The EMBO Journal vol.4 no.12 pp.3281-3288, 1985

Role of the 5′ hairpin structure in the splicing accuracy of the fourth intron of the yeast *cob-box* gene

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Communicated by P.Slonimski

The splicing mechanism of the maturase-coding introns is poorly understood. We have systematically examined the phenotypes of a large number of revertants from the mitochondrial mutation G2457. This mutation results from a single base change near the 5′ splicing site. We show here that this base change does not completely block the splicing of the intron but rather affects the specificity of the splicing process. We examine four classes of revertants which allow us to characterize the crucial role of a stem and loop structure in the accuracy of the intron excision process. An unexpected class of revertant suggests that other elements are involved in this mechanism. Reversion of G2457 can also occur via the excision in the mitochondrial genome of the intron coding sequence. These results are discussed in relation to the possible role fulfilled by the maturase in the control of intron splicing.

Key words: intron/maturase/mitochondria/splicing/yeast

Introduction

One of the most interesting features of some yeast mitochondrial introns is the presence of intron-encoded proteins, called mRNA maturases, which control the splicing process (Lazowska *et al.*, 1980; Anziano *et al.*, 1982; Weiss Brummer *et al.*, 1982). In this respect, the penultimate intron of the gene coding for cytochrome *b* in yeast mitochondria (intron bI4 in the strain 777-3A) is particularly interesting. The bI4 maturase, coded in its sequence, controls the splicing of two different introns: the fourth intron of the cytochrome *b* gene and the fourth intron of the cytochrome oxidase subunit I gene (Dhawale *et al.*, 1981; Labouesse and Slonimski, 1983; Labouesse *et al.*, 1984); furthermore, bI4 maturase is one of the two mRNA maturases whose existence has been confirmed by biochemical studies (Jacq *et al.*, 1984). Since these mRNA maturases constitute today the only class of *trans*-acting factors specifically controlling the splicing of particular introns, it is important to understand how this control is carried out. In this respect, it is crucial to elucidate the mechanism of splicing by which the primary transcripts are processed into mature RNA molecules. In the case of the intron bI4, a genetic approach to this problem has already revealed the existence of two short critical sequences, called *box9* and *box2* and localized 350 bp downstream from the 5′ junction and 25 bp upstream from the 3′ junction, respectively. Mutations in these sequences completely block the splicing of the intron bI4 (De la Salle *et al.*, 1982; Anziano *et al.*, 1982; Weiss Brummer *et al.*, 1982).

A subsequent study of intron sequences and computer analysis of possible secondary structures has led to the convincing classification of mitochondrial introns into two structural groups (Michel *et al.*, 1982; Davies *et al.*, 1982; Waring and Davies, 1984). Cytochrome *b* intron 4 belongs to group I in which the signal sequences *box2* and *box9* occupy typical positions in the secondary structure model. An *in vivo* base pairing involving the *box9* sequence and implicated in the intron bI4 splicing has been shown by Weiss-Brummer *et al.* (1983). The fact that the *Tetrahymena* rRNA intron also belongs to this group has caused some authors to propose a close relationship between the RNA secondary structure and the ribozyme property of *in vitro* splicing (Cech *et al.*, 1983). In other terms, the conservation of an important secondary structure between different nuclear and mitochondrial introns has raised the possibility that all of these introns utilize a similar RNA splicing mechanism. This point is supported by the recent findings that some mitochondrial introns can exhibit *in vitro* splicing (Garriga and Lambowitz, 1984; Van der Horst and Tabak, 1985) or that *Tetrahymena* rRNA intron can be spliced in *Escherichia coli* (Waring *et al.*, 1985). Nevertheless, it is clear that at least in the case of the *Neurospora crassa cob* intron, nuclear-encoded proteins are required for a correct *in vivo* splicing. It must also be emphasized that the secondary structures of group I and group II introns, though undoubtedly conserved in evolution, have received little attention concerning their exact function. It is generally assumed that these structures are involved in the splicing process, but the *in vivo* functions of the different parts of these structures in the different steps of the process remain to be elucidated.

We have focussed our attention on the role of the 5′ exon-intron junction in the splicing process of the cytochrome *b* intron 4. To do this we took advantage of a mutation localized near the junction and we systematically examined the different classes of revertants obtained. A careful molecular analysis of the revertants led to an anambiguous definition of the secondary structure in this region and to a proposition concerning its function in the splicing process. The occurrence of unexpected classes of revertants is discussed with regard to the mechanism of processing of this mRNA maturase-encoding intron.

Results

Isolation of spontaneous revertants of mutant G2457

G2457, a well characterized mitochondrial mutation, consists of a single G to A transition (De la Salle *et al.*, 1982) occurring in the fourth exon of the cytochrome *b* gene (strain 777-3A) 2 bp upstream from the 5′ splicing site. This base change has been shown to be associated with a glycerol minus phenotype. Two possible reasons can be proposed to explain this relationship: either the Gly to Asp substitution provoked by the mutation abolishes the cytochrome *b* activity, or the base change affects an essential signal for the splicing process. The pattern of mitochondrially synthesized proteins in the mutant (Claisse *et al.*, 1978) supports the latter view: proteins accumulate which are typical of mutants blocked in the splicing of the intron bI4.

Independent spontaneous revertants from the mutant G2457 were obtained (see Materials and methods).

Preliminary characterization and classification of revertants

A simple biochemical analysis allowed us to carry out a large-scale classification of the revertants. We took advantage of the two following facts: (i) the G2457 mutation creates a sequence 5′-GATC-3′ which can be recognized by the restriction enzyme *Mbo*I; (ii) on the basis of DNA sequence comparisons between different introns a secondary structure has been proposed in the 5′ region of the intron (Michel *et al.*, 1982; Davies *et al.*, 1982). In this putative secondary structure (Figure 1) the G2457 base change is located in one arm of the hairpin and faces the 5′-GGCC-3′ sequence on the other arm. This sequence can be recognized by the restriction enzyme *Hae*III. G2457 revertants were thus classified on the basis of their restriction map using the restriction enzymes *Mbo*I and *Hae*III. This was done in small-scale preparations of crude mitochondrial DNA, digested with the restriction enzymes and analyzed by blotting with cytochrome

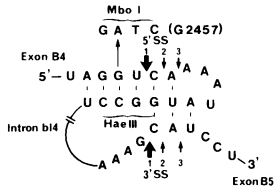

Fig. 1. Localisation of the mutation G2457 in the secondary structure model of cytochrome *b* intron boundaries. Davies *et al.* (1982) and Waring *et al.* (1984) have proposed, on the basis of DNA sequence comparisons, a possible secondary structure model which could be involved in the splicing process. An internal guide sequence (IGS) located just 5′ to the 5′ splicing site would pair with short stretches of RNA sequence at the end of the upstream exon and the beginning of the downstream exon. Due to the sequence of this region, the mRNA sequence studied did not permit us to determine unambiguously the splicing sites (Bonitz *et al.*, 1982). The most likely splicing sites, as suggested by comparisons with other introns, are indicated by the thick arrows (1), whereas the two other possibilities are indicated by small arrows (2) and (3). The mutation G2457 corresponds to a G→A transition as indicated in the figure. This base change generates an *Mbo*I site whereas a *Hae*III site (GGCC) is on the facing arm of the hairpin.

b probes (Figures 2, 3 and 6). In this way, 36 independent revertants were analysed and could be divided into four main classes.

Class I revertants

Two out of 36 revertants had lost the *Mbo*I site created by the G2457 mutation. This could be seen by Southern analysis carried out on *Mbo*I digestions of mitochondrial DNA. In this case (Figure 2, lane C) a typical wild-type pattern (lane A) is restored. The presence of an *Mbo*I site at the wild-type position strongly suggests that these class I revertants are true back revertants. This is in agreement with the fact that their growth on glycerol is identical to that of the wild-type cells.

Class II revertants

In these revertants, the *Mbo*I site of the G2457 is kept whereas the proximal *Hae*III site is lost. This is strongly suggested by the Southern blot presented in Figure 3. The mitochondrial DNA of revertant KM82/R-341 digested with *Hae*III has lost two DNA fragments of 805 and 459 bp and posssesses a new 1264-bp restriction fragment. On the other hand, its *Mbo*I restriction pattern is similar to that of the initial mutant G2457. DNA sequence analysis carried out on one of these Class II revertants was in agreement with these results: the mutated sequence is conserved and the *Hae*III site is lost due to a C to T transition as indicated in Figure 4. Twelve out of 36 revertants are Class II revertants. They were obtained at 36°C and 28°C and exhibit a wild-type phenotype. Their growth rate on glycerol is identical to the growth rate of wild-type cells.

RNA sequence analysis was carried out on these revertants to determine the sequence around the splicing site. To do this, reverse transcriptase elongation of crude mitochondrial RNA was primed with a 74-bp restriction fragment *Bgl*II (exon B6)-*Bst*NI (exon B5) obtained from the intronless form of cytochrome *b* gene (see Materials and methods). This primer, overlapping two fused exonic sequences, selectively hybridizes with the form of cytochrome *b* RNA from which intron bI5 has been spliced out. Figure 4 shows the RNA sequence established by the method

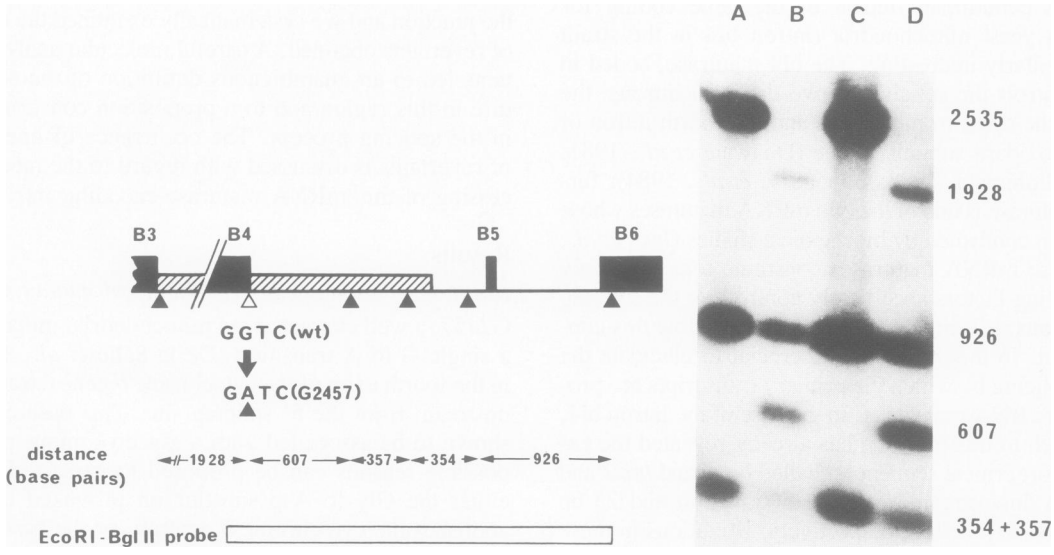

Fig. 2. Southern blot analysis of the *Mbo*I digest of mitochondrial DNA from different strains. The *Mbo*I restriction pattern of the 3′ end of the mitochondrial cytochrome *b* gene from the wild-type strain (KM91), is represented on the left. The localization of the *Mbo*I sites relative to the exonic sequences (black boxes B3−B6) and to the intronic sequences is indicated by the triangles. The hatched areas indicate the intronic open reading frames. The right part of the figure shows a Southern blot of mitochondrial DNA minipreparations (see Materials and methods) digested by *Mbo*I and run through a 1.4% agarose gel. The probe is a nick-translated pBR322 plasmid carrying an *Eco*RI (in exon B4)-*Bgl*II (in exon B6) restriction fragment. The strains analyzed are: **(A)** wild type strain (KM91); **(B)** mutant strain carrying the mitochondria mutation G2457 (KM82); **(C)** Class I revertant strain KM82/R-062; **(D)** Class II revertant strain KM82/R-341 (see text).

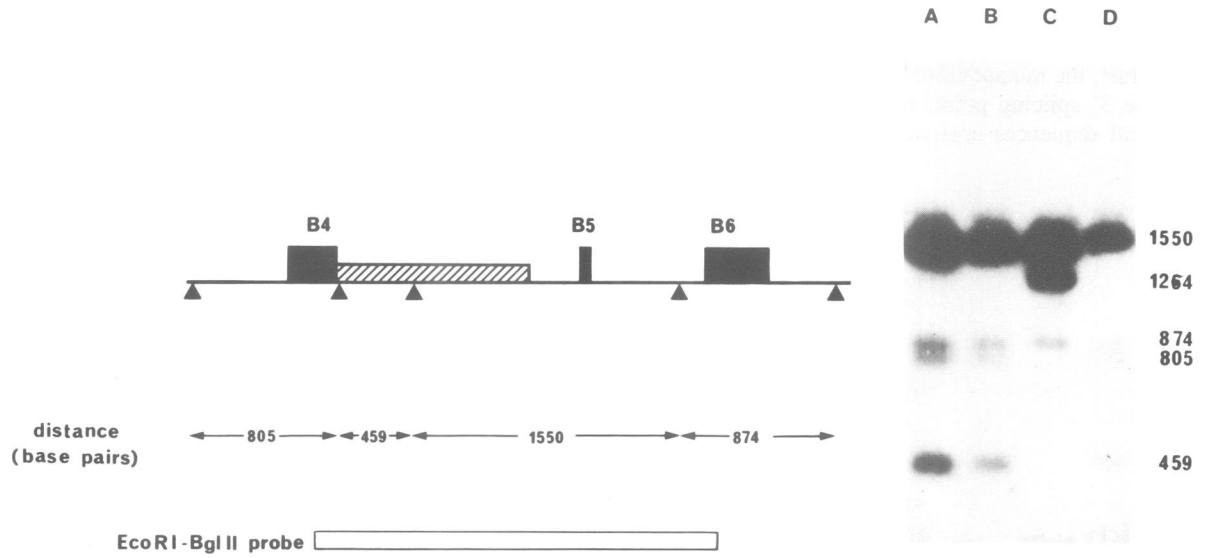

Fig. 3. Southern blot analysis of *Hae*III digests of mitochondrial DNA from different strains. The *Hae*III restriction pattern of the 3′ end of mitochondrial cytochrome *b* gene of wild-type strain KM91 is represented on the left. Symbols are as in Figure 1 except that ▲ are *Hae*III sites. The right of the figure represents a Southern blot of an *Hae*III digest of mitochondrial DNA preparations from the strains: **A**, wild-type (KM91); **b** , G2457 mutant strain (KM82); **C**, Class II revertant (KM82/R-341); **D**, Class III revertant (KM82/R-382).

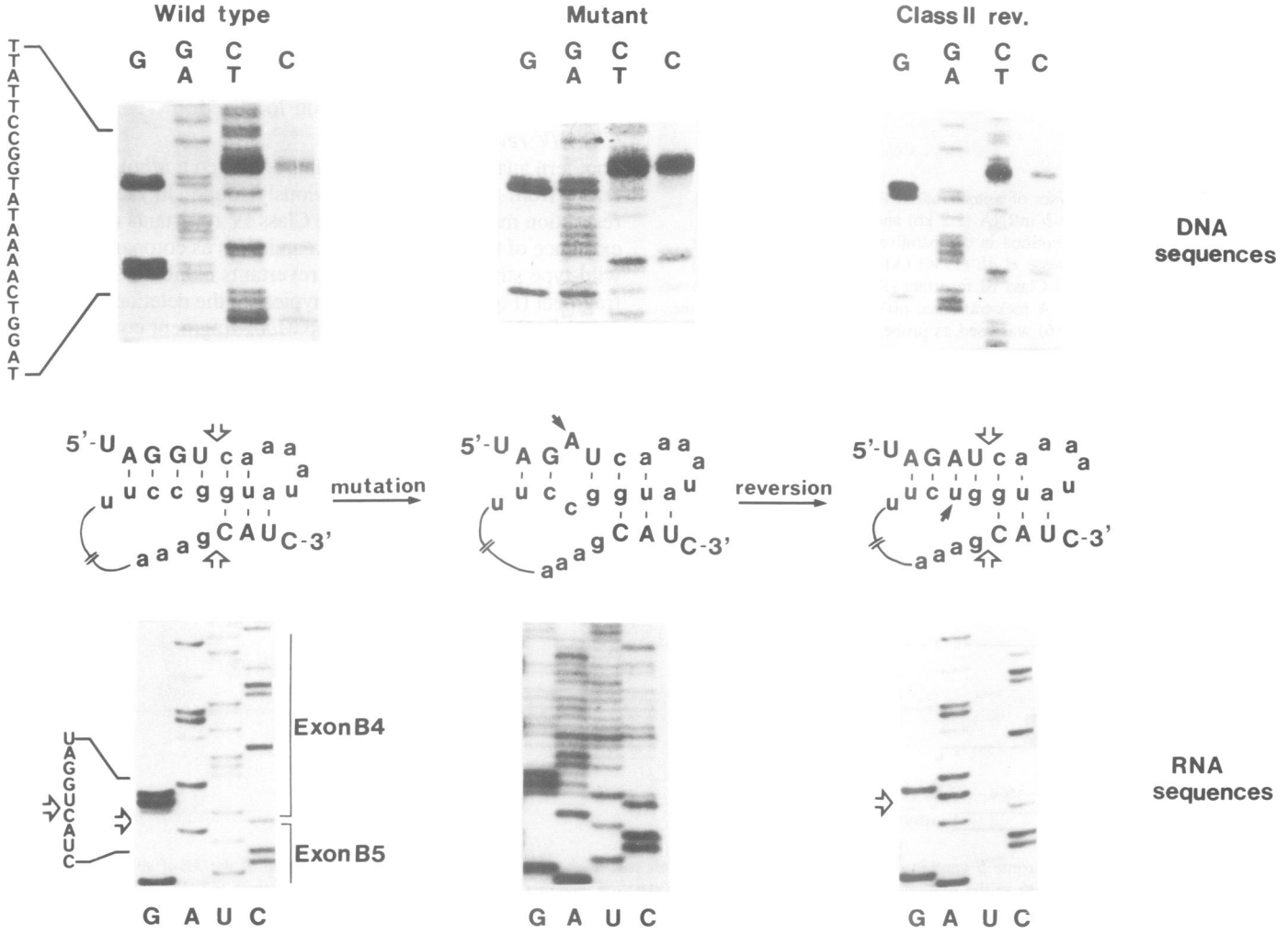

Fig. 4. DNA and RNA sequences of the wild-type strain, mutant G2457 strain and a class II revertant strain (KM82/R-341). DNA and RNA sequence analyses were established as described in Materials and methods. The putative RNA secondary model structure presented (middle line) in the different strains is deduced from the DNA sequence analyses (top line). The RNA sequences (bottom line) are established from primer extension studies. Capital letters in the model are exon sequence; small letters are intron sequence.

of Sanger *et al.*, (1977). Both wild-type (KM91) and Class II revertant (KM82/R-341) exhibit the same unambiguous RNA sequence. By contrast, the mutant G2457 shows an ambiguous sequence up to the 3′ splicing point; its sequence consists of a mixture of several sequences upstream from this point.

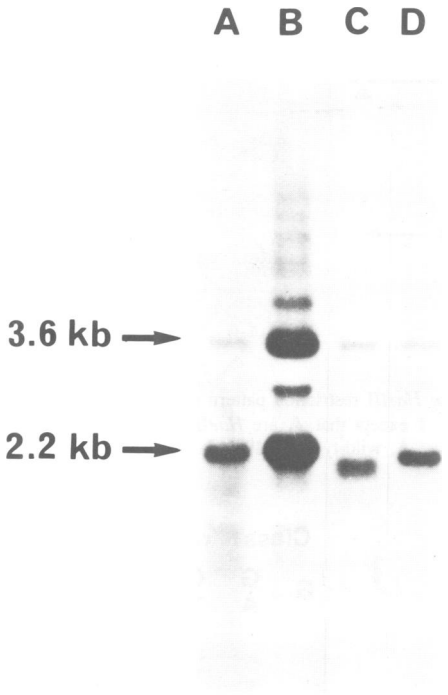

Fig. 5. Northern blot analyses of mitochondrial RNAs from different strains. Mature cytochrome *b* mRNA (2.2 kb) and precursor RNA with intron bI4 (3.6 kb) were identified in comparative experiments with other mutants described in Labouesse *et al.* (1984) **(A)** Wild type (KM91); **(B)** G2457 mutant (KM82); **(C)** Class III revertant (KM82/R-382); **(D)** Class II revertant (KM82/R-341). A nick-translated pBW1 plasmid (a cytochrome *b* exonic probe, see Figure 6) was used as probe.

Class III revertants

Fifteen Class III revertants were obtained at 36°C or 28°C (Table II) in which both *Mbo*I and *Hae*III sites are kept. This is clear in Figure 3 lane D and Figure 6 lane B, where the Class III revertant (KM82/R-382) has the same Southern pattern as the mutant strain (KM82, lane *b* in Figure 2 and lane A in Figure 6).

Most of these Class III revertants are heat and cold sensitive, they grow on N3 plates at 28°C but not at 36°C or 18°C, only one is not temperature sensitive. Class III revertants have a pseudo wild-type phenotype: they grow slowly on glycerol plates on which colonies are visible only after 5 days incubation at 28°C. The most typical features of Class III revertants is shown in Figure 5. Northern blot analysis of one of these revertants (KM82/R-382) shows that they contain an RNA species very similar to that of the wild-type strain (lane A) but migrating slightly faster in an agarose-formaldehyde gel. This reproducible observation was also made for the other Class III revertants.

To see whether this different electrophoretic mobility was due to an aberrant splicing process, we determined the RNA sequence around the splicing site. The sequence around the 3′ junction was determined by primer extension of a restriction fragment as described in Materials and methods. The sequence around the 5′ junction was examined by a similar approach, using the cDNA as a template and a synthetic oligonucleotide as a primer. These experiments revealed an important ambiguity in the splicing process of these revertants but the 5′ and 3′ exonic sequences next to the splicing point remain unaltered. The ambiguity is apparent in the 3′ intronic sequence (data not shown), indicating that the cryptic splicing points are intron localized.

Class IV revertants

Southern analyses of six revertants exhibited a *Mbo*I pattern different from the two types previously found. In fact, a careful restriction map analysis of these Class IV revertants revealed the existence of two subclasses (IVa and IVb) as compared with the wild-type structure. Class IVa revertants exhibit a 94-bp *Mbo*I fragment (Figure 6-2) which is typical of the deletions of introns 4 and 5. Conversely, the 926-bp *Mbo*I fragment corresponding

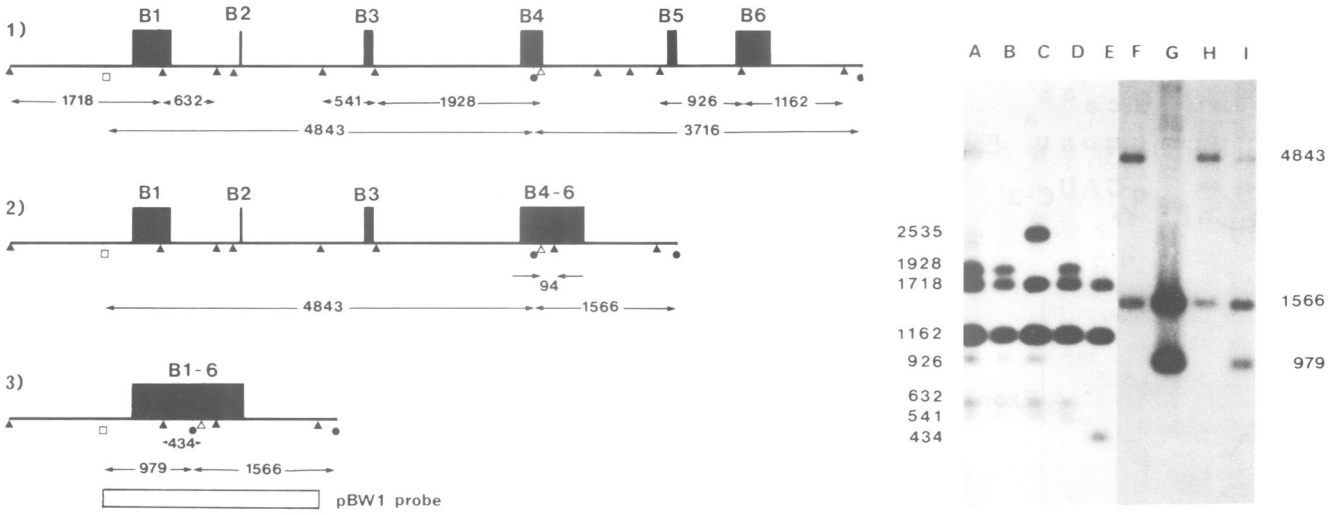

Fig. 6. Structure of the cytochrome *b* gene in class IV revertants. The top left part of the figure (**1**) represents the localization of the *Mbo*I sites relative to the cytochrome *b* exons B1 – B6 in the mutant strain KM82 (G2457). The restriction enzymes *Hha*I (□) and *Eco*RI (●) delineate two DNA fragments in the gene. Southern blot analyses of mitochondrial DNAs from different strains obtained with exonic probe pBW1 are presented on the right, **A – E** are *Mbo*I digestions of: **(A)** G2457 mutant KM82; **(b)** Class III revertant KM82/R-382; **(C)** Class I revertant KM82/R-062; **(D)** Class IVa revertant KM82/R-285; **(E)** Class IVb revertant KM82/R-212. **F – I** are *Eco*RI-*Hha*I digestions of: **(F)** SR30 (*rho*$^-$ issued from an intron bI4-bI5 deleted strain); **(G)** HM51 (a cytochrome *b* intronless strain constructed by Labouesse and Slonimski, 1983); **(H)** KM82/R-285 (Class IVa revertant); **(I)** KM82/R-212 (Class IVb revertant). Restriction maps corresponding to **lanes 2** and **3** are in agreement with the restriction fragments detected by Southern analysis of Class IV revertant strains (see text).

to the presence of intron 5 is lost (Figure 6, lane D) concomitantly with the appearance of a new 1566-bp *Eco*RI-*Hha*I fragment (Figure 6, lane H). This last restriction fragment is also present in an intronless form of the cytochrome *b* gene (lane G, Labouesse and Slonimski, 1983), its presence in IVa revertants strongly suggest that exons B4, B5 and B6 sequences are fused in these strains. Moreover, the presence of the 4843-bp *Eco*RI-*Hha*I restriction fragment as in the SR30 strain (lane F) suggests that this revertant has kept the three introns bI1, bI2 and bI3.

The restriction enzyme patterns of Class IVb revertants fit with the structure presented in Figure 6-3. These revertants have a new 434-bp *Mbo* fragment (lane E) and a new 979-bp *Eco*RI-*Hha*I fragment (lane I) also shown by the intronless cytochrome *b* strain (lane G). This strongly suggests that this Class IVb revertant KM82/R212 is devoid of introns in the cytochrome *b* gene.

DNA sequence anlaysis of Class IVa revertants showed that the DNA splicing of the different introns is strictly identical to the RNA splicing process, since it reproduces at the DNA level the exact RNA sequence of mature RNA (J.Perea and P.Pajot, unpublished results).

Discussion

The aim of the present work was to elucidate the putative role of the 5′ exon-intron junction in the splicing process of an mRNA maturase-encoding intron of the mitochondrial cytochrome *b* gene. To this end, we started from a mit$^-$ mutation, G2457, localized 2 bp upstream from the intron-exon junction (Figure 1) and looked for revertants able to grow on non-fermentable media. The molecular analysis of 36 independent revertants revealed that two of them are true back revertants with a wild-type sequence. This confirms the previous observation (De la Salle *et al.*, 1982) that the G to A transition of the G2457 mutation is responsible for the mit$^-$ phenotype observed in this mutant. Two reasons can be postulated *a priori* to explain this phenotype: either the G to A transition creates a drastic missense change in the cytochrome *b*, or/and, due to its proximity to the splicing site, it affects the splicing activity. Analyses of revertants produced information to answer this question.

The amino acid change created by G2457 mutation has no effect on cytochrome b activity

Class II and Class IV revertants, which have kept the G2457 base change, exhibit a clear wild-type phenotype. The existence of these two classes of revertants, in which the cytochrome *b* coding sequence has an aspartic acid codon instead of a glycine codon (GGU to GAU) proves that this modified cytochrome *b* has maintained its wild-type activity. This is all the more surprising since the cytochrome *b* sequence appears to be highly conserved in this region with an apparently invariant glycine residue at position 252 (Table I). Though unlikely, the possibility that a secondary compensatory mutation could have occurred in the coding sequence was envisaged and the cytochrome *b* coding sequences of a class IV revertant have been examined from the *Eco*RI site to the C-terminal end without noticing any base change other than the G to A G2457 mutation (J.Perea and P.Pajot, unpublished results). It is thus clear that the presence of an aspartic residue at position 252 does not affect the cytochrome *b* activity *per se*.

The G2457 mutation affects the accuracy of intron 4 splicing

G2457 is a new type of splicing-deficient mitochondrial mutation. All the splicing-deficient mitochondrial mutants obtained so far completely block the splicing of at least one intron. This is equally the case for *cis*-dominant mutations in the *box9* or *box2* loci and for *trans*-active mutations in mRNA maturases. These

Table I. Amino acid alignment of several cytochrome *b* sequences around the position 252 in yeast

Saccharomyces cerevisiae	N	T	L	G	H	P	D
Kluyveromyces lactis	N	T	N	G	H	P	D
Neurospora crassa	N	V	L	G	D	S	E
Aspergillus nidulans	N	A	L	G	D	S	E
Oenothera villaricae	N	V	L	G	H	P	D
Zea mays	N	V	L	G	H	P	D
Bos taurus	D	L	L	G	D	P	D
Homo sapiens	D	L	L	G	D	P	D
Mus musculus	D	M	L	G	D	P	D
Rattus rattus	D	L	L	G	D	P	D

three groups of mutants, which lead to the exclusive accumulation of the 3.6-kb mRNA precursor containing the fourth intron, indicate that the critical sequences *box9* and *box2* are involved in the preliminary steps of the splicing process. The mutation G2457 also leads to the accumulation of the 3.6-kb RNA species but we can also see (Figure 5) the presence, in similar amounts, of a 2.2-kb RNA species apparently identical to the mature mRNA. This is surprising if one considers the fact that G2457 is a stringent mutation which completely blocks the growth on glycerol. The sequence of this RNA was analysed by primed extension with a probe located in the fifth exon and it revealed that the corresponding RNA species are homogeneous up to the 3′ splicing site. From that point (Figure 4) the sequencing gel is no longer readable and a mixture of several different sequences can be detected. Precursor mRNA molecules present in this mutant (Figure 5) certainly contribute to this result but other unidentified sequences are also present and reflect the presence of aberrantly spliced RNA species. The base change of G2457 thus alters the specificity of the splicing process activating cryptic splicing sites which do not lead to functional mRNA species. This point is confirmed by two previous observations: (i) the translation products of this mRNA-like species do not lead to an active cytochrome *b* since the G2457 mutant has a stringent phenotype; cytochrome *b* is not detected by spectral techniques (data not shown) and (ii) analysis of mitochondrially synthesized proteins of G2457 has shown (Claisse *et al.*, 1978) that truncated proteins shorter than cytochrome *b* are made. The size of these proteins could fit with the expected size of products translated from frameshift alterations of mRNA generated by a defective splicing.

Function of the 5′ hairpin structure in the splicing process

All the 13 Class II revertants have the same suppressor mutation located 12 bp away from the G2457 mutation. This suppressor mutation affects the intron open reading frame and change an Ala codon (GGC) into a Val codon (GUC). Taken at face value this result suggests that the mRNA maturase coded in this intron has its activity modified so as to be able to recognize the new splicing site. This appealing hypothesis is certainly not valid due to the fact that the mRNA maturase-coding sequence has been localized in the middle and the 3′ part of the intron open reading frame (De la Salle *et al.*, 1982; Anziano *et al.*, 1982), >300 bp downstream from the suppressor mutation. In fact, no *trans*-acting mutations have been found in the upstream part of the intron open reading frame (Jacq *et al.*, 1982).

The most likely explanation for the role of the suppressor mutation is related to the possible existence of a 5′ hairpin structure. Computer analyses of homologies between mitochondrial intron sequences have revealed the presence of a conserved helical structure in the 5′ region of group I mitochondrial introns to which the cytochrome *b* intron 4 belongs. The G2457 mutational change

clearly affects this structure (Figure 1) by decreasing the stability of the stem in the secondary structure models proposed (Michel *et al.*, 1982; Davies *et al.*, 1982).

This observation, associated with our demonstration (see above) that the G2457 base change has no effect on cytochrome *b* activity, strongly suggests that this 5′ helical structure is an essential splicing signal for the cytochrome *b* intron 4. This point is strengthened by the Class II revertants, in which a compensatory change has restored most of the stem stability (Figure 4). Thus, it appears that, in this region, the maintenance of secondary structure has precedence over a conservation of the primary sequences. According to the model proposed by Davies *et al.* (1982), this hairpin would be a part of a more complex structure called the Internal Guide Sequence (IGS) in which, the 5′ hairpin is closed to a 3′ hairpin bringing the two ends of the intron in close proximity (Figure 4). That we detected inappropriate cleavages at several locations suggests that the mispairing created by the G2457 mutation activates some cryptic cleavage sites. This situation is reminiscent of that of rabbit β-globin (Wieringa *et al.*, 1983). The effects of the destabilization of the 5′ intramolecular helix would be similar to the effects of the destabilization of the intermolecular helix formed between U1 RNA and the 5′ junction (Mount and Steitz, 1983). No U1 like species have been found in yeast mitochondria and we would like to propose the idea that the 5′ hairpin loop of the mitochondrial intron could compensate for the absence of U1 RNA. In wild-type strains or in compensatory revertants, the 5′ endonuclease would recognize a double-stranded RNA structure creating a single- or a double-stranded cleavage. It must be emphasized that if this 5′ cleavage is not correctly made, for example in the G2457 mutant, the subsequent steps of the splicing process are still possible and mRNA-like species are synthesized although they cannot be translated into a functional cytochrome *b*.

Intron DNA deletion is not prevented by the mutation G2457

DNA splicing is a phenomenon which has been discovered by analysing revertant genomes of mitochondrial mutations localized within intron sequences. Several combinations of intron deletions have been characterized (Jacq *et al.*, 1982; Labouesse and Slonimski, 1983; Gargouri *et al.*, 1983). In this study two groups of Class IV revertants have been found; one in which all the cytochrome *b* gene introns are deleted and one in which only introns 4 and 5 are deleted (Figure 6). Both classes have the property of growing on glycerol and DNA sequencing carried out on one revertant indicates a clean intron excision (Perea and Pajot, unpublished results).

As noted by Pajot (Jacq *et al.*, 1982), deletion of the intron bI4 which encodes a pleiotropic mRNA maturase, should preclude the expression of subunit I cytochrome oxidase gene by blocking the excision of its fourth intron. At least three reasons can be envisaged to explain why the intron deletion revertants have a wild-type phenotype. (i) The excised intron could be maintained as a stable replicative structure; a mitochondrial plasmid recently described in *N. crassa* (Nargang *et al.*, 1984) suggests that mitochondrial introns may behave as mobile elements. In that case, however, we should have been able to detect the intron sequence in Southern blots which is not the case (Figure 4). (ii) Intron 4 of cytochrome oxidase subunit I gene could also have been cleanly deleted; Southern analysis allowed us to discard this hypothesis. (iii) A nuclear mutation, such as the NAM mutations already described (Dujardin *et al.*, 1980; Jacq *et al.*, 1982; Labouesse *et al.*, 1985), has occurred concomitantly with the intron DNA deletion. This last possibility, which has been well documented in similar cases constitutes the most reasonable explanation. Through this interesting property of DNA deletions, yeast mitochondrial introns belong to the important class of mobile genetic elements.

Table II. Classes of revertants obtained from the mutation G2457

Classes of revertants	Temperature of isolation		Revertant features
	36°C	28°C	
I	1	1	Back mutant
II	9	3	Compensatory mutant
III (*cts*)	0	13	Slow growing revertants
III (not *cts*)	1	1	
IV a	1	0	Introns 4 and 5 deleted
IV b	6	0	All introns deleted

The different classes of revertants are characterized by their DNA restriction pattern (see text). In each class, revertants were selected on glycerol medium (N3 plates) at 28°C or 36°C. *cts* revertants do not grow on N3 plates at 36°C or 18°C.

Several hypothesis have been envisaged to explain the DNA splicing phenomenon (Gargouri *et al.*, 1983). Some of them imply a similarity between the mRNA splicing and DNA splicing processes. The fact that we obtained a high frequency of intron deletion revertants supports the idea that the mutation G2457 affects the RNA splicing process more drastically than the DNA splicing process. This favors the existence of two different mechanisms for the two processes. Other hypotheses invoke a template role of precursor mRNA to make a cDNA by a reverse transcription under the control of a reverse transcriptase activity which would be present in this cell in which the mRNA maturase accumulates. In this case a very small proportion of mature mRNA in the mutant could be selected by the ability to confer a respiration-positive phenotype. The recent finding of a reverse transcriptase activity associated with Ty elements in yeast gives credence to this last hypothesis (Boeke *et al.*, 1985).

Analysis of pseudowild-type revertants

Is the 5′ stem and loop structure the only element to dictate the 5′ cleavage? Analysis of Class III revertants may suggest an answer to this question. In the three preceding classes of revertants the new base change gives rise to a wild-type phenotype. By contrast, Class III revertants exhibit a pseudowild-type phenotype, they grow on glycerol as the primary carbon source but slowly and they are thermo- and cryosensitive (Table II). The common, striking feature of these revertants is the slight modification of electrophoretic mobility of their cytochrome *b* mRNA (Figure 5, lane C). RNA sequence analysis of these revertants gave results apparently similar to those found in the mutant strain (Figure 7). Basically, these strains still have a defect in the accuracy of RNA splicing. The mixture of mRNA species obtained hampered a correct reading of the RNA sequence. Nevertheless these strains grow on glycerol and certainly possess enough active cytochrome *b* to carry out respiratory functions. We are thus forced to conclude that the loss of splicing accuracy in these revertants is less crucial than in the original mutant. Where is the suppressor mutation decreasing the ambiguity of the splicing process?. DNA sequence analysis over 300 bp around the 5′ and 3′ exon-intron boundaries did not show any base change. Genetic localization of this suppressor mutation is being carried out but these results already suggest that an element, distinct from the intron-exon junctions, can modulate the accuracy of splicing.

Finally, these results lead us to speculate as to the putative *trans*-acting elements controlling the 5′ cleavage of this intron. The best known *trans*-acting element involved in the splicing process of this intron is certainly the bI4 maturase; its exact role in this process is still a matter for speculation. The fact that the 1.85-kb cleavage product of the mRNA precursor encompassing the fourth intron and the two last exons is not present in the maturase mutants (Labouesse *et al.*, 1984), suggests that the bI4 maturase is involved in the recognition of the cleavage site. The affinity of bI4 maturase to precursor RNA structures is now being examined by *in vitro* experiments.

Materials and methods

Strains and media

KM82, a diploid strain carrying the G2457 mutation was used to obtain spontaneous revertants. KM82 is the result of a cross between 777-3A/G2457 (α *ade1 op1* [*rho*$^+$ *box1*$^+$]) (De la Salle *et al.*, 1982) and KL14-4A60 (*a his1 trp2* [*rho*^{0}]) (Wolf *et al.*, 1973).

KM91 is a wild-type diploid, isonuclear and isomitochondrial with KM82. It is the result of the cross between 777-3A (α *ade1 op1* [*rho*$^+$ *mit*$^+$] (Kotylak and Slonimski, 1977) and KL14/60.

HM51 is a diploid (*a*/α, *ura1*/+, +/*met*, *NAM2-6*/+ [*rho*$^+$]) without *box* introns constructed by Labouesse and Slonimski (1983).

SR30 is a *rho*$^-$ strain issued from WR27-27/1 (*a ura1* [*rho*$^+$ *mit*$^+$]) and carrying all the *box* genes. This strain is devoid of introns bI4 and bI5 (Jacq *et al.*, 1982).

Media were made as follows: YPGal 1% yeast extract, 1% peptone and 2% galactose. N3 1% yeast extract, 1% peptone and 2% glycerol. Solid media were prepared by adding 2% agar to liquid media.

Isolation of spontaneous revertants

The diploid strain KM82 carrying the mitochondrial mutation G2457 was subcloned in glucose plates. Isolated colonies ($\sim 10^8$ cells) were diluted in 0.1 ml of water and spread on glycerol plates. The plates were incubated at 28°C or 36°C for 21 days. Spontaneous revertants occur at a frequency of $10^{-6}-10^{-7}$. The resulting independent revertants, one from each plate, were subcloned and analysed.

Analysis of restriction patterns of mitochondrial DNA

Small-scale preparations of mitchondrial DNA were made from 1.5 ml YPGal overnight cultures. The cells were treated with zymolyase 6000 (Kirin Brewery) and mitochondrial DNA was prepared as in Dujon and Blanc (1980). After agarose gel electrophoresis the DNA was blotted to nitrocellulose (Southern, 1975) and hybridized with the nick-translated probes described in the text.

Mitochondrial RNA isolation and electrophoresis

Mitochondrial RNA was isolated from 800 ml of YPGal cultures grown until the beginning of stationary phase. Washed cells were digested by Zymolyase 60000 0.5 mg/g wet weight of cells in 1.35 M sorbitol, 0.1 M EDTA, 10 mM Tris/HCl pH 7.4 and 15 nM mercaptoethanol for 30 min at 37°C. The resulting protoplasts were lysed, mitochondria were purified and nucleic acids extracted according to the method of Locker *et al.* (1979). After a phenol extraction, nucleic acids were ethanol precipitated, washed with 70% ethanol and digested with 0.25 μg/ml of RNase-free DNase I (PL-Biochemicals) in the presence of 20 mM Tris/acetate, 10 mM $MgCl_2$ and 5 mM of vanadyl-ribonucleosides (Berger and Birkenheier, 1979).

RNA was electrophoresed through 1% agarose, 6% formaldehyde gels in 20 mM MOPS pH 7.0, 5 mM Na-acetate and 1 mM EDTA as described by Lehrach *et al.* (1977) and Goldberg *et al.* (1980). After electrophoresis, the gel was soaked for 15 min in three changes of water, for 1 h in 20 x SSC and blotted overnight to nitrocellulose filters (BA 0.45 μm Scheleicher and Schull). The filters were placed on a sheet of Whatman 3MM soaked in 3 x SSC in order to desalt them and baked for 2 h at 80°C under vacuum. Filters were prehybridized, hybridized with a nick-translated probe and washed as described by Thomas (1980).

DNA sequencing

The sequence of the suppressor mutation of KM82/R-341, one of the Class II revertants was established as follows: mitochondrial DNA of this strain was isolated and the 2344-bp *Eco*RI-*Bgl*II fragment was cloned in the *Eco*RI-*Bam*HI sites of pBR322. The resulting plasmid was cut with *Eco*RI, 5′ labeled and recut with *Hind*II. The 1244-bp labeled fragment was purified through a polyacrylamide gel and sequenced by the chemical modification method of Maxam and Gilbert (1980).

RNA sequencing

RNA sequencing was accomplished by primed extension with AMV reverse transcriptase in the presence of each of the four dideoxynucleotide triphosphates as inhibitors of elongation.

The primer was a 74-base *Bgl*II (exon 6)-*Bst*NI (exon 5) restriction fragment issued from plasmid pBW1 (p14 in Labouesse and Slonimski, 1983). This plasmid (carrying the cytochrome *b* intronless gene) was cut at the *Bgl*II site, 5′ labeled with [^{32}P]ATP and recut by *Bst*NI. The 72-bp labeled fragment was purified through a 6% polyacrylamide gel. 4 pmol of labeled double-stranded fragment was ethanol co-precipitated with 10 μg of total mitochondrial RNA prepared as described above. The pellet was washed with 70% ethanol and vacuum dried. The DNA-RNA mix was dissolved in 80 μl of 40 mM Pipes pH 6.4, 1 mM EDTA, 0.4 M NaCl, and 80% formamide and denatured 5 min at 68°C. The tube was plunged into a large volume of water at 42°C and the temperature of the bath was allowed to decrease to 12°C in 2 h. After hybridization, 240 μl of cold ethanol was added and DNA-RNA hybrids precipitated, washed twice with 70% ethanol and dried under vacuum.

The dried pellet was dissolved in a 40-μl reaction mixture consisting of 2 × reverse transcriptase buffer (10 × reverse transcriptase buffer is 1 M Tris/HCl pH 8.4 at 42°C, 1.4 M KCl, 0.1 M $MgCl_2$ and 40 mM dithiothreitol), 240 μM each of dATP, dGTP, dTTP, dCTP and 10 units of human placental ribonuclease inhibitor (Amersham). 10 μl of this mix were added to each of four Eppendorf tubes containing 10 μl of 85 μM ddCTP, 35 μM ddTTP, 35 μM ddATP and 85 μM ddGTP, respectively and 10 units of AMV reverse transcriptase (P.H. Steheling and Cie A.G.). The four reactions were carried out for 30 min at 42°C, chased by addition of 1 μl chase mix (6 mM each of four dNPTs) and incubated for another 10 min.

Nucleic acids were precipitated by addition of 20 μl of 5 M ammonium acetate and 120 μl ethanol. Pellets were washed with 70% ethanol, dried and dissolved in 10 μl of loading buffer (80% formamide, 50 mM Tris-borate pH 8.3, 1 mM EDTA, 0.1% xylenecyanol and 0.1% bromophenol blue). 1.5 μl of each sample were denatured for 2 min at 90°C and loaded in a 6% polyacrylamide, 50% urea sequencing gel (0.35 mm thickness). The gel was run at 40 W until the blue was at the bottom of the gel (2.5 h), dried and autoradiographed with a Kodak X-0 mat R film for 15 h.

Acknowledgements

We are grateful to Professor P.P.Slonimski for his continuous interest and support during the course of this work. We thank J.Banroques, A.M.Becam, A.Delahodde and V.Goguel for their friendly collaboration and encouragement. We thank also M.Labouesse for the generous gift of the plasmid pBW1, C.Herbert for checking the English and B.Poirier for expert help in manuscript preparation. This work was supported by grants from the CNRS (ATP 'Biologie Moléculaire du Géne'). J.P. acknowledges the receipt of an EMBO long-term fellowship. J.P. receives a grant from the Plan de Formacion de Personal Investigador (Spain).

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Received on 8 August 1985