Mol Gen Genet (1989) 216:37–43

MGG



# Accumulation of the cytochrome *c* oxidase subunits I and II in yeast requires a mitochondrial membrane-associated protein, encoded by the nuclear *SCO1* gene

**Marion Schulze and Gerhard Rödel***

Institut für Genetik und Mikrobiologie der Universität München, Maria-Ward-Strasse 1a, D-8000 München 19, Federal Republic of Germany

**Summary.** The yeast nuclear *SCO1* gene is required for accumulation of the mitochondrially synthesized cytochrome *c* oxidase subunits I and II (COXI and COXII). We cloned and characterized the *SCO1* gene. It codes for a 0.9 kb transcript. DNA sequence analysis predicts a 33 kDa protein. As shown by in vitro transcription and translation experiments in combination with import studies on isolated mitochondria, this protein is matured into a 30 kDa polypeptide which is tightly associated with a mitochondrial membrane. The possible function of the *SCO1* gene product in the assembly of cytochrome *c* oxidase is discussed.

**Key words:** DNA sequence – *PET* gene – *Saccharomyces cerevisiae* – Mitochondrial import – cytochrome *c* oxidase

## Introduction

Yeast cytochrome *c* oxidase is a multi-protein complex, composed of nine different subunits (Power et al. 1984). The three large subunits (COXI, COXII, COXIII), which are generally believed to represent the catalytic domain of cytochrome *c* oxidase (Capaldi et al. 1983), are coded by mitochondrial DNA, while the six smaller subunits (COXIV, COXV, COXVI, COXVII, COXVIIa, COXVIII) are coded by nuclear genes (Schatz and Mason 1974). With the exception of COXVII, all the respective structural genes have been isolated and characterized (Bonitz et al. 1980; Coruzzi and Tzagoloff 1979; Fox 1979; Thalenfeld and Tzagoloff 1980; Maarse et al. 1984; McEwen et al. 1986; Koerner et al. 1985; Séraphin et al. 1985; Wright et al. 1984, 1986; Patterson and Poyton 1986).

Biogenesis of cytochrome *c* oxidase is subjected to complex regulation. Heme and oxygen have been shown to be essential for accumulation and assembly of the subunits (Saltzgaber-Müller and Schatz 1978; Woodrow and Schatz 1979). In addition, synthesis of cytochrome *c* oxidase is inhibited by the presence of glucose ("glucose-repression"; Mahler et al. 1975). Several mutations have been described which interfere with the formation of cytochrome *c* oxidase. Beside mutations in structural genes, mutations have been identified in genes which are somehow involved in the synthesis and/or assembly of the subunits, preferentially of the three mitochondrially synthesized polypeptides.

Of 34 nuclear complementation groups, which are minimally required for formation of cytochrome *c* oxidase, 17 are specifically involved in expression of either COXI, COXII, COXIII or COXI and cytochrome *b* (COB; McEwen et al. 1986). With the exception of mutants in three complementation groups, which lack *COX1* or *COX2* transcripts, all these mutants show mature-sized mitochondrial *COX* transcripts and are therefore affected in a post-transcriptional step in the expression of the mitochondrially-coded cytochrome *c* oxidase subunits (Kloeckener-Gruissem et al. 1987). This suggests that the mutant phenotype is caused by a failure to translate the respective mRNAs or by a rapid degradation of the newly synthesized protein. In case of *pet494* and *pet54* mutants, which both lack COXIII, synthesis of COXIII can be restored by mitochondrial gene rearrangements, which fuse the COXIII structural gene to 5′-untranslated sequences of other mitochondrial genes (Costanzo and Fox 1986; Costanzo et al. 1986). This result clearly demonstrates that the gene products of *PET494* and *PET54* are necessary to activate *COX3* mRNA translation rather than to prevent rapid degradation of newly synthesized COXIII. Similar specific translation factors for *COX2* mRNA (*PET111*; Poutre and Fox 1987) and *COB* mRNA (*CBS1* and *CBS2*; Rödel et al. 1985; Rödel 1986) have been reported.

We recently described a yeast nuclear gene, *SCO1*, which is required for COXII accumulation (Schulze and Rödel 1988). Deletion of a 1.7 kb DNA fragment of chromosome II, which contained the complete *SCO1* gene, resulted in a concomittant loss of COXII and COXI. This experiment gave no answer as to whether the observed phenotype resulted from loss of *SCO1* alone or whether (partial) deletion of another gene, immediately adjacent to *SCO1*, is responsible for loss of COXI.

In this paper we report the nucleotide sequence of the *SCO1* gene and show that it is required for both COXI and COXII accumulation. In vitro transcription and translation yields a polypeptide of molecular weight 33000 dalton, which is in excellent agreement with the molecular weight of the protein predicted from the nucleotide sequence. We further show by in vitro import studies into isolated mitochondria and subsequent carbonate extraction that this protein represents the precursor form of a mito-

* *Present address:* Institut für Pathologie und Rechtsmedizin der Universität Ulm, Labor für Molekulare Biologie und Allgemeine Pathologie, Neuherbergstrasse 11, D-8000 München 45, Federal Republic of Germany

*Offprint requests to:* M. Schulze

chondrial membrane protein of molecular weight 30000 dalton.

## Materials and methods

*Strains, media and plasmids.* Media and genetic techniques were as described (Sherman et al. 1986). The following yeast strains were used in this study: D273-10B (ATCC 25657); DL1 (*MATα, leu2-3, leu2-112, his3-11, his3-15, ura3-251, ura3-372, ura3-228*); MK20 (*MATa, ura1, met, sco1-1*); NP3 (*MATα, leu2, ura3, sco1-1*); GR20 (*MATα, ura3-251, ura3-228, ura3-372, leu2-3, leu2-112, his3-11, his3-15, sco1::URA3*) (Schulze and Rödel 1988). The *Escherichia coli* strains 490 (hsdR, hsdM, *met*$^-$, *thr*$^-$, *leu*$^-$, *recA*$^-$, *lac*$^-$, *su*$^+$) and BMH71-18 (*Δ(lac-proAB), thi*$^-$, *supE*; F′ *lacI*$^q$, ZΔM15, *proA*$^+$*B*$^+$) (Kramer et al. 1984) were used for *E. coli* transformation and phage M13 amplification.

Plasmid pB10A is identical to plasmid pB10E (Schulze and Rödel 1988) except for a 150 bp *ApaI–Eco*RI fragment (see Fig. 1) which has been deleted. Plasmid pBSB10 results from ligation of the 1615 bp *Dra*I–*Eco*RI fragment (see Fig. 1) of plasmid pB10E into the plasmid bluescript M13+ (Stratagene, San Diego), cut with *Eco*RI and *Hin*dII.

*DNA sequence analysis.* The 1.7 kb *Eco*RI fragment of plasmid pB10 (Schulze and Rödel 1988) was cut with either *Rsa*I, *Hae*III, *Alu*I or *Sau*3A; the resulting fragments were cloned into the phage derivatives M13mp8 or M13mp10 and sequenced by the dideoxy chain termination method (Sanger et al. 1977) with [$^{35}$S]dATP and [$^{32}$P]dATP (Amersham, Braunschweig). Reaction products were resolved on 6% acrylamide gels in the presence of 8 M urea. Both DNA strands of the 1.7 kb fragment were completely sequenced in overlapping fragments. The DNA sequence and the deduced protein sequence were compared with the DNA data base Genebank$^R$ (release number 52) and the NBRF protein data base (release number 13) by use of the microgenie$^R$ program (Beckman, Munich) on an IBM-AT computer.

*In vitro import into isolated mitochondria.* Plasmid pBSCB10 was cut with *Bam*HI and transcribed in vitro with T3 RNA polymerase. The resulting RNA was used to direct protein synthesis in the presence of [$^{35}$S]methionine in a reticulocyte lysate (Amersham, Braunschweig). Mitochondria were prepared from lactate-grown cells of strain D273-10B according to Daum et al. (1982) and added to the labelled proteins in the presence of NADH (2 mM) and ATP (1 mM). After incubation at room temperature for 30 min, protease resistance of imported proteins was tested by addition of proteinase K (150 μg/ml) on ice for 30 min. In parallel experiments, the membrane potential of mitochondria was destroyed by addition of valinomycin (1 μM) and KCl (70 mM). Proteinase K digestion was stopped with phenylmethylsulfonyl fluoride (1 mM) and mitochondria were recovered by centrifugation (10000 × *g*) and washed with SEM buffer (Hartl et al. 1986) prior to gel electrophoresis on a 13.5% or 15% acrylamide gel in the presence of SDS (Laemmli 1970).

*Alkaline extraction.* Alkaline extraction with sodium carbonate was done as described by Fujiki et al. (1982). After in vitro import, mitochondria were diluted 30-fold with 100 mM sodium carbonate (pH 11.5) to a final protein concentration of 150 μg/ml. After incubation at 0° C for 60 min the suspension was centrifuged for 1 h at 100000 × *g*. Membrane proteins (in the pellet) were separated from soluble proteins (in the supernatant), which were recovered by centrifugation (10 min; 15000 × *g*) after addition of trichloroacetate (final concentration 10%). Membrane proteins and soluble proteins were washed with ice-cold acetone and solubilized in 1.5% SDS for polyacrylamide gel electrophoresis.

*Miscellaneous.* Transformation procedures, Northern blot hybridization and nick-translation were done as previously described (Schulze and Rödel 1988).

## Results

### *DNA sequence analysis of the* SCO1 *gene*

We recently described the molecular cloning of a 1.7 kb DNA fragment from a yeast gene bank, which contains the complete *SCO1* gene (Schulze and Rödel 1988). DNA sequence analysis (Fig. 1) revealed an open reading frame (orf) of 881 nucleotides which codes for a polypeptide of 33 kDa (Fig. 2). This protein is characterized by a net positive charge (15% positively versus 11% negatively charged amino acids, see Fig. 3). The codon bias index of 0.06 can be taken as evidence for low expression in yeast (Bennetzen and Hall 1982). Comparison of both the amino acid and nucleotide sequence with the data of a protein and DNA data base (Material and methods) revealed no significant homology to any known sequence.

### SCO1 *is required for both COXI and COXII accumulation*

At a distance of 251 bp downstream of the *SCO1* gene, a second incomplete orf of 276 nucleotides was identified (Fig. 2). As deletion of the complete 1.7 kb *Eco*RI fragment leads to a concomittant deficiency of COXI and COXII

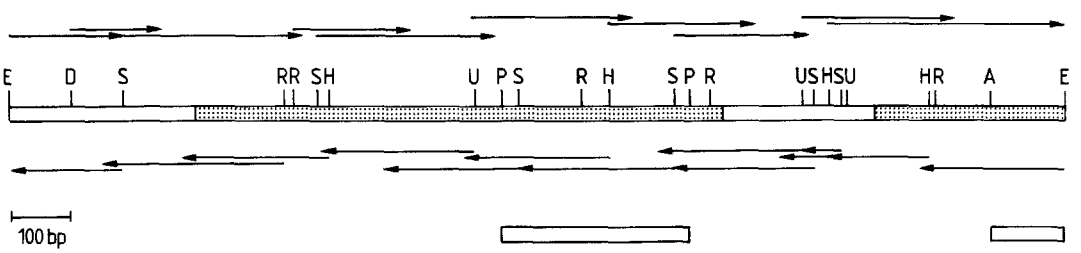


**Fig. 1.** Restriction map and sequencing strategy for the 1.7 kb *Eco*RI fragment. Restriction sites are denoted as follows: A, *Apa*I; D, *Dra*I; E, *Eco*RI; H, *Hae*III; P, *Pst*I; R, *Rsa*I; S, *Sau*3AI; U, *Alu*I. The *arrows* show the direction and approximate length of the fragments sequenced. *Dotted bars* indicate the open reading frames of *SCO1* and of a downstream localized gene. *Open bars* in the lower part of the picture represent gene-specific DNA fragments used for Northern hybridizations

```
   ggaattcccaattgaaactaaattgaagttctgtaaatttcaatatcgatgctatcatactcaaataggaaatatataaacaaatagtataggggtttatactag
-311

     ggttttaaaccccaaaaaactaaccctactatcttcttttcagaccctaaattgaaggaaataaacgggaataataatcaatagcgatctcccgaagagaaaa
  -206

     gtgagaaaatagtataattgtgtctattgtgtttataatcggcattagaaacgtatgttcgtataaggcaagggaaacagaaaaaacagcaaacgtagaaaag
  -103
     Met Leu Lys Leu Ser Arg Ser Ala Asn Leu Arg Leu Val Gln Leu Pro Ala Ala Arg Leu Ser Gly Asn Gly Ala Lys
     ATG CTG AAG TTG TCA AGA AGT GCC AAT CTA AGA TTG GTC CAA TTG CCA GCC GCA AGA TTA AGT GGC AAT GGC GCT AAA
    +1
     Leu Leu Thr Gln Arg Gly Phe Phe Thr Val Thr Arg Leu Trp Gln Ser Asn Gly Lys Lys Pro Leu Ser Arg Val Pro
     TTG CTC ACT CAA AGG GGA TTC TTT ACT GTA ACG CGC TTA TGG CAG TCA AAT GGC AAG AAA CCA TTA AGC AGA GTA CCT
   +79
     Val Gly Gly Thr Pro Ile Lys Asp Asn Gly Lys Val Arg Glu Gly Ser Ile Glu Phe Ser Thr Gly Lys Ala Ile Ala
     GTG GGC GGT ACT CCC ATT AAG GAT AAC GGC AAA GTG CGA GAA GGC TCG ATC GAG TTT TCC ACG GGA AAG GCC ATT GCT
  +157
     Leu Phe Leu Ala Val Gly Gly Ala Leu Ser Tyr Phe Phe Asn Arg Glu Lys Arg Arg Leu Glu Thr Gln Lys Glu Ala
     CTA TTC CTA GCA GTC GGT GGG GCA CTT TCT TAT TTC TTC AAC AGG GAG AAA CGC AGA TTG GAA ACA CAG AAG GAG GCT
  +235
     Glu Ala Asn Arg Gly Tyr Gly Lys Pro Ser Leu Gly Gly Pro Phe His Leu Glu Asp Met Tyr Gly Asn Glu Phe Thr
     GAA GCA AAC AGA GGA TAC GGT AAA CCT TCA CTT GGG GGA CCC TTC CAT CTG GAG GAT ATG TAT GGC AAT GAG TTT ACG
  +313
     Glu Lys Asn Leu Leu Gly Lys Phe Ser Ile Ile Tyr Phe Gly Phe Ser Asn Cys Pro Asp Ile Cys Pro Asp Glu Leu
     GAG AAA AAC CTT CTC GGT AAG TTT TCT ATA ATA TAC TTT GGG TTT AGT AAC TGT CCT GAC ATC TGT CCT GAT GAA CTG
  +391
     Asp Lys Leu Gly Leu Trp Leu Asn Thr Leu Ser Ser Lys Tyr Gly Ile Thr Leu Gln Pro Leu Phe Ile Thr Cys Asp
     GAT AAG CTA GGT CTA TGG CTT AAT ACA CTC TCT TCA AAG TAT GGT ATT ACT CTG CAG CCA TTA TTT ATA ACT TGT GAT
  +469
     Pro Ala Arg Asp Ser Pro Ala Val Leu Lys Glu Tyr Leu Ser Asp Phe His Pro Ser Ile Leu Gly Leu Thr Gly Thr
     CCA GCA AGA GAC TCC CCT GCT GTA TTG AAA GAG TAT TTG AGC GAC TTT CAT CCC TCC ATC CTG GGT TTG ACG GGG ACG
  +547
     Phe Asp Glu Val Lys Asn Ala Cys Lys Lys Tyr Arg Val Tyr Phe Ser Thr Pro Pro Asn Val Lys Pro Gly Gln Asp
     TTC GAT GAG GTG AAG AAC GCA TGC AAG AAG TAC AGA GTA TAC TTT TCT ACG CCT CCA AAC GTC AAA CCG GGC CAA GAT
  +625
     Tyr Leu Val Asp His Ser Ile Phe Phe Tyr Leu Met Asp Pro Glu Gly Gln Phe Val Asp Ala Leu Gly Arg Asn Tyr
     TAT TTG GTA GAC CAT TCC ATC TTC TTT TAT CTC ATG GAC CCT GAA GGA CAG TTT GTT GAT GCT TTG GGT AGA AAT TAT
  +703
     Asp Glu Lys Thr Gly Val Asp Lys Ile Val Glu His Val Lys Ser Tyr Val Pro Ala Glu Gln Arg Ala Lys Gln Lys
     GAT GAA AAA ACG GGC GTG GAC AAG ATC GTG GAA CAC GTT AAG AGT TAT GTG CCT GCA GAG CAG CGC GCC AAG CAG AAG
  +781
     Glu Ala Trp Tyr Ser Phe Leu Phe Lys OCH
     GAG GCA TGG TAC TCC TTC TTA TTC AAA TAA gattgtaaatatatgctactttcttccttgtatatacatatatacttataacgctataacccg
  +859

     ttcttgtaatatcggctatcacccggcgaaggtgtatcgcaagaaaaaaaaaagctctctcactctaaaggaggctatgtgaacgtgggaagatcaagtgaaa
  +952
                                                                                                  Met Ser Thr Thr
     gaaatgtttgtaggccattttcttccagaacagatccgctcttgagctggtgagttagcacgataacaaacaaagatacagcgtc  ATG TCT ACC ACA
  +1055
     Leu Leu Trp Phe Ser Ser Val Ile Gly Tyr Val Ile Gln Thr Lys Cys Leu Ser Asn Ile Gln Ser Lys Lys Glu Ile
     CTA CTT TGG TTT TCA AGT GTA ATA GGC TAC GTG ATT CAA ACA AAA TGT TTG TCT AAC ATA CAA TCT AAA AAG GAA ATC
  +1152
     Ser Val Gly Pro Asn Gly Thr Ile Ala Thr Pro Glu Thr Asn Gly Asp Asn Gly Asn Ser Ser Ser Leu Thr Phe Tyr
     TCC GTG GGG CCC AAT GGT ACA ATT GCA ACG CCT GAA ACT AAC GGC GAC AAC GGA AAC TCA AGT TCA TTA ACC TTC TAT
  +1230
     Leu Thr Phe Met Tyr Phe Ala Ser Trp Leu Leu Leu Val Pro Ala Ser Arg Leu Trp Glu Lys Met Arg Pro Met Phe
     CTG ACC TTT ATG TAT TTT GCT TCG TGG CTG CTC TTG GTG CCT GCA TCT CGA CTT TGG GAG AAG ATG AGA CCG ATG TTT
  +1308
     Val Ser Asp Ser Asp Ser Asn Arg Asn Ser
     GTC TCT GAC TCA GAC TCG AAC AGG AAT TCC
  +1386
```

**Fig. 2.** Nucleotide sequence of the 1.7 kb *Eco*RI fragment. The non-coding DNA strand is shown. *Numbers* refer to the distance (in nucleotides) from the ATG initiation codon of the *SCO1* gene. The amino acid sequences of *SCO1* and the downstream localized gene, deduced from the DNA sequence, are given above the nucleotide sequence

(in contrast to the phenotype of the original *sco1-1* mutant, which only is defective in accumulation of COXII), we asked whether the partial deletion of this downstream orf could be the cause of COXI deficiency. To test this, we transformed strain GR20 (in which the entire 1.7 kb *SCO1* fragment is deleted and replaced by the yeast *URA3* gene) with the autonomously replicating plasmid pB10A (Material and methods). This plasmid carries the *Eco*RI–*Apa*I fragment of pB10E (Fig. 1) which contains the complete *SCO1* reading frame, but only 99 bp of the downstream orf. Therefore, GR20 transformants lack about 180 bp of the downstream gene. All of the transformants obtained were able to grow on non-fermentable substrates like glycerol, i.e. they were respiratory competent. This indicates that formation of a functional cytochrome *c* oxidase is possible in the transformants and therefore independent of the deleted downstream orf sequences. Thus the *SCO1* gene product is a prerequisite for accumulation of both COXI and COXII.

### SCO1 *codes for a 0.9 kb transcript*

We have recently shown that two RNA species of about 1.2 kb and 0.9 kb, respectively, hybridize with the 1.7 kb

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| UUU | phe | 11 | UCU | ser | 4 | UAU | tyr | 8 | UGU | cys | 3 |
| UUC | phe | 9 | UCC | ser | 5 | UAC | tyr | 5 | UGC | cys | 1 |
| UUA | leu | 5 | UCA | ser | 4 | UAA | och | 1 | UGA | opa | |
| UUG | leu | 10 | UCG | ser | 1 | UAG | amb | | UGG | trp | 3 |
| CUU | leu | 4 | CCU | pro | 8 | CAU | his | 3 | CGU | arg | |
| CUC | leu | 4 | CCC | pro | 3 | CAC | his | 1 | CGC | arg | 3 |
| CUA | leu | 5 | CCA | pro | 5 | CAA | gln | 3 | CGA | arg | 1 |
| CUG | leu | 5 | CCG | pro | 1 | CAG | gln | 6 | CGG | arg | |
| AUU | ile | 3 | ACU | thr | 5 | AAU | asn | 6 | AGU | ser | 4 |
| AUC | ile | 5 | ACC | thr | | AAC | asn | 7 | AGC | ser | 2 |
| AUA | ile | 3 | ACA | thr | 2 | AAA | lys | 10 | AGA | arg | 9 |
| AUG | met | 3 | ACG | thr | 7 | AAG | lys | 15 | AGG | arg | 2 |
| GUU | val | 2 | GCU | ala | 5 | GAU | asp | 9 | GGU | gly | 8 |
| GUC | val | 3 | GCC | ala | 4 | GAC | asp | 6 | GGC | gly | 9 |
| GUA | val | 5 | GCA | ala | 8 | GAA | glu | 7 | GGA | gly | 5 |
| GUG | val | 6 | GCG | ala | | GAG | glu | 10 | GGG | gly | 4 |

**Fig. 3.** Codon usage of the SCO1 protein

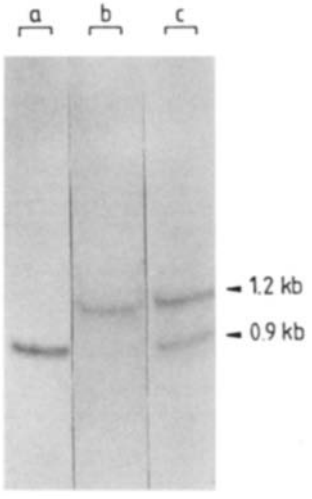


**Fig. 4.** Transcript analysis of the *SCO1* gene region. Total RNA from wild-type DL1 (transformed with plasmid pB10; Schulze and Rödel 1988) was separated by gel electrophoresis, blotted and hybridized with the labelled 1.7 kb *Eco*RI fragment (lane c); the 300 bp *Pst*I fragment (lane a); or the 170 bp *Apa*I–*Eco*RI fragment (lane b). The positions and lengths of the hybridizing RNA species are indicated by *arrowheads*

*Eco*RI fragment, which carries the *SCO1* gene (Schulze and Rödel 1988; Fig. 4, lane c). To obtain a more detailed transcription map, we analyzed total RNA of wild-type strain DL1 by use of gene-specific DNA probes. The nick-translated 300 bp *Pst*I fragment (see Fig. 1), which exclusively originates from the *SCO1* orf, picks up a single transcript of 0.9 kb (Fig. 4, lane a). A 170 bp *Apa*I–*Eco*RI fragment specific for the downstream orf (Fig. 1) specifically hybridizes to a 1.2 kb RNA species (Fig. 4, lane b). This result shows that *SCO1* is transcribed as an RNA species of about 0.9 kb and that the 1.2 kb species results from transcription of the downstream gene, which is not essential for cytochrome *c* oxidase formation (see above).

### *Post-translational import into mitochondria of the* SCO1 *protein*

The *SCO1* gene product is essential for the proper accumulation of the mitochondrially synthesized subunits I and II of cytochrome oxidase. From the localization of cytochrome *c* oxidase in the inner mitochondrial membrane it is likely that the SCO1 protein acts within mitochondria. Inspection of the amino terminal sequence of the SCO1 protein revealed several of the traits usually found in mitochondrial targeting sequences (for reviews see: Hurt and van Loon 1986; von Heijne 1986; Schatz 1987): it is devoid of acidic amino acids, while the content of positively charged amino acids, especially arginine, and of hydrophobic residues is high. Leucine and serine residues are significantly enriched, while valine and isoleucine are almost absent. Moreover, it can potentially fold as an amphipathic $\beta$-sheet structure.

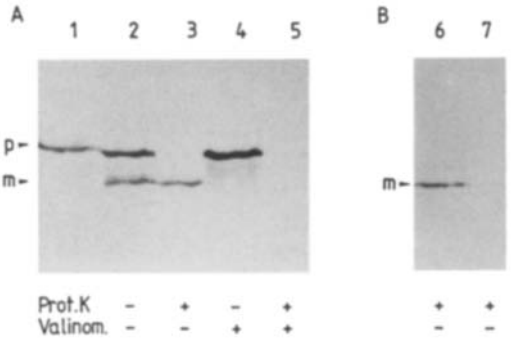


**Fig. 5A and B.** In vitro import and membrane association of the SCO1 protein. **A** In vitro import. [$^{35}$S] labelled SCO1 protein, synthesized in vitro in a reticulocyte lysate, was separated on a 13.5% polyacrylamide gel in the presence of SDS (lane 1). Lanes 2–5, incubation of SCO1 with mitochondria; lane 2, no additives; lane 3, addition of proteinase K after import; lane 4, incubation in the presence of valinomycin; lane 5, as in the lane 4 with subsequent proteinase K treatment. **B** Alkaline treatment. Membrane association was tested after import and proteinase K treatment by the extractibility of proteinase K-resistant proteins with sodium carbonate. Lane 6, membrane fraction; lane 7, soluble fraction; precursor and processed forms of the SCO1 protein are denoted by p and m, respectively

In order to test whether the SCO1 protein is imported into mitochondria, *SCO1* RNA was synthesized from plasmid pBSB10 in vitro. This RNA was then used to direct protein synthesis in a reticulocyte lysate in the presence of [$^{35}$S]methionine. The resulting polypeptide with an apparent molecular weight of 33 kDa (Fig. 5, lane 1) was incubated with isolated energized mitochondria (see Material and methods). After incubation for 30 min about half of the protein was found to be converted into a polypeptide with an apparent molecular weight of 30 kDa (Fig. 5, lane 2). In contrast to the larger protein, this 30 kDa polypeptide is resistant to a subsequent proteinase K treatment for 30 min (Fig. 5, lane 3). This result shows that the primary translation product is the precursor form of the 30 kDa protein. The latter is protected from proteinase K cleavage and thus must have been imported into mitochondria.

Studies on mitochondrial import (for review see Douglas et al. 1986) have shown that translocation of a protein across the mitochondrial membrane requires a membrane potential and that the addition of the ionophore valinomycin in the presence of KCl completely abolishes import of a given protein. As shown in Fig. 5 (lane 4) the 33 kDa protein remains in its precursor form, if valinomycin is included in the incubation medium. Under these conditions, subsequent proteinase K treatment results in complete degradation of SCO1 protein (Fig. 5, lane 5).

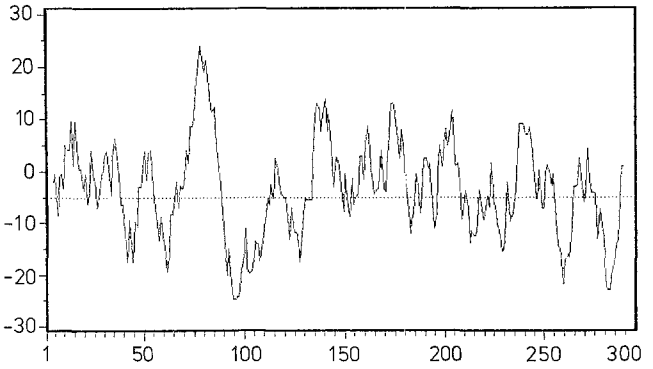


**Fig. 6.** Hydropathy profile of the SCO1 protein. The hydropathic index of the SCO1 protein was calculated according to the algorithm of Kyte and Doolittle (1982) using an interval of nine amino acids. Ordinate, the hydropathic index value; abscissa, SCO1 protein residue number

In summary, the results of the in vitro import experiments show that *SCO1* encodes the precursor form of a mitochondrial protein.

*The* SCO1 *protein is tightly membrane-associated*

To gain further insight into the localization of the mature SCO1 protein, we tested its resistance to alkaline treatment. Integral membrane proteins persist during this extraction procedure and they consequently co-sediment with the membrane fraction (Fujiki et al. 1982). Labelled SCO1 protein was used for an in vitro import experiment as described above. After treatment with proteinase K, mitochondria were treated with sodium carbonate. The membraneous fractions were sedimented by centrifugation, while the soluble and extracted proteins were recovered and analyzed separately. As shown in Fig. 5B, most of the labelled SCO1 protein co-sediments with the membraneous fraction. Only a minimal portion of the SCO1 protein is extracted from the membranes and is therefore found in the soluble fraction. Thus, the SCO1 protein is tightly associated with membrane components of mitochondria. This result is not surprising in view of the hydropathy profile of the SCO1 protein: it contains in the amino terminal third a hydrophobic domain of about 20 amino acids, which according to Rao and Argos (1986) represents a membrane-spanning $\alpha$-helix (Fig. 6).

## Discussion

We have recently identified a yeast nuclear mutant (*sco1-1*) which showed a deficiency of COXII in the mitochondrial translation products. The *SCO1* gene was isolated by functional complementation and used to construct a strain with a completely deleted *SCO1* gene. Our present data demonstrate that this deletion leads to a concomitant loss of COXI and COXII. The lack of COXI accumulation does not result from the absence of COXII (a conclusion already evident from the phenotype of various other nuclear or mitochondrial COXII-deficient mutants, e.g. Klöckener-Gruissem et al. 1987; Weiss-Brummer et al. 1979), but instead results from the absence of the nuclear *SCO1* gene product which is required for accumulation of both cytochrome *c* oxidase subunits. This result suggests that the SCO1 protein, encoded by the originally described *sco1-1* allele, has lost only a partial function, i.e. the ability to accumulate COXII, while it is still functional with respect to COXI accumulation. Thus the analysis of the *sco1-1* allele should allow the protein domain which is specifically required for COXII accumulation to be identified in future experiments.

Mitochondrial translation of COXII has been shown to require an independent nuclear coded factor, PET111, which specifically stimulates translation of *COXII* mRNA (Poutre and Fox 1987). Similar gene-specific translation factors have been reported for the mitochondrial RNAs of the cytochrome b gene (*COB*) (Dieckmann and Tzagoloff 1985; Rödel 1986) and of the *COXIII* gene (Costanzo and Fox 1988). Fusion of the respective mitochondrial target genes to untranslated leader sequences of other mitochondrial genes made their translation independent of the nuclear gene products. This suggests that the nuclear coded factors act on the RNA leader sequences to allow translation. This proposal is in agreement with the observation that the *CBS1* gene product is necessary for translation of a heterologous gene fused to the *COB* leader (Rödel and Fox 1987). The inability of a chimeric gene (the *COXII* structural gene fused to the *COXI* leader) to suppress the *sco1-1* mutation suggests that *SCO1* might act on a post-translational level such as subunit assembly, rather than via the leader sequence and through translational control. This assumption implies that unassembled subunits would be rapidly degraded as in the case of unassembled subunits of some oligomeric complexes (for review see: Rechsteiner et al. 1987).

Candidates to fulfill some role in the process of cytochrome *c* oxidase assembly are the nuclear coded subunits whose function is still unclear. Recently, the role of one of the cytochrome *c* oxidase subunits, COXIV, in the assembly process has been documented (Dowhan et al. 1985). Both the size of the SCO1 protein and the *SCO1* DNA sequence are different from the known cytochrome *c* oxidase subunits which therefore excludes the possibility that SCO1 might represent one of them. Furthermore, the phenotype obtained by disruption of the *COXIV* gene (Dowhan et al. 1985) is different from that of the *SCO1* deletion. This observation excludes the possibility that *SCO1* might represent a factor controlling COXIV expression.

Other factors required for formation of a functional cytochrome *c* oxidase are heme and oxygen. Studies on a heme-deficient mutant revealed that, in the absence of heme, COXII (as well as COXIII and COXIV) is still detectable, while all the other subunits are severely reduced or undetectable (Saltzgaber-Müller and Schatz 1978). Again this divergent phenotype argues against the possibility of *SCO1* being involved in the biosynthesis of heme or functioning as a heme-lyase. Moreover, the other mitochondrial heme-proteins (b, $c_1$, c) are present in normal concentrations in *sco1*-mutants.

Pulse-labelling studies of intact cells showed that COXI and COXII were immunoprecipitable with an antiserum directed against holo cytochrome *c* oxidase only if cells were labeled in the presence of oxygen (Groot and Poyton 1975). From detailed studies, Woodrow and Schatz (1979) conclude that the primary effect of oxygen is to induce association of subunits I and II with subunits VI and VII rather than to control the synthesis of these subunits. As a possible molecular mechanism of the oxygen effect, which is independent of cytoplasmic and mitochondrial protein synthesis, its effects on heme *a* formation or on the oxida-

tion/reduction state of heme *a* or copper was discussed. In view of our data presented here it is reasonable to propose that SCO1 is part of a pre-complex of COXI and II, which allows integration of the two subunits into holo cytochrome *c* oxidase in the presence of oxygen (or an oxygen-mediated effector molecule). Under anaerobic conditions (in the absence of the assembly-mediating effector), newly synthesized COXI and II would not be assembled, but nevertheless be detectable, if formation of a pre-complex confers (partial) protease resistance. In the absence of SCO1 (as a result of *SCO1* gene deletion), however, formation of a pre-complex would be impossible and – as a consequence – newly synthesized subunits I and II would be degraded. This hypothesis would be in line with the observation that the level of *SCO1* RNA is not affected in glucose-repressed or anaerobically grown cells (Schulze and Rödel 1988; T. Pillar, personal communication)

SCO1 is located in a mitochondrial membrane. Since integration of a mitochondrial protein into the outer membrane has not yet been shown to be accompanied by a processing event, SCO1 is likely to be an inner mitochondrial membrane protein. In the light of the above proposal it may function at its location to form the cytochrome *c* oxidase pre-complex or as a receptor protein, targetting individual cytochrome *c* oxidase subunits to their proper position in the membrane. Evidence for receptor-like proteins for some cytoplasmically synthesized precursor proteins at the outer face of mitochondria comes from the observation that in vitro import can be abolished by pretreatment of mitochondria with trypsin or with antisera directed against outer membrane proteins (Riezmann et al. 1983; Zwizinski et al. 1984). It is unknown to date whether similar receptor-like proteins exist in the inner membrane which target mitochondrial translation products or imported matrix-delivered intermediate proteins to their proper location.

SCO1 is synthesized as a larger precursor protein which is cleaved during import. It is most likely to be the amino terminal end, which fits common features of mitochondrial targeting sequences, which is cleaved off. Interestingly, if import was allowed to occur for only 10 min, we observed an additional polypeptide of a electrophoretic mobility slightly slower than the mature form (data not shown). Whether this polypeptide represents a bona fide intermediate of a two-step processing event as documented for proteins of the inner membrane (e.g. Fe/S protein; Hartl et al. 1986) and the intermembrane space (e.g. cytochrome $b_2$, Hartl et al. 1987) remains to be elucidated.

*Acknowledgements.* We thank E. Prätzl for excellent technical assistance, Dr. F.-U. Hartl and A. Körte, Munich, for advice in the in vitro import experiments, A. Candussio for help in computer analysis and Dr. H. Jäckle for critically reading of the manuscript. This work was supported by the Deutsche Forschungsgemeinschaft (SFB 184, project A7).

## References

Bennetzen JL, Hall BD (1982) Codon selection in yeast. J Biol Chem 257:3026–3031

Bonitz GS, Coruzzi G, Thalenfeld BE, Tzagoloff A, Macino G (1980) Assembly of the mitochondrial membrane system: structure and nucleotide sequence of the gene coding for subunit 1 of yeast cytochrome oxidase. J Biol Chem 255:11927–11941

Capaldi RA, Malatesta F, Darley-Usmar VM (1983) Structure of cytochrome *c* oxidase. Biochim Biophys Acta 726:135–148

Corruzi G and Tzagoloff A (1979) Assembly of the mitochondrial membrane system: DNA sequence of subunit 2 of yeast cytochrome oxidase. J Biol Chem 254:9324–9330

Costanzo MC, Fox TD (1986) Product of *Saccharomyces cerevisiae* nuclear gene PET494 activates translation of specific mitochondrial mRNA. Mol Cell Biol 6:3694–3703

Costanzo MC, Fox TD (1988) Specific translational activation by nuclear gene products occurs in the 5′ untranslated leader of a yeast mitochondrial mRNA. Proc Natl Acad Sci USA 85:2677–2681

Costanzo MC, Seaver EC, Fox TD (1986) At least two nuclear gene products are specifically required for translation of a single yeast mitochondrial mRNA. EMBO J 5:3637–3641

Daum G, Böhni PC, Schatz G (1982) Import of proteins into mitochondria: cytochrome $b_2$ and cytochrome *c* peroxidase are located in the intermembrane space of yeast mitochondria. J Biol Chem 257:13028–13033

Dieckmann CL, Tzagoloff A (1985) Assembly of the mitochondrial membrane system: *CBP6*, a yeast nuclear gene necessary for synthesis of cytochrome *b*. J Biol Chem 260:1513–1520

Douglas MG, McCammon MT, Vassarotti A (1986) Targeting proteins into mitochondria. Microbiol Rev 50:166–178

Dowhan W, Bibus RC, Schatz G (1985) The cytoplasmically-made subunit IV is necessary for assembly of cytochrome *c* oxidase in yeast. EMBO J 4:179–184

Fox TD (1979) Five TGA "stop" codons occur within the translated sequence of the yeast mitochondrial gene for cytochrome *c* oxidase subunit II. Proc Natl Acad Sci USA 76:6534–6538

Fujiki Y, Hubbard AL, Fowlern S, Lazarow PB (1982) Isolation of intracellular membranes by means of sodium carbonate treatment: application to endoplasmic reticulum. J Cell Biol 93:97–102

Groot GSP, Poyton RO (1975) Oxygen control of cytochrome *c* oxidase synthesis in isolated mitochondria from *Saccharomyces cerevisiae*. Nature 255:238–240

Hartl F-U, Schmidt B, Wachter E, Weiss H, Neupert W (1986) Transport into mitochondria and intramitochondrial sorting of the Fe/S protein of ubiquinol-cytochrome *c* reductase. Cell 47:939–951

Hartl F-U, Ostermann J, Guiard B, Neupert W (1987) Successive translocation into and out of the mitochondrial matrix: targeting of proteins to the intermembrane space by a bipartite signal peptide. Cell 51:1027–1037

Hurt EC, van Loon APGM (1986) How proteins find mitochondria and intramitochondrial compartments. Trends Biochem Sci 11:204–207

Hurt EC, Pesold-Hurt B, Suda K, Oppliger W, Schatz G (1985) The first twelve amino acids (less than half of the pre-sequence) of an imported mitochondrial protein can direct mouse cytosolic dihydrofolate reductase into the yeast mitochondrial matrix. EMBO J 4:2061–2068

Kloeckener-Gruissem B, McEwen JE, Poyton RO (1987) Nuclear functions required for cytochrome *c* oxidase biogenesis in *Saccharomyces cerevisiae*: multiple trans-acting nuclear genes exert specific effects on expression of each of the cytochrome *c* oxidase subunits encoded on mitochondrial DNA. Curr Genet 12:311–322

Koerner TJ, Hill J, Tzagoloff A (1985) Cloning and characterization of the yeast nuclear gene for subunit 5 of cytochrome oxidase. J Biol Chem 260:9513–9515

Kramer W, Drutsa V, Jansen HW, Kramer B, Pflugfelder M, Fritz HJ (1984) The gapped duplex DNA approach to oligonucleotide-directed mutation construction. Nucleic Acids Res 12:9441–9456

Kyte J, Doolittle RF (1982) A simple method for displaying the hydropathic character of a protein. J Mol Biol 157:105–132

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685

Maarse AC, Van Loon APGM, Riezman H, Gregor I, Schatz

G, Grivell LA (1984) Subunit IV of yeast cytochrome *c* oxidase: cloning and nucleotide sequencing of the gene and partial amino acid sequencing of the mature protein. EMBO J 3:2831–2837

Mahler HR, Bastos RN, Feldman F, Flury U, Lin CC, Perlman PS, Phan SH (1975) Biogenetic autonomy of mitochondrial and its limits. In: Tzagoloff A (ed) Membrane biogenesis. Plenum Press, New York, pp 15–61

McEwen JE, Ko C, Kloeckner-Gruissem B, Poyton RO (1986) Nuclear functions required for cytochrome *c* oxidase biogenesis in *Saccharomyces cerevisiae*. J Biol Chem 261:11872–11879

Patterson TE, Poyton RO (1986) COX8, the structural gene for yeast cytochrome *c* oxidase subunit VIII. J Biol Chem 261:17192–17197

Poutre CG, Fox TD (1987) PET111, a *Saccharomyces cerevisiae* nuclear gene required for translation of the mitochondrial mRNA encoding oxidase subunit II. Genetics 115:637–647

Power SD, Lochrie MA, Sevario KA, Patterson TE, Poyton RO (1984) The nuclear-coded subunits of yeast cytochrome *c* oxidase: I. Fractionation of the holoenzyme into chemically pure polypeptides and the identification of two new subunits using solvent extraction and reversed phase high performance liquid chromatography. J Biol Chem 259:6564–6570

Rao JKM, Argos P (1986) A conformational preference parameter to predict helices in integral membrane proteins. Biochim Biophys Acta 869:197–214

Rechsteiner M, Rogers S, Rote K (1987) Protein structure and intracellular stability. Trends Biochem Sci 12:390–394

Riezmann H, Hay R, Witte C, Nelson N, Schatz G (1983) Yeast mitochondrial outer membrane specifically binds cytoplasmically synthesized precursors of mitochondrial proteins. EMBO J 2:1113–1118

Rödel G (1986) Two nuclear genes, *CBS1* and *CBS2*, are required for translation of mitochondrial transcripts bearing the 5′ untranslated *COB* leader. Curr Genet 11:41–45

Rödel G, Fox TD (1987) The yeast nuclear gene CBS1 is required for translation of mitochondrial mRNAs bearing the COB 5′ untranslated leader. Mol Gen Genet 206:45–50

Rödel G, Körte A, Kaudewitz F (1985) Mitochondrial suppression of a yeast nuclear mutation which affects the translation of the mitochondrial apocytochrome *b* transcript. Curr Genet 9:641–648

Saltzgaber-Müller J, Schatz G (1978) Heme is necessary for accumulation and assembly of cytochrome *c* oxidase subunits in *Saccharomyces cerevisiae*. J Biol Chem 253:305–310

Sanger F, Nicklen S, Coulson AR (1977) DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA 74:5463–5467

Schatz G (1987) Signals guiding proteins to their correct locations in mitochondria. Eur J Biochem 165:1–6

Schatz G, Mason TL (1974) The biosynthesis of mitochondrial proteins. Annu Rev Biochem 43:51–87

Schulze M, Rödel G (1988) SCO1, a yeast nuclear gene essential for accumulation of mitochondrial cytochrome *c* oxidase subunit II. Mol Gen Genet 211:492–498

Séraphin B, Simon M, Faye G (1985) Primary structure of a gene for subunit V of the cytochrome *c* oxidase from *Saccharomyces cerevisiae*. Curr Genet 9:435–439

Sherman F, Fink GR, Lawrence CW (1986) In: Methods in Yeast Genetics, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York

Thalenfeld BE, Tzagoloff A (1980) Assembly of the mitochondrial membrane system: sequence of the oxi2 gene of yeast mitochondrial DNA. J Biol Chem 255:6173–6180

Van Heijne G (1986) Mitochondrial targeting sequences may form amphiphilic helices. EMBO J 5:1335–1342

Weiss-Brummer B, Guba R, Haid A, Schweyen RJ (1979) Fine structure of *OXI1*, the mitochondrial gene coding for subunit II of yeast cytochrome *c* oxidase. Curr Genet 1:75–83

Woodrow G, Schatz G (1979) The role of oxygen in the biosynthesis of cytochrome *c* oxidase of yeast mitochondria. J Biol Chem 254:6088–6093

Wright RM, Ko C, Cumsky MG, Poyton RO (1984) Isolation and sequence of the structural gene for cytochrome *c* oxidase subunit VI from *Saccharomyces cerevisiae*. J Biol Chem 259:15401–15407

Wright RM, Dircks LK, Poyton RO (1986) Characterization of *COX9*, the nuclear gene encoding the yeast mitochondrial protein cytochrome *c* oxidase subunit VIIa. J Biol Chem 261:17183–17191

Zwizinski C, Schleyer M, Neupert W (1984) Proteinaceous receptors for the import of mitochondrial precursor proteins. J Biol Chem 259:7850–7856

Communicated by W. Gajewski

Received August 14, 1988