Expression of the "Split Gene" cob in Yeast mtDNA

NUCLEAR MUTATIONS SPECIFICALLY BLOCK THE EXCISION OF DIFFERENT INTRONS FROM ITS PRIMARY TRANSCRIPT*

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Five nuclear mutants falling into five different complementation groups are shown to block the maturation of long form mitochondrial cob RNA at five different processing steps. At the same time they prevent complete processing of the oxi3 RNA, thus exhibiting the same phenotype as mitochondrial box mutants (cyt b" and oxi3"). The different nuclear factors in question have varying ranges of specificity for the removal of introns from cob RNA, from only one to at the most three introns. Two mutated nuclear elements are shown to be specific for the processing of introns present only in the long form cob gene. One such mutation shows, as expected, no deleterious effect on the processing of the short form cob RNA exchanged into the mutant via cytoduction. The role of nuclear coded factors in the possible translation or activity of intron-coded products ("maturases") is discussed for two mutants. Striking parallels are found between diverse polypeptide products, presumably translated from accumulated cob RNA intermediates, in pet" and mit" mutants blocked in the excision of the same intron.

The mitochondrial genome of bakers' yeast Saccharomyces cerevisiae contains, among other genes, three of mosaic structure. Two of them, oxi3 and cob, code for polypeptides namely for subunit I of cytochrome c oxidase and apocytochrome b, respectively. Their mature messenger RNAs result from the excision of intron sequences from the primary transcripts. The oxi3 transcript harbors five or seven introns (strain-dependent). The cob RNA contains five introns in the long form. The only two introns of the short form (Fig. 1) are equivalent to I4 and I5 of the long form (for review see Refs. 1 and 2).

The study of mit" mutants with lesions within the mitochondrial mosaic genes themselves has yielded much information about their processing. The cob RNA processing has been most closely investigated. Among the five introns of the cob long form RNA, three (I2, I3, and I4) contain open reading frames in phase with the preceding exon sequence (3, 4). The excision of I2 and I4 appears to be dependent on the translation of their open reading frames to yield the so called I2 and I4 "maturases" (4-7). Mutations preventing the translation of the I2 or I4 maturase inhibit complete processing of the cob RNA (cob" phenotype) (7-11). The failure to translate the I4 product not only blocks cob RNA maturation but also prevents correct processing of the oxi3 RNA (box" phenotype) (6-8).

Recently, attention has shifted to the effect of nuclear mutations on the processing of mitochondrial split genes (12, 13). Studying the splicing of the D273-10B short form cob RNA (two introns) (14), Dieckman et al. (15) have isolated nuclear mutants affecting the processing of this RNA. We have concentrated on nuclear mutations preventing the maturation of the long form cob RNA (five introns) as well as the processing of the oxi3 gene (box" phenotype). From a greater number of mutants of this type, which were isolated in this laboratory, five (MK 1-5) were selected for description in this paper.

MATERIALS AND METHODS

Yeast Strains and Mutants—Mutations in nuclear genes affecting the function of the mitochondrial respiratory chain (pet") were derived by mutagenesis with sodium nitrite from isogenic long form wild type strains of S. cerevisiae, SM 11-6c a and FM 8c. They were defined as respiratory-deficient by lack of growth on glycerol medium and as pet" by crosses with rho" tester strains. Spectral and protein analysis further established the nature of the mutants used in this work as cytochrome b" and cytochrome oxidase subunit I (this phenotype being equivalent to mit" box mutants).

Long Form-Short Form Mitochondrial Genome Exchange—The exchange of the long form with the short form (D273-B) mitochondrial genomes was carried out by cytoduction (16).

Mitochondrial RNA Preparation—Yeast strains were cultured in galactose medium (2% yeast extract, 3% galactose) and harvested in the early log phase. Lysis of the cells was achieved using zymolase 5000 (Kirin Brewery, Japan) in a lysis buffer containing 1.5 M sorbitol, 10 mM EDTA, 10 mM Na citrate buffer, pH 7.3, and 1% mercaptoethanol. Mitochondria were isolated by layering onto 10 mM Tris, 1 mM EDTA/glycerin gradients (40-80%) and centrifuged at 40,000 rpm in an SW 60 Ti Beckman rotor. Mitochondria were lysed in 2% sodium dodecyl sulfate, and the mtRNA was separated kinetically through CsCl (1 g/ml of 10 mM Tris, 1 mM EDTA) at 40,000 rpm in an SW 60 Ti rotor.

Gel Analysis and Immobilization of RNA Pattern on Diamobenzyloxymethyl Paper—1.2-μg aliquots of mtRNA were analyzed on 5% mercuric gels (dimethylmercury hydroxide) containing 1.5% agarose. They were run at 140 V for 3-4 h at room temperature (17). Diamobenzyloxymethyl paper was prepared according to Alwine et al. (18). The resulting RNA pattern was blotted onto activated azobenzyloxymethyl paper using 0.2 M sodium acetate buffer, pH 4.0.

Visualization of mtRNA Transcripts by Hybridization with Specific Probes—Specific DNA probes were derived from the mtDNA of the rho- strain dD22 spanning the whole long form cob gene of yeast mitochondrial DNA (10). Cloned fragments were 5'-labeled by nick translation (19). The reaction was catalyzed with Pol I (Boehringer Mannheim) at 10 °C for 3-4 h. Hybridization was carried out in a buffer containing 50% deionized Fornamid (Fluka) 5 x SSC (20 x SSC; 3 μM NaCl, 0.3 μM Na Citrate), 50 μM NaH2PO4 adjusted to pH 7.4 plus 0.2% sodium dodecyl sulfate, 0.02% Denhardt's (18), and 50 μg/ml of sonified herring sperm DNA, at 40 °C for 48 h. The resulting hybridization pattern was visualized on x-ray film using intensifying screens (Kodak Curix) at -60 °C.

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The open reading frame in I3 is as yet unpublished. The RNA transcribed correspond to known open reading frames in phase with preceding exons (3, 4), open areas are closed reading frames. The extent of the form (D273-10B) 5' and 3' extensions (11,151.

cellulose prior to autoradiography produce apocytochrome mitochondrial proteins were separated on sodium dodecyl sulfate gradient gels are calculated from sodium dodecyl sulfate gels are approximately 30% less than the "real" molecular weight (6,23). This, combined with the observation that I1 is excised (Fig. 1). Thus, it appears to be the mature maturase, the 42-kDa maturase polypeptide reacts with the nuclear coded factor is not necessary for the excision of I2 (4,5). Since the exons B1 and B2 code for the proximal part of both apocytochrome b and this maturase, the 42-kDa maturase polypeptide reacts with anticytochrome b (25). However, despite the fact that the full RNA sequence coding for the maturase in mutant MK 1 is enriched, no such polypeptide can be detected (Fig. 4).

Mutant MK 1 (Long Form)—This shows two enriched intermediate cob transcripts of 34 S (7.5 kb1) and 33 S (6.8 kb) (Fig. 2). Further processing of the 33 S intermediate is blocked. Transcript mapping with specific DNA probes reveals that only I1 is excised (Fig. 3 and Table I). The transcript accumulated has established the open reading frame consisting of B1-B2 and extending into I2. In mit+ mutants enriching this sequence in cob RNA intermediates blocked in further processing, a corresponding 42-kDa polypeptide was detected (25). It is suggested that this 42-kDa I2 maturase is necessary for the excision of I2 (4, 5). Since the exons B1 and B2 code for the proximal part of both apocytochrome b and this maturase, the 42-kDa maturase polypeptide reacts with anticytochrome b (25). However, despite the fact that the full RNA sequence coding for the maturase in mutant MK 1 is enriched, no such polypeptide can be detected (Fig. 4).

Mutant MK 1 (Short Form)—This shows a wild type splicing pattern of the cob primary transcript (Fig. 3) indicating that the splicing blockage seen in the long form is released. Thus, the nuclear coded factor is not necessary for the excision of I4 and I5, the only introns found in the short form (Fig. 1). This, combined with the observation that I1 is excised in the long form (see above), shows that the nuclear factor inactive in MK 1 is required for the processing of the cob primary transcript in the excision of the long form introns I2 and/or I3. The 18 S cob RNA seen in this short form is translated to produce apocytochrome b, shown to be enzymatically active. Thus, it appears to be the mature cob mRNA. The mutant, however, remains respiratory-deficient, since, as in the long form, subunit I of cytochrome c oxidase is lacking (Fig. 4). Thus, this factor defective in MK 1 is also engaged in the processing of the ox13 primary transcript. In addition to apocytochrome b, a polypeptide of 20 kDa was detected.

Mutant MK 2 (Long Form)—This reveals two enriched cob RNA intermediates of 33 S (6.8 kb) and 32 S (6.2 kb) (Fig. 2). Further processing of the 32 S intermediate is blocked. Transcript mapping shows that I1 and I5 have been removed (Fig. 5 and Table I). As in mutant MK 1 (long form), this establishes the B1-B2-I2 coding sequence. However, as with mutant MK 1 (long form), no polypeptide reacting with anticytochrome b was detected (Fig. 4).

**RESULTS**

**Mutant MK 1 (Long Form)—**This shows two enriched intermediate cob transcripts of 34 S (7.5 kb) and 33 S (6.8 kb) (Fig. 2). Further processing of the 33 S intermediate is blocked. Transcript mapping with specific DNA probes reveals that only I1 is excised (Fig. 3 and Table I). The transcript accumulated has established the open reading frame consisting of B1-B2 and extending into I2. In mit+ mutants enriching this sequence in cob RNA intermediates blocked in further processing, a corresponding 42-kDa polypeptide was detected (25). It is suggested that this 42-kDa I2 maturase is necessary for the excision of I2 (4, 5). Since the exons B1 and B2 code for the proximal part of both apocytochrome b and this maturase, the 42-kDa maturase polypeptide reacts with anticytochrome b (25). However, despite the fact that the full RNA sequence coding for the maturase in mutant MK 1 is enriched, no such polypeptide can be detected (Fig. 4).

**Mutant MK 1 (Short Form)—**This shows a wild type splicing pattern of the cob primary transcript (Fig. 3) indicating that the splicing blockage seen in the long form is released. Thus, the nuclear coded factor is not necessary for the excision of I4 and I5, the only introns found in the short form (Fig. 1). This, combined with the observation that I1 is excised in the long form (see above), shows that the nuclear factor inactive in MK 1 is required for the processing of the cob primary transcript in the excision of the long form introns I2 and/or I3. The 18 S cob RNA seen in this short form is translated to produce apocytochrome b, shown to be enzymatically active. Thus, it appears to be the mature cob mRNA. The mutant, however, remains respiratory-deficient, since, as in the long form, subunit I of cytochrome c oxidase is lacking (Fig. 4). Thus, this factor defective in MK 1 is also engaged in the

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1 The abbreviation used is: kb, kilobase.

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**Nuclear Mutations**

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**Mutant MK 2 (Long Form)—**This reveals two enriched cob RNA intermediates of 33 S (6.8 kb) and 32 S (6.2 kb) (Fig. 2). Further processing of the 32 S intermediate is blocked. Transcript mapping shows that I1 and I5 have been removed (Fig. 5 and Table I). As in mutant MK 1 (long form), this establishes the B1-B2-I2 coding sequence. However, as with mutant MK 1 (long form), no polypeptide reacting with anticytochrome b was detected (Fig. 4).
Nuclear Mutations Blocking the Excision of cob RNA Introns

TABLE I
Exon/intron configuration of cob RNA intermediates blocked in further processing

Nuclear mutants MK 1-5 block the processing of cob RNA at different steps, producing intermediates of varying exon/intron configurations. The Svedberg value and the diagrammatic representation of the intermediates are given in this table. Black areas denote exon sequences, and white boxes designate introns. Excised introns are given below each intermediate. The mitochondrial genome contained in each mutant is given below the mutant's number as long or short (cf. Fig. 1). Small i denotes intron.

<table>
<thead>
<tr>
<th></th>
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<tbody>
<tr>
<td>MK 1</td>
<td>long</td>
<td>-</td>
<td>-</td>
<td>n.d.</td>
</tr>
<tr>
<td></td>
<td>short</td>
<td>-</td>
<td>-</td>
<td>20 kDa</td>
</tr>
<tr>
<td>MK 2</td>
<td>long</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>short</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>MK 3</td>
<td>long</td>
<td>(+)</td>
<td>(-)</td>
<td>15, 18, 42, 52 kDa</td>
</tr>
<tr>
<td></td>
<td>leaky</td>
<td>(+)</td>
<td>(+)</td>
<td>15, 17, 19, 42, 52 kDa</td>
</tr>
<tr>
<td>MK 4</td>
<td>long</td>
<td>-</td>
<td>-</td>
<td>23 kDa</td>
</tr>
</tbody>
</table>

Mutant MK 2 (Short Form)—This shows two cob RNA intermediates of 26 S (4.2 kb) and 24 S (3.6 kb) (Fig. 2). As already demonstrated for the long form, I5 is excised. Thus, the 24 S intermediate is blocked exclusively in the excision of I4 (Fig. 5 and Table I). The nuclear coded element inactive in this mutant is necessary for both the excision of I2 and/or I3 (present only in the long form) as well as I4. No apocytochrome b can be translated since no messenger is present (Fig. 2). The respiratory deficiency is therefore maintained in the short form. However, unlike the long form, subunit I cytochrome c oxidase is detectable on protein gels (Fig. 4).

Mutant MK 3 (Long Form)—This exhibits two enriched cob RNA intermediates of 32 S (6.2 kb) and 28 S (4.9 kb) (Fig. 2). Intermediates of 33 and 30 S are detectable at much lower concentrations. Further processing of the 28 S product is inhibited. Transcript mapping shows that only I1, I2, and I5 (Fig. 6 and Table I) but not I3 and I4 are removed by this mutant. This indicates that the nuclear coded factor non-
Nuclear Mutations Blocking the Excision of cob RNA Introns

Polypeptides reacting with anti-cytochrome b of nuclear mutants MK 1-5

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Diverse polypeptides reacting with anti-cytochrome b</th>
<th>Introns not excised from cob RNA (see also Table I)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Long form RNA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MK 4</td>
<td>15, 17, 19, 42, and 52 kDa</td>
<td>I3</td>
</tr>
<tr>
<td>MK 5</td>
<td>23 kDa</td>
<td>I5</td>
</tr>
<tr>
<td>MK 3</td>
<td>15, 18, 42, and 52 kDa</td>
<td>I3 and I4</td>
</tr>
<tr>
<td>MK 2</td>
<td>ND*</td>
<td>I2, I3, and I4</td>
</tr>
<tr>
<td>MK 1</td>
<td>ND</td>
<td>I2, I3, and I4</td>
</tr>
<tr>
<td>Short form RNA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MK 1</td>
<td>20 and 32 kDa (apocytochrome b)</td>
<td>All are excised</td>
</tr>
<tr>
<td>MK 2</td>
<td>ND</td>
<td>I4</td>
</tr>
</tbody>
</table>

*ND, none detected.

Fig. 7. Transcript mapping with specific DNA probes. MK 4 accumulates long form cob RNA intermediates (lane dD22); these have been mapped using DNA probes specific for different introns (cf. Fig. 3).

functional in mutant MK 3 is necessary for the excision of I3 and/or I4. An open reading frame comprised of B1-B2-B3 and extending into I3 is established in the 28 S intermediate by the excision of I1 and I2. This sequence would code for a 52-kDa product as measured on polyacrylamide gels. Due to artifacts in sodium dodecyl sulfate-disc electrophoresis, the actual kilodalton value is closer to 68, e.g. 30% more (see “Materials and Methods” and De La Salle (6) for discussion). The exon content of the polypeptide would allow precipitation with anti-cytochrome b. Such an immunologically positive product of 52 kDa indeed is detected (Fig. 4). As described above for MK 2, an additional open reading frame composed of B1-B2-I2 is contained in the 32 S intermediate. Possibly it is translated as a 42-kDa species. Such a polypeptide reacting with anti-cytochrome b is found in MK 3 together with two products of 15 and 18 kDa (Fig. 4 and Table II). Interestingly, these smaller products also occur together in mit mutants blocked in the excision of I3 (see Table III).

Mutant MK 4 (Long Form)—This shows one enriched cob RNA intermediate of 25 S (4.4 kb) (Fig. 2). Intermediates preceding the 25 S product are present at much lower concentrations. Their pattern corresponds to that observed in the wild type (Fig. 2). Transcript mapping reveals that I1, I2, I4, and I5 have been removed from the 25 S intermediate, and that I3 is present (Fig. 7 and Table I). The nuclear encoded element, inactive in this mutant, therefore is required for the excision of I3. The absence of I1 and I2 in the 25 S intermediate creates an open reading frame consisting of B1-B2-B3-I3. As discussed above, this sequence codes for a 52-kDa product, and as in mutant MK 3 a corresponding 52-kDa polypeptide was detected (Fig. 4). There were no additional enriched open reading frames in other intermediates of this mutant. However, a 42-kDa product reacting with anti-cytochrome b is observed in addition to three smaller polypeptides of 15, 17, and 19 kDa, also seen in mit mutants blocked in the removal of I3 (Fig. 4 and Tables II and III). Leakiness in the processing blockage in mutant MK 4 is revealed by a faint wild type splicing pattern and manifested by slow growth on glycerol medium.

Mutant MK 5 (Long Form)—This produces two enriched cob RNA intermediates of 26 S (4.3 kb) and 20 S (2.9 kb) (Fig. 2). Preceding intermediates are of lower concentrations and reveal a wild type pattern. Transcript mapping shows that in the smallest RNA of 20 S of all introns only I5 is present (Fig. 8 and Table I). Thus, the mutant is deficient in

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TABLE II

Polypeptides reacting with anti-cytochrome b of nuclear mutants MK 1-5

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Diverse polypeptides reacting with anti-cytochrome b</th>
<th>Introns not excised from cob RNA (see also Table I)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Long form RNA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MK 4</td>
<td>15, 17, 19, 42, and 52 kDa</td>
<td>I3</td>
</tr>
<tr>
<td>MK 5</td>
<td>23 kDa</td>
<td>I5</td>
</tr>
<tr>
<td>MK 3</td>
<td>15, 18, 42, and 52 kDa</td>
<td>I3 and I4</td>
</tr>
<tr>
<td>MK 2</td>
<td>ND*</td>
<td>I2, I3, and I4</td>
</tr>
<tr>
<td>MK 1</td>
<td>ND</td>
<td>I2, I3, and I4</td>
</tr>
<tr>
<td>Short form RNA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MK 1</td>
<td>20 and 32 kDa (apocytochrome b)</td>
<td>All are excised</td>
</tr>
<tr>
<td>MK 2</td>
<td>ND</td>
<td>I4</td>
</tr>
</tbody>
</table>

*ND, none detected.

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TABLE III

Polypeptides reacting with anti-cytochrome b of mit mutants

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Diverse polypeptides reacting with anti-cytochrome b</th>
<th>Introns not excised from cob RNA*</th>
</tr>
</thead>
<tbody>
<tr>
<td>I1</td>
<td>15 kDa</td>
<td>I1, I2, I3, I4, and I5</td>
</tr>
<tr>
<td>I2</td>
<td>18 and 42 kDa</td>
<td>I2, I3, and I4</td>
</tr>
<tr>
<td>I3</td>
<td>(15), 16, 19, and (50) kDa</td>
<td>I3</td>
</tr>
<tr>
<td>I4</td>
<td>23 and 36 kDa</td>
<td>I4</td>
</tr>
</tbody>
</table>

*Compiled from data in Bechmann et al. (25) and Schmelzer and Schweyen (28), where the introns I1, I2, I3, I4, and I5 correspond to sequences α/β, 10 S, α/β', 10 S, and 6/t, respectively. Parentheses denote weak signal.
the excision of I5. The larger enriched intermediate of 26 S contains I4 and I5 (Fig. 8) and harbors an open reading frame composed of B1-B2-B3-B4-I4. Mit mutants blocked in the removal of I4 also contain this open reading frame, and both pet and such mit mutants exhibit a 23-kDa polypeptide (Tables II and III). It is then probable that the 23-kDa product originates from the larger 26 S intermediate in this pet-mutant.

**DISCUSSION**

Each of the pet mutants studied in this work is characterized by a specific splicing pattern distinguishing it from that of the wild type. Their accumulated cob RNA intermediates correspond to those seen in the wild type. After the excision of one or several of the introns, the splicing process is blocked in a specific step varying from mutant to mutant. The defects observed in these mutants are shown to have contrasting ranges of specificity (long strain of Table I). All but one of the introns of the cob region in mutants MK 4 (I3 retained) and MK 5 (I5 retained) are excised. The three other mutants are characterized by the retention of possibly more than one intron. In mutant MK 1 (long form), the processing of the cob transcript is blocked in the removal of I2 and/or I3, and in mutant MK 3 in the excision of I3 and/or I4. The mutation of MK 2 inhibits the removal of I2 and/or I3 and I4, thus precluding the splicing of two probably three intron sequences. Interestingly, the three introns in question (I2, I3, and I4) all contain open reading frames in phase with the preceding exon (3-6). Nuclear factors are then necessary for the excision of all introns of the long cob form transcript. Two different factors mutated in MK 1 and MK 4 are specific for the excision of introns found only in the long form, and are thus not needed for the processing of the short form cob RNA. The other three mutants (MK 2, 3, and 5) have defective factors necessary for the maturation of both long and short form cob transcripts.

The same nuclear coded factors which, when defective, prevent the processing of the long form cob transcript in all five examples, are needed too for the splicing of the ox13 transcript. Also necessary for the maturation of the ox13 transcript is the correct translation of the cob I4 product which is at the same time required for the excision of I4 from the cob RNA (6, 23). Hence, mutations preventing the expression of the I4 product show a parallel inhibition of cob and ox13 RNA maturation. Since, however, in MK 5 the excision of I4 is undisturbed, e.g. the matrass RNA is translated, the box phenotype of this mutant is caused by an additional effect and cannot be due to the inactivity of the I4-coded product. A similar conclusion is drawn from the investigation of another mutant. The cob- phenotype of mutant MK 1 long form is changed to cob+ by introducing the short form, the cob RNA being fully processed and translated; meanwhile, the box- phenotype is maintained (cf. Fig. 4). This proves that the influence of the same nuclear coded factor, defective in this mutant and thereby preventing the excision of I2 and/or I3 from the long form, but not being engaged in the processing of the short form cob RNA, is also needed for the processing of the ox13 primary transcript. Intron sequence homologies between the cob gene and ox13 have been established (6, 26). This suggests that the activity of the nuclear coded element in question, common to the processing of cob and ox13 RNA, is directed at homologous sequences and/or structures of both cob and ox13 transcripts. The complete processing of the short form in mutant MK 1 characterizes the specificity of the nuclear factor for introns found only in the long form cob RNA (I2 and/or I3).

Mutant MK 2 short form demonstrates that the blockade of translational access to the cob I4 maturase sequence, caused by a karyotically induced splicing defect, is accompanied by the box phenotype. The long form of MK 2 is ox13- and cob+ and I1 and I5 are excised. The short form maintains the cob- phenotype due to the failure to remove I4, and is ox13+ (Fig. 4). The improper processing of I2 and/or I3 therefore leads to the ox13- phenotype. As described previously, nonexcision of I2 and I3 in the long form has a polar effect such that the open reading frame consisting of B1-B2-B3-B4 and I4 is not established, and therefore the I4 maturase cannot be translated (23). In the short form, because of the absence of I2 and I3, this reading frame from the very beginning is accessible to translation and accompanied by the complete processing of the ox13 RNA.

The failure to excise exclusively I3 from cob RNA results in the enrichment of a 25 S intermediate in mutant MK 4. The removal of I3, however, is not completely inhibited in this mutant, as shown by a faint wild type cob RNA splicing pattern in RNA hybridization analysis, and manifested by slow growth on glycerol medium. The 25 S intermediate enriched in MK 4 is observed in the wild type splicing pattern at very low concentrations (Fig. 2). Consequently, the component defect in MK 4 does not completely prevent the removal of I3 from a cob RNA intermediate containing additional intron sequences. Clearly the configuration of the 25 S intermediate is inhibitive for the excision of I3 in MK 4. It is a possibility that at the level of individual intermediates the excision of specific intron sequences is dependent upon a favorable tertiary configuration. Not difficult to imagine is that in the wild type cob RNA a flux in tertiary structures due to translational events and other protein-RNA interactions would lead to a random splicing process, but does not exclude preferential, but not exclusive, tertiary structures, favoring the excision of specific introns in individual intermediates. These observations are in agreement with the conclusions of van Ommen et al. (11) that the excision of introns 2, 4, and 5, prior to the removal of I3 is unfavorable in the wild type cob RNA processing.

The accumulation of cob RNA intermediates in mutants MK 3-5, is accompanied by the appearance of diverse polypeptides reacting with anti-cytochrome b and thus harboring amino acid sequences coded for by exons (Tables II and III and Fig. 4). A comparison with mit- mutants containing lesions within the cob gene, which prevent the excision of individual introns (see Table III) (25, 27), leads to the following points.

1) Both pet- mutants MK 4 and MK 3 retaining I3 translate a 52-kDa product (Table II and Fig. 4). Their RNA intermediates contain an open reading frame comprised of B1-B2-B3 extending into I3 (28), which is suited to code for a 52-kDa polypeptide. A similar 50-kDa product, reacting with anti-cytochrome b, is also detected in mit- mutants blocked in the excision of I3 (Table 3).

2) Despite the enrichment of a B1-B2-I2 open reading frame in the 33 S intermediate of MK 1 and the 52 S product of MK 2, the expected 42-kDa product, the I2 maturase, is not detectable (Table II and Fig. 4). mit- mutants, however, enriching this same sequence due to failure of I2 excision, do exhibit the corresponding 42-kDa product (Table III). This suggests that either mutant MK 1 does not translate the enriched sequence, or that the 42-kDa product is present at a concentration too low to be detected by the methods used. In addition, work on mit- mutants has shown that lesions preventing the correct translation of the B1-B2-I2 open reading frames have a polar effect hindering the processing of the cob transcript further than 32 S (10, 11). Therefore, the processing block caused by the nuclear mutation of MK 1 may be due to
(a) the total absence of the I2 maturase owing to translational error; or (b) the inactivity of the maturase splicing complex due to a defective or absent nuclear component.

3) The 23-kDa polypeptide seen in mutant MK 5, defective in the removal of I5, might appear difficult to reconcile with the appearance of a 23-kDa product in mit- mutants unable to excise I4 (see Table III). However, MK 5 exhibits two enriched cob RNA intermediates of 26 and 20 S. The larger product contains only I4 and I5, thus yielding the open reading frame also present in the mit- mutants blocked in the removal of I4.

4) The smaller polypeptides accompanying the failure to excise I3 from the cob RNA long form transcript in MK 4 (15, 17, and 19 kDa) and MK 3 (15 and 18 kDa) presumably originate from the open reading frame composed of B1-B2-B3 and I3 (Table I). mit- mutants, blocked in the excision of I3, also exhibit smaller products (15, 16, and 19 kDa) comparing favorably with those seen in the equivalent pet- mutants (Tables II and III). These results are not contradictory to the ideas discussed by Behemann et al. (25) that such smaller polypeptides are the product of premature translational termination, with the one distinction that this process may occur on intermediates harboring larger open reading frames. Thus, premature termination would accompany the complete translation of the larger sequence yielding the corresponding larger and smaller products. An additional explanation is the possibility that proteolytic cleavage of larger polypeptides is responsible for the smaller products. Worthing in connection with the origin of smaller polypeptides is the degree of specificity of the process yielding them, as seen by similar, if not identical, polypeptide products of pet- and mit- mutants. (Small differences between some species may be explained by the experimental variation as the results originate from two completely different sets of experiments.) It is thus possible that they either originate from larger polypeptides containing specific sites of proteolytic cleavage, or larger RNA sequences with sites of premature translational termination, or even from smaller RNA sequences corresponding to the size of the polypeptides.

On the basis of these results and those published Dieckmann et al. (13, 15) and Bonitz et al. (14), it is possible to picture several, not mutually exclusive mechanisms responsible for the processing of cob RNA primary transcripts, long and short form. Among those are (a) the splicing of each intron is catalyzed by an enzyme complex (or complexes) comprised of nuclear coded components of proven specificity and for I2, 3, and 4 also requiring mitochondrially translated polypeptides. (b) Processing takes place in close connection with translation on the ribosome or another complex (6, 26, 28). The mutation of a nuclear coded ribosomal polypeptide involved in this activity could lead to a specific block in cob RNA maturation, without necessarily interfering with the process of translation. (c) Nuclear coded factors may be involved in the formation of complexes in the mitochondria similar to those of small nuclear ribonucleoproteins and small cytoplasmic ribonucleoproteins found in the nuclei and cytoplasm of higher eukaryotes (29) and likewise involved in the processing of mosaic genes.

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