondrial ribosome?

To investigate this possibility, we set out to isolate mitochondrial ribosomes from the *mss51-null* strain (W303(167)Δmss51) transformed with a multi-copy construct of the HA-tagged *MSS51* gene. To check the fractionation from crude mitochondria to ribosome fraction these samples were analyzed by SDS-PAGE and Western blotting. Using different antibodies, we determined whether Mss51p co-localizes with the mitochondrial ribosome (data not shown). A low yield caused by partial lysis of mitochondria and incomplete resuspension of the ribosomal pellet, and a high degree of cross-contamination by membrane material, mean that the location of Mss51p could not be established with any degree of certainty.
From the characterization of various properties of Mss51p, including genetic and physical interactions and biochemical characterization, we hypothesized that this protein is different from other translational activators (Chapter 11 / Chapter IV). We were unable to detect an interaction with Pet309p, the integral membrane translational activator for COX1 mRNA. Mitochondrial revertants that arise spontaneously in mutant strains of other translational activators, could not be found for Mss51p, indicating that Mss51p either does not interact with the 5'-UTR or that it recognizes some other part of the mRNA. Characterization of mutant mss51-3 showed that the phenotype of this mutant strain is enhanced in a strain carrying the pR454 mutation (Decoster et al., 1990; Siep et al., 2000). The pR454 mutation is located in a conserved hairpin in the 15S mitochondrial ribosomal RNA and interferes with the decoding process at the ribosomal A site (Li et al., 1982). This mutation, a C to G transversion, confers resistance to paromomycin, an inhibitor of prokaryotic protein synthesis. In the paromomycin-resistant background, an enhancing effect on two other factors involved in mitochondrial protein synthesis has been reported. Mss1 mutants fail to splice the COX1 pre-mRNA (Decoster et al., 1993), the mutant phenotype was interpreted to indicate that Mss1p normally interacts with the 15S rRNA. The paromomycin-resistance allele was also sufficient for the expression of respiratory deficiency in mto1 mutants (Colby et al., 1998). Both Mss1p and Mto1p are mitochondrial proteins that on the grounds of co-sedimentation have been proposed to form a complex (Colby et al., 1998). No association of these proteins with the mitoribosome was observed, transient interactions could not be excluded. Since the effect of the paromomycin-resistance mutation on the mss51-3 mutant is comparable with the situation for MSS1 and MTO1, we hypothesized that Mss51p might interact with the mitoribosome. Alternatively, it would mean that the pR454 mutation diminishes ribosome function and makes mss51 mutants grow more poorly for indirect reasons. For the mitoribosome of S. cerevisiae 65 proteins have been characterized so far (YPD database, Hodges et al., 1999). Mitoribosomes differ from their counterparts in yeast cytoplasm and in other organisms mainly in the protein/RNA ratio, containing many more ribosomal proteins (MRPs). Still not all MRPs are identified, but the availability of a complete sequence of the yeast genome has greatly facilitated the identification of new MRPs (Graack and Wittmann-Liebold, 1998). To investigate a possible interaction of Mss51p with the mitoribosome, we isolated ribosomes from a strain expressing HA-tagged Mss51p. The results we obtained with the antibody against the HA-epitope suggested that Mss51p co-localized to some extent with the mitoribosome, Mss51p could be detected in mitochondrial subfractions that contain ribosomes. Unfortunately, the ribosomes obtained in this experiment are atypical. A low yield caused by partial lysis of mitochondria and incomplete resuspension of the ribosomal pellet, and a high degree of cross-contamination by membrane material, mean that the location of Mss51p could not be established with any degree of certainty.

So far, our experiments still have to reveal the function of Mss51p. Based on the results obtained, we suggest three possible functions. Using pulse-labeling experiments, we showed that MSS51 is specific for COX1 mRNA and Mss51p is required for the synthesis of Cox1p. The simplest explanation is that MSS51 interacts with a component of the translational apparatus that is specifically involved in Cox1p synthesis. This explanation leaves us with three possibilities. First, Mss51p might interact with COX1 mRNA. If this is the case, then interaction is most probably with a part of the mRNA that is distinct from the 5'-UTR, since we and others have been unsuccessful in searches for mitochondrial pseudo-revertants, in which translation has been restored as a result of leader exchange. The fact that the paromomycin-resistance mutation, which interferes with both initiation and elongation of translation, enhances the effect of the mss51-3 mutation, opens the
possibility that Mss51p interacts downstream of the initiator-AUG. Second, while we could not show an interaction with Pet309p, Mss51p might associate with another, yet unidentified, COX1-specific translational activator. Also, our results on the ribosome-association of Mss51p, do not exclude that Mss51p interacts transiently with the mitoribosome. These two possibilities imply that Mss51p may promote efficient elongation of the nascent Cox1p subunit. Third, an interaction with the growing nascent peptide chain might exist. For this last possibility, two interactions could be envisaged. In analogy with bacterial ribosomal trigger factor (Hesterkamp, 1996; 1997), Mss51p, might act to promote folding of the nascent polypeptide as it exits the ribosome. Through interaction with the growing peptide chain, Mss51p might act as a co-translational docking protein, which co-translationally inserts the hydrophobic Cox1 protein in the inner membrane.

The amino acid substitutions in the thermosensitive mutants are located within a 100 amino acid region in the N-terminal part of the Mss51 protein. Since all mutant proteins created were expressed at normal levels and located to mitochondria, this indicates the importance of this part of the protein for its function. Based on amino acid properties the A76V and T167S changes appear not to be very drastic, since they exchange one neutral amino acid for another (Wienholds, 1998), but it should be remembered that these changes do affect side-chain length. The other changes are more drastic, changing the hydrophobicity or charge of amino acids. Comparing the wild type sequence with the sequences of the ts alleles, using secondary structure prediction programs (Levin et al., 1986; Deleage and Roux, 1987; Rost and Sander, 1993; Geourjon and Deleage, 1994; Frischman and Argos, 1996; Garnier et al., 1996; King and Sternberg, 1996), suggested that the single amino acid substitutions might influence the secondary structure of Mss51p (Rijnberg, 1999). On basis of these predictions an area predicted to form an extended strand appeared to have a higher probability of forming an alpha helix in mutant ts3 (F165S), and in mutant ts13 (G193R) a region of random coil might assume an alpha helical conformation. These secondary structure changes might lead to loss of an interaction necessary for the function of Mss51p. Taken together, for the mss51 point mutants described here this could mean that at the restrictive temperature the substitutions in amino acids caused by the point mutations lead to loss of an interaction necessary for proper translation of COX1 mRNA.

Through a database search (Psi BLAST, NCBI), we found an homologue for MSS51 in Schizosaccharomyces pombe. This protein most probably represents the S. pombe orthologue of Mss51p. Four of the amino acids that were mutagenized in our mutagenesis approach (D134, F165, T167 and G193) appeared to be conserved between S. cerevisiae Mss51p and the S. pombe orthologue (Fig. 2). Although the function of this S. pombe orthologue of MSS51 has to be determined, it is striking that we find an homologue through database search in a distantly related yeast. For the other translational activator protein for COX1 mRNA, Pet309p, a protein with significant sequence similarity has been found: the Neurospora crassa gene CYA-5, which is required in a post-transcriptional step for the expression of Cox1p (Coffin et al., 1997). Functional homologues of PET309 have also been found in Candida albicans and S. pombe (Costanzo et al., 2000), while the maize chloroplast translational activator protein Crp1p exhibits sequence similarity to Pet309p (Fisk et al., 1999). The fact that for the COX1 translational activators MSS51 and PET309 homologues with significant similarity could be found in distantly related fungi, while for the COX2 and COX3 translational activators no significant matches could be found, indicates that proteins involved in synthesis of Cox1p may be subjected to more stringent evolutionary constraints.

Recently, it was shown that in the closely related yeasts Saccharomyces kluyveri and Kluyveromyces lactis the mRNA-specific translational activators PET111 and PET122 are
highly diverged, while their functions are orthologous (Costanzo et al., 2000). These homologues were found by screening for complementation of mutations in genes adjacent to the PET genes and failed to function in S. cerevisiae. Not only the translational activator genes, but also the 5'-UTRs of the COX2 and COX3 mRNAs in S. kluyveri and K. lactis have little similarity to each other or to those of S. cerevisiae. This implies that through co-evolution of the yeast mitochondrial mRNA translational activators and the yeast mitochondrial mRNAs membrane-bound translation has been retained, while the factors involved have diverged.
The amino acid substitutions in the translationally activated region of the cytochrome c oxidase chain in the S. cerevisiae and B. subtilis N351 strains were determined at nucleotide and amino acid levels. Amino acid substitutions at positions 196, 207, 224, 287, and 291 were observed in both strains. These changes are important for the proper translation of COX1 mRNA.

The S. cerevisiae cytochrome c oxidase chain is composed of 423 amino acids, with a hydrophobic domain and a hydrophilic domain. The hydrophobic domain is rich in tyrosine and tryptophan residues, whereas the hydrophilic domain is rich in proline, asparagine, and serine residues. The hydrophobic domain is involved in the assembly of the cytochrome c oxidase complex, while the hydrophilic domain is involved in the electron transfer process.

Through a database search, the COX1 mRNA sequence was identified as a likely candidate for the S. cerevisiae cytochrome c oxidase chain. This protein may be necessary for the proper translation of COX1 mRNA. The S. cerevisiae cytochrome c oxidase chain is composed of 423 amino acids, with a hydrophobic domain and a hydrophilic domain. The hydrophobic domain is rich in tyrosine and tryptophan residues, whereas the hydrophilic domain is rich in proline, asparagine, and serine residues. The hydrophobic domain is involved in the assembly of the cytochrome c oxidase complex, while the hydrophilic domain is involved in the electron transfer process.

Recently, it was shown that in the closely related yeasts Z. bailii and B. subtilis, a high degree of similarity exists in the mRNA sequence specific for the translation of COX1 mRNA. This suggests that the COX1 mRNA sequence may be conserved across different species.