The EMBO Journal vol.15 no.14 pp.3758–3767, 1996

Replacement of two non-adjacent amino acids in the *S.cerevisiae* bi2 intron-encoded RNA maturase is sufficient to gain a homing-endonuclease activity

Tomasz Szczepanek and Jaga Lazowska[1]

Centre de Génétique Moléculaire CNRS, 91198 Gif-sur-Yvette cedex, France

[1]Corresponding author

Two homologous group I introns, the second intron of the *cyt b* gene, from related *Saccharomyces* species differ in their mobility. The *S.capensis* intron is mobile and encodes the I-*Sca*I endonuclease promoting intron homing, whilst the homologous *S.cerevisiae* intron is not mobile, but functions as an RNA maturase promoting splicing. These two intron-encoded proteins differ by only four amino acid substitutions. Taking advantage of the remarkable similarity of the two intron open reading frames and using biolistic transformation of mitochondria, we show that the replacement of only two non-adjacent residues in the *S.cerevisiae* maturase carboxy-terminal sequence is sufficient to induce a homing-endonuclease activity without losing the splicing function. Also, we demonstrate that these two activities reside in the *S.capensis* bi2-encoded protein which functions in both splicing and intron mobility in the wild-type cells. These results provide new insight into our understanding of the activity and the evolution of group I intron-encoded proteins.

Keywords: group I intron/homing-endonuclease/maturase/yeast

Introduction

Some group I intron-encoded proteins which belong to a large family sharing conserved dodecapeptide sequences (also called P1-P2 or LAGLI-DADG, Michel *et al.*, 1982; Waring *et al.*, 1982; Hensgens *et al.*, 1983), have been shown to function either as RNA maturases, promoting splicing, or site-specific DNA endonucleases promoting intron homing. These proteins have a wide phylogenetic distribution, and mobile group I introns have been found in organelle, bacteriophage and nuclear genomes. However, only three proteins have been shown to have a maturase activity and these are all encoded by introns of the yeast mitochondrial *cyt b* gene (reviewed by Dujon, 1989; Cech, 1990; Lambowitz and Belfort, 1993). In all these cases, the maturase function has been demonstrated genetically by showing that mutations in the intron's open reading frame (ORF) result in defective splicing, which can be complemented in *trans* by the wild-type protein *in vivo*. Two maturases, those encoded by the second (Lazowska *et al.*, 1980) and third intron (Lazowska *et al.*, 1989) of the *cyt b* gene in *Saccharomyces cerevisiae*, are intron-specific, participating only in the splicing of the intron by which they are encoded. The third, encoded by the fourth intron of the *cyt b* gene is required for splicing of its own intron (Anziano *et al.*, 1982; De la Salle *et al.*, 1982; Weiss-Brümmer *et al.*, 1982) and the related ai4 intron of the gene coding for subunit I of cytochrome oxidase (*coxI*) (Labouesse *et al.*, 1984). The RNA maturase activity of the bi4 intron-encoded protein was also confirmed by showing that engineered forms of this protein, translated in the cytoplasm and imported into mitochondria, were able to complement maturase-deficient mutations (Banroques *et al.*, 1986, 1987). Although the participation of RNA maturases in the maturation of mitochondrial pre-mRNA is well documented genetically, their activity has not been tested biochemically, thus the exact role of these proteins in RNA processing is still speculative.

In contrast to the poorly understood maturase-mediated group I intron splicing, the endonuclease-mediated group I intron homing pathway is now well understood. Group I intron homing is a strictly DNA-based process, initiated by the double strand cleavage of an intronless allele by an intron-encoded endonuclease. Acquisition of the intron has been proposed to proceed by a double strand break repair pathway, originally postulated for gene conversion in yeast (Szostak *et al.*, 1983). According to this model, gene conversion initiated by the intron-specific endonuclease is accompanied by co-conversion of flanking exon sequences. In the mitochondrial genome of *S.cerevisiae* (strain 777-3A), four group I introns are known to be active in gene conversion. The first to be identified was the intron ω present in the *LSUrRNA* gene and encoding the I-*Sce*I homing endonuclease (Jacquier and Dujon, 1985; Macreadie *et al.*, 1985; Colleaux *et al.*, 1986). Other mobile introns are present in the *coxI* gene: ai3 encodes the I-*Sce*III endonuclease (Sargueil *et al.*, 1991; Schapira *et al.*, 1993; Szczepanek *et al.*, 1994), ai4 encodes the I-*Sce*II endonuclease (Delahodde *et al.*, 1989; Wenzlau *et al.*, 1989) and ai5α encodes the I-*Sce*IV endonuclease (Moran *et al.*, 1992; Séraphin *et al.*, 1992). A distinctive feature of the LAGLI-DADG type homing-endonucleases is the high specificity for their recognition sites (at or near the intron insertion site) that have an overall asymmetry.

The possibility that site-specific endonuclease and maturase activities could reside in the same protein is attractive from both a mechanistic and evolutionary standpoint: the DNA target sequences for the endonuclease correspond to the RNA flanking sequences in the 5′ and 3′ exons that may also participate in base pairing interactions required for splicing; spreading of the intron DNA should be facilitated by the RNA splicing promoted in the target gene. However, the presence of both activities in one molecule has never been observed in nature. Interestingly, a latent maturase activity can be revealed in the mobile ai4 intron (which encodes the I-*Sce*II homing endonuclease) by selection of mutants having either a single amino acid change, the *mim2* mutation (Dujardin

© Oxford University Press

et al., 1982), or a single amino acid replacement in the nuclear *NAM2/MLRS* gene (Labouesse *et al.*, 1987). Nevertheless, in the wild-type cells, the ai4-encoded protein is a potent endonuclease required for intron mobility and is not involved in splicing. Furthermore, none of the hybrid bi4 maturase–ai4 endonuclease proteins generated *in vivo* were found to be bifunctional, suggesting that in the case of these proteins (sharing 60% identity) the two activities are mutually exclusive (Goguel *et al.*, 1992).

Recently we reported that the second intron (bi2) of the *cyt b* gene from *Saccharomyces capensis*, which is homologous to its *S.cerevisiae* counterpart, is extremely mobile (Lazowska *et al.*, 1992). Remarkably, the *S.capensis* intronic ORF differs from that encoding *S.cerevisiae* maturase by only four amino acid replacements which must therefore play a crucial role in group I intron homing since the *S.cerevisiae* intron is not mobile (Meunier *et al.*, 1990; Lazowska *et al.*, 1992). In this study, we have asked which amino acids are responsible for the mobility. To this end, we have replaced these four amino acids and different combinations of them in the *S.cerevisiae* bi2 maturase sequence by biolistic transformation and tested the ability of modified introns to invade an intronless allele. We show that the replacement of only two residues, alanine and histidine at positions 212 and 239 instead of two threonines, is neccessary and sufficient for the acquisition of an endonuclease activity promoting intron homing. We also show that the *S.capensis* bi2 intron-encoded protein acts as a DNA endonuclease (I-*Sca*I) and an RNA maturase. It is the first case of a LAGLI-DADG bifunctional protein in the wild-type cells.

Results

The sequences of the S.capensis mobile intron and the S.cerevisiae non-mobile intron are remarkably conserved

We have previously shown that the *S.capensis* bi2 intron of the *cyt b* gene is able to invade an intronless allele with a very high efficiency reaching nearly 100%, whereas its homologous intron from *S.cerevisiae* was transmitted in a non-polar fashion (Lazowska *et al.*, 1992). The *S.capensis cyt b* gene has the same structure (Figure 1A) as the long variant of this gene present in the *S.cerevisiae* strain 777-3A (Lazowska *et al.*, 1980). The determination of the sequence of the *S.capensis* bi2 intron (Figure 1B) and its comparison with that of the *S.cerevisiae* intron has revealed an extraordinary conservation. These two introns are inserted at the same position in the *cyt b* gene, have the same length and their primary sequences differ by nine nucleotide substitutions (denoted by triangles in Figure 1B). There are six nucleotide substitutions in the intron ORF and four of them lead to amino acid replacements: aspartic acid at position 78 in *S.capensis* instead of asparagine in *S.cerevisiae*, threonine at position 172 instead of methionine, alanine and histidine at positions 212 and 239, respectively, instead of two threonines present at these positions in the *S.cerevisiae* intron. The substitution in the methionine codon at position 31 is silent and position 181 is already polymorphic in different *S.cerevisiae* strains and can be occupied by isoleucine (strain KL14-4A/B231, Gargouri, 1989) as in *S.capensis*, or methionine (strain 777-3A, Lazowska *et al.*, 1980).

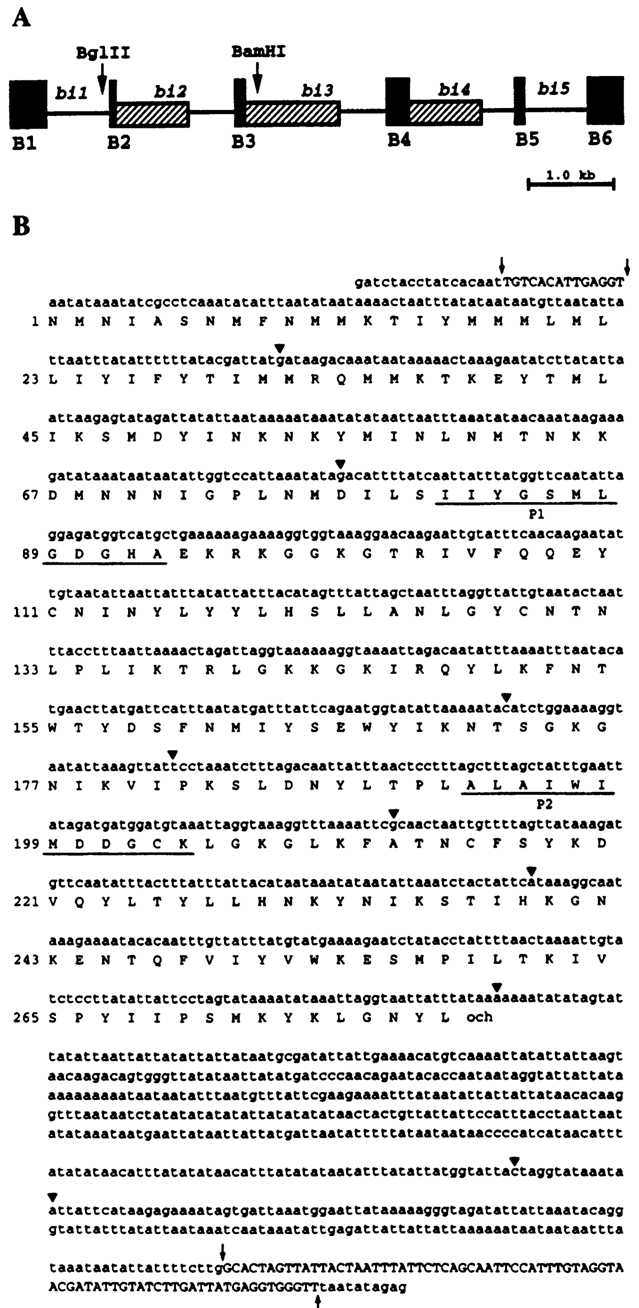


Fig. 1. Structure of the *S.capensis cyt b* gene and sequence of the second intron and its surrounding regions. (**A**) Black boxes denote exon sequences, hatched boxes denote intron coding sequences and horizontal lines denote intron non-coding sequences. Only the restriction sites used for cloning and DNA sequencing are indicated. (**B**) The sequence of the *Bgl*II–*Bam*HI fragment is shown; exonic and intronic sequences are indicated by upper and lower case letters, respectively. The exon–intron junctions are indicated by arrows. The *S.cerevisiae* mitochondrial genetic code is used for translation. Only the intronic amino acids are numbered. Two conserved dodecapeptide sequences, P1 and P2, are underlined. Black triangles indicate the polymorphic nucleotide substitutions compared with the *S.cerevisiae* sequence (data from Lazowska *et al.*, 1980; Gargouri, 1989). The sequence has been deposited in the EMBL database under accession no. X95974.

According to the secondary structure model of the *S.cerevisiae* bi2 intron (Tian *et al.*, 1991), the three substitutions occurring in the non-coding 3′ part of the *S.capensis* intron do not affect the sequences important for RNA folding.

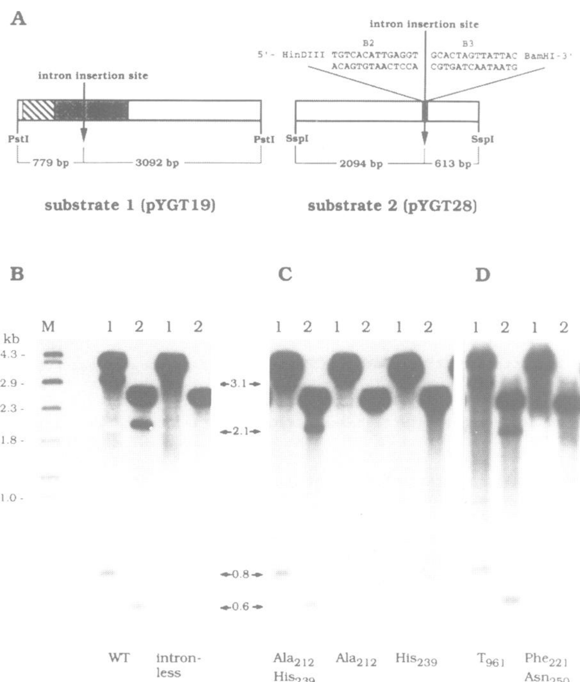


Fig. 2. *In vitro* assay of the endonuclease activity. (**A**) Structure of the substrates. Substrate 1 (pYGT19): the filled box represents the *cyt b* exonic sequences (B1–B6) and the hatched box represents a part of the upstream *cyt b* gene sequence. Substrate 2 (pYGT28): carries only the exonic sequences flanking the bi2 insertion point (14 bp on either side, see Materials and methods). In both diagrams, open boxes denote pUC13 plasmid sequences. The restriction sites indicated were used to make the substrates linear. The calculated lengths of fragments expected from cuts at or in the vicinity of the intron insertion site are indicated. The endonuclease assays were carried out as described in Materials and methods. The cleavage products were revealed by Southern blot analysis using labelled pYGT19. The linearized substrates 1 and 2 were incubated with the mitochondrial extracts from: (**B**) the *S.capensis* wild-type strain and the *S.cerevisiae* wild-type strain carrying an intronless mtDNA; (**C**) *S.cerevisiae* strains carrying an Ala212,His239-substituted bi2 maturase (strain ST25-44), Ala212-substituted maturase (strain ST28-39) and His239-substituted maturase (strain ST16-3); (**D**) *S.capensis* mutant strains carrying a T961 instead of a C in the non-coding sequence of the bi2 intron (strain ST29/5) and a Phe221 and Asn250 instead of valine and isoleucine respectively, in the bi2 ORF (strain ST30/1). Lane M in (B) shows molecular weight markers (Raoul, Appligene). The observed lengths (in kb) of cleavage products are indicated.

The S.capensis bi2 intron-encoded protein has a double strand DNA endonuclease activity

In the majority of cases, group I intron homing was associated with the presence of a site-specific endonuclease cleaving the intronless allele close to the intron insertion site (Lambowitz and Belfort, 1993). To test the possible endonucleolytic activity of the *S.capensis* bi2 intron-encoded protein, we used specific substrates containing exonic sequences surrounding the bi2 intron insertion site, and mitochondrial extracts from strains containing this intron (see Materials and methods).

The structure of substrates used is showed in Figure 2A. The first (substrate 1) carries almost an entire intronless *cyt b* gene and the second (substrate 2) is more specific and contains only the 28 nucleotides encompassing the bi2 intron insertion point. After incubation of linearized substrates with the mitochondrial extracts, the cleavage products were revealed by Southern blot analysis as described in Materials and methods. As shown in Figure 2B, double strand DNA cuts are observed in both substrates when incubated with the mitochondrial extract from the wild-type *S.capensis* strain. The observed lengths of cleavage products, 3.1 and 0.8 kb for the larger substrate and 2.1 and 0.6 kb for the smaller one, are in perfect agreement with the calculated length of fragments expected from cuts at or in the vicinity of the bi2 insertion site (Figure 2A and B). The cleavage products were not detected when the substrates were incubated with an extract from the *S.cerevisiae* strain CKYL2 (Szczepanek *et al.*, 1994) carrying an intronless mitochondrial genome which was used as a control (Figure 2B).

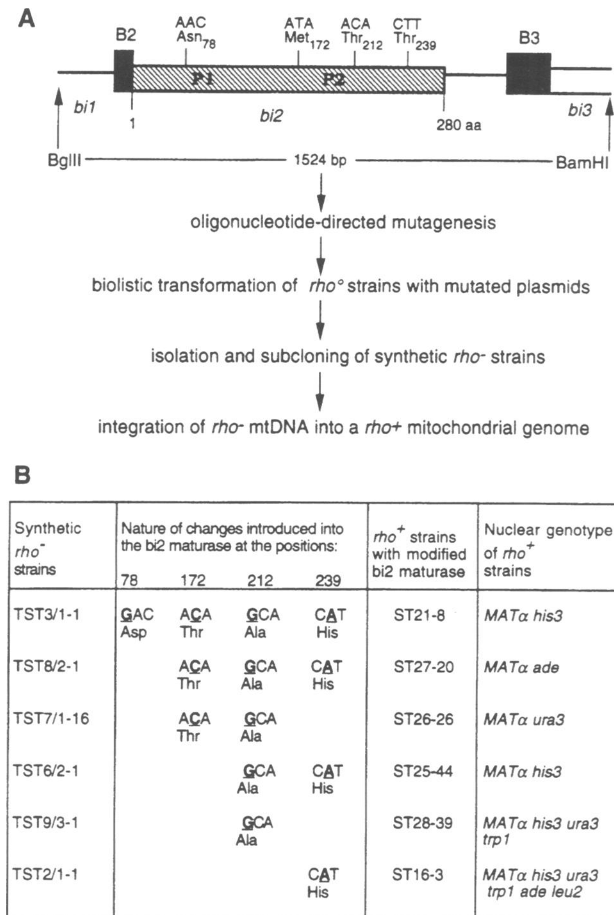
Taken together, these results demonstrate that the *S.capensis* bi2-encoded protein has an endonucleolytic activity which specifically recognizes the exon sequences flanking the insertion site of the intron which encodes it. This new homing-endonuclease is named I-*Sca*I and is the first intron-encoded enzyme from the yeast *S.capensis*.

Two amino acids substitutions in the S.cerevisiae bi2-encoded RNA maturase are needed to induce intron mobility

Taking advantage of the conservation of sequence between *S.cerevisiae* maturase and *S.capensis* I-*Sca*I endonuclease and using *in vitro* mutagenesis and biolistic transformation of mitochondria, we introduced all four polymorphic changes occurring in the *S.capensis* sequence as well as different combinations of them into the *S.cerevisiae* sequence to see if we could make this intron mobile.

The *S.cerevisiae* rho^+ strains carrying alterations in the bi2 maturase sequence were constructed in several steps which are represented schematicaly in Figure 3A and described in detail in Materials and methods. Six variants of the recombinant plasmids that carry either all four substitutions (Asp78, Thr172, Ala212, His239), three of them (Thr172, Ala212, His239), two different combinations of two (Thr172, Ala212, and Ala212, His239) or two different mono-substitutions (Ala212 and His239) were constructed and used for biolistic transformation of mitochondria. The alterations of the bi2 sequence present in the mitochondrial DNA (mtDNA) of the resulting rho^- strains (listed in Figure 3B, first two columns) were integrated into the rho^+ mitochondrial genome by homologous recombination. The rho^+ haploid strains (listed in Figure 3B, last two columns) which are all respiratory competent, were used as bi2 intron donors in order to determine whether the maturase modifications could lead to intron mobility and, if so, which ones are necessary.

To study the *S.cerevisiae* modified bi2 intron homing, the intron donor strains were crossed to the same recipient strain having an intronless mtDNA, and the transmission of mitochondrial markers to the progeny was analysed as described in Materials and methods. The donor and recipient strains carry a mitochondrial marker, located in the *oli1* gene, conferring sensivity (O^S, allele present in donor strains) or resistance (O^R, allele present in recipient strain) to oligomycin which is known to be transmitted in a non-polar fashion (Dujon *et al.*, 1974; Lazowska *et al.*, 1994). The transmission of this marker was used as a control in order to assess the respective contributions of the parental mtDNA to the mating pool. Thus, the mobility of the altered bi2 intron can be deduced from its polar transmission when compared with the transmission of a



Synthetic rho^- strains	Nature of changes introduced into the bi2 maturase at the positions: 78	172	212	239	rho^+ strains with modified bi2 maturase	Nuclear genotype of rho^+ strains
TST3/1-1	**G**AC Asp	AC**A** Thr	**G**CA Ala	C**A**T His	ST21-8	*MATα his3*
TST8/2-1		AC**A** Thr	**G**CA Ala	C**A**T His	ST27-20	*MATα ade*
TST7/1-16		AC**A** Thr	**G**CA Ala		ST26-26	*MATα ura3*
TST6/2-1			**G**CA Ala	C**A**T His	ST25-44	*MATα his3*
TST9/3-1			**G**CA Ala		ST28-39	*MATα his3 ura3 trp1*
TST2/1-1				C**A**T His	ST16-3	*MATα his3 ura3 trp1 ade leu2*

Fig. 3. Construction and description of the *S.cerevisiae* strains carrying various substitutions in the bi2 maturase. (**A**) Structure of the mitochondrial *BglII–BamHI* fragment carrying the wild-type sequence of the *S.cerevisiae* bi2 intron which is cloned in the *BamHI* site of pUC19. Exonic and intronic sequences are depicted as in Figure 1. The position and nature of *S.cerevisiae* codons which are different in the homologous *S.capensis* intron are indicated. The steps involved in strain constructions were carried out as described in Materials and methods. (**B**) The synthetic rho^- strains have the nuclear genotype of the transformed rho° strain (W303-1B/A/50, *MAT***a** *his3 ura3 trp1 ade2 leu2*). The modified nucleotides present in mitochondrial genomes of constructed rho^- and rho^+ strains are in bold and underlined. The haploid rho^+ strains having the appropriate nuclear genotype were isolated after sporulation of diploids from crosses between the rho^- strains and the strain G1909 (*MATα ade1 op1*) as described in Materials and methods.

non-polar marker carried by the same donor genome. Alternatively, the mobility or immobility of the tested intron can also be deduced by the comparison of its transmission with that of a known mobile intron present in the same genome.

Table I shows the results of a quantitative analysis of the diploids obtained from crosses between the wild-type strain (used as control) and six donor strains carrying various modifications of the bi2 maturase sequence and the same recipient intronless strain. In three cases, when the *S.cerevisiae* bi2 maturase has been modified to have either four (Asp78, Thr172, Ala212 and His239; strain ST21-8), three (Thr172, Ala212 and His239; strain ST27-20) or only two substitutions (Ala212 and His239; strain ST25-44), the *S.cerevisiae* bi2 intron became mobile. This is demonstrated by the observation that the modified bi2 intron present in these strains is transmitted with a very high frequency (reaching or nearly reaching 100%), while the transmission of a marker located in a non-polar gene (O^S) is much lower. It should be emphasized that the replacement of two non-adjacent amino acids (two threonines by alanine and histidine) separated by 29 residues in the *S.cerevisiae* maturase C-terminal sequence leads to the acquisition by this intron of the capacity to invade an intronless allele in the same manner as the *S.capensis* homologous intron (Lazowska *et al.*, 1992) encoding the I-*Sca*I endonuclease.

In three cases, when the *S.cerevisiae* bi2 maturase has been modified to have either Thr172 and Ala212 (strain ST26-26), only Ala212 (strain ST28-39) or only His239 (strain ST16-3), the acquisition of intron mobility is not observed. The results shown in Table I clearly indicate that the transmission of the modified bi2 intron present in these strains is non-polar when compared with transmision of the mobile ai4 intron encoding I-*Sce*II homing-endonuclease present in the *coxI* gene (Delahodde *et al.*, 1989; Wenzlau *et al.*, 1989). Indeed, it is the same as that of the wild-type intron bi2 from the strain 777-3A (used as a control) which has been shown previously to be transmitted in a non-polar fashion (Meunier *et al.*, 1990; Lazowska *et al.*, 1992). The transmission of a non-polar genetic marker (O^S) cannot be measured in these cases since the ai4 intron homing to the intronless mitochondrial genome led to a respiratory-deficient phenotype due to the absence in these mtDNAs of the bi4 maturase necessary for splicing of the ai4 intron (Labouesse *et al.*, 1984). Obviously this situation is not observed when the altered bi2 intron has the capacity to invade an intronless allele and the bi4 intron is preferentially transmitted at the same time by co-conversion events (Szczepanek, 1995).

To relate the intron mobility to the endonuclease activity encoded by the modified intron, *in vitro* endonuclease assays were carried out on mitochondrial extracts from the strain (ST25-44) carrying an Ala212,His239-substituted maturase. As shown in Figure 2C, double strand cuts are detected in both substrates. We have also examined the endonuclease activity using extracts from strains carrying either an Ala212- or a His239-substituted maturase. These strains have no detectable mobility in crosses (Table I) and they also lack detectable I-*Sca*I activity (Figure 2C).

Taken together, the results presented above demonstrate that the replacement of only two threonine residues at positions 212 and 239 in the *S.cerevisiae* bi2 intron-encoded maturase by alanine and histidine (Figure 2C, Table I) respectively, is sufficient to gain the homing-endonuclease activity. Importantly, this acquired activity requires both residues to be changed; single substitutions (either alanine or histidine) do not activate a homing activity. As expected from the respiratory-competent phenotype of constructed strains, none of the changes leading either to acquisition or loss of intron mobility affect the maturase function. Thus, the simultaneous occurrence of alanine and histidine at positions 212 and 239 must be crucial for the homing-endonuclease function.

The S.capensis bi2 intron-encoded homing-endonuclease also has an RNA maturase activity in wild-type cells

Having demonstrated that the *S.capensis* bi2 intron-encoded protein has a DNA endonuclease activity, we

Table I. Transmission of the modified bi2 introns to the *S.cerevisiae* intronless *cyt b* gene

Donor strains	Amino acid at position				Transmission of markers (%)			No. of diploids analysed	Intron mobility induced
	78	172	212	239	$bi2^+$	$ai4^+$	O^S		
777-3A	N	M	T	T	55	90	–	180	no
ST21-8	D	T	**A**	**H**	**98**	–	69	182	**yes**
ST27-20	N	T	**A**	**H**	**100**	–	49	174	**yes**
ST26-26	N	T	A	T	64	97	–	175	no
ST25-44	N	M	**A**	**H**	**96**	–	61	182	**yes**
ST28-39	N	M	A	T	48	92	–	175	no
ST16-3	N	M	T	H	33	99	–	176	no

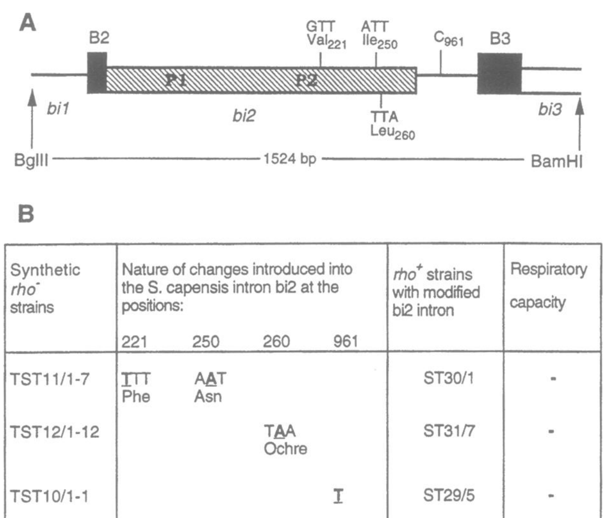
All donor strains were crossed with the same recipient strain CKYL2 (*MATa leu1 kar1-1*) having an intronless mitochondrial genome which carries a mitochondrial marker conferring resistance to oligomycin (O^R) (Szczepanek *et al.*, 1994). The nuclear genotypes of donor strains are given in Figure 3B. All donor strains have the mitochondrial genome of the strain 777-3A (see Materials and methods) in which the different amino acid modifications in the bi2 intron were introduced at the indicated positions (second column). The wild-type *S.cerevisiae* strain 777-3A (*MATα ade1 op1*), was used as a control. The transmission of genetic markers present in donor mitochondrial genomes was measured as described in Materials and methods. Intron mobility is deduced from the polar transmission of the bi2 intron when compared with the transmission of a non-polar marker (sensivity to oligomycin, O^S). Intron immobility is deduced from the non-polar transmission of the bi2 intron when compared with that of the mobile intron ai4 of the *coxI* gene.

have addressed the question of whether this protein has an RNA maturase activity. The fact that the *S.capensis* is respiratory competent suggests but does not prove this point. The only way to identify such an activity is by a mutational genetic analysis.

Using the same experimental approaches as those described above with some modifications, which are described in Materials and methods, we have introduced into the wild-type sequence of the *S.capensis* bi2 intron (Figure 4A) the mutational changes known to occur in *S.cerevisiae* mutants: the substitution of Val221 by Phe and Ile250 by Asn found in the *trans*-active splicing-deficient mutant G1909 (referred to as box3-2 in Lazowska *et al.*, 1980), the substitution of Leu260 by a stop codon found in the *trans*-active splicing-deficient mutant W91 (referred to as box3-5 in Lazowska *et al.*, 1980) and C to a T transition at position 961 found in the *cis*-acting splicing-deficient mutant P144 (Gargouri, 1989). The resulting *rho*⁻ and *rho*⁺ *S.capensis* strains carrying mutational changes are listed in Figure 4B. All *rho*⁺ *S.capensis* mutants have respiratory-deficient phenotypes, suggesting that the *S.capensis* bi2 intron-encoded protein is involved in splicing of its own intron.

To confirm this, we carried out the analyses of mitochondrial transcripts and mitochondrial translation products from the wild-type and mutant strains of *S.capensis* and *S.cerevisiae* (used as controls). The results of Northern blot analysis, shown in Figure 5, clearly indicate that all mutants accumulate bi2-containing intermediate RNAs (7.4, 5.3 and 3.6 kb) and do not make the mature mRNA. The splicing deficiency is strongly pronounced in the case of *trans*-active mutations G1909 and W91 but less so in the case of the somewhat leaky, *cis*-acting mutation P144.

Consistently, analysis of mitochondrial translation products, presented in Figure 6, revealed accumulation of the 42 and 38 kDa polypeptides in the *trans*-acting mutants G1909 and W91, which correspond to the upstream exon–bi2 maturase fusion protein (carrying two missense mutations) and its truncated form respectively (Claisse *et al.*, 1978; Lazowska *et al.*, 1980). The same 42 kDa band (wild-type protein) is present in both yeasts carrying a *cis*-acting mutation affecting intron RNA folding (P144). As expected, the protein accumulated in the *S.capensis*



Synthetic rho⁻ strains	Nature of changes introduced into the S. capensis intron bi2 at the positions: 221	250	260	961	rho⁺ strains with modified bi2 intron	Respiratory capacity
TST11/1-7	TTT Phe	AAT Asn			ST30/1	-
TST12/1-12			TAA Ochre		ST31/7	-
TST10/1-1				T	ST29/5	-

Fig. 4. Construction and description of the *S.capensis* strains carrying splicing-deficient mutations of the *S.cerevisiae* intron bi2. (**A**) Structure of the mitochondrial *BglII–BamHI* fragment carrying the wild-type sequence of *S.capensis* intron bi2. The position and nature of codons which when mutated in the *S.cerevisiae* homologue intron abolish maturase activity as well as the nature of the nucleotide 961 essential for ribozyme activity are indicated. The principal steps involved in the construction of the strains are the same as those indicated in Figure 3, with some modifications which are described in Materials and methods. (**B**) The alterations carried by mtDNA of stable synthetic *rho*⁻ clones (nucleotides marked in bold and underlined) were introduced, by mating, to the wild-type strain (ST13-43, *MATα lys2*) having in its mitochondrial genome only the first three introns (bi1, 2 and 3) of the *S.capensis cyt b* gene. All *rho*⁺ diploids (see Materials and methods for selection) do not grow on glycerol media.

mutant carrying this mutation possesses the I-*Sca*I activity as shown in Figure 2D. Due to the organization of mitochondrial genomes in the two yeasts, the chimeric proteins accumulated in the *S.capensis* mutants are partially (48 kDa band) or totally (38 kDa band) hidden by a large coxI protein band which is not expressed in the corresponding *S.cerevisiae* mutants (De la Salle *et al.*, 1982). The truncated 38 kDa maturase form in the *S.capensis* mutant was easily detected when the conditions

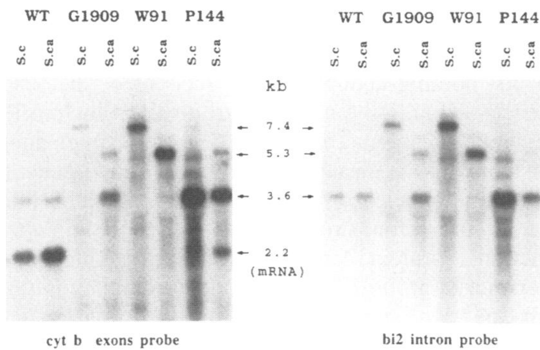


Fig. 5. Northern blot analysis of mutant strains. Total mtRNA was purified from isolated mitochondria, separated on a 1.2% denaturing agarose gel, transferred to Hybond C filters and hybridized with exon and bi2 probes as described in Materials and methods. Total mtRNA was extracted from wild-type *S.cerevisiae* (S.c) and *S.capensis* (S.ca) strains 777-3A and ST13 respectively; and from the mutant strains obtained *in vivo* (S.c) and *in vitro* (S.ca) carrying the same mutational changes. The names of the mutations are given in the upper line and refer to the *S.cerevisiae* mutant. The corresponding *S.capensis* mutant strains that carry the G1909, W91 and P144 mutations are ST30/1, ST1/7 and ST29/5 respectively. The length of the main transcripts revealed by cyt b exon probes and bi2 intron probes are indicated. The content of the main transcripts has been deduced by additional hybridizations with a full set of *cyt b* intron-specific probes (not shown). The 7.4 kb transcripts contain the unexcised introns bi2, bi3, bi4 and bi5 and are present in the *S.cerevisiae* mutants. Those of 5.3 kb (bi2 and bi3) and 3.6 kb (bi2) are detected in both yeasts according to the *cyt b* intron configuration in the mutant strains.

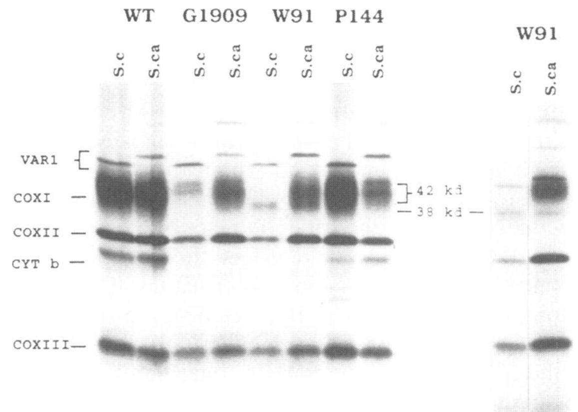


Fig. 6. Mitochondrial translation products accumulated in mutant strains. The mitochondrial translation products of the *S.cerevisiae* and *S.capensis* mutant strains were labelled *in vivo* with $^{35}SO_4$ in the presence of cycloheximide and separated on an SDS–polyacrylamide gel (10–15% exponential gradient) (left part of the figure). The same set of wild-type and mutant strains used for Northern blot analysis was used to analyse the labelled translation products (see description in Figure 5). COXI, II, III, CYT b and VAR1 denote the three mitochondrially synthesized subunits of cytochrome oxidase, apocytochrome b and the variant 1 ribosomal protein respectively. The 42 and 38 kDa polypeptides correspond to the bi2 maturase–upstream exons fusion protein (G1909, P144) and its truncated form (W91) respectively. The panel on the right represents a separation of the translation products accumulated in mutants W91 (S.c and S.ca) on an SDS–polyacrylamide (15%) gel.

of electrophoretic separation were modified (Figure 6, autoradiograph on the right).

Altogether, analysis of mitochondrial transcription and translation products from the mutant strains clearly shows that the *S.capensis* bi2 intron-encoded protein is also necessary for the excision of the intron itself and for the correct splicing of the pre-mRNA into *cyt b* mRNA. Thus, the wild-type *S.capensis* bi2 intron-encoded protein is the first group I intron-encoded protein that functions simultaneously as a DNA endonuclease and as an RNA maturase.

Having demonstrated that the wild-type *S.capensis* bi2 intron-encoded protein is bifunctional, we have adressed the question of whether the two missense mutations (Phe221 and Asn250) present in strain ST30/1 (Figure 4B) which abolish maturase activity also affect the I-*Sca*I endonuclease activity. As shown in Figure 2D, there was no detectable double strand cleavage when specific substrates were incubated with mitochondrial extract from this strain that accumulates the mutated protein (Figure 6, lane G1909 S.ca). These results indicate that the Val221 and Ile250 residues located in the carboxy-terminal part of the bi2 intron-encoded proteins are important for both activities. It remains to be seen whether individual mutations of each of the two residues can affect one or both functions of the protein.

Discussion

We have shown that the *S.cerevisiae* bi2 intron encoding an RNA maturase gains a homing-endonuclease activity after the replacement of only two non-adjacent threonine residues at positions 212 and 239 by alanine and histidine respectively. These amino acids are located in the C-terminal part of the bi2 ORF which encodes a protein of 280 residues. Importantly, they are located outside of the evolutionary conserved P1-P2 (or LAGLI-DADG) motifs.

It should be remembered that these motifs have been found in all mitochondrial intron-encoded proteins shown to have a maturase activity, as well as in nearly all experimentally demonstrated intron- and intein-encoded homing-endonucleases (reviewed by Lambowitz and Belfort, 1993; Cooper and Stevens, 1995). Until now, most of the mutations analysed which lead to the loss of the maturase or homing-endonuclease activities have been located in these motifs. However, there is a somewhat confusing situation concerning the involvement of P1 or P2 in one or other of the activities. Early studies of mutants selected *in vivo* and located in the bi3 and bi4 maturases of *S.cerevisiae* strengthened the idea that the P2 motif is important for this activity (Anziano *et al.*, 1982; De la Salle *et al.*, 1982; Lamb *et al.*, 1983; Lazowska *et al.*, 1989). Later, it was shown that the introduction of missense mutations (by *in vitro* mutagenesis) in the P1 motif of the bi4-encoded maturase which splices its own intron and the intron ai4 of the *coxI* gene, completely abolished the maturase activity (Perea *et al.*, 1990). Thus, in the case of the bi4 intron-encoded protein, the integrity of both motifs seems to be necessary for intron splicing. Mutational analysis of P1-P2 motifs in the intein-encoded homing-endonucleases (PI-*Tli*I and PI-*Sce*I), has provided strong evidence that these dodecapeptides may comprise a part of the catalytic centre (Hodges *et al.*, 1992; Gimble and Stephen, 1995). Finally, a specialization of function between the two motifs was reported for the ai4 intron-encoded protein which shares 60% identity with bi4 maturase, showing that the P1 motif is important for endonuclease activity while the P2 motif is important for maturase functions (Henke *et al.*, 1995).

Although, it is clear that most changes in the conserved dodecapeptides abolish or considerably diminish the activities, it remains to be seen in which way, directly or indirectly, they could affect the maturase or endonuclease functions. It is likely that intron-encoded proteins, depending on the function in which they assist, may be needed to stabilize structural elements, or may be needed to actually promote the catalytic reactions. The mutations in the P1-P2 motifs could affect the folding and thus globally perturb the protein conformation. These motifs, although separated by ~100 residues in the primary sequence, may be close to each other in the tertiary folding of the protein. A mutation in one motif could alter the structural properties of the second and consequently modify the affinity/activity of the protein for one or both polynucleotides (DNA, RNA). Obviously, knowledge of the structure of the LAGLI-DADG-type proteins will be of crucial importance to understand the structure–function relationships of group I intron-encoded proteins. However, in no single instance has a gain of function been observed by mutations located in P1-P2 motifs.

Our studies emphasize the involvement of the region C-terminal to the P2 motif in the gain of endonuclease function and the maintenance of maturase function. This is evidenced by the fact that the two residues (Ala212,His239) are needed for the acquisition of homing activity. Importantly, they do not affect the maturase function. Furthermore, the mutational changes, Val221 to Phe and Ile250 to Asn, abolish not only the maturase function but also the I-*Sca*I endonuclease activity. We note that positions 212 and 239 which are crucial for homing function are in close proximity to positions 221 and 250 which are essential for both activities and not too distant from the P2 motif (located from position 193 to 204). It is tempting to speculate, at least for Ala212 and His239, that although not adjacent in the primary sequence they could be in close proximity in the folded protein and might be involved in the catalytic site. The structural specificity needed to facilitate RNA catalysis is not affected, since the Ala212,His239-substituted maturase splices its own intron.

In this work, we present the first case of a group I intron-encoded protein which ordinarily functions as a specific DNA endonuclease promoting its own intron homing and as an RNA maturase promoting its own intron splicing. Based on what is known about DNA endonucleases recognizing the duplex DNA target site during homing and what is presumed about RNA maturase recognizing the duplex RNA splice junctions during splicing, it could be suggested that the *S.capensis* bifunctional protein may be able to recognize and act upon both RNA and DNA substrates. Alternatively, this protein might recognize the DNA target sequence only. This possibility is suggested by the observations that I-*Sce*I and I-*Sce*III exhibit asymmetrical binding to DNA exon sequences (Perrin *et al.*, 1993; Schapira *et al.*, 1993). Such asymmetrical binding conceivably could modulate the RNA transcription, leading to a productive folding of ribozyme and RNA splicing. However, neither I-*Sce*I nor I-*Sce*III display an RNA maturase activity.

The demonstration of two homologous intron-encoded proteins, one of which is bifunctional and the other unifunctional but able to acquire the second function by only two amino acid substitutions, is also important in evolutionary terms. The fact that mitochondrial intron-encoded homing-endonucleases recognize the exonic sequences flanking the intron insertion site which in RNA are essential elements that define 5′ and 3′ splice sites, is consistent with the idea that the maturase function evolved from the endonuclease function which originated first as a means of intron propagation. Thus, the *S.capensis* bifunctional protein can be considered as incipient of mitochondrial group I intron-encoded protein evolution in which the two activities have not yet diverged. The *S.cerevisiae* protein with two mutations fixed, Thr212 and Thr239, has just lost the original function and maintained the acquired one.

Materials and methods

Media

The following media were used for the growth of yeast: YPGA (1% yeast extract, 1% peptone, 2% glucose, 20 mg/l adenine); YP10 (1% yeast extract, 1% peptone, 10% glucose); N3 (as YP10 but 2% glycerol instead of glucose); YPGal (1% yeast extract, 1% peptone, 0.1% glucose, 2% galactose); WO (0.67% yeast nitrogen base without amino acids, 2% glucose); WO10 (as WO but 10% glucose); and WOT (WO supplemented with 2 mg/l adenine, 10 mg/l histidine, 10 mg/l tyrosine, 60 mg/l leucine, 0.75% sorbitol and 0.75% mannitol). When required, N3 medium was supplemented with appropriate antibiotics.

Extract preparation and endonuclease assay

Cells were grown on 2% galactose (YPGal) and harvested at the early exponential phase. Mitochondria were isolated as described by Hudspeth *et al.* (1980) and purified on a discontinous sucrose gradient according to Sargueil *et al.* (1990). Collected mitochondria were lysed with 1 M KCl and 1% NP-40 (Wenzlau *et al.*, 1989) and extracts were dialysed overnight against 50 mM Tris–HCl, pH 7.5, 2 mM EDTA, 100 mM NaCl, 2 mM dithiothreitol (DTT), 0.1 mM phenylmethylsulfonyl fluoride (PMSF) and 10% glycerol.

All mitochondrial extracts were made from diploid strains. For this purpose, the *S.cerevisiae* and *S.capensis* wild-type haploid strains (CKYL2 and ST13, respectively) were crossed with the *rho*° KL14-4A/60 (Groudinsky *et al.*, 1981).

The following two recombinant plasmids (provided by Dr G.L.Tian) were used as substrates for endonuclease assay. pYGT19 contains a 1.19 kb fragment of an intronless *cyt b* gene (strain CKYL2, Szczepanek *et al.*, 1994) from an *Hpa*II site 344 bp upstream of the start codon of B1 through the *Bgl*II site in exon B6, cloned in the *Acc*I–*Bam*HI sites of pUC13. pYGT28 contains the 28 bp insert which carries the exonic sequences flanking the bi2 intron in the *cyt b* gene from strain 777-3A (Lazowska *et al.*, 1980). Two complementary oligonucleotides (sequences shown in Figure 2A) were synthesized, hybridized and cloned into the *Hin*dIII–*Bam*HI sites of pUC13.

For the endonuclease assay, the mitochondrial extracts (15–20 μg of protein) were incubated with ~100 ng of linearized substrates at 30°C for 5 min as described by Wenzlau *et al.* (1989). The cleavage products were separated on a 1% agarose gel, blotted and hybridized to pYGT19 labelled by nick translation using three [α-^{32}P]dNTPs.

Oligonucleotide-directed mutagenesis

The *Bgl*II–*Bam*HI mitochondrial fragment carrying the wild-type sequence of the bi2 intron (from *S.cerevisiae* or *S.capensis*), was cloned into the *Bam*HI site of pUC19, then transfered to M13mp18 phage (using the *Eco*RI and *Pst*I sites of the polylinker which flank the mitochondrial insert). The mutagenesis was performed using Muta-Gene M13 Kit (Bio-Rad) according to the manufacturer's recommendations.

To introduce the nucleotide changes corresponding to the *S.capensis* amino acids into the *S.cerevisiae* bi2 ORF, the following oligonucleotides were used (changed bases in bold and underlined): aspartic acid at position 78, 5′-GGTCCATTAAATATA**G**ACATTTTATCAATTA-3′; threonine at position 172, 5′-GGTATATTAAAAATA**C**ATCTGGAAAAGGTAA-3′; alanine at position 212, 5′-TAAAGGTTTAAAATTC**G**CAACTAATT-GTTTTAG-3′; histidine at position 239, 5′-TATTAAATCTACTATT-CA**T**AAAGGCAATAAAGAAA-3′. After verification by sequencing, the

fragment was recloned into the *Eco*RI–*Pst*I sites of pUC19 and used for transformation of mitochondria.

To introduce into the *S.capensis* bi2 intron the nucleotide changes corresponding to *S.cerevisiae* splicing-deficient mutations, the following oligonucleotides were used: phenylalanine at position 221, 5′-TTTA-GTTATATAAAGAT**T**TTCAATATTTAC-3′; asparagine at position 250, 5′-ATACACAATTTGTAA**A**TTATGTATGAAAAGA-3′; ochre at position 260, 5′-AATCTATCCTATTT**A**AACTAAAATTGTATC-3′; and a T at position 961, 5′-ATTATATGATCCCAA**T**AGAATACACCAATAA-3′. After verification by sequencing, the mutated fragments were inserted into the *Xba*I–*Eco*RI sites of the plasmid pJM2 which carries the *coxII* gene (Mulero and Fox, 1993).

Biolistic transformation of mitochondria and screening of transformants

For biolistic transformation, 30 μg of mutant plasmid DNA and 5 μg of YEP352 plasmid DNA (which contains the *URA3* gene allowing the scoring of nuclear transformants) were co-precipitated, resuspended in 10 μl of sterile water and mixed with 50 μl of 1.0 μm gold particles at a concentration of 60 mg/ml (supplied in the Standard Pressure Kit Particle Delivery System, Bio-Rad). Microcarrier preparation and coating DNA onto microcarriers were according to the manufacturer's protocol. Aliquots (6 μl) of microcarriers were used for a high-velocity microprojectile bombardment co-transformation of the recipient strain, (W303-1B/A/50, *rho°*, *MAT***a**, *ura3*, *his3*, *leu2*, *ade2*, *trp1*) (Szczepanek, 1995) with a Biolistic PDS-1000/He Particle Delivery System (Bio-Rad). The lawn of the *rho°* strain (~ 10^8 cells/ml) was evenly spread onto the WOT plates. After 5–6 days incubation at 28°C, the plates with URA^+ nuclear transformants were replica mated to a lawn of different tester strains.

For screening mitochondrial transformants containing modifications in the *S.cerevisiae* intron bi2, the tester strain was G1909 (*rho⁺*, *mit⁻* *MAT*α, *ade1*, *op1*) which derives from the wild-type strain 777-3A and carries two non-reverting missense bi2 mutations (Lazowska *et al.*, 1980). After 2 days at 28°C, the cells were replica plated to the plates containing 2% glycerol (N3). The identification of mitochondrial transformants was based upon their ability to produce respiratory-competent diploids. The presence of such diploids is expected from the fact that the mtDNA of all rho^- strains carries the wild-type alleles for the G1909 mutations, and the equivalent *S.capensis* rho^+ strain is able to grow on glycerol-containing medium. The haploid synthetic rho^- strains were purified through at least three rounds of subcloning and screening by the replica mating assay. The relevant regions of mtDNA of subcloned diploids were then confirmed by DNA sequencing. The chosen diploids were sporulated and the appropriate haploids were selected to test the mobility of the modified bi2 intron.

The identification of mitochondrial transformants containing splicing-deficient mutations in the *S.capensis* intron bi2 was based upon their ability to produce respiratory-competent diploids when mated to a tester strain (TF145, *MAT*α *ade2*) bearing a *coxII* deletion mutant (Mulero and Fox, 1993). The mtDNA of subcloned stable synthetic rho^- clones was integrated, by mating, to the wild-type strain ST13-43, (*MAT*α *lys2*, mitotic segregant of strain ST13, Golik *et al.*, 1995). The diploids carrying mutations in the *S.capensis* bi2 intron were selected in three steps. First, for each cross, ~180 individual diploids were picked at random, grown on microtitration plates (96-well) in a complete medium (YPGA, 300 μl/well) and replicated on N3 plates to check their ability to respire. Total DNA was extracted in the plates in which the diploids were grown (according to di Rago *et al.*, 1990), loaded on Hybond filters and analysed by dot–blot hybridizations with the bi2 intron and coxI exon probes. The genetic and molecular analysis allowed us to distinguish between the rho^+, rho^+ mit^- and rho^- mtDNAs. Next, the respiratory-deficient diploids having a rho^+ mitochondrial genome and the parental haploids (as controls) were selected and analysed by a rapid screening technique based on hybridization with synthetic oligonucleotides overlapping the mutational changes and the relative stability of homo- and heteroduplexes during differential dehybridization. The four oligonucleotides carrying mutations at positions 221, 250, 260 and 961, described above, were used in hybridization and dehybridization experiments which were carried out as described in di Rago *et al.* (1990) and Lazowska *et al.* (1994). Finally, the introduction of mutational changes was confirmed by direct sequencing of total mtRNA.

Measurement of the transmission of mitochondrial markers

Haploid cells with homoplasmic genomes containing a modified bi2 intron (donor genomes) were crossed to haploid cells with an intronless genome (recipient genome). After synchronous mating performed in a complete non-selective medium (YP10) and segregation (~15 generations) in minimum anaerobic medium (WO10), the progeny of the cross were subjected to quantitative genetic and molecular analysis as described in Szczepanek *et al.* (1994).

The cells were plated on minimal medium (WO), and individual prototrophic diploid colonies were then picked at random and grown on complete medium (YPGA, 300 μl/well) for 2 days in 96-well microtitration plates. Plates were replicated on selective media (WO, N3 and glycerol with appropiate antibiotics) to determine the transmission of genetic non-polar markers. Total DNA extracted from the same colonies was checked for transmission of individual introns by dot–blot hybridizations using specific exon and intron probes.

Molecular probes

The coxI exon probe was pYGT21, a recombinant plasmid which contains most of the intronless *S.cerevisiae coxI* gene (provided by Dr G.L.Tian). The cyt b exon probe was pYGT19 (see description above). The bi2 probe was pYJL5 (Lazowska *et al.*, 1989) and the ai4 probe was puBO3 (provided by Dr P.Netter). DNA was made radioactive by nick-translation (Sambrook *et al.*, 1989) with [α-^{32}P]dATP.

Sequence determination

For DNA sequencing, the mitochondrial *Bgl*II–*Bam*HI fragment from the wild-type or mutant strains was cloned in the *Bam*HI site of pUC19, transferred to M13mp18 and M13mp19 phages and sequenced using the Sequenase Version 2.0 kit (U.S. Biochemical).

For RNA sequencing, the total mtRNA was extracted and purified as described below. RNA was sequenced by primer extension with avian myeloblastosis virus reverse transcriptase (from Amersham) according to Tian *et al.* (1991).

Analysis of mitochondrial transcripts

Isolation and purification of total mtRNA from galactose-grown cells (100 ml liquid culture) were performed essentially according to di Rago *et al.* (1990) with minor modifications. Cell walls were digested at 37°C for 1 h in 12 ml of 1.35 M sorbitol, 100 mM EDTA, 20 mM Tris–HCl pH 7.4, 0.3% β-mercaptoethanol and 0.5 mg/ml of zymolyase 100 000. After the spheroplast lysis and purification of mitochondria, the mitochondrial pellet was transferred to Eppendorf tubes and lysed in 500 μl of 2% SDS, 10 mM Tris–HCl pH 7.4 and 1 mM EDTA. Then, proteins were phenol extracted and nucleic acids were ethanol precipitated.

Total mtRNA was separated through denaturating formaldehyde–1.0% agarose gel, transferred to Hybond C filters (Amersham), and Northern hybridizations were essentially as described by Sambrook *et al.* (1989).

Analysis of mitochondrial translation products

Aerobic cultures and labelling of cells with $^{35}SO_4$ (500 μCi/ml) in the presence of cycloheximide (0.6 mg/ml) were carried out as described by Groudinsky *et al.* (1981). The proteins were separated by electrophoresis on SDS–polyacrylamide gradient gels (10–15%). Labelled translation products were visualized by autoradiography of the dried slab gels.

Acknowledgements

We thank P.P.Slonimski, C.J.Herbert and C.Monteilhet for critical reading of the manuscript and helpful discussions, G.L.Tian for construction of pYGT19 and pYGT28 plasmids and T.D.Fox (Cornell University, Ithaca) for providing the pJM2 plasmid and tester strain. T.S. is the recipient of a postdoctoral fellowship from the CNRS within the framework of the Jumelage Franco-Polonais. This study was partly supported by grants from G.R.E.G. no.15/94 and 76/94.

References

Anziano,P.Q., Hanson,D.K., Mahler,H.R. and Perlman,P.S. (1982) Functional domains in introns: *trans*-acting and *cis*-acting regions of intron 4 of the *cob* gene. *Cell*, **30**, 925–932.

Banroques,J., Delahodde,A. and Jacq,C. (1986) A mitochondrial RNA maturase gene transferred to the yeast nucleus can control mitochondrial mRNA splicing. *Cell*, **46**, 837–844.

Banroques,J., Perea,J. and Jacq,C. (1987) Efficient splicing of two yeast mitochondrial introns controlled by a nuclear encoded maturase. *EMBO J.*, **6**, 1085–1091.

Cech,T.R. (1990) Self-splicing of group I introns. *Annu. Rev. Biochem.*, **59**, 543–568.

Claisse,M.L., Spyridakis,A., Wambier-Kluppel,M.L., Pajot,P. and Slonimski,P.P. (1978) Mosaic organization and expression of the mitochondrial DNA region controlling cytochrome *c* reductase and oxidase. II—Analysis of proteins translated from the box region. In Bacila,M., Horecker,B.L. and Stoppani,A.O.M. (eds), *Biochemistry and Genetics of Yeasts*. Academic Press, pp. 369–390.

Colleaux,L., d'Auriol,L., Betermier,M., Cottarel,G., Jacquier,A., Galibert,F. and Dujon,B. (1986) Universal code equivalent of a yeast mitochondrial intron reading frame is expressed into *E.coli* as a specific double strand endonuclease. *Cell*, **44**, 521–533.

Cooper,A.A. and Stevens,T.H. (1995) Protein splicing: self-splicing of genetically mobile elements at the protein level. *Trends Biochem. Sci.*, **20**, 351–356.

Delahodde,A., Goguel,V., Becam,A.M., Creusot,F., Perea,J., Banroques,J. and Jacq,C. (1989) Site-specific DNA endonuclease and RNA maturase activities of two homologous intron-encoded proteins from yeast mitochondria. *Cell*, **56**, 431–441.

De la Salle,H., Jacq,C. and Slonimski,P.P. (1982) Critical sequences within mitochondrial introns: pleiotropic mRNA maturase and *cis*-dominant signals of box intron controlling reductase and oxidase. *Cell*, **28**, 721–732.

di Rago,J.P., Netter,P. and Slonimski,P.P. (1990) Pseudo-wild-type type revertants from inactive apocytochrome *b* mutants as a tool for the analysis of the structure/function relationships of the mitochondrial ubiquinol-cytochrome *c* reductase of *Saccharomyces cerevisiae*. *J. Biol. Chem.*, **265**, 3332–3339.

Dujardin,G., Jacq,C. and Slonimski,P.P. (1982) Single base substitution in an intron of oxidase gene compensates splicing defect in the cytochrome *b* gene. *Nature*, **298**, 628–632.

Dujon,B. (1989) Group I introns as mobile genetic elements: facts and mechanistic speculations—a review. *Gene*, **82**, 91–114.

Dujon,B., Slonimski,P.P. and Weill,I. (1974) Mitochondrial genetics, IX. A model for recombination and segregation of mitochondrial genomes in *Saccharomyces cerevisiae*. *Genetics*, **78**, 415–437.

Gargouri,A. (1989) Recherches sur les introns de l'ADN mitochondrial chez la levure *Saccharomyces cerevisiae*: mutations, suppressions et deletions genomiques d'introns. Ph.D. Thesis, l'Université Pierre et Marie Curie, Paris VI, Paris.

Gimble,F.S. and Stephen,B.W. (1995) Substitutions in conserved dodecapeptide motifs that uncouple the DNA cleavage activities of PI-*Sce*I endonuclease. *J. Biol. Chem.*, **270**, 5849–5856.

Goguel,V., Delahodde,A. and Jacq,C. (1992) Connections between RNA splicing and DNA intron mobility in yeast mitochondria: RNA maturase and DNA endonuclease switching experiments. *Mol. Cell. Biol.*, **12**, 696–705.

Golik,P., Szczepanek,T., Bartnik,E., Stepien,P.P. and Lazowska,J. (1995) The *S.cerevisiae* nuclear gene *SUV3* encoding a putative RNA helicase is necessary for the stability of mitochondrial transcripts containing multiple introns. *Curr. Genet.*, **28**, 217–224.

Groudinsky,O., Dujardin,G. and Slonimski,P.P. (1981) Long range control circuits within mitochondria and between nucleus and mitochondria. II. Genetic and biochemical analyses of suppressors which selectively alleviate the mitochondrial intron mutations. *Mol. Gen. Genet.*, **184**, 493–503.

Henke,R.M., Butow,R.A. and Perlman,P.S. (1995) Maturase and endonuclease functions depend on separate conserved domains of the bifunctional protein encoded by the group I intron ai4α of yeast mitochondrial DNA. *EMBO J.*, **14**, 5094–5099.

Hensgens,L.A.M., Bonen,L., de Haan,M., van der Horst,G. and Grivell,L.A. (1983) Two introns in yeast mitochondrial gene: homology among URF containing introns and strain dependent variation flanking exons. *Cell*, **32**, 379–389.

Hodges,R.A., Perler,F.B., Noren,J. and Jack,W.E. (1992) Protein splicing removes intervening sequences in an archaea DNA polymerase. *Nucleic Acids Res.*, **20**, 6153–6157.

Hudspeth,M.E.S., Shumard,D.S., Tatti,K.M. and Grossman,L.I. (1980) Rapid purification of yeast mitochondrial DNA in high yield. *Biochim. Biophys. Acta*, **610**, 221–228.

Jacquier,A. and Dujon,B. (1985) An intron-encoded protein is active in a gene conversion process that spreads an intron into a mitochondrial gene. *Cell*, **41**, 383–394.

Labouesse,M., Netter,P. and Schroeder,R. (1984) Molecular basis of the 'box effect'. A maturase deficiency leading to the absence of splicing of two introns located in two split genes of yeast mitochondrial DNA. *Eur. J. Biochem.*, **144**, 85–93.

Labouesse,M., Herbert,C.J., Dujardin,G. and Slonimski,P.P. (1987) Three mutations which cure a mitochondrial RNA maturase deficiency occur at the same codon in the open reading frame of the nuclear *NAM2* gene. *EMBO J.*, **6**, 713–721.

Lamb,M.R., Anziano,P.Q., Glaus,K.R., Hanson,D.K., Klapper,H.J., Perlman,P.S. and Mahler,H.R. (1983) Functional domains in introns, RNA processing intermediates in *cis*- and *trans*-acting mutants in the penultimate intron of the mitochondrial gene for cytochrome *b*. *J. Biol. Chem.*, **258**, 1991–1999.

Lambowitz,A.M. and Belfort,M. (1993) Introns as mobile genetic elements. *Annu. Rev. Biochem.*, **62**, 587–622.

Lazowska,J., Jacq,C. and Slonimski,P.P. (1980) Sequence of introns and flanking exons in wild-type and *box3* mutants of cytochrome *b* reveals an interlaced splicing protein coded by an intron. *Cell*, **22**, 333–348.

Lazowska,J., Claisse,M., Gargouri,A., Kotylak,Z., Spyridakis,A. and Slonimski,P.P. (1989) Protein encoded by the third intron of cytochrome *b* gene in *Saccharomyces cerevisiae* is an mRNA maturase. Analysis of mitochondrial mutants, RNA transcripts, proteins and evolutionary relationships. *J. Mol. Biol.*, **205**, 275–289.

Lazowska,J., Szczepanek,T., Macadre,C. and Dokova,M. (1992) Two homologous mitochondrial introns from closely related *Saccharomyces* species differ by only a few amino acid replacements in their open reading frames: one is mobile, the other is not. *C.R. Acad. Sci. Paris*, **315**, 37–41.

Lazowska,J., Meunier,B. and Macadre,C. (1994) Homing of a group II intron in yeast mitochondrial DNA is accompanied by unidirectional co-conversion of upstream-located markers. *EMBO J.*, **13**, 4963–4972.

Macreadie,I.G., Scott,R.M., Zinn,A.R. and Butow,R.A. (1985) Transposition of an intron in yeast mitochondria requires a protein encoded by that intron. *Cell*, **41**, 395–402.

Meunier,B., Tian,G.L. Macadre,C., Slonimski,P.P. and Lazowska,J. (1990) Group II introns transpose in yeast mitochondria. In Quagliariello,E., Papa,S., Palmieri,F. and Saccone,C. (eds), *Structure, Function and Biogenesis of Energy Transfer Systems*. Elsevier, Amsterdam, pp. 169–174.

Michel,F., Jacquier,A. and Dujon,B. (1982) Comparison of fungal mitochondrial introns reveals extensive homologies in RNA secondary structure. *Biochimie*, **64**, 867–881.

Moran,J.V., Wernette,C.M., Mecklenburg,K.L., Butow,R.A. and Perlman,P.S. (1992) Intron ai5a of the *coxI* gene of yeast mitochondrial DNA is a mobile group I intron. *Nucleic Acids Res.*, **15**, 4069–4076.

Mulero,J.J. and Fox,T.D. (1993) Alteration of the *Saccharomyces cerevisiae COX2* mRNA 5′-untranslated leader by mitochondrial gene replacement and functional interaction with the translational activator protein PET111. *Mol. Biol. Cell*, **4**, 1327–1335.

Perea,J., Delahodde,A., Goguel,V., Hatat,D., Sargueil,B. and Jacq,C. (1990) Function of intron-encoded proteins from yeast mitochondrial genome. RNA maturase and DNA endonuclease. In Quagliariello,E., Papa,S., Palmieri,F. and Saccone,C. (eds), *Structure, Function and Biogenesis of Energy Transfer Systems*. Elsevier, Amsterdam, pp. 205–208.

Perrin,A., Buckle,M. and Dujon,B. (1993) Asymmetrical recognition and activity of the I-*Sce*I endonuclease on its site and on intron–exon junctions. *EMBO J.*, **12**, 2939–2947.

Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning: A Laboratory Manual*. 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sargueil,B., Hatat,D., Delahodde,A. and Jacq,C. (1990) *In vivo* and *in vitro* analyses of an intron-encoded DNA endonuclease from yeast mitochondria. Recognition site by site-directed mutagenesis. *Nucleic Acids Res.*, **18**, 5659–5665.

Sargueil,B., Delahodde,A., Hatat,D., Tian,G.L., Lazowska,J. and Jacq,C. (1991) A new specific endonuclease activity in yeast mitochondria. *Mol. Gen. Genet.*, **225**, 340–341.

Schapira,M., Desdouets,C., Jacq,C. and Perea,J. (1993) I-*Sce*III: an intron-encoded DNA endonuclease from yeast mitochondria. Asymmetrical DNA binding properties and cleavage reaction. *Nucleic Acids Res.*, **21**, 3683–3689.

Séraphin,B., Faye,G., Hatat,D. and Jacq,C. (1992) The yeast mitochondrial intron ai5α: associated endonuclease activity and *in vivo* mobility. *Gene*, **113**, 1–8.

Szczepanek,T. (1995) Mobilité des introns de groupe I: recherches sur les domaines fonctionnels des activités endonucléasique et maturasique des protéines introniques des mitochondries de levure. Ph.D. Thesis, l'Université Paris XI, Orsay.

Szczepanek,T., Macadre,C., Meunier,B. and Lazowska,J. (1994) Two homologous introns from related *Saccharomyces* species differ in their mobility. *Gene*, **139**, 1–7.

Szostak,J.W., Orr-Weaver,T.L., Rothstein,R.J. and Stahl,W.F. (1983) The double-strand-break repair model for recombination. *Cell*, **33**, 25–35.
Tian,G.L., Michel,F., Macadre,C., Slonimski,P.P. and Lazowska,J. (1991) Incipient mitochondrial evolution in yeasts. II. The complete sequence of the gene coding for cytochrome *b* in *Saccharomyces douglasii* reveals the presence of both new and conserved introns and discloses major differences in the fixation of mutations in evolution. *J. Mol. Biol.*, **218**, 747–760.
Waring,R.B., Davies,R.W., Scazzocchio,C. and Brown,T.A. (1982) Internal structure of a mitochondrial intron in *A.nidulans*. *Proc. Natl Acad. Sci. USA*, **79**, 6332–6336.
Weiss-Brümmer,B., Rüdel,G., Schweyen,R.J. and Kaudewitz,F. (1982) Expression of the split gene *cob* in yeast: evidence for a precursor of a 'maturase' protein translated from intron 4 and preceding exons. *Cell*, **29**, 527–536.
Wenzlau,J.M., Saldanha,R.J., Butow,R.A. and Perlman,P.S. (1989) A latent intron-encoded maturase is also an endonuclease needed for intron mobility. *Cell*, **56**, 421–430.

Received on February 5, 1996