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Intron 5α of the *COXI* gene of yeast mitochondrial DNA is a mobile group I intron

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ABSTRACT

We have found that intron 5α of the *COXI* gene (aI5α) of yeast mtDNA is a mobile group I intron in crosses between strains having or lacking the intron. We have demonstrated the following hallmarks of that process: 1) co-conversion of flanking optional intron markers; 2) mutations that truncate the intron open reading frame block intron mobility; and 3) the intron open reading frame encodes an endonuclease activity that is required for intron movement. The endonuclease activity, termed I-*Sce* IV, cleaves the *COXI* allele lacking aI5α near the site of intron insertion, making a four-base staggered cut with 3′ OH overhangs. Three cloned DNAs derived from different forms of the *COXI* gene, which differ in primary sequence at up to seven nucleotides around the cleavage site, are all good substrates for *in vitro* I-*Sce* IV cleavage activity. Two of the strains from which these substrates were derived were tested in crosses and are comparably efficient as aI5α recipients. When compared with ω mobility occurring simultaneously in one cross, aI5α is less efficient as a mobile element.

INTRODUCTION

The ω intron of the large rRNA gene of yeast mitochondrial DNA (mtDNA) was the first group I intron shown to be mobile in crosses between yeast strains having or lacking that intron (1–3). Recently, a number of other examples of mobile group I introns have been reported (4, 5). All mobile group I introns studied thus far encode a site-specific endonuclease activity that cleaves intronless alleles in the vicinity of the intron insertion site. This cleavage initiates a process of double-strand break-gap repair, similar to mating-type switching in the yeast nucleus (6, 7). Group I intron mobility, also called 'intron homing' (4), is a site-specific process which leads to the loss of intronless alleles and their replacement, sometimes quantitatively, by intron-containing ones.

The second example of a mobile group I intron in yeast mtDNA is intron 4α (aI4α) of the cytochrome c oxidase subunit I (*COXI*) gene (8, 9). Like ω, this intron encodes an endonuclease that has a number of conserved amino acid motifs (LAGLI-DADG sequences) typical of the majority of group I intron-encoded proteins (10). Three introns from the cytochrome b (*COB*) gene, bI2, bI3 and bI4, encode a splicing, or maturase, function (reviewed in ref. 11) but are not mobile (12). Intron 4α of the *COXI* gene is unique in that it not only encodes an active endonuclease, but also a latent maturase (13, 14). Other mobile group I introns are found in chloroplasts (15–19), as well as in bacteriophage T4 (20–23), and in the nuclear large rRNA gene of *Physarum polycephalum* (24, 25). In some of these other cases the site-specific endonuclease activities are encoded by reading frames that represent a minor subclass of group I intron ORFs: they lack the LAGLI-DADG motifs and have a different motif, GIY-YIG (26–28). Thus, there are at least two distinct classes of proteins encoded by group I introns; some of each type have endonuclease activity but, so far, maturase functions are associated only with the majority class.

Recent data suggest that three additional introns of the *COXI* gene of the yeast mitochondrial genome are mobile. Two of them, aI1 and aI2, are group II introns with open reading frames (29). Their mobility may occur by a different mechanism than group I introns. The group II intron of the cognate of one of those introns in the mtDNA of *Kluyveromyces lactis* also appears to be mobile (30). Preliminary evidence has been presented for an endonuclease activity (I-*Sce* III) that cleaves the exon sequence in which aI3 is usually found (31). It was proposed that *I-Sce* III activity is encoded by aI3 and, therefore, that aI3 is mobile.

Among other introns of the yeast mitochondrial genome, the five introns of the yeast *COB* gene do not appear to be mobile. We have previously reported that intron 5β (aI5β) of the *COXI* gene is not mobile (8). In crosses carried out in that study, both ω and aI4α were mobile, but we could not rule out the possibility that the mobility of aI5β might be detected using some other

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cross. However, aI5β probably does not encode a protein because its reading frame is not an extension of the upstream exon and it lacks a suitably situated AUG codon for the start of translation within the intron (10). Thus, intron 5α (aI5α) is the only group I intron of mtDNA of *Saccharomyces cerevisiae* that remains to be tested for mobility. Here, we report that aI5α is mobile in crosses and that it encodes an endonuclease activity necessary for its mobility.

MATERIALS AND METHODS

Yeast strains and plasmids

Each yeast strain used in this study is named according to its source of nuclear and mtDNA. Four different nuclear genomes were used in the genetic experiments: COP-19 (α *ade1 lys1*); 5DSS (**a** *ura*); GRF88 (α *his4-38*); and ID41-6/161 (**a** *ade1 lys1*). The mtDNA of strain ID41-6/161 (referred to as 161) contains seven introns in the *COXI* gene (aI1, aI2, aI3α, aI4α, aI5α, a5β and aI5γ) and five introns of the *COB* gene (bI1−bI5). It was mutagenized with Mn^{2+} and mutants C1017, C1047 and C1056, mapping to the part of the *COXI* gene containing aI5α and aI5β were isolated. The mtDNA of strain Δ1, 2 is a derivative of strain 161 that lacks aI1 and aI2; it was obtained as a revertant of a mutant in aI1 that had excised the two introns from mtDNA (32). The mtDNA denoted Δ1-3 resulted from the same screen and lacks introns 1, 2 and 3α. The mtDNA referred to as GII-0, containing *COXI* introns 3α and 4α and the *COB* gene of strain D273-10B, was constructed as described previously (8). Finally, the mtDNA of strain D273-10B (lacking introns aI5α, aI5β and bI1−3) was used. Standard cytoduction methods (33, 34) were used to construct the combinations of nuclear and mitochondrial genomes used here: 5DSS/GII-0; GRF88/GII-0; COP-19/Δ1,2; 5DSS/D273; 161/161. The respiration deficient mutants of strain 161 were analyzed in the nuclear background of strain 161. A derivative of GII-0 mtDNA containing *COXI* introns 3α, 4α and 5α was obtained as a diploid progeny of a cross; that mtDNA was characterized and is used as a control in Fig. 2 and Fig. 4B. All mitochondrial extracts analyzed for endonuclease activity were prepared from strains having the WA12 nuclear background (*a ade2 ura3 trp1 his3 leu2 nuc1::LEU2 IMP1*) (see ref. 8, 35), which contains a disruption of the *NUC1* gene encoding a mitochondrial nuclease activity (36); the mtDNAs noted were transferred into that background by cytoduction.

Plasmid pJVM134+ and pJVM135− contain the aI5α homing site of mtDNA of strain GII-0 cloned in pBLSKS+ and pBLSKS− (respectively) as a 2.1 kb *Bam* HI to *Eco* RI fragment (see Figure 1). Plasmid pSMB-R1 contains the aI5α homing site of mtDNA of strain D273-10B cloned in pBS+ as a 1.35 kb *Eco* RI fragment (from pJD20 (37) (see Figure 1). Plasmid pΔ5α, β, γ contains the aI5α homing site, cloned in pBS+ as a 730 bp Hind III to *Eco* RI fragment of a derivative of strain 161, which had lost introns aI5α, aI5β and aI5γ by reversion of a point mutant in intron 5γ. Plasmid pRSX contains the homing site for aI4α (35).

Mating conditions and media used

Matings for intron transmission measurements were done in patches on solid YPD medium (1% yeast extract, 1% peptone, 2% dextrose). A sample of the mating mixture containing at least 10^4 diploids was subjected to prototroph selection through two cycles of growth in minimal medium (0.67% yeast nitrogen base) containing 10% dextrose. An aliquot of diploid cells from each cross was then expanded in 100 ml of YPD medium containing 10% glucose, harvested in early stationary phase and reserved for mtDNA extraction.

Analysis of mitochondrial DNA and RNA

DNA was extracted from harvested cells using the mini-prep procedure of Sherman *et al.* (38) and was banded in CsCl containing bis-benzimide to enrich for mtDNA (39). DNA samples were digested with the restriction enzymes (Promega and Boehringer-Mannheim) noted in the text and fractionated on 0.7% agarose gels; the gels were blotted onto Nytran paper (Schleicher and Scheull) using a Stratagene Posiblot^{tm} apparatus, hybridized with 5′ end labeled oligonucleotide probes and the signal detected by autoradiography. Most blots were quantified using a Molecular Dynamics Phosphorimager. To obtain mitochondrial RNA samples, mitochondria were purified as described in ref. 39 and extracted with 4M guanadinium isothiocyanate and 75 mM sodium citrate, pH 7.0 according to ref. 40. RNA samples were fractionated on 1.2% agarose gels containing 6% formaldehyde; hybridizations with various probes were carried out in the gel (41) and the signals detected by autoradiography. All probes were 5′ end-labeled oligonucleotides containing the following short sequences of mtDNA: the probe specific for *COXI* exon 4 contains nt 8313−8329 of ref. 42; probes for *COXI* exons 5 and 6 are complementary to nt 6754−6771 and 9560−9579 of ref. 42, respectively. The aI5α and aI5β specific probes are complementary to nt 670−687 and 1992−2012 of ref. 10. The probe for *COB* alleles contains nt 1309−1333 of ref. 43. The probe for ω alleles is complementary to nt 75255−75276 of ref. 44.

Extract preparation and endonuclease assay

Crude mitochondria were prepared as previously described (35) and were lysed with 1M KCl and 1% NP40. Lysates were centrifuged at 40,000×g for 30 min. The supernatant fraction was dialyzed in buffer containing 50 mM potassium phosphate (pH 7.5), 50 mM KCl, 10% glycerol, 2 mM EDTA, 1 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin and 2 mM DTT. For endonuclease assays, substrate plasmids were linearized at a unique *Sca*I site in the vector and 3′-end labelled with (^{35}S) α-dATP as described previously (35). In preliminary experiments it was found that I-*Sce* IV activity has a broad pH optimum between pH 6.5 and 9.5, is inhibited by ≥150 mM KCl and requires Mg^{++} for activity. Reaction mixtures contained 25 mM Tris-HCl, pH 9.5, 10 mM $MgCl_2$, 50 mM KCl, 2 mM DTT, 100 ng of linearized plasmid DNA and an aliquot of enzyme sample. I-*Sce*II assays were carried out similarly, except that the pH was 7.5. Further details are provided in the figure legends.

Other biochemical methods

Cloned fragments of mtDNA were sequenced using the Sequenase Version 2.0 kit (United States Biochemical). The position of cleavage of pJVM134+ and pJVM135− by I-*Sce*IV was determined using these reagents and the approach developed by Wenzlau *et al* (8). Oligonucleotides were 5′ end-labeled for use as probes as described in Sambrook *et al.* (45).

RESULTS

Intron 5α is mobile in crosses

We analyzed several crosses to test aI5α for mobility using *COXI* alleles diagramed in Figure 1A. In the first cross, Δ1,2×GII-0, the mobile group II introns aI1 and aI2 are not present in either

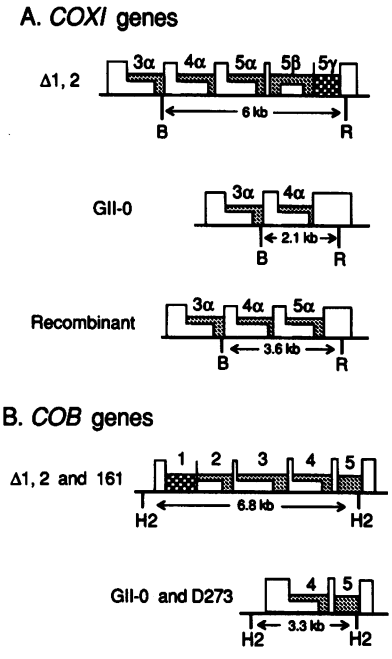

Figure 1. Intron configurations in *COXI* and *COB* genes used in crosses. A. *COXI* genes. The *COXI* genes of strains Δ1, 2 and GII-0 contain five and two introns, respectively; they are readily distinguished on DNA blots using *Bam* HI (B) plus *Eco* RI (R) digests of purified mtDNA followed by hybridization with a probe specific for the exon preceeding intron 4α of both strains (see Figure 2). Consistent with previous studies of this sort (8) movement of aI5α into the GII-0 *COXI* gene will sometimes yield a non-parental form of the gene indicated in the figure as 'recombinant' (see also Figure 2 and 4B) that is readily detected as a 3.6 kb fragment (arrow). The *COXI* gene of strain 161 and the mutants derived from it (see Fig. 3) have the same intron configuration as does strain Δ1, 2 except that two additional introns, aI1 and aI2, are upstream of aI3α; the digest analyzed in Fig. 4B yields the same 6 kb fragment. The *COXI* gene of strain D273-10B resembles that of strain 161 except that it lacks introns 5α and 5β; therefore, its *Bam HI* to *Eco RI* fragment detected with the exon 4 probe is only 3.1 kb and the recombinant resulting from movement of aI5α without co-conversion of aI5β is 4.4 kb. The nomenclature of these introns follows suggestions made in Ref 8. Intron 3α is sometimes referred to as intron 3 (31) and intron 4α is sometimes called intron 4 (52). Group I introns are shaded with diagonal lines and group II introns are shaded by a checkerboard pattern. Exons are tall unfilled rectangles or thin lines and intron reading frames are short unfilled rectangles within the introns. B. *COB* genes. The *COB* gene of strains Δ1, 2 and 161 have five introns while that of strains GII-0 and D273 have two introns. These alleles are distinguished by Hinc II (H2) digests when probed with an oligonucleotide specific for *COB* intron 4 present in both fragments. *COB* introns 4 and 5 are sometimes referred to as introns 1 and 2 (43). Shading and reading frame designations are as in panel A.

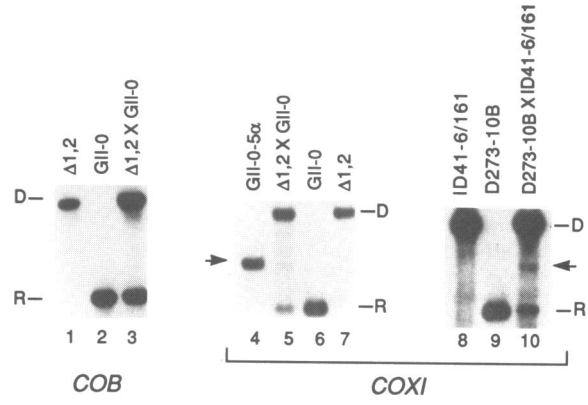

Figure 2. Transmission of *COB* and *COXI* alleles in crosses. Cross of Δ1, 2×GII-0: Alleles of the *COB* gene, detected as outlined in the legend to Figure 1B, are shown in lanes 1 and 2 and the amount of each allele in the progeny of the cross is shown in lane 3. The level of transmission of alleles to the progeny in this and all subsequent hybridizations was quantified using Phosphorimager scanning and the results are summarized in Table 1. The labels D and R identify each parent as either a potential donor or recipient of aI5α. The same DNA samples as in lanes 1–3 were cleaved with *Bam* HI and *Eco* RI and blots hybridized with the *COXI* exon 4 probe. The parental alleles are shown in lanes 6 and 7 and the output of the cross is shown in lane 5. Lane 4 defines the location of the predicted recombinant *COXI* gene formed in this cross (see arrow and Fig. 1A); note a faint signal in the output of the cross (lane 5) that comigrates with this control sample. Cross of 161×D273: The *COXI* alleles of the parental strains are shown in lanes 8 and 9 and the output of the cross is shown in lane 10 and Table 1 (cross 2). The *COB* alleles were analyzed as in lanes 1–3, and the outputs are summarized in Table 1. The arrow denotes the location of the predicted 4.4 kb recombinant form of the gene containing intron 5α but not 5β.

parental genome (Fig. 1A); also, both parents have aI3α and aI4α, so that in this cross, neither of those introns will be mobile. In addition, the parent containing aI5α (Δ1, 2) also has aI5β and aI5γ that can serve as flanking markers for aI5α movement. To correct for mitochondrial genome input bias in these crosses, we used polymorphisms in the *COB* gene as outside markers (Fig. 1B). Each cross was carried out and analyzed as previously described (8); in addition the radioactive filters were analyzed quantitatively using a Molecular Dynamics Phosphorimager. If aI5α is mobile, the 2.1 kb recipient (GII-0) *Bam* HI to *Eco* RI fragment should be absent or reduced in relative intensity, and a novel 3.6 kb *Bam* HI-*Eco* RI recombinant fragment should be present. If there is coconversion of the nearby introns aI5β and aI5γ, then those products of intron mobility will be like the donor allele, so that there may be an excess of the 6.0 kb donor-like (Δ1,2) fragment (see Fig. 1A).

The results of this cross are shown in Figure 2 for alleles of the *COB* gene (lane 3) and for *COXI* alleles (lane 5) and the quantitative data are summarized in Table 1 (cross 1). From analysis of the outside *COB* marker, it is clear that both parental genomes are present among the progeny of the cross, yielding outputs of the *COB* alleles of 59% from Δ1,2 (aI5α donor) and 41% from GII-0 (aI5α recipient). In contrast, the pattern of *COXI* gene fragments shows outputs of 78% for the donor and 18% for the recipient allele. Four percent of the output signal is non-parental, and is the size of the anticipated recombinant in which only aI5α moved into the GII-0 recipient allele. We have screened progeny of a related cross (not shown) and have isolated a recombinant in which the *COXI* gene contains only introns 3α, 4α and 5α. The *COXI Bam* HI to *Eco* RI fragment of that DNA (lane 4) comigrates with the minor, non-parental band appearing in the output of the cross in lane 5. From these data it appears that 56% of the recipient *COXI* allele has been converted to other forms; 17% of the converted alleles appear as a non-parental form and the remainder is detected as donor-like molecules. These data show that aI5α is mobile and that the majority of conversion events are associated with the co-conversion of the flanking markers (intron 5β and 5γ). Intron 5β is 134 bp from the insertion site for aI5α and intron 5γ is only an additional 25 bp away. Thus, the extent of co-conversion detected here is comparable to the reported co-conversion of sites flanking the ω and aI4α homing sites of yeast mtDNA (3, 4, 8).

Conversion of aI5α appears less efficient in this cross than expected from our previous experience studying ω and aI4α

Table 1. Outputs of crosses used to evaluated the mobility of aI5α.

Cross	Strain	Outputs (%) *COB*	*aI5α*	ω
	COP19/GII-0	41 ± 3	18 ± 2	
1	Rec	–	4 ± 0.6	
	5DSS/Δ1,2	59 ± 3	78 ± 3	
	COP19/D273-10B	10.5 ± 1	4 ± 0.3	44 ± 1
2	Rec	–	0.9	–
	5DSS/161	89.5 ± 1	95.2 ± 0.3	56 ± 1
	GRF88/GII-0	58 ± 2	38 ± 4	
3A	Rec	–	6 ± 1	
	161/161	42 ± 2	56 ± 5	
	GRF88/GII-0	77 ± 0.7	61 ± 2.3	
3B	Rec	–	6 ± 0.1	
	161/C1017	23 ± 0.7	33 ± 1.3	
	GRF88/GII-0	73 ± 1.7	76 ± 2.1	
3C	Rec	–	0	
	161/C1047	27 ± 1.7	24 ± 2.1	
	GRF88/GII-0	77.6 ± 0.5	80 ± 1	
3D	Rec	–	0	
	161/C1056	22.4 ± 0.5	20 ± 1	

DNA blots shown in Figures 2 and 4B (and others referred to in the text but not shown as primary data) were analyzed using a Phosphorimager scanner to determine the ratio of parental restriction fragments and the percentage of non-parental fragments (denoted as Rec, when present) resulting from the indicated crosses. The results are presented as the average of two or three independent crosses. Restriction digests and probes used to detect specific alleles are all summarized in the legends to Figures 2 and 4B and in 'Materials and Methods'.

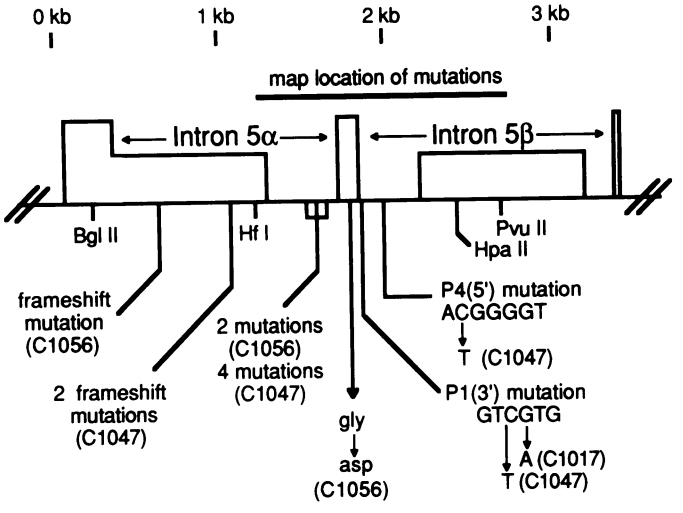

Figure 3. Map location and relevant sequence changes in *COXI* mutants. The portion of the *COXI* gene containing mutations in strains C1056, C1047 and C1017 is shown according to sequence coordinates reported by Hensgens *et al.* (10). The mutations were mapped using petite mutants to the region between the *Hinf* I (HfI) site in aI5α and the *Pvu* II site in aI5β as indicated by the bar above the diagram (see ref. 47). From mutants C1056 and C1017, the *Bgl* II to *Hpa* II fragment between the sites shown on the figure was cloned. For mutant C1056 the entire region was sequenced, revealing 4 point mutations: −1 deletion of nucleotide 280 (a frameshift that truncates the intron reading frame after 9 triplets read out of frame); a −1 deletion of base 1208; A1350 to T; and G1448 to A (gly to asp in the exon). For mutant C1017 only the region of the clone coinciding with the map location was sequenced, revealing a single mutation (G1519 to A) which alters the P1 pairing of aI5β. For mutant C1047 the *Hpa* II site in aI5β was absent due to a mutation so that the fragment cloned included most of aI5β up to the next closest *Hpa* II site. That clone was sequenced from position 687 to 1737 (10), revealing fourteen point mutations. The ones probably most relevant to the phenotype of that strain are as follows: +T between positions 700−701 and between 714−715, leading to truncation of the reading frame; C1021 to T, C1038 to T, C1147 to T and C1231 to T, all altering sites in and around the conserved intron structures of aI5α; and C1518 to T altering the P1(3′) element of aI5β. Also, there are four missense changes in the exon (C1367 to T (ala to val), C1403 to A (pro to his), C1415 to T (ala to val) and C1473 to A (asn to lys)) and two silent mutations in the exon (C1431 to T and C1437 to T). Finally, the change of C1702 to T alters the P4−5′ sequence of aI5β; that change probably does not disrupt the P4 pairing but it may interfere with the triple helix pairing recently proposed by Michel and Westhof (53). Five strain specific sequence changes were also noted in the region sequenced: deletion of A1568, T1592 to A and TTT1707-09 to AAA.

conversion in similar crosses. For that reason we analyzed a second cross (161×D273-10B) in which mobility of a second mobile group I intron (w) can be studied. Strain 161 is the parent of the Δ1, 2 strain used as the donor in the cross described above and it is used here in the same nuclear background as was Δ1, 2; it differs from Δ1, 2 only in that it contains wild-type forms of *COXI* introns 1 and 2. The *COXI* gene of strain D273-10B contains five of the same introns as the donor strain, lacking only aI5α and aI5β; it is analyzed here in the same nuclear background as was the recipient genome (GII-0) in the first cross. It should be noted that the nucleotide sequence of the exons flanking the insertion site for aI5α differs by one nucleotide between GII-0 and D273-10B (summarized in Figure 6A). Also, the 21S rRNA gene of D273-10B mtDNA contains the ω intron while that of strain 161 lacks it; probing *Hinc* II digests of mtDNA with an oligonucleotide specific for exon 1 of the 21S rRNA gene distinguishes the w^+ and ω^- alleles.

The *COXI* alleles of the progeny and parental strains of this cross are shown in Figure 2, lanes 8−10 and the quantitative data for *COB*, *COXI*, and ω alleles are summarized in Table 1 (Cross 2). This cross has a more extreme parental input bias than was the case in the first cross but the data show clearly that there is significant conversion of aI5α: the fraction of donor alleles increases from 89.5% to 95.2% and the fraction of recipient alleles decreases from 12% to 3.6%. Also, 0.9% of the *COXI* alleles are the expected non-parental fragment that results from movement of aI5α without co-conversion of aI5β (Fig. 2, lane 10 and Table 1 (Cross 2)). As was the case in the first cross, the recipient *COXI* allele was not eliminated from the progeny by quantitative conversion of aI5α even though the more extreme input bias in the second cross means that fewer conversion events are needed to eliminate that allele. Next, the output of ω alleles was scored using the same mtDNA samples (see Table 1). In this cross even though the input bias is strongly in favor of the ω^- allele, the fraction of donor (ω^+) genomes increases nearly four-fold, from 10.5% to 44%. These quantitative data show clearly that ω is a more efficient mobile element in this cross than is aI5α.

Effects of aI5α mutations on mobility

Mutants of the ω and aI4α introns were useful in the analysis of their mobility (5, 8, 46). We have a number of respiration deficient mutants that map to the aI5α-aI5β region of the *COXI* gene of the donor strain 161 (47) and we analyzed some of them in detail. As shown in Figure 3, mutants C1017, C1056 and

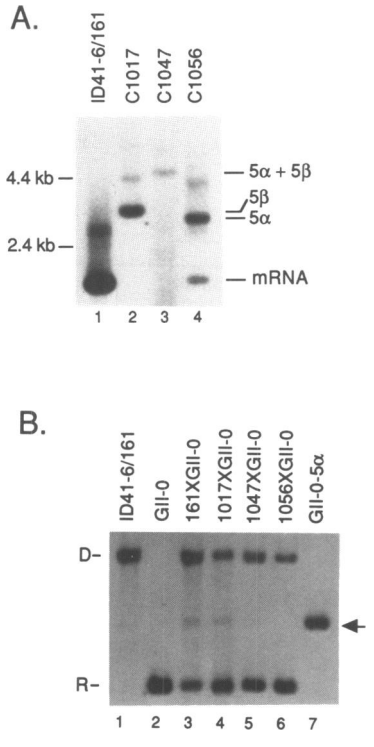

Figure 4. Splicing and mobility phenotypes of mutant strains. A. *COXI* transcripts that accumulate in mutant strains. Mitochondrial RNAs were fractionated on an agarose/formaldehyde gel and hybridized in the dried gel with a *COXI* exon 4 specific probe (see Materials and Methods). RNAs of known length were run in an adjacent lane to calibrate the sizes of the molecules detected with the probe. RNAs from the wild-type parent and the three mutant strains, C1017, C1047 and C1056, were analyzed in lanes 1–4, respectively. Similar dried gels were also hybridized with aI5α and aI5β specific probes to define the sequences present in the transcripts specific to each mutant (see text). The minor large RNA species contain exon sequences, the intron blocked by the mutation and one or more other introns. B. Effects of coxI gene mutations on mobility of aI5α. Crosses between the recipient strain GII-0 (in the GRF88 nuclear background) and the control and mutant strains (in the 161 nuclear background) were analyzed as described in the legend of Figure 2. Blots of *COXI* alleles of the parental strains (161 and GII-0) and progeny from crosses using 161, C1017, C1047 and C1056 are shown in lanes 1–6, respectively. Lane 7 contains mtDNA from a recombinant strain GII-0-5α that marks the location of that recombinant allele in the outputs (see lanes 3 and 4). These data and findings from a parallel analysis of *COB* alleles are summarized in Table 1 (crosses 3A–3D).

C1047 map to the 3′ half of aI5α or the 5′ half of aI5β. Complementation experiments show them to be *cis*-dominant mutants (47). Northern blot analysis (Figure 4A, lanes 2–4) shows that all three mutants are clearly splicing-defective and lack or are severely deficient in the prominent 1.9 kb *COXI* mRNA band that is present in the control (wild-type) sample (lane 1). The blot, using an exon 4-specific probe, shows that mutant C1056 accumulates some mRNA and an abundant precursor RNA about 3.3 kb long (lane 4). Using aI5α and aI5β-specific probes, that precursor hybridizes only with the aI5α probe (not shown), indicating that C1056 has a leaky defect in the splicing of aI5α. As shown in lane 2, the exon probe detects a single major 3.5 kb transcript in mutant C1017. Using the two intron specific probes, we found that C1017 is blocked for aI5β splicing. Finally, the exon probe shows that mutant C1047 accumulates a single major transcript, which is larger than that found in the other mutants by about 1.5 kb (lane 3). That RNA species from C1047 also hybridizes with the aI5α and aI5β probes (not shown), indicating that it is at least a double mutant affecting the splicing of the two adjacent introns. Consistent with this interpretation is the finding that C1047 does not revert; the other two mutants yield spontaneous revertants indicating that the splicing defects may result from a single mutation.

Next, we assessed the ability of each mutant to serve as a donor of aI5α in a cross with strain GII-0 (Figure 4B and Table 1, crosses 3A–D). These crosses are analogous to the one in Figure 1 except that the potential aI5α donor strains also contain introns aI1 and aI2. Also, we chose a different pair of nuclear backgrounds for these crosses, genomes that we have already shown to support efficient aI4α mobility (8). The output of the control cross (161×GII-0) confirms that aI5α is mobile (Fig. 4B, lane 3 and Table 1, cross 3A). Mutant C1017 (lane 4) is an active donor of aI5α while both C1047 and C1056 (lanes 5 and 6, respectively) are blocked for aI5α mobility (see also Table 1, crosses 3B–3D).

To learn the molecular basis of the splicing and mobility phenotypes of the three mutants, we cloned the *Bgl* II to *Hpa* II fragment of mtDNA containing all of aI5α and part of aI5β from those strains and sequenced a relevant portion of each clone (see Figure 3). Mutant C1017 contains the wild-type sequence of the region of aI5α from the *Hinf* I site to the end of the intron; however, there is a point mutation in aI5β that alters the internal guide sequence (P1–3′), which readily accounts for the aI5β splicing defect and the absence of any effects on aI5α mobility. Mutant C1047 contains six point mutations in aI5α, two mutations in aI5β, and six mutations in the exon between them. A mutation of the P1(3′) sequence of aI5β would explain the splicing defect of that intron, and any of a number of the changes in and around the *cis*-acting splicing signals of aI5α (listed in the legend to Fig. 3) would explain its splicing defect. Two of the aI5α mutations of strain C1047 are +1 frameshift mutations of the aI5α reading frame that are probably responsible for the mobility defect; note that the reading frame mutations lie upstream of the map location of the defect responsible for blocking respiratory growth. Finally, mutant C1056 contains a missense mutation in the exon between introns 5α and 5β. That mutation is responsible for the glycerol growth defect because it returned to the wild-type sequence in one spontaneous revertant that was sequenced. C1056 also contains two mutations in the P9 region of aI5α that probably account for its partial splicing defect (see Fig. 4A). There is a frameshift mutation in the open reading frame upstream of the map location of the C1056 mutations, which would readily account for the aI5α mobility defect.

Mitochondrial extracts contain an endonuclease activity that cleaves aI5α homing sites

To determine whether there is an aI5α-encoded endonuclease activity, we prepared plasmid pJVM134+, which contains the insertion site for aI5α in the 2.1 kb *Bam HI* to *Eco RI* fragment of the *COXI* gene from strain GII-0 (see Fig. 1). The sequence of the homing site in that substrate is given in Figure 6A. In preliminary experiments using extracts of several strains that have an intact aI5α reading frame we detected an activity that cleaves that substrate in the expected location. Assay requirements were determined using extracts from those control strains (see Materials and Methods). Next, in order to verify that the endonuclease activity we detected is associated with aI5α, we analyzed mitochondrial extracts from several strains that could have the

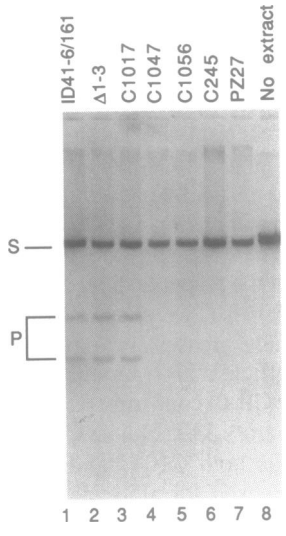

Figure 5. Survey of yeast strains for I-SceIV endonuclease activity. High salt/detergent extracts of mitochondrial fractions from the indicated strains were prepared and tested for their ability to cleave pJVM134+ (GII-0) DNA as described in 'Materials and Methods'. Reactions were incubated for 60 min with 80, 90, 90, 200, 80, 80 and 110 μg of mitochondrial protein for lanes 1–7, respectively. Lane 8 is a control in which protein extract was omitted. The locations of the substrate (S) and products (P) are noted. The substrate molecule is 5.1 kb long and when cleaved near the aI5α homing site is expected to yield the 2.1 and 3.0 kb products seen in lanes 1–3.

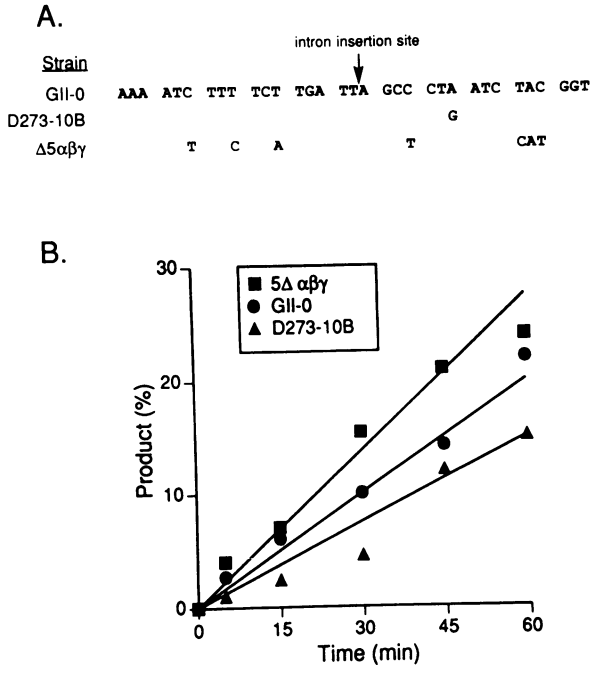

Figure 6. Cleavage of three different spliced exon sequences. A. Sequence of the aI5α insertion region from three yeast strains. The sequence of the aI5α insertion region from three yeast strains is shown. Each sequence was determined experimentally from the clone used in the next panel. The standard homing site for this study is the sequence of spliced exons from the *COXI* gene of strain GII-0 (top line). That same coding region from strain D273-10B differs at the one site shown in the second line. The sequence from a derivative of the donor strain that lacks introns 5α, β, and γ (strain Δ5αβγ) is shown in the last line; it differs from the GII-0 sequence at the seven positions indicated. B. *I-Sce* IV cleavage of cloned aI5α sequences. The rate of cleavage of plasmids containing one of the three different insertion sites shown in Panel A. A high salt/detergent extract of purified mitochondria from strain Δ1-3 (90 μg/sample) was used as the source of I-*Sce* IV. Samples were incubated for 0, 5, 15, 30, 45 and 60 minutes and analyzed as described in 'Materials and Methods'.

aI5α-encoded endonuclease activity and several others that should lack it (Figure 5). There are three positive controls: 1) ϱ^+ strain 161. Its *COXI* gene contains seven introns, including aI4α and aI5α. It is known to have some I-*Sce* II activity and is expected to have the activity encoded by the aI5α reading frame (this activity is termed hereafter I-*Sce* IV). 2) Δ1-3. It is a ϱ^+ derivative of strain 161, deleted for aI1, aI2 and aI3α and is expected to have both activities. 3) Mutant C1017. It is a *cis*-dominant splicing defective mutant of aI5β, and is expected to have levels of I-*Sce* IV and I-*Sce* II similar to those of strain 161. Two negative controls were analyzed: 1) C245. It is deleted for most of the *COXI* gene, including aI5α and, so, cannot express I-*Sce* IV. 2) PZ27. It lacks the bI4-encoded maturase due to the deletion of the *COB* gene; therefore, it fails to splice aI4α and overexpresses the aI4α reading frame. It should not be able to express the aI5α ORF. Experimental samples are from the two mutants, C1056 and C1047, which have a truncated aI5α reading frame and do not exhibit aI5α mobility in crosses.

As shown in Figure 5, lanes 1 and 2, the extracts from the parental strain, 161 and the related ϱ^+ strain, Δ1-3, cleave the substrate for I-*Sce* IV. The sizes of the products generated with the aI5α substrate (2.1 and 3.0 kb) suggest that cleavage occurs in the vicinity of the aI5α insertion site. The extract from C1017 also cleaves the I-*Sce* IV substrate to yield the expected products of cleavage (lane 3). The extract from strain C245, however, does not cleave the aI5α substrate (lane 6), nor does the extract of strain PZ27 (lane 7). Finally, the extracts from strains C1047 and C1056 also do not cleave the aI5α substrate (lanes 4 and 5, respectively). We have assayed all of the above extracts for the aI4α-encoded I-*Sce* II activity using a substrate, pRSX, previously described by us (35). In all strains where aI4α was present, whether those extracts contained I-*Sce* IV activity or not, we found I-*Sce* II activity (data not shown). We conclude from the above experiments that yeast mitochondria contain an endonuclease activity, I-*Sce* IV, that cleaves *COXI* gene exons in the vicinity of the aI5α insertion site. The endonuclease activity is not detected in strains where translation of the ORF is blocked or when the intron is absent. We did not find any simple *cis*-dominant mutants of aI5α that would be expected to block the splicing of aI5α but not its mobility. Also, we did not find any mutants that are purely *trans*-recessive, blocking splicing solely due to a block in expression of the intron encoded protein (maturase); in fact, the available data do not permit any strong conclusion as to whether aI5α encodes a maturase function.

We also tested several other cloned aI5α homing sites to determine if they are substrates for I-*Sce* IV activity. These include the homing site of strain D273-10B, which differs by one nucleotide from the GII-0 substrate analyzed above, and a clone of spliced exons from the donor strain 161 (see Figure 6A). The latter substrate is especially interesting because it differs at 7 of the 25 nucleotides flanking the site of aI5α insertion. Kinetic analysis shows that the rates of cleavage of these substrates by I-*Sce* IV do not differ by more than two-fold (Figure 6B). Thus, at this level of analysis, I-*Sce* IV appears to be more like I-*Sce* II than like I-*Sce* I in that it is tolerant of base changes near its cleavage site.

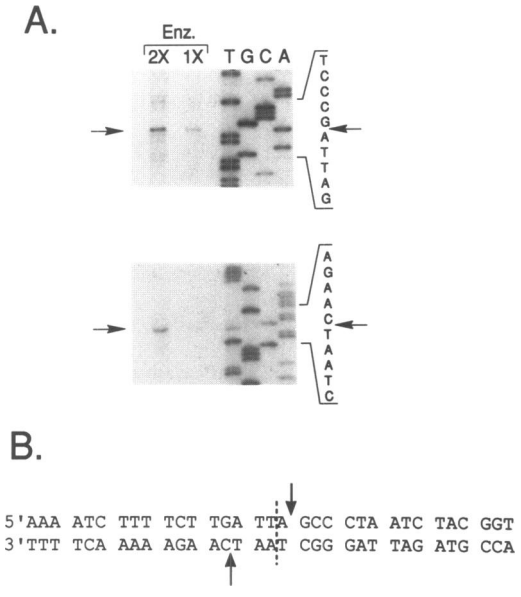

Figure 7. The cleavage site of the aI5α encoded endonuclease, *I-Sce* IV. A. Cleavage of primer extended labeled + and − strands. Single-stranded DNA from plasmids pJVM134+ (top section) and pJVM135− (bottom section) was annealed with an oligonucleotide primer, labeled and extended as described by Wenzlau et al. (8). Each sample was then incubated with a mitochondrial extract from strain Δ1-3 containing 90 or 180 μg of protein (1× and 2× samples, respectively) for cleavage of the *I-Sce* IV site. The reactions were phenol extracted, passed through a small G25 Sephadex column, ethanol precipitated, denatured and analyzed on standard sequencing gels alongside of a dideoxynucleotide sequencing ladder prepared using the same templates and primers. A portion of the sequence flanking the cleavage site is shown. The main product of cleavage is denoted by an arrow. B. Site of cleavage of the GII-0 substrate. Both strands of the sequence of the intron insertion site from strain GII-0 are shown in the figure. The insertion site is denoted by a vertical dashed line and the site of cleavage on each strand (based on the data of panel A) is denoted by an arrow.

Mapping the I-*Sce* IV cleavage site

We used an extract from strain Δ1-3 to map the cleavage site more accurately. The cleavage site was located on each strand using the mapping method developed by Wenzlau *et al.* (8) (Figure 7A). As is summarized in Figure 7B, the enzyme makes the same kind of staggered cut leaving a 4 base 3′ OH overhang made by three other LAGLIDADG-type endonucleases already characterized in yeast mitochondria (8, 48, 49). The intron insertion site is within the cleavage site as noted in Fig. 7B; however, this location of the insertion site relative to the cleavage sites on the two strands is unique. I-*Sce* I cleaves symmetrically on both sides of the insertion site for the ω intron (48) while I-*Sce* II cleaves both strands to one side of the insertion site for aI4α (8).

DISCUSSION

The results presented here show that the group I intron 5α of the yeast mitochondrial *COXI* gene is mobile in crosses. Further, we identified an endonuclease activity, termed I-*Sce* IV, that is probably responsible for initiating conversion events. The absence of aI5α conversion and I-*Sce* IV activity in two mutants that contain frameshift mutations in the aI5α reading frame, provides strong evidence that the aI5α reading frame encodes a homing endonuclease. This conclusion is supported further by the observation that there is no detectable I-*Sce* IV activity in a strain that is deleted for the *COXI* gene or in another strain that should not be able to translate the aI5α reading frame due to a block in splicing of the intron upstream of aI5α. However, a block in splicing of the adjacent downstream intron, aI5β (mutant C1017) has no effect on either aI5α mobility or the production of I-*Sce* IV activity. It should be noted that the genetic mapping data show that the reading frame truncations that block I-*Sce* IV activity are probably not responsible for the respiration deficient phenotype of the strains that contain them. This suggests that aI5α may not encode a maturase function though that issue requires further study before a definitive conclusion can be reached.

In none of the crosses we have analyzed, using several different nuclear backgrounds and intron configurations of the *COXI* gene, has aI5α movement led to the quantitative conversion of the intronless allele (see Fig. 2, Fig. 4B and Table 1 and compare with ref. 8). Our data include one cross in which both aI5α and ω are able to move and in that case ω converted much more efficiently than did aI5α. This suggests that aI5α is less active in mobility than are either of the other two mobile group I introns in yeast mtDNA. Clearly, the process must require the introduction of a double-strand break in the intron homing site, but it is not known whether that step is rate limiting for the overall process of intron conversion. Our ability to find I-*Sce* IV activity in crude mitochondrial extracts, at levels comparable in terms of relative substrate cleavage to I-*Sce* II activity, would suggest that I-*Sce* IV activity is not limiting for conversion. However, detailed studies of the purified protein may identify some feature of it that may account for the relatively low level of mobility. At present there is little detailed information on other steps that may be needed for group I intron mobility, such as possible donor-recipient pairing reactions, heteroduplex formation, replicative repair of the double-strand break and resolution of joint molecules. Any of those steps could be rate limiting for aI5α movement relative to that of the other introns.

The cleavage of the aI5α homing site by I-*Sce* IV generates a 4 bp staggered cut. This pattern of cleavage is the same as the yeast mitochondrial I-*Sce* I and I-*Sce* II homing endonucleases. However, the location of the intron insertion site relative to the cleavage sites on both strands is unique for I-*Sce* IV. Although we have not determined the I-*Sce* IV recognition sequence, i. e., the minimal sequence required for I-*Sce* IV cleavage, we have shown comparable I-*Sce* IV cleavage activity using three different substrates containing aI5α homing sites that differ in sequence by up to seven positions within 25 bp flanking the intron insertion site. The closest sequence changes lie four bp downstream and six bp upstream of that site. These data suggest that the sequence specificity for I-*Sce* IV cleavage will be more like I-*Sce* II than like I-*Sce* I. I-*Sce* II is tolerant of nucleotide substitutions within its recognition site (50), while I-*Sce* I has a very stringent nucleotide sequence requirement for substrate cleavage (51). Further studies will be required to define the I-*Sce* IV recognition sequence and to evaluate its nucleotide specificity and the frequency that it will cleave complex genomes.

The aI5α reading frame is probably expressed as a 632 amino acid long fusion protein more than half of which is encoded by the upstream *COXI* exons. It is likely that the primary translation product expressing the aI5α ORF is processed, as is the case for aI4α and bI4 (35, 54). However, it is not certain that all such proteins are processed (e.g., ref. 55) and this point warrants a direct test. The level of I-*Sce* IV activity detected here using crude mitochondrial extracts is low, compared with comparable extracts

of an overproducer of I-*Sce* II, so that purifying the protein from available strains is not an attractive means of solving this problem. One approach would be to use site-directed mutagenesis to create a strong *cis*-dominant splicing defect in aI5α, followed by mitochondrial transformation to make a mutant yeast strain that overproduces the active protein.

Note added in proof

After this paper was submitted for publication, a paper appeared by Séraphin,B., Faye,G., Hatat,D. and Jacq,C. (1992) *Gene*, **113**, 1–8, which shows *in vivo* mobility and associated endonuclease activity for intron 5α of the *COXI* gene of yeast mitochondrial DNA.

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