Splicing defective mutants of the *COXI* gene of yeast mitochondrial DNA: initial definition of the maturase domain of the group II intron Al2

John V. Moran, Kirk L. Mecklenburg^{1,+}, Philip Sass^{2,§}, Scott M. Belcher, Donna Mahnke³, Alfred Lewin³ and Philip Perlman*

Department of Biochemistry, University of Texas Southwestern Medical Center, Dallas, TX 75235,

¹Department of Molecular Genetics, The Ohio State University, Columbus, OH 43210, ²Department of Chemistry, Indiana University, Bloomington, IN 47405 and ³Department of Immunology and Medical Microbiology, University of Florida, College of Medicine, Gainesville, FL 32610, USA

Received February 23, 1994; Revised and Accepted April 25, 1994

ABSTRACT

Six mutations blocking the function of a seven intron form of the mitochondrial gene encoding subunit I of cytochrome c oxidase (*COXI*) and mapping upstream of exon 3 were isolated and characterized. A *cis*-dominant mutant of the group IIA intron 1 defines a helical portion of the C1 substructure of domain 1 as essential for splicing. A *trans*-recessive mutant confirms that the intron 1 reading frame encodes a maturase function. A *cis*-dominant mutant in exon 2 was found to have no effect on the splicing of intron 1 or 2. A *trans*-recessive mutant, located in the group IIA intron 2, demonstrates for the first time that intron 2 encodes a maturase. A genetic dissection of the five missense mutations present in the intron 2 reading frame of that strain demonstrates that the maturase defect results from one or both of the missense mutations in a newly-recognized conserved sequence called domain X.

INTRODUCTION

Genetic studies have been valuable in studying the splicing of group I and group II introns in yeast mitochondria (reviewed in 1, 2). *Cis*-dominant mutations have defined RNA sequences or structures required for splicing. *Trans*-recessive mutations mapping in intron reading frames led to the concept of maturases, proteins encoded by introns that are required for their splicing (3). While both types of introns are inherently self-splicing, specific introns are known to require a maturase or a nuclear encoded protein for splicing. *Trans*-recessive mutations that define maturase function have been reported only for the group I introns 2, 3 and 4 of the cytochrome b (*COB*) gene (reviewed in 2) and for the group II intron 1 of the *COXI* gene (aI1) (4,

5). Some yeast group I introns are mobile in crosses and they encode sequence-specific endonucleases that initiate intron transposition by making a double strand break in mitochondrial DNA (reviewed in 6, 7). One of those mobile introns does not encode a maturase, while another encodes a bifunctional protein with a latent maturase activity. Recently, a variant of *COB* intron 2 was found that has both maturase and endonuclease activity (8).

Nearly all reading frames of group II introns resemble those of retroelements. Most have a long domain that is most closely related to the reverse transcriptases (RTs) of the Mauriceville and Varkud plasmids of *Neurospora* mitochondria, bacterial retrons, and various non-LTR retrotransposons (9–13). Genetic data indicate that the proteins encoded by one or both of the two yeast group II introns with reading frames (aI1 and aI2) are required for intron excision from DNA, a genetic phenomenon that appears to involve reverse transcription (14, 15). Meunier *et al.* have reported that those two introns are mobile in crosses and have inferred that reverse transcription may be involved (16; see also 17). The reading frames of some group II introns contain three other conserved sequences that resemble motifs present in some retroelements (11, 13). Recently, Mohr *et al.* (18) defined a new, roughly 100 amino acid long sequence, called domain X. Although some group II intron reading frames lack the RT motif or have only remnants of it, nearly all contain domain X.

Clearly group II intron reading frames could encode a number of functions. However, no missense mutants have been reported that might permit a phenotype to be associated with a specific protein domain. The two most obvious functions to consider are maturase and RT; the latter activity has been demonstrated biochemically (20), and has been shown to be encoded by aI2 (see 21). In this paper we describe the analysis of mutations mapping in the 5' end of an allele of the *COXI* gene that contains the group II introns aI1 and aI2. Key findings of this study are

*To whom correspondence should be addressed

Present addresses: ⁺Department of Biological Sciences, Indiana University, South Bend, IN 46634 and [§]American Cyanamide Corp., Pearl River, NY 10965, USA

the demonstration that *al2* encodes a maturase function and the discovery that domain X of the intron reading frame is needed for that function.

MATERIALS AND METHODS

Yeast strains and mitochondrial mutants

Petite mutants DS6/A400 and DS6/A401 (*MAT α met*) are defined in ref. 22 and were gifts of Dr. Alexander Tzagoloff. All of the *mit*⁻ mutations were isolated by treatment of *Saccharomyces cerevisiae* strain ID41/6-161 (*MAT α ade1 lys1 q⁺*) (23) with MnCl₂ (2, 24). Four *mit*⁻ deletions were used as mapping reagents (see 25, 26 and Fig. 1). Mutant C1006 is deleted for approximately 4.6 kb upstream of the first coding region of the gene. C2107 is deleted for approximately 7.5 kb starting in exon 2 through the first half of intron 5 β . Deletions of about 2500 bp in mutants 5B and AD1 fuse the remnants of the *al1* and *al2* open reading frames at the positions shown (26, 27).

For mapping and complementation studies, mitochondrial genomes carrying the C1006, 5B, AD1, C2107, C1036 and C1082 mutations were transferred by cytoduction (28, 29) to the nuclear background of strain JC8/55 (*MAT α kar1 leu1*) (30). Mutant PZ27 is deleted for the *COB* gene of strain ID41-6/161 (31) and mutant C245 is deleted for most of the *COXI* gene of that strain (from upstream of the promoter to within intron 5 β) (32). Both deletions were studied in the ID41-6/161 and JC8/55 nuclear backgrounds and *q ϕ* derivatives of those strains were used as noted.

Strain C1082rec1 (see Fig. 5) was constructed in the following fashion. Biolistic transformation was used to construct a 'synthetic' petite strain (pMIT *al2* Δ D5) containing the portion of the *COXI* gene from the wild-type strain 161 from an Eco R1 site in *al2* through the Bam HI site in *al3* (see Fig. 1) in the nuclear background of strain DBY947 (*MAT α ade2-101 ura3-52*) (27, 33). Unrelated to its use here, the domain 5 sequence of *al2* was deleted from the plasmid prior to transformation (see 21, 34). The petite was mated to mutant C1082 and a *gly*⁺ diploid containing the C1082rec1 allele of *al2* was isolated; *gly*⁺ spore clones were isolated and cytoduction was used to place that mtDNA in the nuclear background of strain 161 to yield the construct used here. DNA sequencing was used to determine which of the mutations of C1082 remain in C1082rec1 (see Figure 5). C1082-AD1rec3 and C1082-5Brec1 are other derivatives of C1082 that were constructed as described previously (20); we have recently found that those two strains also contain a missense mutation in the Zn²⁺ finger-like domain, beyond of the region analyzed previously (C₄₇₉₆ to T [Pro to Thr]). That mutation originated in *al2* of strain C1036 and, by itself, has no effect on the splicing of *al2* (21, 34). Strain C1036 Δ 1 is a revertant of C1036 in which *al1* was excised from mtDNA (see 20 and Fig. 6).

Mapping and complementation methods

Marker rescue with genetically defined, physically mapped, petite mutants and *mit*⁻ deletions was used to map the new mutations (see 24). Transient complementation experiments were performed as described in ref. 35. Oxygen uptake measurements reported here were obtained using 10 hour matings. These respiration rates (nanoatoms of oxygen/min/10⁷ diploids) are about ten times higher than those reported by other groups (e.g., 35); since the *kar1* mutation of strain JC8/55 blocks nuclear fusion in about 90% of zygotes, our measurement of the number of prototrophic

diploids in the samples, which is roughly 10% of the number of zygotes (30), leads to that apparent overestimation of the respiration rate per diploid.

Analysis of labeled mitochondrial proteins

Mitochondrial proteins were labeled with ³⁵SO₄²⁻ in the presence of cycloheximide and proteins were separated by gradient gel electrophoresis as described in ref. 36. One-dimensional fingerprinting of labeled proteins excised from SDS polyacrylamide gels was carried out as described in ref. 37.

RNA blots

Mitochondrial RNA was extracted from isolated mitochondria or cell homogenates as described in 38 and 39, respectively. RNA was denatured at 65°C and separated on 1.2% agarose gels containing 6% formaldehyde. The RNA was then transferred to Hybond-N filters (Amersham) or gels were dried and probed directly (40). Probes were 5' end labeled synthetic oligonucleotides: probes *al1* 400AS and *al2* 3560AS are complementary to nt 396–414 and 3550–3567 of ref. 22, respectively; probe exon 4 H7 is complementary to nt 6754–6771 of ref. 22; and probe *OLI2* is complementary to nt 469–487 of ref. 41. Hybridization was done in the presence of 6 $\times$ SSC (SSC is 0.15 M NaCl and 0.015 M Na citrate), 0.1% SDS, 10 $\times$ Denhardt's solution with 50 μ g/ml of salmon sperm DNA at 55°C using between 10⁶ and 10⁷ cpm of probe per filter. Filters were washed in 6 $\times$ SSC and then 2 $\times$ SSC, each containing 0.1% SDS. The filters were then dried and exposed to Kodak XAR5 medical X-ray film with a Dupont Cronex intensifying screen. In some cases hybridization was obtained in 2 hr at 42°C using Rapid-Hyb rate enhanced hybridization buffer (Amersham). Some blots were quantitated using a Molecular Dynamics PhosphorImager.

DNA sequencing

For sequence analysis of portions of the *COXI* gene of mutant and wild-type strains, restriction fragments were cloned in M13mp18 or mp19 or pBLS KS⁺ and sequenced using universal or synthetic oligonucleotide primers. For all mutants we sequenced at least the entire portion of the gene to which the mutation was mapped (as defined in Figure 1). The wild-type sequence of the *COXI* gene of strain D273-10B is published in ref. 22 (Genbank # JO 1481); however, these mutants are derived from strain 161, so we determined the wild-type sequence of exons 1–3 and introns 1 and 2 from that strain. Sequence differences between those two wild-type strains are summarized in the legend to Figure 3.

RESULTS

Physical mapping of mutations in the 5' half of the *COXI* gene

Six mutants mapping to the 5' half of the *COXI* gene were characterized. Each mutant was mated to each tester strain shown in Figure 1 and the crosses were scored for the appearance of respiring (*gly*⁺) progeny. The results of these crosses define the physical location of each mutation as summarized in Figure 1. For example, mutants C1007, C1087 and C2126 are restored to a *gly*⁺ phenotype in crosses with the *mit*⁻ tester strain C1006 but not by the petite mutant strain DS6/A400. Since the deletion breakpoints of each tester strain are known quite accurately, most having been sequenced, it follows that those three mutants map to the 541 bp interval between breakpoints b and c.

Figure 1. Physical map of mutations in the *COXI* gene. The lengths of the first four exons and three introns of the *COXI* gene of yeast mtDNA are diagrammed at the top of the figure; coordinates in kbp correspond to the published sequence of this gene from strain D273-10B (22; Genbank # J01481). Six mutant genomes used to map point mutations in this region of the gene are described. For each, the thin line indicates portions of the gene that are present in the strain. Unfilled rectangles indicate portions of the *COXI* gene deleted in mit⁻ mutants; vertical lines denote the breakpoints of the two petite mutants used. In several cases, the precise location of a deletion is known to be between two restriction sites; in those cases the uncertainty is indicated by a filled box. In all other cases the breakpoints were determined by DNA sequencing. Each breakpoint is assigned a letter and the map of segments of the *COXI* gene distinguishable by crosses using these strains is summarized at the bottom of the figure. Each mutant maps to a discrete interval of the gene and sequencing that interval identifies the responsible mutation(s). The two restriction sites indicated define the portion of yeast mtDNA present in a 'synthetic' petite mutant, pMIT-a12Δd5 used in a strain construction.

None of these six mit⁻ mutants has a detectable deletion based on restriction site mapping (not shown). Mutants C1007, C2126, C2108 and C1082 revert spontaneously, indicating that one mutation is responsible for the splicing defect. Mutant C1087 does not revertant, indicating that it probably contains two or more mutations. Mutant C1036 yields only a class of pseudorevertants (see 26) implying that several mutations contribute to the respiration deficient phenotype. Manganese mutagenesis of yeast mtDNA often yields clusters of mutations (see 2 and below). In many cases a combination of detailed physical mapping and analysis of revertant strains identifies which mutation is responsible for the respiration defect (see below).

Complementation experiments define an a12-encoded maturase

Transient complementation experiments were conducted to determine whether each mutant is *cis*-dominant or *trans*-recessive. Negative controls include matings with ρ^o strains and some matings between strains having the same mutation. All negative controls yield essentially the same low respiration rate that is at the limit of detection (Table 1, line 1 and column 5). As a positive control we mated each mutant to PZ27, a deletion of the *COB* gene. While there is substantial variation among the positive controls (shaded positions in Table 1) all have azide-sensitive respiration rates at least ten times that of the negative controls.

Initial experiments involved crosses with mutant coxI-7, an exon mutant mapping near the end of the *COXI* gene. Since that mutant has no splicing defect (42) it has all *COXI* gene maturases

Table 1. Complementation analysis of *COXI* gene mutants

		nanoatoms oxygen/min/10 ⁷ diploids					
	<i>MATα</i>		coxI-7	PZ27	ρ ^o	C1036	C1082
	strains	N					
Row	<i>MATα</i> strains						
1	ρ ^o	5	11	9	11	6	7
2	PZ27	5	145	10	11	295	260
3	C1007	2	10	270	12	10	6
4	C1087	2	12	215	12	16	8
5	C2126	2	5	310	6	270	6
6	C1036	9	202	438	10	9	225
7	C2108	2	11	102	7	198	74
8	C1082	3	79	256	12	171	9

Column: 1 2 3 4 5 6 7

All values shown represent respiration rates of diploids formed by mating the strains indicated. Because these data are uncorrected for a very low rate of oxygen consumption in control experiments in which cells were omitted, all of the negative controls (and negative experimental samples) yield a non-zero value (from 6–16). In all experiments, cultures of each strain were tested for their content of petite mutants and revertants and all of the cultures used had fewer than 10% petites and fewer than 0.01% revertants. The number of independent replicates of each cross is summarized for the first three tester strains in column 2. Columns 6 and 7 report the average of duplicate crosses. Variation in respiration rate among replicate experiments was less than 20% in nearly all cases. Shaded rectangles identify positive controls.

and, so, should complement all *trans*-recessive mutants of this gene. All *cis*-dominant intron mutants and all other exon mutants should fail to complement when mated to this tester (see 24). Table 1, column 3 summarizes respiration in diploids from crosses with mutant coxI-7. By this test, only C1036 and C1082 are *trans*-recessive; this finding is a benchmark for defining an intron-encoded maturase function. The other four mutants (C1007, C1087, C2126, and C2108) yield respiration values like those of the negative controls, showing that they contain *cis*-dominant mutations belonging to the same complementation group as mutant coxI-7.

These findings are extended by crosses in which *MATα* strains containing mtDNA of C1036 (column 6) and C1082 (column 7) were mated to each of the *MATα* strains used above. These data show that C1036 and C1082 are in different complementation groups from each other and from the rest of the mutants. C2108 complements both C1036 and C1082, showing that it has maturase activity for both introns even though it maps between them. Mutant C2126 appears to be a *cis*-dominant mutant of a11 because it has only the a11 maturase by this test. Mutants C1007 and C1087 lack both maturases since they fail to complement both *trans*-recessive mutants; they have *cis*-dominant defects and also lack the intron 1 maturase.

Novel mitochondrial proteins are accumulated by some mutants

Mitochondrial intron mutants often accumulate detectable levels of intron encoded proteins (e.g., 3, 35, 43–46). As shown in Figure 2, all of these mutants have the normal array of mitochondrial translation products and lack COXI protein. Mutants C1007, C1087, and C2108 accumulate no novel proteins (lanes 3, 4 and 6, respectively). Mutant C2126 accumulates two large proteins, a major one estimated to be 68 kdal (p68) and a minor one of about 66 kdal (lane 2). Since C2126 complements

Figure 2. Mitochondrial translation products accumulated in mutant strains. Mitochondrial proteins were labeled *in vivo* with ³⁵S-sulfate as cited in Materials and Methods, fractionated on a 10–15% polyacrylamide gel and detected by autoradiography. Lane 1 is a control using the ρ⁺ parent of the mutants. Proteins accumulated by mutants C2126, C1007, C1087, C1036, C2108 and C1082 are illustrated in lanes 2–7, respectively. The major proteins present in the control lanes are labeled alongside of lane 1 and the estimated sizes of prominent novel proteins present in some mutants are indicated alongside of lanes 1 and 8.

Table 2. Transcripts accumulated in *COXI* mutants

Transcript Accumulated							
RNA SIZE (kb)	>7	7	4.3-4.4	2.4-2.5	1.9	0.9	
RNA identity	pre	pre	pre	aI1	aI2	mRNA	aI5γ
ρ ⁺	-	-	-	+++	+++	+++	+++
C2126	-	+	+	-	+	-	+++
C1007	+/-	+	-	-	-	-	+++
C1087	+/-	+	-	-	-	-	+++
C1036	+/-	+	-	-	-	-	+++
C2108	-	-	-	+++	+++	+++	+++
C1082	-	-	+	+++	-	-	+++

The Table summarizes the main transcripts of the *COXI* gene accumulated in these strains based on RNA blot experiments using several RNA preparations from each strain and probes specific for *COXI* exons and introns 1, 2 and 5g. Figures 4 and 6 contain some of the primary data (see also ref. 34 for additional data). The abundant RNAs present in mitochondria of ρ⁺ strains (2.4–2.5 kb, excised aI1 and aI2 RNA lariats; 1.9 kb, mRNA; and 0.9 kb, excised aI5γ RNA lariat) are already well-characterized (49). Relative amounts of transcripts are estimated based on experiments in which the RNA from each strain was first balanced by determining the level of *OLI2* mRNA, a stable mitochondrial transcript that does not appear to be affected by any of these mutations; as balanced, all of the strains have roughly the same amount of the 0.9 kb aI5γ intron RNA. Strains that splice aI1 or aI2 accumulate essentially wild-type levels of the 2.4–2.5 kb excised intron lariat RNAs. However, none of the mutants with a splicing defect has a high level of any precursor RNA (see text). Symbols used are defined as follows: + + +, an abundant transcript detected using relevant specific probes; +, a relatively minor transcript; +/-, a barely detectable transcript; -, no detectable signal.

the *trans*-recessive mutant C1036, one of the two large proteins accumulated in C2126 may be the functional aI1-encoded maturase. Mutant C1036 accumulates a major protein of about 54 kdal (p54) (lane 5); partial protease digests of p68 and p54

Figure 3. Sequence analysis of *COXI* gene mutations. The figure illustrates the location of base changes in each mutant strain. Each mutant basepair is indicated along with the sequence coordinate relative to the published sequence of this gene from strain D273-10B (22). A number of mutations distinguish the wild-type sequence of strain 161 from that of strain D273-10B. The following are changes in the untranslated 5' leader: C₋₁₁₈ to A; +C at position -105; deletion of TTAAA from -95 to -91; deletion of twoTs between T₋₅₆ and T₋₆₆; and deletion of an A between A₋₇ and A₋₁₅. There are two silent mutations in the intron reading frames: A₁₅₉ to G and C₄₀₈₆ to T. Finally, six mutations alter five amino acids of the intron reading frames: A₃₄₉ to T (Asn to Phe); A₃₅₀ to T (Asn to Phe); G₅₅₀ to T (Gly to Trp); T₇₀₅ to A (Phe to Leu); C₃₁₅₅ to T (Ala to Val); and T₄₆₂₀ to A (Ser to Arg).

show that they are closely related (see 42), so p54 is probably a shortened form of the aI1-encoded protein (see below). The *trans*-recessive aI2 mutant, C1082, has a major new protein of about 62 kdal (p62) (lane 7).

RNA blots define splicing defects

Mitochondrial RNA was analyzed by northern hybridizations with exon and intron-specific probes and the results are summarized in Table 2. We conclude that C2108 is probably an exon 2 mutant because it has a normal level of *COXI* mRNA and accumulates no abundant precursor RNA. The other mutants lack the 1.9 kb *COXI* mRNA and have larger precursor RNAs so they clearly are splicing mutants. With exon and intron probes, the upstream mutants (C1007, C1087, C1036 and C2126) yield only weak signals in the region of the gel containing very large (>7 kb) RNAs. Blots using exon and aI2 probes show that mutant C1082 has a 4.3 kb precursor RNA the size expected to result from an aI2 splicing block. Figures 4B and 6 illustrate these points for C2126 and C1082, respectively.

Excised group II intron RNAs accumulate in yeast mitochondria and are detected as strong signals on RNA blots with intron-specific probes (e.g., 47–49). Using an aI1 probe, mutants C2108 and C1082 accumulate the excised aI1 intron RNA (Table 2). In the other mutants, only large precursor RNAs were detected with this probe. Using an aI2 probe, mutants C1007, C1087, C1036 and C1082 had no detectable excised intron 2 RNA; C2108 contains excised aI2 RNA as a strong signal and C2126 has a weak signal at that position (see Fig. 4B and below). The sizes of the major precursor RNAs present in the splicing defective mutants indicate that mutations in aI1 and aI2 do not block the splicing of any of the five downstream introns. Thus, it appears that aI1 and aI2 are the only introns of this allele of the *COXI* gene that encode maturases.

Figure 4. C2126 is a mutation in the C1 helix of a11. **A.** The secondary structure of the C1 region of domain 1 of a11: The secondary structure of the C1 portion of domain 1 of a11 is shown; portions of the substructure are labeled according to conventions developed in ref. 61. The two nucleotides labeled ϵ' participate in 5' splice site definition by basepairing with the third and fourth nucleotides of the intron. C2126 has the missense mutation in the C1⁽ⁱ⁾ helix shown in the figure and another missense change in the domain 1 helix B nearby. The revertant strain C2126R1:5B contains a suppressor mutation C₂₉₂ to T that restores basepairing with A₂₄₉. **B.** RNA blots: RNA was isolated from partially purified mitochondria from the following strains: wild-type (161 q⁺), lanes 1, 4 and 7; C2126, lanes 2, 5 and 8; and revertant R1:5B, lanes 3, 6 and 9. The RNAs were fractionated and analyzed as described in Materials and Methods using probes specific exon 4 (lanes 1–3), intron 1 (lanes 4–6), and intron 2 (lanes 7–9). RNA inputs were balanced for roughly equal amounts of *OL12* mRNA (not shown). Sizes of relevant RNAs are identified on the left side of the figure (see Table 2).

DNA sequence analysis of mutant strains

DNA sequence analysis of relevant portions of the *COXI* gene from strains C2126, C1036, C2108 and C1082 confirms most of the tentative conclusions made in the previous sections. The key mutations present in each strain are summarized in Figure 3, relative to the sequence of the *COXI* gene of strain D273-10B (22; Genbank # JO1481).

Mutant C1036 has six point mutations, four missense mutations, one silent third position substitution, and one nonsense mutation, all clustered within a 300 bp region of the a11 reading frame (see 26). Any of the five changes that alter the protein sequence might inactivate the maturase function. However, the T to A transition at position 2018 (not 2128 as reported in ref. 26 due to a typographical error) creates an in-frame TAA (stop)

Figure 5. A genetic dissection of the mutations present in mutant C1082. The diagram at the top shows the structure of the 5' part of the *COXI* gene. The locations and relative sizes of the portions of the a2 reading frames that contain distinct sequence motifs are denoted by shaded rectangles (P, retroviral protease-like domain; Z, non-LTR retrotransposon-like domain; RT, reverse transcriptase-like domain; X, maturase domain of group II intron reading frames; Zn, Zn²⁺ finger-like domain). The positions of the five missense mutations present in the a2 reading frame in mutant C1082 are indicated by vertical lines. Below the diagram, the sequence of a2 at those five sites (and a sixth site in the Zn region) is summarized for the wild-type strain, C1082 and three gly⁺ derivatives of C1082 (+ = wild-type sequence; - = mutant sequence [see the text for the specific mutations]). The splicing phenotype of each strain is summarized in the right-hand column.

codon in domain X that is sufficient to account for the accumulation of the shortened protein (p54) and the maturase deficiency.

Mutant C2126 contains two point mutations near the beginning of a11 (G₂₁₄ to A [Val to Met] and G₂₄₉ to A [silent 3rd position change]). Each of those mutations can destabilize a different highly conserved RNA helix in the domain 1 secondary structure of a11 (see Fig. 4A). We characterized a spontaneous gly⁺ revertant (strain C2126-R1:5B) that restores efficient splicing to both introns (Fig. 4B). DNA sequencing shows that reversion results from a nearby suppressor mutation (C₂₉₂ to T [His to Tyr]) that compensates for the mutation at position 249 by restoring basepairing in the middle of the helix that initiates the C1 substructure of domain 1 (Fig. 4A). This result demonstrates that this highly conserved helix is important for splicing of this intron.

Mutant C2108 has one base change in exon 2, A₂₆₃₃ to T, changing a histidine to threonine. We verified that this mutation is responsible for the mutant phenotype by characterizing a spontaneous revertant that proved to be a back-mutation at that site. That His codon is highly conserved in *COXI* genes from diverse sources; since we were unable to find second-site suppressors of the mutation, that histidine appears to be important for subunit I function (see ref. 52). We also found a missense mutation in the RT domain of the a2 reading frame of C2108 (G₃₆₄₆ to A [Ala to Thr]); that mutation does not affect splicing and its effect on RT activity and intron mobility will be reported elsewhere (21).

Sequencing all of a2 from mutant C1082 revealed one silent mutation (C₃₁₁₇ to T) and five missense mutations in the intron reading frame (summarized in Figures 3 and 5). None of the mutations alters a nucleotide that has been proposed to be part of a *cis*-acting splicing signal for group II introns. Three mutations (G₂₆₈₉ to A [Val to Met]; C₃₁₇₀ to T [Thr to Ile]; and T₃₈₉₆ to

Figure 6. *COXI* splicing in C1082 and strains derived from it. Mitochondrial RNAs were isolated from cultures of the six strains listed above the lanes in Panel A. Denaturing gels were used to fractionate the RNAs and a separate gel was prepared for each probe. Samples were first roughly balanced by quantifying the amount of signal obtained with an *OLI2*-specific probe (Panel D). The gel in Panel A was hybridized with a probe specific for *COXI* exon 4. Panels B and C were hybridized with probes specific for aI1 and aI2, respectively. The sizes of the main RNAs detected in each panel are indicated on the left and the identities of those RNAs are noted on the right.

A [Ile to Asn]) are not responsible for the respiration deficient phenotype because they are located upstream of the location of the relevant mutation (see Fig. 1). The mutation at nt 3896 is intriguing because it is a non-conservative change in block 4 of the RT-like portion of this intron reading frame (see 10, 12). The remaining missense changes (C₄₅₉₈ to T [Ser to Leu] and G₄₆₇₈ to A [Asp to Asn]) (see Fig. 3), are within the map location of the defect and, so, appear responsible for blocking the maturase function of this protein (see below). Those mutations are located within the recently-defined 'domain X' of this group II intron reading frame (18).

Domain X is a maturase domain of the aI2 reading frame

Analysis of strain C1082rec1 confirmed our inference that the domain X mutations block the aI2 maturase function. It is a derivative of C1082 in which the two missense mutations in domain X have been restored to the wild-type sequence (see Materials and Methods and Figure 5). The RNA blot data of Figure 6A show that C1082rec1 has a nearly normal amount of *COXI* mRNA. Some 4.3 kb precursor RNA accumulates in C1082rec1, indicating that one or more of the remaining mutations caused a partial splicing defect. Using intron-specific probes we found that the amount of excised aI2 RNA was unexpectedly low (Fig. 6C) (relative to aI1 RNA [Fig. 6B] and *OLI2* mRNA [Fig. 6D]). This result suggests that one or more

of the remaining mutations in aI2 of that strain alters the stability of the excised aI2 RNA.

The phenotypic consequence of each of the five aI2 mutations in C1082 is not yet known. However, studies of two more derivatives of that mutant support the conclusion that the mutation in the RT domain causes the remaining partial splicing defect. Strain C1082-AD1rec3 is identical to C1082rec1 except that it has an additional mutation in the Zn²⁺ finger-like region of the aI2 reading frame (C₄₇₉₆ to T [Pro to Thr]) that was placed there inadvertently during the strain construction (see ref. 20 and Figure 5). The RNA phenotypes of strains C1082-AD1rec3 and C1082rec1 are very similar (Fig. 6A and C). This shows that the mutation in the Zn²⁺ finger-like region does not alter the splicing phenotype associated with the first four mutations of the intron reading frame. That inference was confirmed by analysis of strain C1036D1 (see Materials and Methods) that contains only the Pro to Thr mutation (lane 6 in each panel). Because C1036D1 splices aI2 efficiently (Fig. 6A) and accumulates excised aI2 RNA (Fig. 6C) we conclude that this mutation of the Zn²⁺ finger-like region does not affect either process. Strain C1082-5Brec1 is identical to C1082-AD1rec3 except that it is wild-type in the RT domain (see Fig. 5). The northern blots show that removing the RT domain mutation restores the efficiency of splicing based on the level of mRNA and the absence of the 4.3 kb precursor RNA (Fig. 6A). Because strain C1082-5Brec1 still has less excised aI2 RNA than the control strains (Fig. 6C), it appears that one or both of the remaining mutations (see Fig. 5) influences the stability of the aI2 lariet.

DISCUSSION

We have characterized six respiration deficient mutants mapping in or near the group IIA introns of the *COXI* gene of yeast mtDNA. Four mutants map to aI1 and block the splicing of aI1 and aI2. One mutant is in exon 2 and has no effect on splicing of either intron flanking it. Finally, one mutant maps to aI2 and blocks its splicing. The analysis of the aI2 mutant, C1082, shows for the first time that the aI2 reading frame encodes a maturase needed for splicing that intron. Transient complementation experiments demonstrate that the C1082 mutation is *trans*-recessive with respect to mutations in the exons of the *COXI* gene. We also characterized a new *trans*-recessive mutation (C1036) of the aI1 reading frame. Crosses between mutants C1036 and C1082 exhibit complementation, showing that the two intron reading frames represent separate complementation groups.

In three mutants we detected novel mitochondrial translation products in place of the missing *COXI*. The predicted size of the protein specified by exon 1 plus the aI1 reading frame is about 96 kD. Our estimate of 68 kdal for the aI1-encoded protein present in C2126 probably indicates that the protein is processed. Since mutant C1036 has a stop codon in domain X, near the end of the intron reading frame and accumulates a p54 species that is related to the p68 of C2126, we conclude that p68 is probably the C-terminal product of processing the precursor protein. Other workers have suggested that the aI1-encoded precursor is processed to yield a large protein about the size reported here and a 20 kdal protein (4) that we have not observed. The protein encoded by exons 1 and 2 plus the aI2 reading frame should be about 98 kdal. The main protein accumulated by the aI2 mutant, C1082, is about 62 kd and is probably processed from the precursor. Another lab has prepared a polyclonal antibody to the 70 amino acids at the C-terminal end of the aI2 reading frame

and reported the presence of a 65 kb protein in *cis*-dominant mutants of that intron (53).

Kennell *et al.* (20) have shown that both aI1 and aI2 are associated with an RT activity and it is now clear that each intron encodes a maturase function. Studies are underway to determine whether the 68 kd protein encoded by aI1 and the 62 kd protein encoded by aI2 have RT activity. It is possible that several pathways of processing of the precursor protein could lead to different-sized products that might have different functions (e.g., RT, maturase, or other activities involved in splicing or the several RNA-mediated processes exhibited by these introns [see 15–17, 21]). Several nuclear genes are needed for splicing of these group II introns (54, 55) so that it is likely that a multi-protein complex including the maturase splices the introns *in vivo*.

Previous studies indicate that the C1 region of a group IIB intron contains an unpaired sequence (ϵ') that may pair with two nucleotides near the 5' end of the intron (ϵ) and that the ϵ – ϵ' pairing may be needed for splicing (50). Our analysis of the C2126 mutant of aI1 shows that a mutation of the RNA helix adjacent to the ϵ' sequence of that group IIA intron blocks splicing *in vivo*. We have also found that the C2126 mutation significantly blocks the self-splicing of aI1 *in vitro* and that the suppressor mutation reported here restores self-splicing (51). Our working hypothesis is that the C1 substructure is responsible for positioning the ϵ' nucleotides for their role in defining the 5' splice site.

Mutant C2108 is a missense mutation in exon 2. It is surprising that it does not accumulate COXI protein since it has processed mRNA. Many missense and chain termination mutants of the exons of the *COB* gene have been analyzed by the protein labeling methods used here and nearly all accumulate of non-functional cytochrome *b* apoprotein or fragments of it, as appropriate for the mutation (35, 56–59). Those studies include missense mutants that lack a cytochrome *b* spectrum and therefore cannot be assumed to have assembled apocytochrome *b* correctly with other components of the respiratory chain. It appears that the inactive form of COXI protein made in mutant C2108 is unstable, perhaps because it fails to assemble with other membrane proteins. Interestingly, a missense mutation of an invariant Histidine encoded by exon 5 γ of the COXI protein does not block its accumulation (52).

We were surprised to find that mutants blocked for aI1 and aI2 splicing accumulate little COXI pre-mRNA since earlier studies of splicing blocks of other COXI introns lead to readily detected pre-mRNA (34, 51, 60). Thus, it is possible that precursor RNAs containing aI1 and aI2 or just aI2 are inherently somewhat unstable. Reduced transcription of the COXI gene does not explain the low level of precursor because each mutant has a normal level of excised aI5 γ , a non-maturase-requiring group II intron (not shown) and all of these mutant strains have comparable levels of *OLI2* mRNA which is cotranscribed with the COXI gene (e.g., Fig. 6D).

While both aI1 and aI2 encode maturase proteins with related primary sequences, the extent to which they are intron-specific is still uncertain. Other researchers have noted that some *cis*-dominant mutants of aI1 splice aI2 to some extent (4, 5). They suggested that high levels of active aI1 maturase accumulated by such mutants may permit some splicing of the closely-related aI2. We show that the *cis*-dominant aI1 mutant C2126 splices aI2 to some extent, but further studies of that strain indicate that the splicing of aI2 probably results from a low level of aI1 splicing (see 34). Since C2126 does not complement the aI2 maturase

mutant, it follows that even overproduction of the aI1 maturase does not effectively substitute for the aI2 protein.

Independent evidence indicating that these group II intron maturases may be partially interchangeable comes from our earlier studies of the COXI deletion mutants 5B and AD1 (26, 27 and Fig. 1). In those strains, aI1 and aI2 are fused by a deletion; the hybrid intron does not splice but encodes a chimeric protein that substitutes, probably inefficiently, for the aI1 maturase (26, 27). While each strain makes a somewhat different hybrid protein, each contains the entire X and Zn²⁺ finger-like domains of aI2 and each has sequences upstream of the RT domain from aI1. Thus, a protein containing the domain X of aI2 can participate in splicing of aI1, at least when expressed at a high level. A more direct test of the specificity of the aI1 and aI2 encoded maturases, however, is still needed.

Finally, an important outcome of this study is the initial definition of a domain of the aI2 reading frame that contributes to the maturase function. Our analysis of mutant C1082 demonstrates that one or both of the missense mutations in domain X block the splicing function (see Fig. 5). One of them changes a highly conserved Ser to Leu and the other changes a non-conserved Asp at the end of the domain to Asn (see Fig. 3 and ref. 18). Kennell *et al.* (20) reported that mutant C1082 has no RT activity; because that strain has three other missense mutations in the aI2 reading frame, the available data do not test whether the domain X function is needed for RT activity. As was speculated in ref. 20, since both RT and maturase should require an RNA binding function, it is possible that RNA binding is the function of domain X. Mutant C1082 also contains a missense mutation in block 4 of the RT domain (12). Our analysis of gly⁺ derivatives of C1082 indicates that the RT domain mutation affects splicing somewhat and that mutations of the aI2-encoded protein influence the stability of the excised aI2 RNA.

ACKNOWLEDGEMENTS

This research was supported by NIH research grants GM31480 (to PSP) and GM12228 (to AL). SMB was a Fellow of the Robert A. Welch Foundation (Grant I-1211). We thank Alan Lambowitz and Ron Butow for helpful comments about this manuscript. Troy Schmidt assisted in some of the DNA sequencing experiments reported here. This study was initiated in a collaboration with Professor Henry R. Mahler (Indiana University).

REFERENCES

1. Perlman, P. S., Daniels, C., and Peebles, C. L. (1990) in: *Intervening Sequences in Evolution and Development* (Stone, E. M. and Schwartz, R. J., eds.) pp. 112–161. Oxford University Press, New York.
2. Perlman, P. S. (1990) *Meth. Enzymol.* **181**, 539–558.
3. Lazowska, J., Jacq, C., and Slonimski, P. P. (1980) *Cell* **22**, 333–348.
4. Carignani, G., Groudinsky, O., Frezza, D., Schiavon, E., Bergantino, E., and Slonimski, P. P. (1983) *Cell* **35**, 733–742.
5. Carignani, G., Netter, P., Bergantino, E., and Robineau, S. (1986) *Curr. Genet.* **11**, 55–63.
6. Perlman, P. S. and Butow, R. A. (1989) *Science* **246**, 1106–1109.
7. Lambowitz, A. M. and Belfort, M. (1993) *Ann. Rev. Biochem.* **62**, 587–622.
8. Lazowska, J., Szczepanek, T., Macadre, C., and Dokova, M. (1992) *Comptes rendus de l'Academie des Science* **315**, 37–41.
9. Michel, F. and Lang, B. F. (1985) *Nature* **316**, 641–643.
10. Xiong, Y. and Eickbush, T. H. (1988) *Mol. Biol. Evol.* **5**, 675–690.
11. Doolittle, R. F., Feng, D. F., Johnson, M. S., and McClure, M. A. (1989) *Quart. Rev. Biol.* **64**, 1–30.
12. Xiong, Y. and Eickbush, T. H. (1990) *EMBO J.* **9**, 3353–3362.
13. McClure, M. A. (1991) *Mol. Biol. Evol.* **8**, 835–856.

14. Kotylak, Z., Lazowska, J., Hawthorne, D. C., and Slonimski, P. P. (1985) in: *Achievements and Perspectives of Mitochondrial Research, Volume I, Biogenesis* (Quagliariello, E., Slater, E. C., Palmieri, R., Saccone, C., and Kroon, A. M., eds.) pp. 1–20, Elsevier Science Publishers, B. V., Amsterdam.
15. Levra-Juillet, E., Boulet, A., Seraphin, B., Simon, M., and Faye, G. (1989) *Mol. Gen. Genet.* **217**, 168–171.
16. Meunier, B., Tian, G. L., Macadre, C., Slonimski, P. P., and Lazowska, J. (1990) in *Structure, Function and Biogenesis of Energy Transfer Systems* (Quagliariello, E., Papa, S., Palmieri, F., and Saccone, C., eds.) pp. 169–174. Elsevier Press, Amsterdam.
17. Skelly, P. J., Hardy, C. M., and Clark-Walker, G. D. (1991) *Curr. Genet.* **20**, 115–120.
18. Mohr, G., Perlman, P. S., and Lambowitz, A. M. (1993) *Nucleic Acids Res.* in press.
19. Saldanha, R., Mohr, G., Belfort, M., and Lambowitz, A. M. (1993) *FASEB Journal* **7**, 15–24.
20. Kennell, J. C., Moran, J. V., Perlman, P. S., Butow, R. A., and Lambowitz, A. M. (1993) *Cell* **73**, 133–146.
21. Moran, J. V., Zimmerly, S. J., Eskes, R., Kennell, J. C., Lambowitz, A. M., Butow, R. A., and Perlman, P. S. (1994) in preparation.
22. Bonitz, S. G., Coruzzi, G., Thalenfeld, B. E., Tzagoloff, A., and Macino, G. (1980) *J. Biol. Chem.* **255**, 11927–11941.
23. Alexander, N. J., Vincent, R. D., Perlman, P. S., Miller, D. H., Hanson, D. K., and Mahler, H. R. (1979) *J. Biol. Chem.* **254**, 2471–2479.
24. Perlman, P. S. and Mahler, H. R. (1983) *Meth. Enzymol.* **97**, 374–395.
25. Mecklenburg, K. L. (1987) *RNA processing in yeast mitochondria: genetic and molecular analysis of the *oxi3* gene*. Ph.D. Dissertation, The Ohio State University.
26. Anziano, P. Q., Moran, J. V., Gerber, D., and Perlman, P. S. (1990) *Nucleic Acids Res.* **18**, 2133–2329.
27. Anziano, P. Q., and Butow, R. A. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 5592–5596.
28. Nagley, P. and Linnane, A. W. (1978) *Biochem. Biophys. Res. Commun.* **85**, 585–592.
29. Lancashire, W. and Mattoon, J. (1979) *Mol. Gen. Genet.* **176**, 255–264.
30. Conde, J. and Fink, G. R. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 3651–3655.
31. Perlman, P. S., Alexander, N. J., Hanson, D., and Mahler, H. R. (1980) in: *Gene Structure and Expression* (Dean, D. B., Johnson, L. F., Kimball, P. C., and Perlman, P. S., eds.) pp. 211–253. The Ohio State University Press, Columbus, Ohio.
32. Mahler, H. R., Hanson, D., Miller, D., Bilinski, T., Ellis, D. M., Alexander, N. J., and Perlman, P. S. (1977) in *Genetics and Biogenesis of Mitochondria* (F. Kaudewitz, Schweyen, R. J., Bandlow, W., and Wolf, K., eds.) pp. 345–370. Walter de Gruyter Press, Berlin.
33. Peebles, C. L., Belcher, S. M., Zhang, M., Dietrich, R. C., and Perlman, P. S. (1993) *J. Biol. Chem.* **268**, 11929–11938.
34. Moran, J. V. (1994) Studies of mobile introns in the yeast mitochondrial genome. Ph. D. Dissertation. University of Texas Southwestern Medical Center, Dallas, TX.
35. Haid, A., Schweyen, R. J., Bechmann, H., Kaudewitz, F., Solioz, M., and Schatz, G. (1979) *Eur. J. Biochem.* **94**, 451–464.
36. Douglas, M., Finkelstein, D., and Butow, R. A. (1979) *Meth. Enzymol.* **56**, 56–65.
37. Cleveland, D. W., Fischer, G. S., Kirschner, M. W., and Laemmli, U. K. (1977) *J. Biol. Chem.* **252**, 1102–1106.
38. Chomczynski, P. and Sacchi, N. (1987) *Analyt. Biochem.* **162**, 156–159.
39. Schmitt, M. E., Brown, T. A., and Trumpower, B. L. (1990) *Nucleic Acids Res.* **18**, 3091.
40. Ahmad, I., Finkelstein, J. A., and Steggle, A. W. (1990) *BioTechniques* **8**, 162–165.
41. Macino, G. and Tzagoloff, A. (1980) *Cell* **20**, 507–517.
42. Sass, P. M. (1984) *Mutations in the mosaic *cox1* gene in the mitochondrial genome of the yeast *Saccharomyces cerevisiae**. Ph. D. Dissertation, Indiana University, Bloomington, Indiana.
43. Schmelzer, C., Haid, A., Grisch, G., Schweyen, R. J., and Kaudewitz, F. (1981) *J. Biol. Chem.* **256**, 7610–7619.
44. de la Salle, H., Jacq, C., and Slonimski, P. P. (1982) *Cell* **28**, 721–732.
45. Hanson, D. K., Lamb, M. R., Mahler, H. R., and Perlman, P. S. (1982) *J. Biol. Chem.* **257**, 3218–3224.
46. Lamb, M. R., Anziano, P. Q., Glaus, K. R., Hanson, D. K., Klapper, H. J., Perlman, P. S., and Mahler, H. R. (1983) *J. Biol. Chem.* **258**, 1991–1999.
47. Arnberg, A. C., van Ommen, G. J. B., Grivell, L. A., van Bruggen, E. F. J., and Borst, P. (1980) *Cell* **19**, 313–319.
48. Halbreich, A., Pajot, P., Foucher, M., Grandchamp, C., and Slonimski, P. P. (1980) *Cell* **19**, 321–329.
49. Hensgens, L. A. M., Arnberg, A. C., Roosendaal, E., van der Horst, G., van der Veen, R., van Ommen, G. J. B., and Grivell, L. A. (1983) *J. Mol. Biol.* **164**, 35–58.
50. Jacquier, A. and Michel, F. (1990) *J. Mol. Biol.* **213**, 437–447.
51. Belcher, S. M. (1993) *In vivo, in vitro* and reverse genetic studies of splicing of group II introns from yeast mtDNA. Ph.D. Dissertation. University of Texas Southwestern Medical Center, Dallas.
52. Netter, P., Robineau, S., Sirand-Pugnet, P. and Pauvarque, M.-O. (1992) *Current Genetics* **21**, 147–151.
53. Bergantino, E. and Carignani, G. (1990) *Mol. Gen. Genet.* **223**, 249–257.
54. Seraphin, B., Simon, A., Boulet, A., and Faye, G. (1989) *Nature* **337**, 84–87.
55. Wiessenberger, G., Waldherr, M., and Schweyen, R. J. (1992) *J. Biol. Chem.* **267**, 6963–6969.
56. Claisse, M. L., Spyridakis, A., Wambier-Kluppel, M. L., Pajot, P., and Slonimski, P. P. (1978) in *Biochemistry and Genetics of Yeast* (M. Bacila, Horecker, B. L., and Stoppani, A. O. M., eds.) pp. 369–390. Academic Press, New York.
57. Hanson, D. K., Miller, D. H., Mahler, H. R., Alexander, N. J., and Perlman, P. S. (1979) *J. Biol. Chem.* **254**, 2480–2490.
58. Kreike, J., Bechmann, H., Van Hemert, F. J., Schweyen, R. J., Boer, P. H., Kaudewitz, F., and Groot, G. S. P. (1979) *Eur. J. Biochem.* **101**, 607–617.
59. Alexander, N. J., Perlman, P. S., Hanson, D. K., and Mahler, H. R. (1980) *Cell* **20**, 199–206.
60. Moran, J. V., Wernette, C. M., Mecklenburg, K. L., Butow, R. A., and Perlman, P. S. (1992) *Nucleic Acids Res.* **20**, 4069–4076.
61. Michel, F., Umeson, K., and Ozeki, H. (1989) *Gene* **82**, 5–30.