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Steps in processing of the mitochondrial cytochrome oxidase subunit I pre-mRNA affected by a nuclear mutation in yeast

(*oxi-3* mitochondrial RNA splicing/cleavage-site S1 nuclease mapping/*Saccharomyces cerevisiae*/*mss51* nuclear mutation)

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ABSTRACT **In *Saccharomyces cerevisiae*, the mitochondrial gene encoding the subunit I of cytochrome *c* oxidase (*oxi-3* gene) is interrupted by intervening sequences. In this report, a nuclear mutation [referred to as *mss51* in Faye, G. & Simon, M. (1983) *Cell* 32, 77–87] that specifically affects the processing of *oxi-3* pre-mRNA was further characterized. DNA probes covering each *oxi-3* exon–intron boundary were individually hybridized to wild-type and mutant mitochondrial RNA. By a technique relying on the S1 nuclease resistance or sensitivity of the RNA·DNA hybrids thereof, we have shown which site needs the MSS51 gene product to be cleaved. The mutation in the *MSS51* gene gave rise to a complex pattern of splicing: the third intron was excised efficiently but the first two introns remained bracketed by their flanking exons. Further, the fourth and fifth introns were only partially split from their common exon and remained fused to their upstream and downstream flanking exon, respectively. Several plausible roles for the MSS51 gene product are discussed.**

In *Saccharomyces cerevisiae*, the mitochondrial gene encoding the subunit I of cytochrome *c* oxidase (*oxi-3* gene) has a mosaic structure. In the wild-type strain D273-10B (a short-gene strain), this gene is interrupted by five large introns and possibly by two very short ones located at its 3′ end (1). Its primary transcript is processed by a cut-and-ligation mechanism into a translatable mature messenger. Though splicing components for some mitochondrial introns are encoded in the mitochondrial genome itself (e.g., intron-encoded maturases; refs. 2 and 3), it is now clear that several other components of the splicing apparatus are specified by the nuclear genome (4–6).

Recently, we have characterized and cloned such a nuclear gene (named *MSS51*) whose mutation drastically impairs the maturation of the *oxi-3* pre-mRNA (7). RNA transfer blots of the mitochondrial RNA of the strain E4-218 carrying the *mss51* mutation disclosed an increased amount of large steady-state transcripts as well as the absence of shorter processing intermediates, mature mRNA included, when compared with blots of wild-type RNA. These results indicate that steps in splicing are blocked and the occurrence in both strains of some identical processing intermediates lets us suggest that the MSS51 protein acts at particular steps of the processing.

In the present report, DNA fragments covering each *oxi-3* exon–intron boundary were individually hybridized to wild-type and mutant mitochondrial RNAs. By analyzing the effects of S1 nuclease on the RNA·DNA hybrids thereof, we were able to determine which splice junction needs the MSS51 product to be cleaved. Furthermore, we have previously shown that the mutation in E4-218 does not affect the overall processing of the cytochrome *b* short-gene transcript (7). By introducing this mutation into a mitochondrial cytochrome *b* long-gene context, we have demonstrated that the additional introns are removed correctly.

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METHODS

Yeast and Bacterial Strains. The yeast strains used were D273-10B/A, α *met* (8); E4-218, α *mss51* (9); 1B-TR-2D, α *leu2 trp1 mss51* (7); KL14-4A, **a** *his1 trp2* (10); HD1 *rho*$^-$ (11). *Escherichia coli* strain RR1 was used for transformation (12).

Cloning of Mitochondrial DNA Fragments. Mitochondria of yeast strains D273-10B/A and HD1 were isolated as described (7). Mitochondrial DNA was purified by bisbenzimide/CsCl buoyant density centrifugation (13). The DNA fragments *Hpa* II–*Eco*RI (I), *Eco*RI–*Eco*RI (II), and *Eco*RI–*Bam*HI (III) were prepared from HD1 mitochondrial DNA and the fragment *Bam*HI–*Eco*RI (IV) was prepared from D273-10B/A (Fig. 1). They represent most of the *oxi-3* gene. Fragment I was cloned in pBR328 (14) between its *Cla* I and *Eco*RI sites. Fragments II–IV were cloned in pBR322 (12). Restriction analysis and cloning in *E. coli* were carried out as described by Montgomery *et al.* (15).

Introduction of the *mss51* Mutation in a Mitochondrial Long-Gene Context. A culture of the yeast strain 1B-TR-2D was diluted 1:10 in 2 ml of YPG medium [1% yeast extract (Difco)/1% bacto-peptone (Difco)/2% glucose] containing 50 mM sodium phosphate buffer (pH 6.5) and 50 μg of ethidium bromide per ml. Cells were grown for 16 hr at 28°C in the dark. After appropriate dilutions, cells were plated on YPG medium. One colony, named 1B-TR-2D/F1, was allowed to grow in 5 ml of YPG liquid medium; a second round of mutagenesis was then carried out, and finally, a colony, named 1B-TR-2D/F11, was isolated, which is a *rho*^{0} (mitochondrial DNA-less) clone. 1B-TR-2D/F11 was crossed with KL14-4A and the obtained diploid LJ1 was induced to sporulate. One spore, LJ1-7B *leu2 trp1 mss51*, was studied further.

RNA Transfer to Nitrocellulose Filter. Mitochondrial RNA was purified as described (7). RNA transfer to nitrocellulose filters and RNA·DNA hybridization were performed according to the Southern method as modified by Thomas (16).

RESULTS

Cloning of the *oxi-3* Gene. A general approach to studying the RNA processing of a mosaic gene blocked by some mutational event is to follow the elimination of different introns with the help of intronic or exonic DNA probes. Because the DNA sequence of the *oxi-3* gene of strain D273-10B is known (1), these probes can be prepared from DNA fragments spanning the *oxi-3* locus and cloned in *E. coli* plasmids. Four DNA fragments, named I–IV (Fig. 1), encompassing most of the *oxi-3* short gene were so cloned. The desired DNA probes were then purified from these recombinant plasmids.

RNA Transfer Blot Analysis of aI1, aI2, and aI5 Excisions. Introns aI1, aI2, and aI5 are released from the *oxi-3* pre-

Abbreviations: kb, kilobase(s); bp, base pair(s).

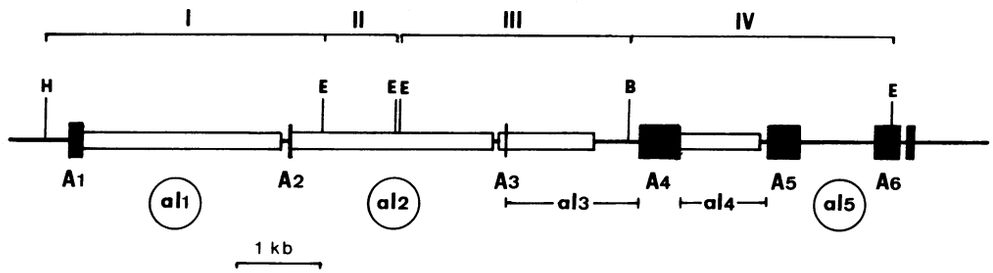


FIG. 1. Organization of the *oxi-3* gene. A1–A6 indicate exonic sequences and aI1–aI5 indicate intronic ones. Solid bars are exons. Intron open reading frames are represented by open bars. The splicing of aI1, aI2, and aI5 leads to the formation of circular RNA molecules. The four DNA fragments cloned in pBR328 or pBR322 are marked by horizontal brackets. H, *Hpa* II; E, *Eco*RI; B, *Bam*HI restriction sites. kb, Kilobase.

mRNA as stable linear and circular RNA forms (17). Using pure intronic probes, we have searched for the presence of these molecules in strains D273-10B/A and E4-218, which possess the wild-type and a mutated *MSS51* gene, respectively. Mitochondrial RNA extracted from purified mitochondria, was denatured with glyoxal (18). After separation by electrophoresis on agarose, it was transferred to a nitrocellulose paper. In Fig. 2, lane a, the mitochondrial RNA of D273-10B/A was probed with the 1,570-base-pair (bp) *Hin*fI–*Hin*fI-labeled DNA fragment isolated from intron aI1. The two major bands seen at 2,450 and 2,250 bases were not detected in the mitochondrial RNA of E4-218 (lane b), and the larger RNA species hybridized more strongly than the equivalent transcripts of the wild-type strain, indicating an accumulation of unspliced RNA precursors.

When both RNAs were probed with the 1.04-kb *Eco*RI–*Eco*RI-labeled DNA fragment originating from intron aI2, similar results were obtained. Two transcripts of D273-10B/A with strong signals were revealed; they had estimated sizes of 2,550 and 2,350 bases. They were absent in E4-218 RNA (Fig. 2, lanes c and d).

Bands with mobilities appropriate for intron aI5 RNAs were identified in the RNA of the wild-type strain when probed with the 665-bp *Hin*fI–*Acc* I DNA fragment prepared from intron aI5. Their sizes were about 1,000 and 940 bases. However, it seems that in E4-218 a unique band with an intermediate size was generated—i.e., the processing of intron aI5 appears to be impeded by the *mss51* mutation.

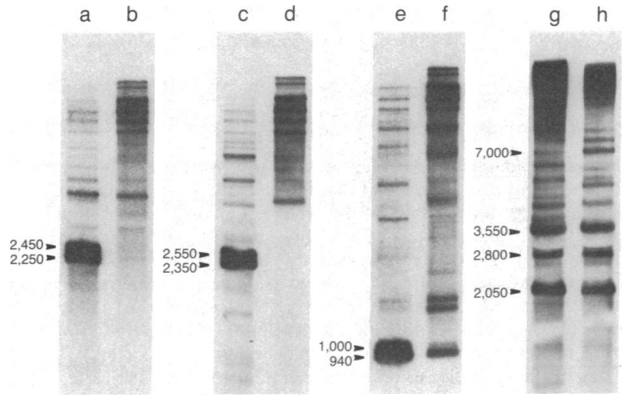


FIG. 2. RNA transfer blot analysis of mitochondrial RNA. Lanes, a, c, and e, mitochondrial RNA purified from D273-10B/A. Lanes b, d, and f, mitochondrial RNA prepared from E4-218. Mitochondrial RNAs were hybridized with a 1,570-bp *Hin*fI–*Hin*fI DNA probe isolated from intron aI1 (lanes a and b), with a 1,040-bp *Eco*RI–*Eco*RI DNA probe from intron aI2 (lanes c and d), and with a 665-bp *Hin*fI–*Acc* I DNA fragment prepared from intron aI5 (lanes e and f). Mitochondrial RNAs of LJ1 (lane g) and LJ1-7B (lane h) were hybridized with an exonic cytochrome *b* probe. Mitochondrial RNA of LJ1 and LJ1-7B were not treated with RNase-free deoxyribonuclease I before electrophoresis. The estimated sizes of the transcripts (in bases) are indicated in the margins and were extrapolated by using 21S (3,270 bases) and 15S rRNA (1,660 bases) as size markers.

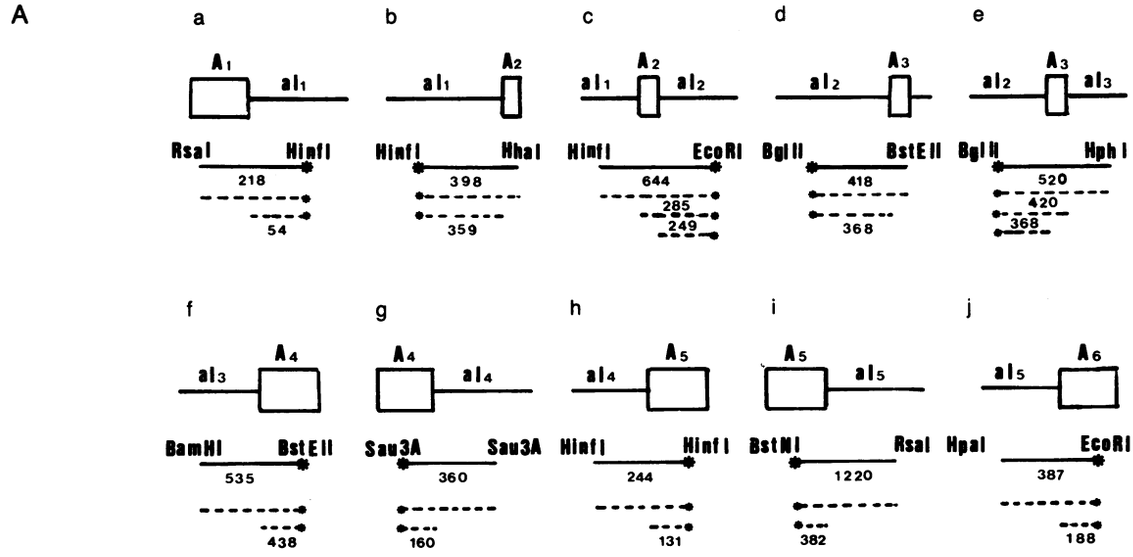
Analysis of the Exon–Intron Splice Junctions. To find out which particular splice junction of the *oxi-3* primary transcript is affected by the *mss51* mutation, mitochondrial RNAs from the wild-type and the mutant strains were hybridized with a series of specific DNA probes overlapping the cleavage site of each individual exon–intron boundary. Each resulting RNA·DNA duplex was then subjected to S1 nuclease trimming and the lengths of the nuclease-resistant DNA fragments were analyzed on polyacrylamide/urea gel slabs. To study upstream or downstream junctions, the coding strand of each probe was labeled at its 5′ or 3′ end, respectively. In all cases, the labeled ends were complementary to a stable part of transcript. In that way, annealing of either unspliced RNA intermediates of the wild strain or unspliced junctions of the mutant RNA with a given probe would set up a labeled hybrid resistant to S1 nuclease on its full length, whereas a hybrid with a spliced RNA intermediate would only be protected from S1 nuclease attack on part of its length. For instance, when the 218-bp 5′-end-labeled *Rsa* I–*Hin*fI fragment is hybridized to wild-type mitochondrial RNA, a 54-base fragment is protected from S1 nuclease; the probe is also protected on its full length by hybridization to as yet uncut splicing intermediates (Fig. 3, probe a). That this latter case is the only one detected with mutant RNA clearly means that the mutant is unable to split the A1–aI1 junction.

The size of the S1 nuclease-resistant DNA fragments, determined on polyacrylamide/urea gels, is shown in Fig. 3*B*. The probes covering each upstream and downstream junction flanking introns aI1 and aI2 are entirely protected when hybridized with mutant RNA, whereas the same probes are partially protected when hybridized with wild-type RNA (Fig. 3 *A* and *B*, lanes a–d). These results, consistent with those presented above (Fig. 2), establish that the excision of the first two introns, aI1 and aI2, is fully blocked in the mutant strain.

The 520-bp 3′-end-labeled *Bgl* II–*Hph* I probe (Fig. 3 *A* and *B*, lane e) hybridized with wild-type and mutant RNA and digested with S1 nuclease allows the analysis of both the aI2–A3 and A3–aI3 junctions. A correct cut at the first or second junction would generate a 368- or 420-base S1 nuclease-resistant fragment, respectively. The occurrence of only this latter 420-base fragment in the mutant strain indicates that junction A3–aI3 is correctly cleaved.

In the same way, the occurrence of the 438-base S1 nuclease-resistant fragment resulting from the 5′-end-labeled *Bam*HI–*Bst*EII fragment covering the aI3–A4 junction (Fig. 3 *A* and *B*, lane f) signifies that intron aI3 is removed normally in the mutant strain.

The analysis of the partially S1 nuclease-protected stretch



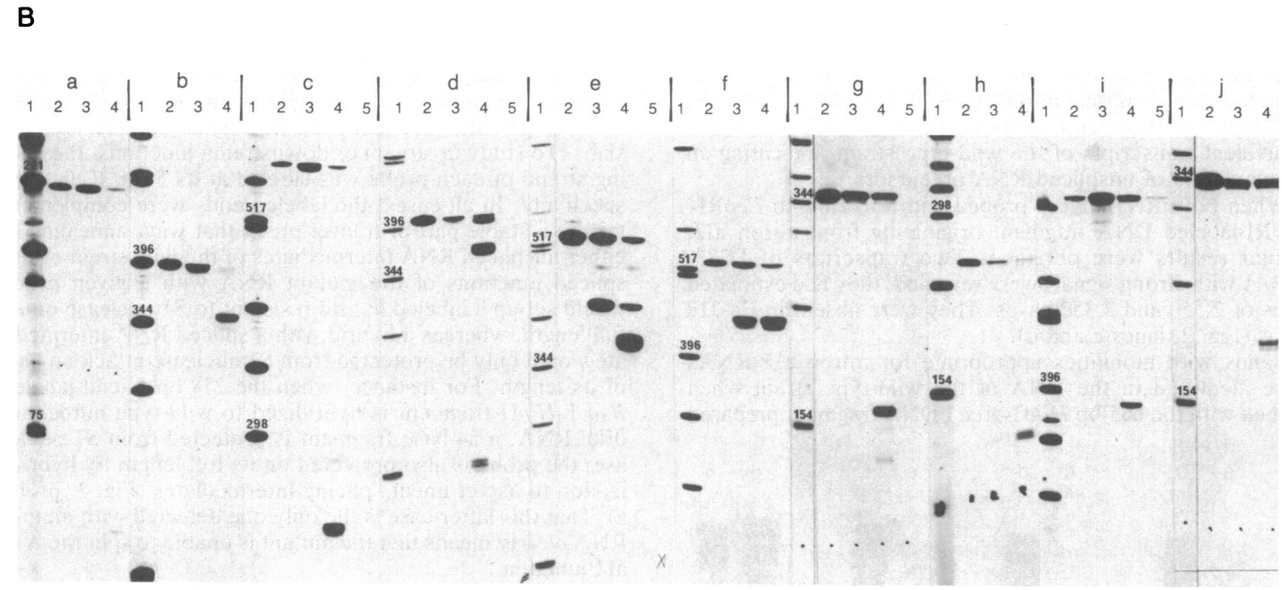


FIG. 3. S1 mapping of the *oxi-3* cleavage sites. By digesting the *oxi-3* cloned fragments (see Fig. 1) with appropriate combinations of restriction endonucleases, single-stranded end-labeled DNA fragments were generated, the labeled end usually residing within an exon. However, when no appropriate restriction site could be found, the labeled end was chosen inside a stable intron. DNA fragments used as hybridization probes and covering each exon (A1–A6)–intron (aI1–aI5) boundary are depicted, from a to j in *A*, by solid lines delimited by their restriction sites. The numbers below the lines indicate their sizes in bp. The study of the uppermost downstream ill-defined part of the *oxi-3* locus was not undertaken. Probes generated by a single endonuclease (g and h) were end labeled and their coding strand was isolated on 6% acrylamide gel slabs (19). Owing to its heterogenous G-C distribution, probe j was also strand separated; its coding strand migrated anomalously fast at 345 bases on a sequencing gel. —*, A 5′ end labeled with T4 polynucleotide kinase (19); *—, a 3′ end labeled with Klenow DNA polymerase (19). Dashed lines indicate stretches of probe DNA predicted to be protected from S1 nuclease by unspliced (upper dashed lines) or correctly spliced mitochondrial RNA (lower dashed lines). (*B*) Autoradiograms showing the sizing of the S1 nuclease-resistant fragments on 6% (lanes a, g, h, and j) or 4% acrylamide/urea sequencing gels (19) run at 75 W for 1 or 3 hr. Gels were then exposed to Kodak XAR films with intensifying screens for several days at −70°C. Lanes 1, End-labeled restriction fragments of pBR322 cut with *Hin*fI and used as size (base) markers; lanes 2, ^{32}P-labeled DNA probe (about 4,000–5,000 dpm) used as a size marker (no S1 digestion); lanes 3, 13 μg of mitochondrial RNA of the mutant strain E4-218 mixed with 10,000–20,000 dpm of end-labeled probe was hybridized in 25 μl of an 80% formamide-containing buffer (20) at a temperature 2°C above the calculated melting point of the DNA probe, as described (7). Hybridization was stopped by a 1:10 dilution in a buffer containing an excess of S1 nuclease (10,000 units/300 μl; Boehringer). Incubation was carried out for 30 min at 37°C or 1 hr at 28°C (lanes 3 and 4 in a) to prevent the destabilization of the short RNA·DNA duplex; lanes 4, same as lanes 3 with RNA from the wild-type D273-10B/A. Hybridization with single-stranded probes (lanes g, h, and j) was carried out in 0.8 M NaCl/0.1 M Pipes, pH 7.8/0.01 M EDTA for 16 hr at 68°C (21); lanes 5, 3′-end-labeled probes were used as a control of DNA reannealing on a parallel hybridization mixture lacking mitochondrial RNA.

of the 360-bp 3′-end-labeled *Sau*3A–*Sau*3A fragment (Fig. 3 *A* and *B*, lane g) and 5′-end-labeled *Hin*fI–*Hin*fI fragment (Fig. 3 *A* and *B*, lane h) of the coding strand covering the junctions A4–aI4 and aI4–A5, respectively, revealed that the 3′ cleavage site of intron aI4 of the mutant strain is cut at a much lower rate than in wild type. The hybridization signal of the 160-base S1 nuclease-resistant fragment expected from the correct cut at its 5′ junction was detected as a trace amount (Fig. 3*B*, lane 3 in g) in the mutant strain.

A similar analysis depicted in Fig. 3*A*, i and j, the results of

which are shown in Fig. 3B, lanes i and j, revealed that intron aI5 is partially cut at its 5′ boundary, whereas the expected S1 nuclease-protected 188-base fragment was not detected in the mutant. This observation means that the 3′ splice junction may not be correctly cut.

Processing of the pre-mRNA of the Cytochrome *b* Long Gene. According to the sequence stretches and the potential RNA secondary conformations they share, *S. cerevisiae* mitochondrial introns can be arranged into two groups (22, 23). Introns aI1, aI2, and aI5 of the *oxi-3* short gene belong to group II along with intron bI1 of the cytochrome *b* long gene. All of them, once excised from pre-mRNA, are circularized. To find out whether the processing of intron bI1 also requires the gene *MSS51*, the *mss51* mutation was introduced in a mitochondrial long-gene context. A *rho*^{0} derivative from yeast strain 1B-TR-2D was crossed with KL14-4A, which carries the long forms of the cytochrome *b* and *oxi-3* genes. From the obtained diploid (LJ1), one spore (LJ1-7B) harboring the *mss51* mutation was isolated. Mitochondrial RNA of LJ1 and LJ1-7B was analyzed by RNA transfer blot hybridization by using an exonic probe prepared from the cytochrome *b* gene. The mature form of the cytochrome *b* mRNA was detected for both LJ1 and LJ1-7B (Fig. 2, lanes g and h), meaning that, despite the structural similarity of bI1 with aI1 and aI2 and especially with aI5, its removal is not affected by the *mss51* mutation. The variabilities observed for the upper bands might be explained by the fact that these two strains are not isochromosomic.

DISCUSSION

We have shown by RNA transfer blot experiments that, in the mutant strain, the first two *oxi-3* introns are not released as stable transcripts. It was not immediately apparent from these results whether this was due to a failure in their stabilization by circularization or to either incorrect or no splicing (or both). By analyzing their splice junctions, we have shown that, in fact, none of them is excised. Only the third intron (aI3), correctly split at its 5′ and 3′ boundaries, is removed normally. Introns aI4 and aI5 are only partially split from exon A5 and intron aI4 remains nearly bound to its upstream exon A4, whereas the aI5–A6 junction is not cleaved at all. This latter result is in apparent discrepancy with the interpretation of our RNA transfer blot data, which indicated that some aI5 release could occur in the mutant strain. Actually, the somewhat too large transcript hybridized to pure intron aI5 probe on RNA blots might represent intron bound to exonic sequence. This could be taken to suggest that the removal of aI5 could occur in several steps and, in the mutant, an alternate splice site may be used. Further, the removal of the fourth intron aI4 is thought to depend on a *trans*-acting element (maturase) encoded in the cytochrome *b* gene (3). Because aI4 is not removed, irrespective of the processing of the upstream introns, this particular maturase may need the MSS51 protein for its activity or specificity on the *oxi-3* pre-mRNA. Alternatively, the abnormally processed *oxi-3* pre-mRNA may not be a "substrate" for this maturase; both interpretations are, of course, not mutually exclusive.

The effects of the *mss51* mutation on splicing thus appears to be specific and multiple. Some plausible explanations of the role played by the MSS51 gene product are discussed below; critical evaluation of these possibilities will need the development of splicing enzymology.

The ligation activity of the splicing apparatus is probably not affected because the accumulation of high molecular weight transcripts detected on RNA transfer blots must result, in part, from the ligation of large splicing intermediates; thus, the strong 8.8-kb signal previously observed (7, see Fig. 1, lane b) may be the ligation intermediate formed after the excision of intron aI3. Rather, it is most probably a splicing component related to cleavage that is impaired by the *mss51* mutation.

Cleavage specificity implies endonucleolytic activity and site recognition, which may or may not be separate entities. At least two situations may be envisaged: either different endonucleases exist for different introns or one endonuclease carries out aspecifically the cleavages while other entities ensure the recognition of the splicing points. In keeping with the first possibility, the MSS51 gene product could be an endonuclease, inactivated in the mutant, that recognizes and splits the exon–intron borders flanking the first or the first two introns. One might then envisage that a block at an upstream junction would give rise, in the mutant, to steady-state intermediates that are not efficient substrates for downstream cleavage by other endonucleases, except for the third intron aI3, which is excised efficiently. However, it remains to be seen whether self-splicing reactions (24) could be involved in the mitochondrial splicing process. According to the second hypothesis, the endonuclease moiety need not be splice-junction specific by itself. Rather, the cleavage specificity could be mediated, through the MSS51 gene product, by the recognition of a structural conformation of the precursor RNA. It has been shown that their primary sequence confers to introns aI1 and aI2 quite similar secondary and, consequently, tertiary structures, bringing the exon–intron borders close together (22, 23, 25). Conceivably, the MSS51 gene product could be necessary to stabilize the folding of these tertiary structures to set up a proper splicing substrate. Whereas its activity is a prerequisite for the scission at both the 5′ and 3′ splice junctions of the first two introns, sufficient conformation would be maintained in the presence of the mutated *mss51* product to cleave, at least partially, the aI4–A5 and A5–aI5 boundaries. Experimental results suggest an involvement of the "mitoribosomes" in the excision of some introns (3, 26); an association of the MSS51 gene product to mitoribosomes could account for this differential effect. We have shown that the MSS51 protein is unnecessary for the correct removal of the third intron aI3. Interestingly, this intron presents a different structure when compared to the other group I mitochondrial introns (22, 23, 25) and it may well be removed by quite a different mechanism that does not preclude self splicing.

Several experimental arguments suggest that the excision of some mitochondrial introns is dependent on intron-encoded functions (2, 3). In this respect, intron aI1 presents a long open reading frame in phase with that of exon A1; the putative fusion protein specified by the sequence A1–aI1 could plausibly possess a maturase activity. If this hypothesis is true, the mutated mss51 product could intervene indirectly in the processing of the *oxi-3* pre-mRNA by preventing the biosynthesis or the activity of a A1–aI1 maturase. Thus, intron aI1 would not be excised; as a corollary, a second putative maturase A1–A2–aI2 would not excise the second intron aI2. As a consequence of the differential effect discussed above, the excision of the downstream introns (except aI3) would be deficient.

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