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Determination of functional domains in intron bI1 of yeast mitochondrial RNA by studies of mitochondrial mutations and a nuclear suppressor

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The sequence of intron 1 in the *cob* gene in mtDNA (bI1) of the yeast strain 777-3A has been determined. Furthermore, we have performed a systematic search for complementary sequence stretches within this intron RNA, and within the RNA of intron 5γ of the *oxi3* gene (aI5γ) which shares distinctive sequences with bI1. Possible secondary structure models derived from this analysis show nearly identical core structures for bI1 and aI5γ RNA with conserved sequence stretches in prominent positions. These core structures are similar to those previously reported for RNAs of introns having very limited sequence homology with bI1 and aI5γ. In two mutants which are defective in bI1 excision from *cob* pre-mRNA, nucleotide sequence alterations in bI1 have been determined. One mutation (G5049) apparently affects the stability of a hybrid stretch in the proposed secondary structure of bI1 RNA whereas the other one (M1301), a deletion of one A in a run of five As, affects a sequence which is conserved in bI1 and aI5γ and is involved in the formation of a distinct secondary structure. Out of seven revertants of M1301, three were found to have restored the wild-type bI1 sequence AAAAA, three others had the related sequence AAAAG which is functionally indistinguishable from wild-type, whereas one revertant had a nuclear mutation which suppresses the splicing defect exerted by the mitochondrial mutation M1301. This nuclear suppressor (SUP-101) is allele specific and dominant. The possible role of the sequence affected by M1301 in terms of a recognition site for a nuclear gene product will be discussed.

Key words: intron mutations/nuclear suppressor/secondary structure/sequencing

Introduction

Split genes with introns interrupting the sequences coding for rRNA, tRNA or protein have been shown to occur in organelle DNA of fungi (L-rRNA, apocytochrome b, cytochrome oxidase subunit I in mtDNA), of plants (cytochrome oxidase subunit II in *Zea mays* mtDNA, L-rRNA, tRNA in *Z. mays* chlDNA) and of algae (L-rRNA in *Chlamydomonas* chlDNA) (Michel and Dujon, 1983, and references therein). These organelle introns are generally thought to be distinct from introns in nuclear protein coding genes largely because exon-intron boundary sequences do not fit the GT...AG rule (Breathnach *et al.*, 1978) and mutations inside organelle introns rather than at the exon-intron boundaries affect splicing (De la Salle *et al.*, 1982; Jacq *et al.*, 1982; Mahler *et al.*, 1982; Netter *et al.*, 1982; Schmelzer *et al.*, 1982; Weiss-Brummer *et al.*, 1982). As revealed by sequence comparisons, organelle introns exhibit considerable conservation of sequences and secondary structure elements. Thus, various organelle intron RNAs can be folded to form secondary structures with similar cores (Davies *et al.*, 1982; Michel *et al.*, 1982; Michel and Dujon, 1983; Waring *et al.*, 1983). Based on these structures, two intron families can be distinguished, group I and group II, both of which include mitochondrial and chloroplast introns. Interestingly, introns in the nuclear rRNA of protists exhibit similarities with introns of group I, which comprises the majority of organelle introns (Michel and Dujon, 1983; Waring *et al.*, 1983).

Experimental data lend strong support to the idea of intramolecular hybrid structures being relevant for splicing of organelle pre-mRNA: (i) mutational alterations of sequences inside mitochondrial introns of *Saccharomyces cerevisiae* at distances of 30 to some 430 bp have been shown to impair splicing, (ii) these sequences are identical with evolutionary conserved stretches apparently involved in hybrid structure formation and (iii) a pair of mutations, one exerting a block in splicing and the other one reversing it, affect complementary sequences in a possible intramolecular hybrid structure (Anziano *et al.*, 1982; De la Salle *et al.*, 1982; Jacq *et al.*, 1982; Mahler *et al.*, 1982; Netter *et al.*, 1982; Schmelzer *et al.*, 1982; Weiss-Brummer *et al.*, 1982, 1983).

In *S. cerevisiae*, four of the 13 introns belong to one group of introns (referred to as group II) having certain sequence stretches conserved and surviving as circular RNAs after excision: introns 1, 2 and 5 of the *oxi3* gene (aI1, aI2 and aI5γ) and intron 1 of the *cob* gene (bI1) (Arnberg *et al.*, 1980; Grivell *et al.*, 1980; Halbreich *et al.*, 1980; Michel *et al.*, 1982; Schmelzer *et al.*, 1982; aI5γ previously was referred to as aI5, for altered nomenclature, see Hensgens *et al.*, 1983).

So far mutational analysis of functional domains in this intron family is restricted to one mutation (M4873) and its reversion in bI1. This mutation affects a stem and loop structure which in group II introns directly precedes the 3′ splice point (Schmelzer *et al.* 1982). This report deals with the wild-type mitochondrial sequence of this intron bI1 in the yeast strain 777-3A which is the parental strain of most bI1 mutants, with the sequence alterations of two mutants which fail to excise bI1 from *cob* pre-mRNA (Schmelzer *et al.*, 1982), and with the characterization of revertants.

Results

Nucleotide sequence of bI1 in strain 777-3A

Intron bI1 has been sequenced by Lazowska *et al.* (1980, 1983) using mtDNA from two *rho*$^-$ clones, L12 and L29, which were derived from strain 777-3A. We have now determined the sequence of the same intron of strain 777-3A, but using DNA fragments isolated from the wild-type *rho*$^+$*mit*$^+$ (cf. Figure 2A), following the strategy given in Materials and methods. We further determined the complete sequence of bI1 using fragments of two *cob*$^-$ mutants of strain 777-3A, G5049 and M1301, which block excision of bI1 from *cob* pre-mRNA (Schmelzer *et al.*, 1981, 1982).

Our three sequences were consistent except for a single deletion in mutant M1301 and for three base pair substitutions in mutant G5049 which are regarded as mutational

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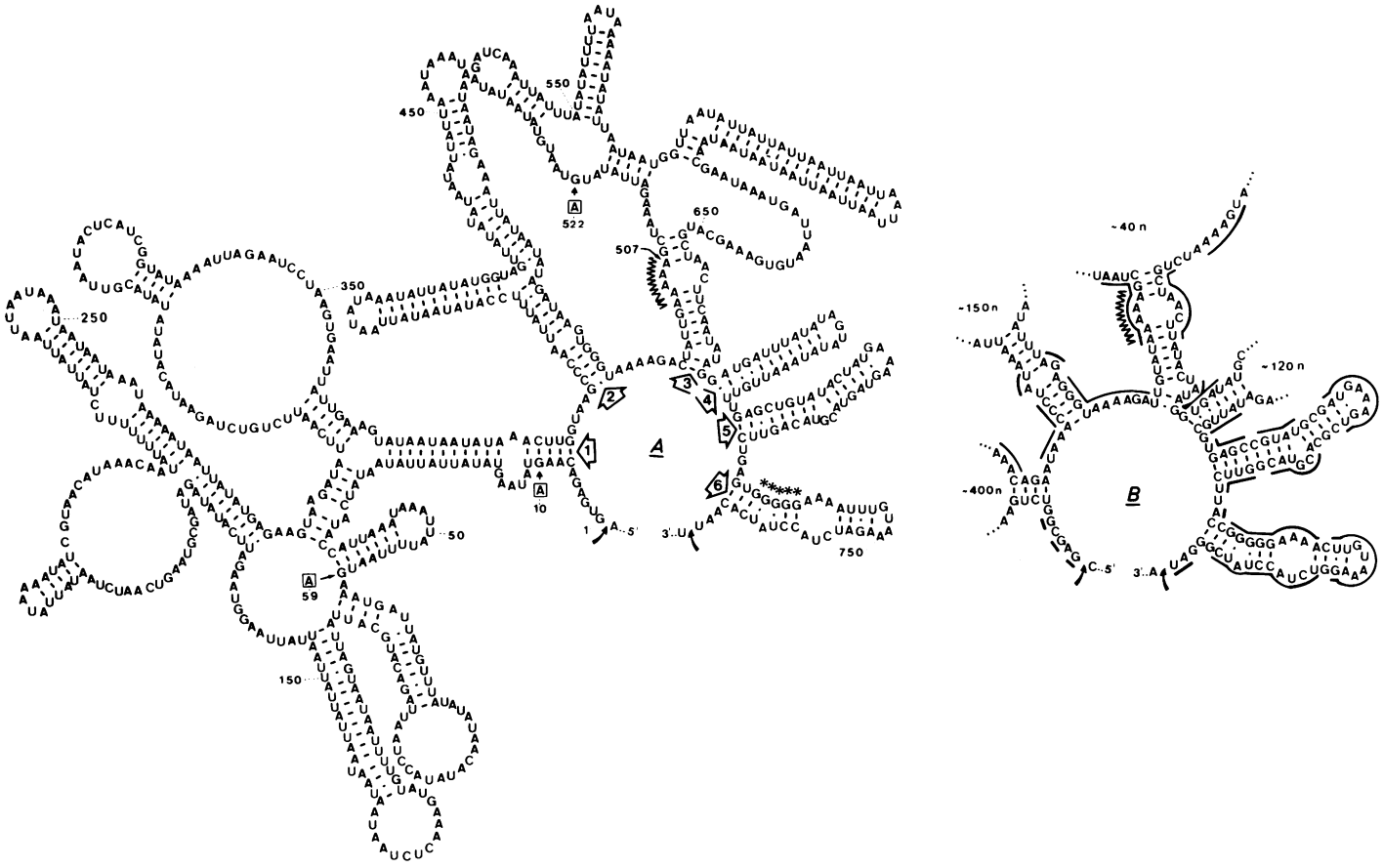

Fig. 1. A, secondary structure model of bI1 RNA from strain 77-3A. Large arrows indicate the proposed 5′ and 3′ splice junctions. Boxed letters correspond to mutations found in G5049 (positions 10, 59, 522). vvv points to the sequence in which the mutation of M1301 was found (positions 503 – 507). * refers to the mutation M4873 (– 1 G deletion in the run of five Gs, positions 735 – 739; cf. Schmelzer *et al.*, 1982). Stems of the core structure are numbered 1 – 6. Comparison of the sequence shown with the sequence of *rho*⁻ DNA L29, which has been determined by Lazowska *et al.* (1983), revealed four differences: position 42: A→G, position 121: G→A, position 175: A→G, position 549: – 1 U deletion. **B,** core structure model of aI5γ. Peripheral arrangements which differ from those in bI1 are not shown. Major sequences conserved in the core structures of bI1 and aI5γ are marked. Sequence according to Bonitz *et al.* (1980).

alterations (see below). The sequence of bI1 in the *rho*⁻ DNA L29, which Lazowska *et al.* (1983) regard as the reliable one, differs from our sequence in four positions (see legend to Figure 1A). The significance of these differences awaits further analyses.

Secondary structure of bI1 and aI5γ

For some group II intron RNAs, secondary structure models have been proposed, but not for bI1 and aI5γ which share some distinctive features (Michel *et al.*, 1982; Schmelzer *et al.*, 1982; Michel and Dujon, 1983). We have performed a computer graphic matrix analysis of bI1 and aI5γ RNA displaying conserved sequences and all potential helices with stems of 4 bp or more and minimal stability (see Materials and methods). Those helical structures were chosen which in both intron RNAs allowed us to build a core structure similar to those previously suggested for group II introns, and which show conserved sequences in identical positions (Figure 1).

For peripheral structures, which in mitochondrial intron RNA are generally variable (Michel and Dujon, 1983), the most stable stems have been chosen. The resulting model for bI1 shows a 60% overall base pairing. Primary sequences building the wheel of the core are well conserved in bI1 and aI5γ; this is less obvious when these two introns are compared with other group II introns, although the central cores are strikingly similar. Sequence conservation appears to be restricted to a GNGNG motif at the 5′ end of these introns and parts of stem 5.

Mutations in bI1

In contrast to group I introns, little is known about mutations that affect excision of group II introns. One such mutation, M4873, is a single deletion in a sequence some 30 bp in front of the 3′ splice point (Schmelzer *et al.*, 1982). Two further mutants affecting bI1 excision, M1301 and G5049, have now been studied in detail. According to genetic mapping (Figure 2A), mutations of both strains affect the sequence between M4873 (the mutation preceding the 3′ end of bI1) and G171 which has been shown to be a substitution of the fourth base pair preceding the presumptive 5′ splice point of bI1 (Lazowska *et al.*, 1980). Physical mapping by *rho*⁻ deletion analysis (Figure 2B) allocates M1301 to a sequence of some 200 bp in the central part of bI1 and G5049 to the most upstream 250 bp of bI1 or the exon B1/intron bI1 junction.

The analysis of the entire bI1 sequence and some 20 bp of the adjacent B1 sequence of mutant G5049 revealed three positions with single G→A transitions, two of them are displayed in Figure 3. The first is located 10 bp downstream from the postulated 5′ splice point, the second and the third occur at a distance of 59 bp and 522 bp, respectively, downstream from this splice junction (cf. Figure 1A). We consider the last mutation to be silent because the wild phenotype is

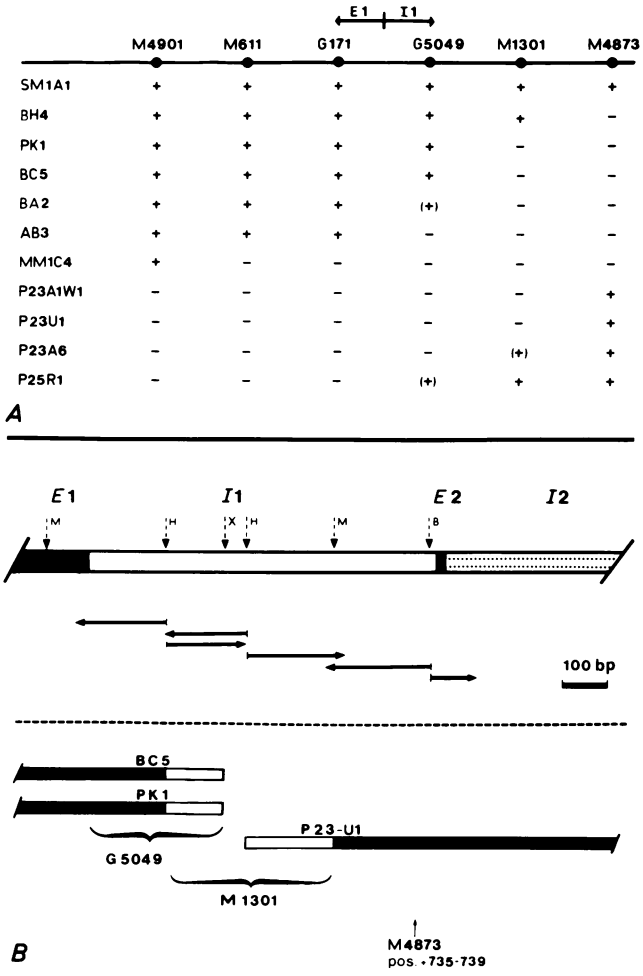

Fig. 2. Genetic and physical maps of bI1. **A**, restoration tests in *rho*$^+$ *cob*$^-$ x *rho*$^-$ *cob*$^+$ crosses. + and (+) denote confluent and papillated growth, respectively, of patches from $>10^5$ cells of diploid progeny on YG plates; both indicate the presence of the *cob*$^+$ allele in question in the *rho*$^-$ clone tested. − denotes absence of growth and indicates loss of the *cob*$^+$ allele in question. **B**, physical map of bI1 and allocation of mutations. **Upper part** (physical map); open bar: intron bI1; open bar with dotted line: part of I2 containing an open reading frame; black bars: exons. Restriction sites for *Mbo*I (M), *Hinf*I (H), *Bgl*II (B) and *Xba*I (X) are given on top of the figure. Horizontal arrows indicate length and direction of sequenced DNA fragments which were derived from strain 777-3A (wild-type and mutants M1301, G5049). **Lower part** (location of mutations G5049 and M1301); horizontal bars represent *rho*$^-$ mtDNA sequences investigated by restriction analysis (black, minimal extension; void, maximal extension). Approximate positions of M1301 and G5049 are indicated (for details see text). Position of M4873 previously was determined (Schmelzer *et al.*, 1982).

restored by crosses of G6059 with *rho*$^-$ clones pK1 and BC5 which according to physical mapping do not extend to this sequence (cf. Figure 2B).

The sequence alteration of M1301 is shown in Figure 3. Only one base change was found within the complete bI1 sequence: it is a deletion of one A in a run of five As located at a distance of 503 – 507 bp from the presumed 5′ splice point. Interestingly, this mutation M1301 lies within a longer sequence which is well conserved in bI1 and aI5γ with regard both to primary sequence (5′..GGGTAAAAGACTATTG-AAAAAGCTAA..3′ in bI1, 5′..GGGTAAAAGATTGTAT-AAAAAGCTAA..3′ in aI5γ) and position in the core structure (stem 2, 3 and downstream loop, cf. Figure 1).

Revertants

In mutant G5049 no spontaneous revertants could be found. Mutagenic treatment also did not yield any revertants. This suggests that G5049 carries two (or more) mutational alterations each causing a separate defect in mitochondrial function.

Mutant M1301, in contrast, spontaneously gave rise to respiratory competent revertants at a frequency of $<10^{-7}$. Treatment with N-methyl-N′-nitro-N′-nitrosoguanidine (MNNG) or methyl-methanesulphonate (see Materials and methods) significantly increased this frequency. Most of these revertants grew on a non-fermentable substrate (glycerol) similar to wild-type whereas some revertants exhibited temperature sensitivity of growth on glycerol medium. Genetic analyses of these revertants (basically following the scheme of Dujardin *et al.*, 1980, see 'Testcross' in Materials and methods) suggested that in the vast majority of revertants the secondary mutation is in mitochondrial DNA, whereas a low percentage of revertants carried nuclear dominant mutations (*SUP*$^-$).

The presence of a nuclear suppressor mutation was further corroborated by crossing a *rho*^{0} clone of one revertant (M1301/R101 *SUP*$^-$) with a *rho*$^+$*sup*$^+$ wild-type strain and by analyzing the meiotic progeny of this cross (tetrad analysis). With the exception of a few spontaneous petites all spores were able to grow on glycerol, suggesting that the *SUP*$^-$ mutation in a *rho*$^+$ *cob*$^+$ cell has no pronouced phenotype. To discriminate between *SUP*$^-$ and *sup*$^+$ spores, *rho*^{0} spore clones (obtained by ethidium bromide treatment, cf. Materials and methods) were crossed with mutant M1301 and phenotypes of the diploid progeny were studied. From eight tetrads analyzed, six showed a 2:2 and two a 3:1 segregation of the phenotype of *cob*$^-$ M1301 (glycerol-negative, *sup*$^+$) and of the original revertant, that is temperature-dependent growth on glycerol (*SUP*$^-$). This result is consistent with the notion that (i) a single nuclear factor *SUP*$^-$*-101* causes suppression of the splicing defect of *cob*$^-$ M1301, (ii) this mutation is dominant and (iii) the degree of suppression by *SUP*$^-$*-101* is temperature dependent.

To determine the action spectrum of this dominant nuclear suppressor, diploids were generated by genetic crosses (cf. Materials and methods) which were heterozygous *SUP*$^-$*/sup*$^+$ and carried one or the other of 135 *cob*$^-$ mutations (including bI1 mutations M1301, M4873, G5049) or of 150 *oxi3* mutations mapping throughout the respective gene. The result points to a strong allele specificity of *SUP-101*: Only when combined with the bI1 mutation did M1301 exhibit suppressor significant activity, in that growth on non-fermentable substrate was restored.

Of the many revertants of M1301 with a secondary mutation in mtDNA, six were chosen for the determination of their bI1 nucleotide sequence. We chose mainly conditional revertants in the hope of finding novel sequences in bI1 that confer temperature-dependent splicing. The results are shown in Table I. Three revertants have the wild-type nucleotide sequence, that means the secondary mutation was a + 1 A insertion into the sequence of M1301 between position 503 and 507 (cf. Figures 1A and 3); the other three revertants compensate the − 1 A deletion of the primary mutation by a + 1 G insertion at position 507 thus creating a novel sequence.

Temperature-sensitive growth on glycerol of the latter revertants might be due to this novel sequence AAAAG;

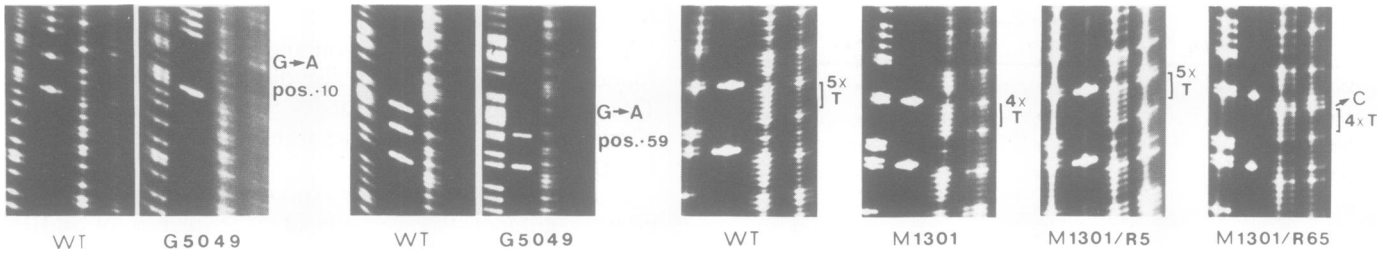

Fig. 3. Sequencing gels (8%) showing the mutational alterations in G5049, M1301 and revertants from M1301. DNA sequences from G5049 correspond to the non-coding strand, sequences from M1301 and revertants correspond to the coding strand.

Table I. Nucleotide sequences of wild-type, mutant M1301 and revertants of this mutant

	Temperature-dependent growth	
wt 777-3A	–	5'..AAAAAGCTAA..3'
M1301	–	...AAAAGCTAA..
M1301/R3	cs/hs	..AAAAGGCTAA..
M1301/R5	–	..AAAAAGCTAA..
M1301/R13	cs	..AAAAAGCTAA..
M1301/R65	cs	..AAAAGGCTAA..
M1301/R140	hs	..AAAAAGCTAA..
M1301/R169	cs/hs	..AAAAGGCTAA..

The sequence shown is located at a distance of 503–513 bp from the presumed 5' splice point. cs, cold-sensitive; hs, heat-sensitive.

however, accumulation of *cob* mRNA precursors as in other conditional bI1 mutants (e.g., M4873, cf. Schmelzer *et al.*, 1981) was not detectable (data not shown). This suggests that the reversion by a + 1 G insertion at mutational site M1301 was accompanied by a new *mit*$^-$ mutation, probably outside of bI1, generating a conditional respiration deficiency.

To find support for this notion the revertant M1301/R65 (cold-sensitive growth on glycerol) was crossed with the original mutant M1301 (glycerol-negative). Several percent of progeny were phenotypically wild-type (glycerol-positive), i.e., they are likely to originate from recombinational events separating a (*mit*$^-$) mutation conferring temperature sensitivity from the revertant sequence AAAAG in bI1.

In revertants M1301/R13 (cold-sensitive) and M1301/R140 (heat-sensitive), with the wild-type sequence at the mutational site M1301 in bI1, the temperature-dependent phenotype must be also due to additional mutations besides the reversion to the wild-type sequence. In one case (M1301/R140) genetic crosses indicate that the heat sensitivity is conferred by a nuclear (*pet*$^-$) mutation.

Apparently the screening of conditionals results in collecting revertants with additional mutations not related to the splicing function disturbed in the original mutant M1301.

Discussion

Secondary structure models of introns bI1 and aI5γ as proposed here are not only very similar among themselves but match the general pattern proposed for group II introns (Michel and Dujon, 1983). The core structure consists of a central wheel of some 30 bases and six typical stem structures leading to a variety of peripheral structures encompassing the bulk of the introns nucleotide sequences (Figure 1). Conservation of primary sequences in bI1 and aI5γ is strong as far as the central core stucture is concerned (cf. Figure 1B), but weak in peripheral sequences (not shown). Only a GNGNG motif at the 5' end, and parts of stem 2 and 5 are also conserved in other introns of group II.

The biological role of these folded intron RNAs apparently is to serve as splicing substrates (although a function of spliced out, long living circular RNAs cannot be excluded *a priori*). For group I introns there is ample evidence, from the study of intron mutations in yeast, in support of this idea. This work also helps to define primary sequences and hybrid stretches of critical importance for splicing (De la Salle *et al.*, 1982; Anziano *et al.*, 1982; Weiss-Brummer *et al.*, 1983). In group II introns only one of these critical sites had been identified by the analysis of a mutation (M4873) and reversions which block and restore, respectively, excision of intron bI1 from *cob* pre-mRNA (Schmelzer *et al.*, 1982). This hybrid happens to be the 3'-terminal stem in the secondary structure model of group II introns (cf. Figure 1A).

One of the mutants studied here, G5049, exhibits three mutational alterations in bI1. The one in the central part of bI1 (position 522) lies outside of the sequence which according to genetic/physical mapping (cf. Figure 2) carries the determinant(s) for the phenotype of G5049. The other two are G→A transitions within this sequence (10 bp and 59 bp from the 5' splice site) and either may have an effect on splicing. The fact that no revertants of G5049 could be found is consistent with the existence of two mutations, both of which cause a splicing defect. The mutation close to the 5' end of bI1 (position 10) apparently weakens the stability of the first conserved stem of the core (ΔG: −7.8 kcal *versus* ΔG: −3.4 kcal) thus facilitating the melting of the helix and enlarging the wheel. The other mutation (position 59) affects a helical region in a lateral, non-conserved area of the proposed secondary structure. Its effect on the folding up of bI1 RNA thus seems less obvious.

Mutation M1301 carries a single alteration – a deletion of one A in a run of five As located in a bI1 internal sequence (positions 503–507). In the secondary structure model it takes part in the formation of stem 3 and of its interior loop. Most interestingly, this sequence is among the stretches conserved in bI1 and aI5γ and occupies the same position relative to the common core structures. Reversion of M1301 can occur at the primary mutational site either by a + 1 A insertion, restoring the wild-type sequence AAAAA, or by a + 1 G insertion (position 507), creating a novel sequence AAAAG which permits splicing similar or identical to the wild-type sequence.

All six revertants studied showed either one or the other of these two fully functional sequences although the selection

was strongly in favor of revertants with novel sequences conferring temperature-dependent splicing (see Results). We regard these results as indicating functional constraint on the sequence in question, in that the wild-type AAAAA or the related AAAAG pentanucleotide can restore splicing, but none of the other pentanucleotides which a + 1 bp insertion into AAAA would generate can do this. According to the secondary structure model of bI1 the − 1 bp deletion in M1301 will not destabilize stem 3; it will, however, reduce the size of its internal loop and the length of the conserved decanucleotide starting with AAAAA. Both types of revertants of M1301 restore the size and the primary sequence of the internal loop.

Mutation M4873, a − 1 G deletion in stem 6 (Schmelzer *et al.*, 1982, cf. Figure 1A) similarly does not dramatically reduce the stability of the stem, but increases the size of a bulge loop conserved in bI1 and aI5γ. Since from both mutations we obtained only two types of reversions, we cannot definitively decide whether the change in the primary sequence *per se*, or the alteration of the interior loop size, or both cause the defect in splicing.

Reversion of mutant M1301 is also possible by a nuclear mutation (*SUP-101*) leading to suppression of the splicing defect exerted by the mitochondrial mutation. Taken at face value this points to the interaction of a product of a nuclear gene with mitochondrial splicing. Interestingly, *SUP-101* is allele specific, leading to significant suppression only when combined with M1301, but not with any of the other *cob*$^-$ or *oxi3*$^-$ mutations tested. Another dominant nuclear suppressor interacting with mitochondrial RNA splicing has been described recently. Unlike *SUP-101* this suppressor *NAM2-1* is not allele specific; its major effect is to suppress a series of mutations in bI4 which affect a putative RNA maturase encoded by this intron, rather than mutations which alter RNA sequences that *per se* are involved in splicing (Dujardin *et al.* 1980; Groudinski *et al.*, 1981).

It is tempting to speculate that the *SUP-101* gene product interacts with the bI1 RNA sequence domain affected by M1301 − the *sup*$^+$ product with the wild-type RNA, the *SUP-101* product with the mutant M1301 RNA − in order to promote splicing. However, less direct effects of *SUP-101* are conceivable. Firmer conclusions await corroboration of the secondary structure model of bI1 RNA and studies on the nature of the nuclear gene product affected by *SUP-101* and its interaction with bI1 RNA splicing.

In conclusion, studies of mutational alterations in introns as presented here are of great value in determining the sequences of critical importance for splicing. As in the case of such studies on group I intron mutations, the mutations in bI1 mark sequence stretches which the other criteria available also reveal as critical: (i) sequences which are conserved in two or more introns, (ii) sequences which participate in the folding up of intron RNA. The study of suppressor mutations, like the nuclear *SUP-101,* finally may help to identify nuclear gene products which interact with intron RNA sequences to promote splicing.

Materials and methods

Strains

Origin of *cob*$^-$ mutations, map positions and phenotypes generated are given in Bechmann *et al.* (1981) and Schmelzer *et al.* (1982) and references therein. All *cob*$^-$ mutants originally were derived from strain 777-3A *ade1 op1*. Transfer of the mutant mitochondrial genomes to strain IC8 *a leu1 kar1* was accomplished by crossing the original mutant strain with IC8/AA1 (*rho*^{0}) according to the cytoduction protocol of Lancashire and Mattoon (1979) which makes use of the *kar1* mutation (Conde and Fink, 1976). Origin and genotypes of the *rho*$^-$ strains used in this study are given in Bechman *et al.* (1981).

MNNG and MMS mutagenesis and selection of revertants

Late log phase cells of strain IC8 (*rho*$^+$*cob*$^-$ M1301) were harvested and resuspended in 0.1 M Sørensen buffer, pH 7.0. MNNG and MMS were added to final concentrations of 100 μg/ml and 5 mg/ml, respectively, and cell suspensions were incubated for 20 min at 30°C. Cells were washed, resuspended in yeast extract (1%) liquid medium containing 5% glucose (YD medium) and grown for ~5 cell generations at 30°C in order to allow phenotypic expression of induced mutations. In order to select revertants, cells were plated at high cell density on yeast extract (1%) solidified media containing glycerol (3%) as the only carbon source (YG plates). Replica plates were incubated at 23°C, 30°C and 35°C for up to 6 days. Revertants that grew well at 30°C but weakly at 23°C or at 36°C or at 23°C and 36°C were designated as 'cold-sensitive' or 'heat-sensitive' or 'cold-sensitive/heat-sensitive', respectively.

Ethidium bromide mutagenesis

In order to obtain *rho*^{0} clones of revertants, cells were grown on YD plates containing 80 μg/ml ethidium bromide (added after autoclaving the medium; YD/EB). To ensure that the revertant cells grew on this medium for ~10 cell generations, patches grown on YD/EB were repeatedly replica plated onto the same media.

'Testcross'

In order to establish the genetic nature of secondary mutations causing reversion or suppression of the primary mutation *cob*$^-$ M1301, *rho*$^+$ clones and *rho*^{0} clones of each revertant (strain IC8 *a leu1 kar1*) were crossed with the original *cob*$^-$ mutant (strain 777-3A α *ade1 op1*). Growth of the diploid progeny of both crosses suggests a nuclear dominant, growth of the progeny from cross *rho*$^+$ revertant x *mit*$^-$ tester only suggests a mitochondrial mutation (for details see Dujardin *et al.*, 1980).

Tetrad analysis

Diploid cells of the cross IC8 *a SUP*$^-$ *(rho*^{0}*)* x K382-19D (*rho*$^+$) were sporulated and tetrads were dissected (Klapholz and Esposito, 1982). The mitochondrial phenotype of spore colonies was established by replica plating on YG medium and growth at 23°C, 30°C and 36°C. The presence of the *SUP*$^-$ mutation in spores was established by 'Testcrosses' (see above) involving *rho*^{0} colonies of spores and *a* or α strains with the mitochondrial phenotype *rho*$^+$ *cob*$^-$ M1301 and α (777-3A) or *a* (IC8) tester strain carrying the original *mit*$^-$ mutation (*rho*$^+$ *cob*$^-$ M1301).

Preparation of mtDNA

Yeast cells grown at 30°C in YD liquid medium were harvested in the early stationary phase. After mechanical treatment of the cells with glass beads, mitochondria were isolated (Lang *et al.*, 1977). Mitochondrial DNA was extracted with 2% SDS and purified by CsCl gradients with bisbenzimide dye (Hoechst 33258).

DNA sequencing

A 4300-bp *Hpa*II restriction fragment extending from the 5′ end of the cob region to bI3 was isolated and digested with *Bgl*II and *Hinf*I (BRL), respectively. The *Bgl*II sites and the *Hinf*I sites were labeled with DNA polymerase 'Klenow fragment' (Boehringer Mannheim) using [^{32}P]dGTP and [^{32}P]dATP (2000−3000 Ci/mmol, NEN), respectively. Labeled *Hinf*I fragments were either treated by secondary restriction enzyme cleavage or strand separation. After separation on 6% polyacrylamide gels, fragments were degraded according to the method of Maxam and Gilbert (1980) and run on 8% and 20% sequencing gels.

Computer calculations of RNA secondary structures

A graphic matrix analysis was performed with a program written in 'basic' for an APPLE II computer and an EPSON graphic printer displaying all potential helices with stems of 4 bp and more as well as their stability (Tinoco *et al.*, 1971). Free energy values were calculated according to Nussinov *et al.* (1982) and Cech *et al.* (1983).

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