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RNA processing in yeast mitochondria: characterization of mit⁻ mutants disturbed in the synthesis of subunit I of cytochrome *c* oxidase

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Summary. Mit⁻ mutants disturbed in the synthesis of cytochrome *c* oxidase subunit I lack the mRNA for this protein and accumulate longer RNAs still containing intron sequences. We have analyzed the patterns of transcripts occurring in several such mutants in an attempt to define a pathway of processing events and to demarcate intron-sequences involved in RNA splicing. We find that processing does not follow a strictly ordered pathway and, in contrast to the situation for the cytochrome *b* gene, that a block in the processing of an intron does not necessarily lead to a block in the processing of introns downstream. Although in some cases, this may result from overlapping specificities of intronic-URF encoded RNA maturases, an internal start of translation on precursor RNAs seems more likely.

M5-16, a mutant deleted for a large part of the central portion of the subunit I gene exhibits delayed processing and a highly simplified pattern of intermediates. The lengths of these indicate that maturation of the mRNA for subunit I involves processing, as well as splicing.

Key words: RNA processing – Yeast mitochondria – mit⁻ mutants – RNA splicing

Introduction

The gene for subunit I of cytochrome *c* oxidase (Fig. 1), localized in the *OXI3* region of yeast mtDNA, is split and contains between 4 and 10 exons, dependent on strain (Bonitz et al. 1980b; Hensgens et al. 1983a). As in the two other split genes of this mitochondrial genome, several of the introns contain long open reading frames (URFs) and both we and others have surmised that these encode proteins required for RNA processing. Thus complete processing of *OXI3* transcripts is dependent on mitochondrial protein synthesis (Hensgens et al. 1983b). Mutations within introns hamper or block RNA splicing; several of the URFs display clear amino acid homology with known RNA maturases (Bonitz et al. 1980b; Hensgens et al. 1983a). Some introns contain sequences closely resembling the cis-acting elements shown to be necessary for the maturase-mediated splicing of transcripts of the cytochrome *b* gene (Davies et al. 1982; Michel et al. 1982; Michel and Dujon 1983; De la Salle et al. 1982; Anziano et al. 1982; Netter et al. 1982; Schmelzer et al. 1982 and Weiss-Brummer et al. 1982). Despite this information, many features of the various steps in the processing of the RNAs transcribed from this gene have not yet been fully resolved. Two URF-containing introns lack both amino acid homology with other URFs and nucleotide homology with previously defined cis-acting elements (Michel and Dujon 1983). Three intronic sequences accumulate after excision as circular RNAs (Arnberg et al. 1980; Hensgens et al. 1983b) and the exact relationship of this circularization to the splicing process remains to be established.

In this paper, we report an initial characterization of a number of mit⁻ mutants that fail to synthesize subunit I of cytochrome *c* oxidase as a result of a block in RNA splicing. As in the *COB* region, study of such mutants should facilitate identification of distinct stages in mRNA maturation and lead to the definition of intron sequences important in splicing.

Materials and methods

Isolation of nucleic acids. Cells of wild type *S. cerevisiae* 777-3A (long version of the subunit I and apocytochrome *b* gene), *S.*

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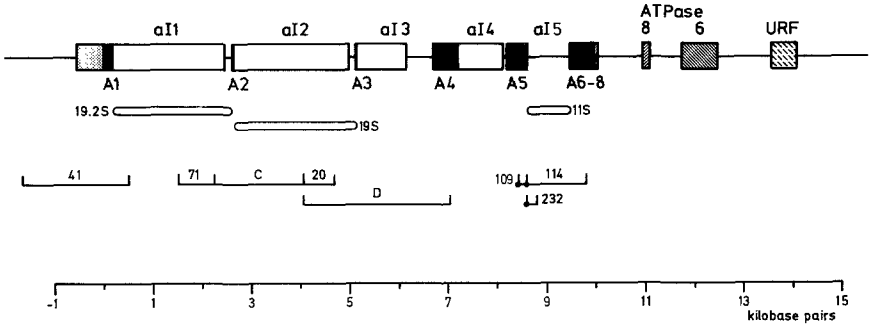

Fig. 1. Organization of the *OXI3-OLI2* region. Black bars represent sequences coding for subunit I (exons A1-A8). The open bars represent the URFs present within the introns (aI1-aI4). Dotted bars represent untranslated 5'-leader and 3'-trailing sequences present in the 18S mRNA of the subunit I. The genes for the ATPase subunits 6 and 8 and the URF2 are indicated by the different striped bars. The positions of the DNA sequences contained within the different clones used as probes in the hybridization experiments are given (see also Hensgens et al. 1983b). Probes 109, 114 and 232 contain additional intron sequences, absent from *S. cerevisiae* D273-10B. The sites of insertion of these introns are indicated by 0. The positions of the sequences coding for the circular RNAs are also indicated

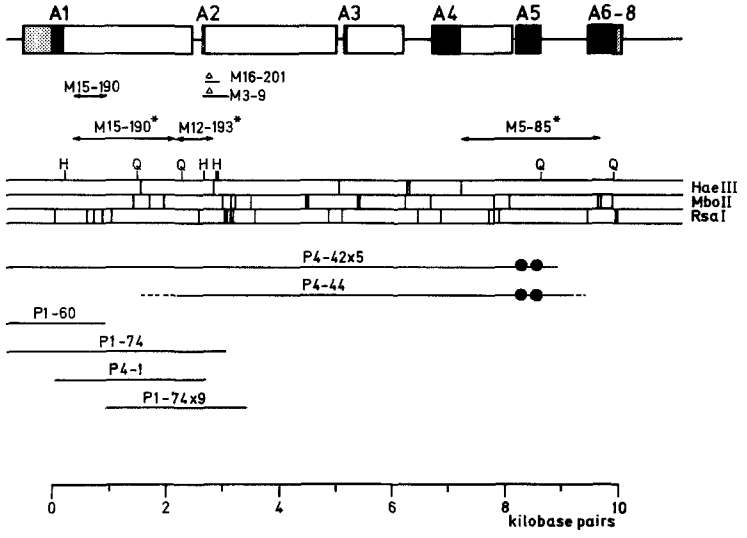

Fig. 2. Genetic and physical map of the cytochrome *c* oxidase subunit I gene. The sites of restriction enzymes used in the physical characterization of the discriminating rho$^-$ mutants are indicated, including HhaI (H) and TaqI (Q). The wild-type sequences retained in the rho$^-$ mutants are shown by solid lines and the uncertainties in the mapping are indicated by broken lines. Triangles pinpoint the mapped deletions present in the *oxi3* mutants M16-201 and M3-9 (see Figs. 3 and 4) with the bars indicating the approximate extent of the deletions. * localization of the *oxi3* mutants as determined by Bonitz et al. 1980a, b. The localization of the mutation present in M15-190 as deduced from the combined data of this study and Bonitz et al. 1980a, b is also indicated. See Fig. 1 for further details

cerevisiae D273-10B and the mutants were grown in 2% (w/v) galactose, 1% (w/v) yeast extract and 1% (w/v) Bactopeptone. Yeast cells were harvested in mid-to-late exponential phase. mtRNA was isolated and treated with pronase and deoxyribonuclease as described by Van Ommen et al. (1979). mtDNA was prepared as described by Moorman et al. (1978).

Electrophoresis and hybridization. Electrophoresis, staining, transfer to diazobenzyloxymethyl (DBM)-cellulose paper and hybridization were performed according to Van Ommen et al. (1979) and Hensgens et al. (1983b). 21S rRNA, 15S rRNA and the *OXI3* 18S mRNA (3,273, 1,686 and 2,219 nucleotides respectively; Grivell, 1984) were used as size markers for calibration of transcripts.

In vitro labelling of nucleic acids. Plasmid preparations and mtDNA of the petite mutant LH30-A5, covering the whole *OXI3-OLI2* region (Hensgens et al. 1983b) were used.

Genetic localization of oxi3 mutants. The restoration of respiratory competence in the *oxi3* mutants (parental type *S. cerevisiae* D273-10B) when crossed with discriminating rho$^-$ clones (derived from the petite mutant LH30-A5; Hensgens et al. 1983b) was carried out as described by Slonimski and Tzagoloff (1976) and Schweyen et al. (1977).

Results

A collection of mit$^-$ mutants, deficient in the synthesis of cytochrome *c* oxidase subunit I (coxI) was screened by RNA blotting for abnormal transcript patterns. Those mutants showing deviation from the wild type pattern in terms of the level of the mature 18S mRNA (2,200 nucleotides; Hensgens et al. 1983b) and accumulation of high molecular weight intermediates were analyzed further with respect to the nature of transcripts accumulated and the nature and position of the lesion present. The main conclusions of this study are summarized in the following sections:

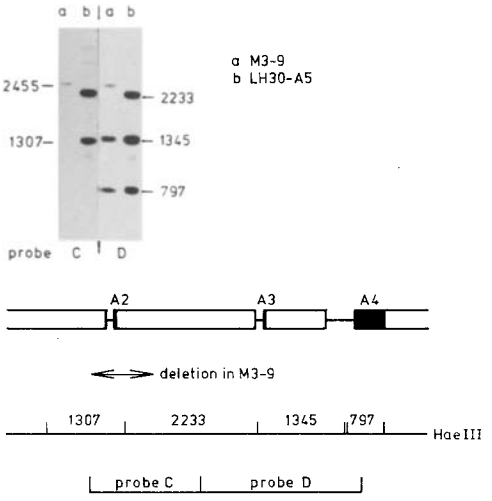

Fig. 3. Mapping of the deletion present in M3-9. mtDNA of the *oxi3* mutant M3-9 and the rho⁻ mutant LH30-A5 was digested with HaeIII. Southern blots were hybridized with two probes of the aI2 region. In the hybridization with probe C two bands of 2,233 and 1,307 bp are missing and a new band of 2,455 bp appears which also hybridizes with probe D. The probable localization of the 1,085 bp deletion in M3-9 is indicated

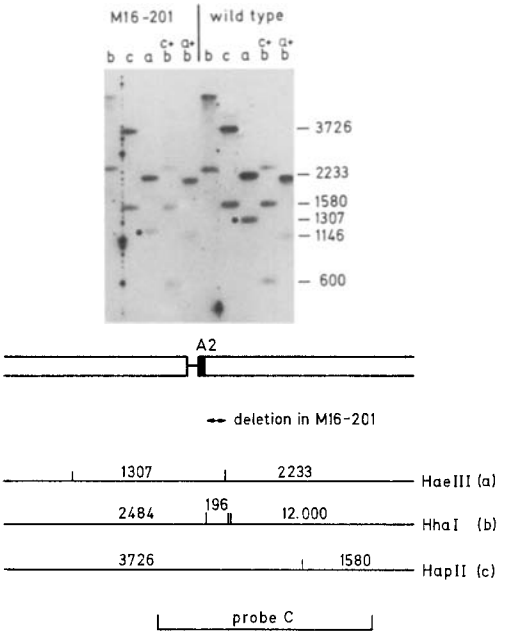

Fig. 4. Mapping of the deletion in M16-201. mtDNA of the *oxi3* mutant M16-201 and the wild type *S. cerevisiae* D273-10B were digested with HaeIII (a), HhaI (b), HapII (c) and a combination of HhaI with HaeIII (a + b) or with HapII (b + c). The DNA fragments were separated on an agarose gel (1.2%), blotted to nitrocellulose and hybridized with probe C. The 1307 bp HaeIII wildtype fragment and the mutant fragment are indicated by a *. The 3,726 bp HapII fragment of wild-type DNA is slightly smaller in M16-201 (lanes c). The 196 bp HhaI wild-type fragment has run off the gel. Double digests do not show any deviation from wild-type indicating that the small deletion is located within the 196 bp HhaI fragment

Physical mapping of mit⁻ mutants

The mutants were first described by Slonimski and Tzagoloff (1976), who used a combination of rho⁻ x mit⁻ and mit⁻ x mit⁻ crosses for mapping. Their results suggested that the mutants M16-201, M12-120, M5-16 and M11-125 are double, or deletion mutants. This has since been confirmed directly for M11-125 which contains mutations at the A1/aI1 boundary and in aI5 (Rabinowitz et al. 1976; Morimoto et al. 1979) and for M5-16 which harbours a large deletion (Rabinowitz et al. 1976; Grivell and Moorman 1977; Morimoto et al. 1979; see also below). We have carried out further mapping studies and the results obtained which clarify some inconsistencies in the early mapping are shown in Figs. 2–4 and Table 1.

M15-190. Bonitz et al. (1980a) localized the mutation within the reading frame of intron aI1 between positions 291 and 2,136. More recent petite deletion mapping (Table 1, Fig. 2) has narrowed this range somewhat to the area between positions 220 and 1,000, but the nature of the changes is still unknown.

Table 1. Restoration to respiratory competence of *oxi3* mit⁻ mutants in crosses with discriminating rho⁻ mutants. +, – indicates restoration or lack of restoration respectively. The rho⁻ mutants were obtained by ethidium bromide mutagenesis of the rho⁻ strain LH30-A5 which was derived from the strain AMR34-17B (Hensgens et al. 1983b)

rho⁻ (a) \ mit⁻ (α)	M15-190	M12-193	M16-201	M3-9	M9-9	M5-85	M5-16
P1-60	+	–	–	–	–	–	–
P1-74	+	+	–	–	–	–	–
P1-74x9	–	+	–	+	–	–	–
P4-1	+	+	–	–	–	–	–
P4-42x5	+	+	+	+	+	+	–
P4-44	–	+	+	+	–	+	+

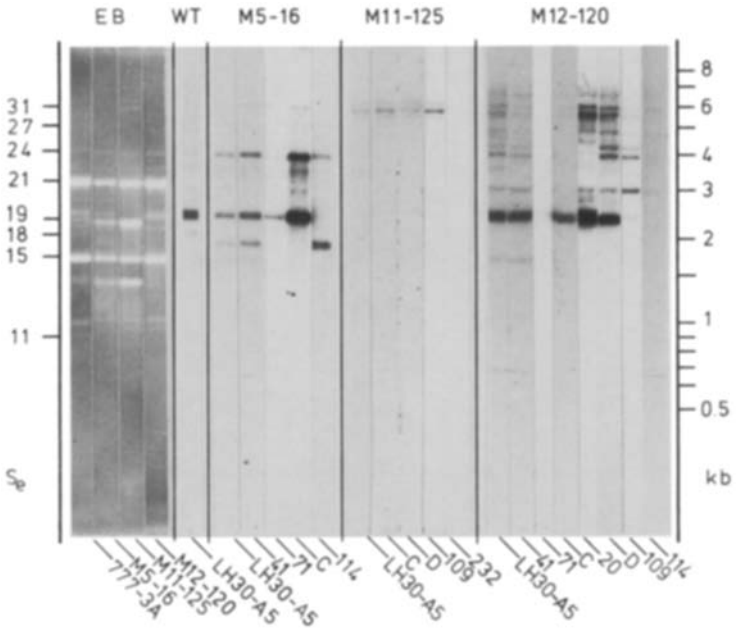

Fig. 5. *OXI3* transcripts in wildtype and *oxi3* mutants. mtRNA was prepared from *S. cerevisiae* 777-3A (WT), and the *oxi3* mutants M5-16, M11-125 and M12-120. 1 μg mtRNA of each strain was electrophoresed through 1.25% (w/v) agarose gels, transferred to DBM paper and hybridized with different probes from the *OXI3* region. The LH30-A5 probe also detects the 22S and 24S transcripts encoded by the genes for ATPase subunits 6 and 8 (Hensgens et al. 1983b). Sequences retained in the *OXI3* clones used as probes are shown in Fig. 1

M5-16. This mutant contains a 6,200 bp deletion which has eradicated the central portion of the coxI gene. Although early estimates suggested the size of the deletion to be around 5,400 bp, our present hybridization results show that all sequences between and including the HhaI site at the boundary of exon A2 with intron aI2 (position 2,652) and a TaqI site at the exon A5-intron aI5 border (Fig. 2) are absent. Since excision of intron aI1 occurs normally (see below), the upstream boundary of the deletion must lie within exon A2 (36 bp); we have no information on the site of the downstream boundary in aI5.

M9-9. Restriction mapping of this mutant has not revealed any differences from wild type (data not shown). Petite mapping suggests, however, that at least two mutations are present (Table 1). The first is located upstream of M15-190 (see above). The second is likely to be between mutants M3-9 (which contains a 1,000 bp deletion in the 5′-part of intron aI2; Fig. 3) and M5-85, localized by Bonitz et al. (1980a, b) between positions 2,844–4,537 and 7,190–9,632 respectively (Fig. 2).

M16-201. The available evidence strongly suggests that this is a double mutant. A small deletion can be mapped by restriction enzyme analysis around the A2/aI2 border (Fig. 4), but petite restoration patterns (Table 1) indicate that this change alone cannot be responsible for the phenotype. The position of the second change is most probably downstream of the deletion in M3-9.

M12-120. No changes with respect to wild type have been detected by restriction mapping (Grivell and Moorman 1977; this study, data not shown). The behaviour of this mutant in the complementation studies of Slonimski and Tzagoloff (1976) is rather complex so that no firm conclusions can be drawn as to the location of the mutation(s). Transcript mapping (see below) suggests that at least one important change lies within intron aI5.

Excision of aI1 is independent of A2-aI5 sequences

Transcripts accumulating in the deletion mutant M5-16 are shown in Fig. 5 and are identified in Table 2. Due to the size of the deletion, 18S mRNA and the excision products of introns aI2 and aI5 are missing. A major transcript comigrates with the wildtype 19.2S circular RNA derived from the splicing of aI1 and hybridizes specifically with probes for this intron. We conclude that this event leads to the formation of a truncated 1,900 nucleotides RNA in which part of exon A2 is fused to part of aI5. Excision of all is thus independent of sequences covered by the deletion.

Processing of introns aI1 and aI2

For the apocytochrome *b* gene, hybridization studies with RNA isolated from splicing-deficient mutants have established that intron excision must be an ordered process, since a mutation which blocks splicing in an upstream intron also blocks excision of the downstream introns, whose splicing is dependent on the translation of an intronic reading frame (Van Ommen et al. 1980;

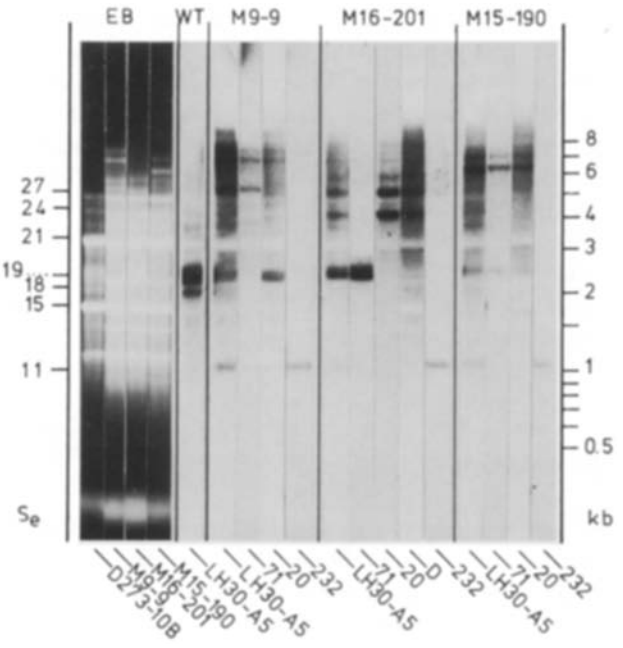

Fig. 6. *OXI3* transcripts in wildtype and *oxi3* mutants. mtRNA was prepared from *S. cerevisiae* D273-10B (WT) and the *oxi3* mutants M9-9, M16-201 and M15-190. See for further details Figs. 1 and 5

Haid et al. 1980; Halbreich et al. 1980 and Schmelzer et al. 1981). Results obtained with the mutant M9-9 suggest that the situation in the *OXI3* gene is different. This mutant lacks 18S mRNA and the 19.2S RNA formed from aI1 as shown in Fig. 6. Yet excision products of aI2 and aI5 (19.0S and 11S respectively) are present at normal levels. This mutant also accumulates a number of abnormal transcripts whose size and hybridization behaviour suggest that excision of aI3 is blocked, but this is probably due to the presence of the second, unmapped lesion in this mutant (see below).

Although the results obtained with M9-9 indicate that excision of aI2 is not necessarily coupled to that of aI1, mutations in the latter intron can prevent splicing of both introns. This is shown in Fig. 6 by the mutant M15-190, which according to petite deletion mapping contains a single clearly-defined lesion in the N-terminal region of the aI1 reading frame. Levels of both 19.0S and 19.2S circular RNAs are severely decreased and high molecular weight transcripts, still containing sequences of aI1 and aI2 accumulate.

Table 2. *OXI3* transcripts in the *oxi3* mutants. The table summarizes the data obtained from Fig. 2 and 3. The sizes of the transcripts are given in nucleotides. (high) and (low) indicate the presence of transcripts in high respectively low (*) relative concentration

Mutants	Intermediates		Normal	Missing
	(high)	(low)	transcripts	transcripts
M5-16	1,900 (17S) 4,100 (24S)	3,000 (20S) 5,200 (27S) 6,200 (30S)	19.2S (aI1)	19.0S (aI2) 11.0S (aI5) 18S mRNA
M11-125		5,600 (28S) 5,800 (29S) 6,000 (29S)		19.2S (aI1) 19.0S (aI2) 11.0S (aI5) 18S mRNA
M12-120	3,000 (20S) 3,900 (23S) 4,200 (24S) 5,500 (28S) 5,900 (29S)	2,400 (19S) 4,800 (26S) 5,200 (27S) 6,400 (30S) 6,900 (31S)	19.2S (aI1) 19.0S (aI2)	11.0S (aI5) 18S mRNA
M15-190	6,300 (30S) 7,100 (32S) 7,700 (33S)		11.0S (aI5) 19.2S (aI1)* 19.0S (aI2)*	18S mRNA
M16-201	4,200 (24S) 5,200 (27S)	5,900 (29S) 6,900 (31S) 7,300 (33S) 7,800 (34S)	19.2S (aI1) 11.0S (aI5)	19.0S (aI2) 18S mRNA
M9-9	5,200 (27S)	6,700 (31S) 7,400 (33S)	19.0S (aI2) 11.0S (aI5)	19.2S (aI1) 18S mRNA

Processing of introns aI3, aI4 and aI5

Introns aI3, aI4 and aI5 can be excised without prior excision of aI1 or aI2. This follows from the transcript pattern observed in M15-190 (Fig. 6), which normally excises introns aI3 through aI5. The 11S RNA from aI5 is clearly visible and the smallest abnormal transcript accumulating corresponds in both size and hybridization to an 18S species still containing aI1 and aI2 only (6,300 nucleotides). This is also observed in other mutants. *OXI3* transcripts present in the mutant M16-201 are shown in Fig. 6 and are identified in Table 2. Probably due to the mutation at the A2/aI3 border, mature 18S mRNA and the excision product of intron aI2 (19.0S) are missing. 19.2S and 11S RNA, derived from introns aI1 and aI5, are present in concentrations similar to wild-type and hybridize specifically with probes for these introns. The smallest abnormal transcript accumulating (24S, approx. 4,200 nucleotides) corresponds both in size and hybridization to an 18S species still containing aI2. This indicates that splicing of aI3 and aI4 occurs normally despite the fact that excision of aI2 is blocked.

A similar observation is made in the complex mutant M11-125 (Fig. 5). This mutant lacks 18S mRNA and the 19.2S, 19.0S and 11S circular RNAs, excision products of introns aI1, aI2 and aI5 respectively. All *OXI3* probes detect three large transcripts of 6,000, 5,800 and 5,600 nucleotides. These sizes are probably underestimates of the true values since the calibration of transcripts larger than 4,000 nucleotides is unreliable. We therefore conclude that the 5,600 nucleotides transcript corresponds to an 18S transcript still containing aI1, aI2 and aI5 sequences (approx. 7,000 nucleotides) and that in this mutant introns aI3 and aI4 are excised without prior excision of aI1 and aI5. In all mutants studied thusfar intron aI5 is excised normally. Only M11-125 and M5-16 in which this intron is mutated, show a blocked aI5 excision. We have shown previously (Hensgens et al. 1983b) that this intron is also spliced in rho$^-$ mutants, which lack mitochondrial protein synthesis. This is consistent with the conclusion that excision of this intron is independent of splice events elsewhere in the *OXI3* region.

OXI3 transcripts in the mutant M12-120 are shown in Fig. 5. 18S mRNA and 11S RNA are absent. The smallest abnormal transcript (3,000 nucleotides) corresponds both in size and hybridization to a pre-mRNA still containing aI5 sequences (3,100 nucleotides). Apparently splicing of the upstream URF-containing introns is normal and independent of the excision of aI5. Furthermore, the 13 transcripts larger than 20S correspond to the various intermediates expected if splicing of introns aI1-aI4 occurs in a random fashion (see also Hensgens et al. 1983b).

Some mutants like M11-125 and M9-9 have multiple lesions in the *OXI3* region, as shown by petite restoration tests. These mutants also display complicated changes in the pattern of *OXI3* transcripts. In mutant M11-125, for example, aI2 is not spliced in addition to the blocked excision of introns aI1 and aI5. In mutant M9-9, aI3 excision is blocked. These observations can be explained by the presence of unmapped mutations in the affected introns.

Processing of introns aI3, aI4 and aI5 in M16-201 proceeds normally. The second lesion in this mutant has apparently no additional effects on RNA processing. The second mutation may therefore be located within aI2 or in sequences not involved in splicing.

Trimming of OXI3 transcripts

In vitro capping and run-off transcription initiation assays have recently established that transcription of the coxI gene starts approximately 500 bp upstream of the initiator AUG codon and that the mature mRNA does not undergo processing at its 5′-end (Osinga et al. 1984a). As for other yeast mitochondrial genes, RNA synthesis is initiated within the conserved nona-nucleotide 5′-ATATAAGTA-3′ first identified by Osinga et al. (1982). This conserved sequence is absent from the region separating the *OXI3* region from the genes for ATPase subunits 6 and 8 and it thus seems likely that these genes are not transcribed independently, but belong to the same transcription unit as the subunit I gene. This view has recently been strengthened by the finding that the 3′-end of the subunit I mRNA abuts the 5′-end of the longest transcript covering the *aap1-OLI2* region within a conserved dodecamer sequence. This sequence probably marks a processing site for mRNAs in yeast mitochondria (Osinga et al. 1984b).

The very large deletion in the mutant M5-16 makes it possible to study processing events other than splicing more precisely. As a consequences of the deletion only one splice event (excision of aI1) is possible, and the theoretical number of splicing intermediates is thus reduced from 31 to 1 (Hensgens et al. 1983b). The size of *OXI3* precursors is also reduced by 6,200 nucleotides, thus allowing a better resolution and size estimation. In addition to the truncated 1,9000-nucleotide (17S) RNA (see above) and excised aI2 (19.2S) only one major transcript of approximately 4,100 nucleotides (24S) is detected (Fig. 5) with all *OXI3* probes. We conclude that this transcript is the unspliced precursor to the 17S and 19.2S transcripts.

Two transcripts of 5,200 (27S) and 6,200 (30S) nucleotides are present in relatively low concentration. Since initiation of transcription occurs at a site 540 bp upstream of exon A1 (Osinga et al. 1984a) we conclude that these transcripts must have 3′-extensions of different lengths. The transcript of 5,200 nucleotides is

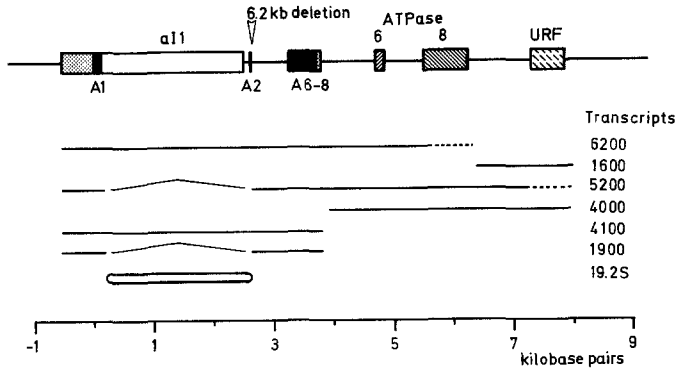

Fig. 7. Transcription map of the *OXI3-OLI2* region in the deletion mutant M5-16. The figure shows the physical organization of the region in this mutant and the position of the 6.2 kb deletion. Sequences coding for the different transcripts are indicated (——) with areas of uncertainty by (·····). (') indicates that intron aI1 is excised from these transcripts. The sizes of the transcripts is given in nucleotides. The sizes of the 1,600 and 4,000 nucleotide transcripts derived from the URF2 and the ATPase genes are re-estimated from those determined by Cobon et al. (1982). The values for the size of the rRNAs used by these authors in the size determination of transcripts were too high. See legend to Fig. 1 for further details

not detected by the aI1 probe 71; it therefore lacks intron aI1 and thus must have a 3'-extension of approx. 5,200 – 1,900 (17S) = 3,300 nucleotides.

The 6,200 nucleotides transcript is detected by the aI1 probe and contains sequences which extend 1,770 nucleotides (6,200-4,430: size of coxI gene in M5-16). The size determination of these long transcripts is imprecise and probably represents an underestimation. We therefore consider the possibility that the 3'-part of the 5,200 nucleotides RNA corresponds to the 24S major *aap2-OLI2* transcript (Van Ommen et al. 1979; Cobon et al. 1982; Hensgens et al. 1983b). The 6,200 nucleotides RNA could arise by cleavage of a longer transcript at a sequence which is also used for the generation of a second major transcript of 1,600 nucleotides covering the free URF (see Fig. 7 and Cobon et al. 1982).

Discussion

We have characterized the patterns of transcripts of the split subunit I gene for cytochrome *c* oxidase in mitochondrial mutants disturbed in the maturation of the mRNA. All mutants display characteristic patterns which are the consequence of a blocked or strongly inhibited splicing of (a) mutated intron(s). The patterns highlight some unique features of RNA processing at the *OXI3* locus which contrast with the situation for *COB*.

Translation of the OXI3 URFs may initiate internally

Processing of *OXI3* URF-containing introns is dependent on mitochondrial protein synthesis (Hensgens et al. 1983b). Recently Carignani et al. (1983) showed that the aI1 URF is likely to encode an RNA maturase involved in the splicing of intron aI1. Since the other *OXI3* intronic URFs (aI2, aI3 and aI4) display clear amino acid homology with known RNA maturases (Bonitz et al. 1980; Hensgens et al. 1983a), it is probable that they also encode maturases involved in RNA splicing. Thus in analogy with the apocytochrome *b* gene one might expect that mutations which block splicing in an upstream intron also block excision of those introns downstream, whose excision is dependent on the translation of the intronic reading frame. In contrast to this we observe that in mutants M11-124, M15-190 and M16-201 splicing of the URF-containing introns aI3 and aI4 proceeds normally despite the fact that the excision of introns aI1 and/or aI2 is blocked or strongly inhibited. In addition, intron aI2 and aI4 are spliced normally in mutant M9-9 while excision of aI1 and aI3 is fully blocked and introns aI3 and aI4 are spliced normally in M16-201 notwithstanding the blocked excision of intron aI2.

These differences can be explained in two ways: First splicing of the *OXI3* introns might be mediated by homologous maturases encoded elsewhere on the mitochondrial genome. Intron aI4 in *S. cerevisiae* D273-10B displays high sequence homology with intron bI1 (intron bI4 in the long version of the apocytochrome *b* gene; Bonitz et al. 1980b) and it has been proposed that a *box7* maturase encoded by bI1 is involved in the splicing of aI4 (Weiss-Brummer et al. 1982; Jacq et al. 1982; Hensgens et al. 1984). A similar explanation (overlapping maturase activity) might be valid for introns aI1 and aI2 which display high sequence homology and similar putative RNA secondary structure (Bonitz et al. 1980; Michel et al. 1982). It is however, difficult to magine why the aI1 and aI4 URFs should have been conserved in evolution if the activity of the maturases they encode can be replaced by the aI2 and bI4 URF-encoded maturases respectively. Furthermore all the aI1 mutants, defective in the trans-acting aI1 maturase (Carignani et al. 1983) accumulate an *OXI3* precursor whose size corresponds to a transcript containing 18S mRNA + intron aI1 sequences. Thus in these mutants intron aI2 can be excised without prior excision of aI1 and in the absence of an active aI1-encoded maturase. We therefore prefer a second possibility – namely that translation of downstream URFs can be initiated internally on intron-containing precursor RNAs that have accumulated as result of the splicing block. Translation of the aI2 URF thus might initiate at the AUG codon present within exon A2 or at the codons present 138 or 147 nucleotides downstream of A2. For the aI3 URF, an AUG codon in A3 can be

used, as can also 3 AUG codons present in the small reading frame preceding and in phase with this exon. For the aI4 URF translation might initiate at the four AUG codons in exon A4 or at the AUG codon 39 nucleotides downstream of this exon. This model implies that the region upstream of the internal start does not contribute to the active maturase. This has in fact been proposed for the bI4 and aI1 maturase (Weiss-Brummer et al. 1982; Jacq et al. 1982; Carignani et al. 1983). For the bI4 maturase it has been suggested that a cleavage occurs at or near an amino acid sequence which is conserved in both aI3 and aI4 (33 respectively 87 amino acids downstream of the upstream exon) (Hensgens et al. 1983a). Any initiation upstream of this sequence in intron or exon could thus yield a maturase precursor which can still be cleaved post- or co-translationally. A similar mechanism, internal start of translation accompanied by proteolytic cleavage, has been proposed for the expression of the *box7* maturase (the p27 protein, encoded by the bI4 URF in the long version of the apocytochrome *b* gene) in mutants with nonsense mutations in the upstream exon (de la Salle et al. 1982).

Since a model involving an internal start of translation can accommodate most of our data, we favour the second alternative to explain the observed effects of mutations on intron splicing. Two observations, however suggest that additional effects play a role. In the mutants M11-125 and M15-190, splicing of aI2 is blocked or strongly reduced by mutations within aI1 in contrast to M9-9 which processes aI2 normally despite a fully blocked splicing of aI1. Although secondary lesions might be responsible for this, accumulation of a (mutated) aI1 maturase might disturb the splicing of the aI2 intron in a fashion similar to the effects that mutated *COB* maturases may have on splicing of *OXI3* transcripts (Hensgens et al. 1984).

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