Mol Gen Genet (2000) 264: 137–144
Digital Object Identifier (DOI) 10.1007/s004380000297

ORIGINAL PAPER

T. Szczepanek · K. Jamoussi · J. Lazowska

Critical base substitutions that affect the splicing and/or homing activities of the group I intron bi2 of yeast mitochondria

Received: 31 January 2000 / Accepted: 30 May 2000 / Published online: 7 July 2000

Abstract The second intron (bi2) of the *cyt b* gene from related *Saccharomyces* species has an extraordinarily conserved sequence and can have different functions in wild-type cells. The protein encoded by the *S. cerevisiae* intron functions as a maturase to promote intron splicing, while the homologous *S. capensis* intron encodes a bifunctional protein that acts both as a maturase and as a homing endonuclease (I-*Sca*I) promoting intron mobility. The protein encoded by intron bi2 belongs to a large gene family characterized by the presence of two conserved LAGLIDADG motifs (P1 and P2). In this study, we analysed a set of splicing-deficient mutants of the *S. cerevisiae* intron bi2 that carry non-directed mutations affecting the maturase activity, and a set of directed missense mutations introduced into the bifunctional protein encoded by the *S. capensis* intron. Analysis of these mutations has allowed identification of the residues in the conserved P1 and P2 motifs which are crucial for splicing and homing activities. Moreover, several mutations which are located in the C-terminal part of the protein have been found to affect both functions.

Key words Group I intron · Maturase/Homing endonuclease · Intronic protein mutations · Yeast · Mitochondria

Introduction

Almost all proteins encoded by group I introns belong to a large family whose members share the two conserved LAGLIDADG amino acid motifs also referred to as P1 and P2 motifs (Dujon 1989; Cech 1990; Lambowitz and Belfort 1993). Some of these proteins are RNA maturases, required for efficient intron splicing, while others are site-specific DNA endonucleases that promote intron (or intein) homing (Lambowitz and Belfort 1993; Belfort and Roberts 1997; Jurica and Stoddard 1999). Among the more than 130 known proteins with LAGLIDADG motifs only five have been shown to function as maturases. This function was demonstrated genetically by showing that mutations in the intron's ORF lead to a splicing deficiency which can be complemented in *trans* by the wild-type protein in vivo. This demonstration was carried out for the proteins encoded by the second (bi2, Lazowska et al. 1980; Szczepanek and Lazowska 1996), third (bi3, Lazowska et al. 1989) and fourth (bi4, Anziano et al. 1982; De la Salle et al. 1982; Weiss-Brümmer et al. 1982) introns of the *cyt b* gene in *Saccharomyces cerevisiae*. The *S. cerevisiae* bi4 maturase activity was also confirmed by showing that engineered forms expressed in the cytoplasm and imported into mitochondria could complement maturase-deficient mutations (Banroques et al. 1986, 1987). Each maturase is required for the splicing of the intron that encodes it. The bi4 maturase is also required for splicing of the intron ai4 of the *coxI* gene and could thus regulate the coordinate expression of the two genes (Labouesse et al. 1984). So far, the activity of yeast maturases has not been tested biochemically and their exact role in the processing of mitochondrial precursors remains to be elucidated. Recently, it has been reported that the maturase encoded by the group I intron (AnCOB) of the *cyt b* gene in *Aspergillus nidulans* mitochondria is required to stabilize the tertiary structure of the RNA and to activate splicing in the presence of low concentration of Mg^{2+} (Ho et al. 1997; Ho and Waring 1999).

Numerous members of the LAGLIDADG family function as homing endonucleases. The cleavage of the target site (an intronless allele) by the intron-encoded endonuclease initiates gene conversion, leading to the propagation of the intron. Several homing endonucleases have been well characterized biochemically, and

Communicated by D. M. Lonsdale

T. Szczepanek · K. Jamoussi · J. Lazowska (✉)
Centre de Génétique Moléculaire du CNRS,
91198 Gif-sur-Yvette Cedex, France
E-mail: lazowska@cgm.cnrs-gif.fr
Fax: +33-1-69075539

recently the structures of the group I intron-encoded I-*Cre*I (Heath et al. 1997), the archeal intron-encoded I-*Dmo*I (Silva et al. 1999) and the intein-encoded PI-*Sce*I endonucleases (Duan et al. 1997) were determined.

An important finding concerning the LAGLIDADG family was the discovery of bifunctional proteins, demonstrating that maturase and endonuclease activities are very closely associated. Both activities have been demonstrated, in vivo in wild-type cells, for the protein encoded by the *S. capensis* intron bi2, and in vitro for the protein encoded by the *A. nidulans* intron AnCOB (Szczepanek and Lazowska 1996; Ho et al. 1997). In *Schizosaccharomyces pombe*, one intron which encodes a maturase is mobile in crosses and is likely to encode a DNA endonuclease (Schäfer et al. 1994). Also, it has been shown that the two unifunctional proteins in wild-type cells, the I-*Sce*II homing endonuclease (encoded by intron ai4 of the *cox1* gene, Delahodde et al. 1989; Wenzlau et al. 1989) and the *S. cerevisiae* bi2 maturase, can acquire the second function by mutation. The ai4-encoded protein becomes a latent maturase as a result of second-site suppressor mutations located either in the intron ORF (mim2-1, Dujardin et al. 1982) or in the nuclear gene *NAM2*/*MLRS* (NAM2-1, Labouesse et al. 1987), while the *S. cerevisiae* bi2-encoded protein acquires endonuclease activity on replacement of two residues in the intron ORF (Szczepanek and Lazowska 1996).

Studies of mutations in intron- or intein-encoded proteins which lead to the loss of the maturase or homing activities show that most of them occur in the conserved P1 and P2 motifs. Analysis of mutants located in the bi4-encoded maturase shows that the integrity of both motifs is necessary for intron splicing (Anziano et al. 1982; Lamb et al. 1983; Perea et al. 1990). The only missense mutation known to inactivate the bi3 maturase alters the invariant glycine residue in the P2 motif (Lazowska et al. 1989) and interestingly, the equivalent mutation was previously reported for the bi4 maturase (Anziano et al. 1982; Lamb et al. 1983). Mutational studies of the P1-P2 motifs of intein-encoded endonucleases (PI-*Tli*I and PI-*Sce*I) have 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-encoded protein when it was shown that the P1 motif is important for the homing function while the P2 motif is important for splicing activity (Henke et al. 1995).

In the case of the bi2 intron-encoded proteins from *S. cerevisiae* and *S. capensis*, the only known missense mutations (occurring in the double mutant) that abolished maturase and endonuclease activities are located outside the conserved motifs (Lazowska et al. 1980; Szczepanek and Lazowska 1996). In this work, we analysed four *trans*-acting splicing-deficient mutations located in the *S. cerevisiae* bi2 maturase and introduced six missense mutations into the bifunctional bi2 protein from *S. capensis*. We identified several residues located in the conserved P1 and P2 motifs which are crucial for both activities. We have also identified a region in the C-terminal part of the protein that is involved in both splicing and homing functions.

Materials and methods

Yeast strains and media

The *S. cerevisiae* mutant strains are isonuclear and isomitochondrial to the wild-type strain 777-3A (*MAT*α, *ade1*, *op1*). Two *rho*$^-$ clones, B231 and B111, are derived from the strain KL14-4A (*MAT***a**, *his1*, *trp2*, *rho*$^+$). The *S. capensis* wild-type strain CST32 (*MAT*α, *his3*, *kar1-1*) was obtained by transfer of mitochondria derived from the previously constructed interspecific hybrid strain FMD3 (*S. cerevisiae* nucleus and *S. capensis* mitochondria; Lazowska et al. 1992) to the *S. cerevisiae rho*° strain.

All the media used to grow yeast cells were as described by Szczepanek and Lazowska (1996).

Construction of mutant strains

Six strains carrying missense mutations were constructed in several steps using the strategy and methodology described in Szczepanek and Lazowska (1996). Oligonucleotide-directed mutagenesis was performed using the Muta-Gene M13 kit (Bio-Rad). To introduce the mutational changes into the *S. capensis* bi2 ORF, the following oligonucleotides were used (altered bases are underlined): 5′-GGTTCAATATTAGGATCTGGTCATGCTGAAAA-3′ (serine at position 90); 5′-CCTTTAGCTTTAGCTGATTGAATTATA-GATGA-3′ (aspartic acid at position 196); 5′-GCTATTTGAAT-TATATCTGATGGATGTAAATT-3′ (serine at position 200); 5′-ATTTGAATTATAGATTCTGGATGTAAATTAGG-3′ (serine at position 201); 5′-TTTAGTTATAAAGATTTTCAATATT-TA CTTT-3′ (phenylalanine at position 221); 5′-ATACACAAT-TTGTTAATTATGTATGAAAAGA-3′ (asparagine at position 250). After verification by sequencing, the mutated fragments were recloned into the *Eco*RI/*Pst*I sites of pUC19 and then transferred into the *Xba*I/*Eco*RI sites of the plasmid pJM2, which carries the *coxII* gene (Mulero and Fox 1993), and used for transformation of mitochondria. Cells of the *rho*° strain W303-1B/A/50 (*MAT***a**, *ura3*, *his3*, *leu2*, *ade2*, *trp1*) were bombarded with a 6:1 mixture of mutant plasmid DNA and YEp352 plasmid DNA (which contains the *URA3* gene to allow the scoring of nuclear transformants). Plates with Ura$^+$ nuclear transformants were replica-mated to lawns of a tester strain (TF145, *MAT*α, *ade2*) bearing a *coxII* deletion mutation (Mulero and Fox 1993). The identification of mitochondrial transformants was based upon their ability to produce respiration-competent diploids. The presence of the relevant regions of mtDNA in subcloned *rho*$^-$ strains (mitochondrial transformants) was confirmed by DNA sequencing. The mtDNA of subcloned stable synthetic *rho*$^-$ clones were integrated, by mating, into the wild-type strain CST32. The diploids carrying mutations in the *S. capensis* bi2 intron were analysed and selected by dot-blot hybridizations using the bi2 intron and coxI exon probes (to distinguish between *rho*$^+$ and *rho*$^-$ mtDNAs) and by a rapid screening technique based on hybridization with synthetic oligonucleotides overlapping the mutated sites and measurement of the relative stability of homo- and heteroduplexes during differential melting (to distinguish between the *rho*$^+$ and *rho*$^+$*mit*$^-$ mtDNAs). The haploid mutant strains were obtained either by sporulation of diploids or by cytoduction. The presence of mutational changes in these strains was confirmed by sequencing the relevant region of their mtDNAs.

DNA and RNA manipulations

For DNA sequencing the mitochondrial fragment from the mutant or *rho*$^-$ strains was either amplified by PCR using appropriate

oligonucleotides or cloned in the appropriate site of pUC19, transferred to M13mp18 and M13mp19 phages and sequenced using the Sequenase Version 2.0 kit (US Biochemical).

Extraction of total mtRNA, purification and Northern blot analysis were carried out as described by Szczepanek and Lazowska (1996). The molecular probes used were the recombinant plasmids pYGT19 carrying the exons of the *cyt b* gene (Szczepanek and Lazowska 1996) and pYJL5 carrying the bi2 intron sequences (Lazowska et al. 1989).

Endonuclease assay

The mitochondrial extracts from the wild-type and mutant strains were prepared as described in Szczepanek and Lazowska (1996). The recombinant plasmid pYGT28 containing the 28-bp insert which carries the exonic sequences flanking the bi2 intron was linearized with *Ssp*I before use as a substrate for endonuclease assay (Szczepanek and Lazowska 1996). In vitro endonuclease assay was performed as described by Wenzlau et al. (1989). The cleavage products were separated on a 1% agarose gel, blotted and hybridized to pYGT28 labelled by nick translation using three [α-^{32}P]dNTPs.

Determination of the transmission of mitochondrial markers

Strains with mitochondrial genomes containing a modified bi2 intron were crossed to a strain with an intronless mitochondrial genome. The progeny of the crosses were subjected to quantitative genetic and molecular analysis according to Szczepanek et al. (1994). For each cross, about 180 randomly picked diploid clones were checked on glycerol-containing media supplemented with appropriate antibiotics to determine the transmission of non-polar markers. Total DNA extracted from the same clones was checked for transmission of individual introns by dot-blot hybridizations using specific intron probes, plasmids pYJL5 for bi2 and BV02 for ai1 (Carignani et al. 1986).

Results

Splicing-deficient mutations in the bi2 intron that affect the maturase activity

Among some fifty *trans*-active splicing-deficient mutants located in the *S. cerevisiae* intron bi2 ORF isolated in vivo, only five carry missense mutations, as deduced from the observation that they accumulated the 42-kDa polypeptide corresponding to the upstream exons-bi2 maturase fusion protein (Lazowska et al. 1980; Bechmann et al. 1981). We have previously shown that one of these mutants, G1909, carries two missense mutations (V221F and I250N) located in the C-terminal part of the maturase (Lazowska et al. 1980).

To identify the residues crucial for the maturase activity, we established the sequence of the remaining four mutants which accumulated the 42-kDa polypeptide (Bechmann et al. 1981). The mutational changes identified in the mutant strains are listed in Table 1. Three out of four mutants carry a modification in the conserved P1 motif. The replacement of the first glycine in the P1 motif by aspartic acid (mutation G85D) was found in two independent mutants, M4111 and M1282, while the third mutant, M4926, carries the mutation H92P (Fig. 1A). These mutations support the idea that the integrity of the P1 motif is important for maturase activity.

The fourth mutant studied, W401, carries four mutations distributed along the maturase sequence (A5T, H120Y, S236Y and V252M) and all lie outside the two conserved motifs (Fig. 1A, Table 1). The splicing defects caused by the W401 mutations were dissected in crosses with two discriminating *rho*$^-$ clones (B111 and B231, Fig. 1A). The fact that respiration-competent diploids are observed in crosses between W401 and *rho*$^-$ B231, which, as expected from its repeat length, can correct only the W401-3 and W401-4 mutations indicates that the mutations S236Y and/or V252M are responsible for the splicing-deficient phenotype (Fig. 1A, Table 1). Interestingly, these mutations are very close to the sites of the first (V221F) and second (I250N) mutations, respectively, observed in the double mutant G1909.

To confirm that the mutations described above affect the maturase function of the bi2-encoded protein, we analysed mitochondrial transcripts from wild-type and mutant strains. The results of Northern analysis, shown in Fig. 1B, clearly indicated that all *trans*-active mutations lead to a very strong splicing defect. All mutants exhibit the same transcript patterns and accumulate only high-molecular-weight bi2-containing intermediates and do not make the mature mRNA.

Table 1 Description of the *trans*-acting splicing-deficient mutants located in the *S. cerevisiae* intron bi2. Mutated (M) nucleotides and amino acids are in *bold*. Restoration tests were performed by mating the mutant strains with two *rho*$^-$ clones, the diploids issued from the crosses were replicated onto glycerol-containing medium. + and − denote that diploids are respiration competent or respiratory deficient, respectively

Strain	Mutation	Base substitution			Codon substitution			Restoration test with *rho*$^-$ clones:	
		No.	WT	M	No.	WT	M	B231	B111
M4111	G85D	254	G	**A**	85	GGT (G)	**GAT (D)**	−	+
M1282	G85D	254	G	**A**	85	GGT (G)	**GAT (D)**	−	+
M4926	H92P	275	A	**C**	92	CAT (H)	**CCT (P)**	−	+
W401-1	A5T	13	G	**A**	5	GCC (A)	**ACC (T)**	−	+
W401-2	H120Y	358	C	**T**	120	CAT (H)	**TAT (Y)**	−	+
W401-3	S236Y	707	C	**A**	236	TCT (S)	**TAT (Y)**	+	+
W401-4	V252M	754	G	**A**	252	GTA (V)	**ATA (M)**	+	+

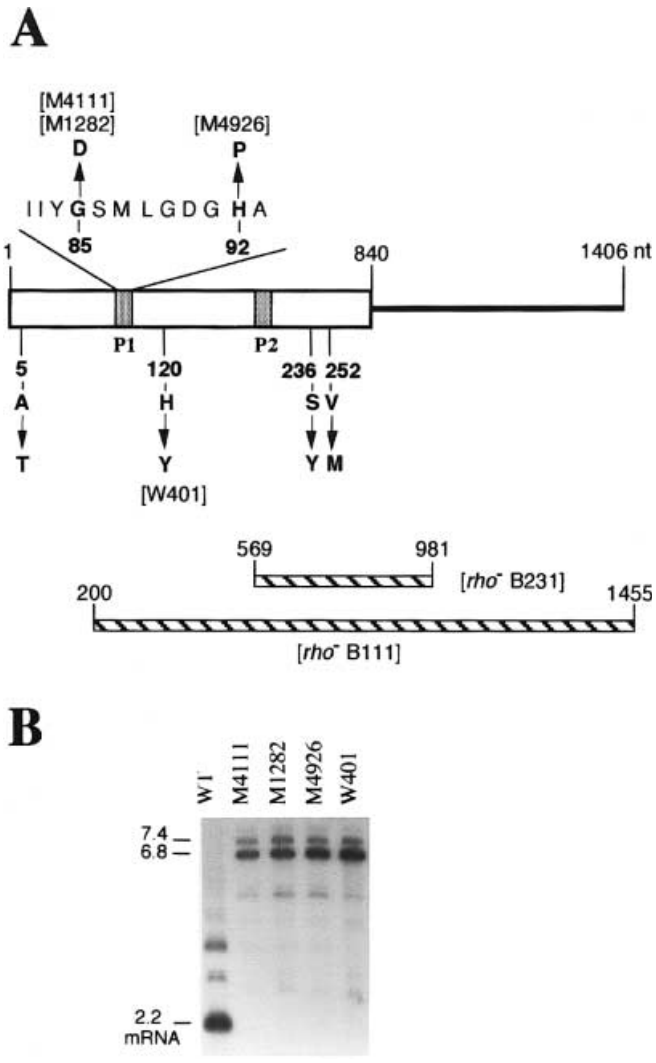


Fig. 1A, B Location and nature of *trans*-active mutations in the *S. cerevisiae* bi2 intron that affect the maturase activity of the protein product. **A** The diagram represents the bi2 intron: the *open box* denotes coding sequences (ORF) in the intron, the *shaded boxes* denote the conserved motifs P1 and P2 and the *horizontal line* indicates non-coding sequences in the intron (nt, nucleotides). The positions (*numbers in bold*) and nature (*in bold*) of mutations observed in four mutant strains (names in *brackets*) are indicated. The *hatched bars* represent the segments of the mtDNA retained in two *rho*$^-$ clones, B231 and B111, used to map the mutants. The positions of the mtDNA segments retained in the *rho*$^-$ clones are indicated. **B** Northern analysis of mutant strains. Total mtRNA was purified from isolated mitochondria, fractionated on a 1.1% denaturing agarose gel, transferred to Hybond C filters and hybridized with a *cyt b* exon probe. WT indicates the total mtRNA extracted from the wild-type *S. cerevisiae* strain 777-3A. The lengths of the transcripts is given in kb

Construction and analysis of mutants that affect the splicing and/or homing functions of the protein encoded by the *S. capensis* bi2 intron

To further study the properties of the intron-encoded proteins we introduced several missense mutations into the *S. capensis* bi2 intron-encoded protein, which differs from that encoded by the homologous intron from *S. cerevisiae* by only four amino acid replacements and acts both as a DNA endonuclease (I-*Sca*I) and an RNA maturase (Szczepanek and Lazowska 1996).

To study the effects of the P1 and P2 motifs of the bi2-encoded protein on the functions that it performs, we introduced four missense mutations and tested their effects on maturase function, endonuclease I-*Sca*I activity and intron mobility. All directed mutations were introduced into mitochondria by biolistic transformation as described in Materials and methods. We have constructed mutant strains carrying mutations that change each of the aspartic acids in the P1 and P2 motifs of bi2 to serine (D90S, D200S and D201S) and the isoleucine in the P2 motif to aspartic acid (I196D, Table 2, Fig. 2A). All strains carrying a D $\rightarrow$ S mutation are able to grow on glycerol-containing media, whereas the mutation I196D leads to the respiration-deficient phenotype. This was confirmed by Northern analysis of mtRNAs from these strains. As shown in Fig. 2B, the transcript patterns in the D $\rightarrow$ S mutants are identical to that of the wild-type strain and the RNA species that accumulate in the I196D mutant are identical to those observed in the *trans*-active mutants for the *S. cerevisiae* intron bi2 (Fig. 1B). Thus, the analysis of non-directed (see preceding paragraph) and directed mutations demonstrates that the presence of hydrophobic residues at the fourth position in the P1 (G85) and P2 (I196) motifs of intron bi2 is essential for maturase activity (Figs. 1 and 2).

We have previously shown that two missense mutations, originally observed in the *S. cerevisiae* double mutant G1909, when introduced into the *S. capensis* intron bi2, abolished its maturase and I-*Sca*I endonuclease activities (Szczepanek and Lazowska 1996). To test whether either of these mutations affects one or both functions of the protein, we constructed two single-site mutants carrying, respectively, the V221F and I250N mutations (see Materials and methods). Interestingly, in contrast to the splicing-deficient phenotype and strongly effect on intron splicing observed in the double mutant, the simple mutants carrying only one of the mutations are respiration and splicing competent (Fig. 2B).

To test the effect of introduced mutations on the I-*Sca*I endonuclease activity, in vitro assays were carried out on mitochondrial extracts from the wild-type and mutant strains as described in Materials and methods. As shown in Fig. 3A, the cleavage products are only observed when substrate is incubated with the wild-type extract and all extracts from the mutant strains have no detectable I-*Sca*I activity.

The homing of mutated introns was tested by mating the intron donor strains and the wild-type strain (used as control) with a recipient strain that has an intronless mitochondrial genome and performing a quantitative analysis of the transmission of mitochondrial markers to the progeny (see Materials and methods). The donor and recipient strains carry two mitochondrial markers conferring resistance (allele in the recipient strain) or sensitivity (allele in the donor strains) to oligomycine and erythromycin, which are known to be transmitted in

Table 2 Description of the *S. capensis* mutant strains carrying the directed mutations introduced into mtDNA

Strain[a]	Nuclear genotype	Mitochondrial mutation	Base substitution			Codon substitution		
			No.	WT	M	No.	WT	M
ST37	*MATα*, *ade2*, *his3*	D90S	268–9	GA	**TC**	90	GAT (D)	**TCT (S)**
CST21	*MATα*, *his3*, *kar1-1*	I196D	586–7	AT	**GA**	196	ATT (I)	**GAT (D)**
ST38	*MATα*, *his3*	D200S	598–9	GA	**TC**	200	GAT (D)	**TCT (S)**
ST39	*MATα*, *ura3*, *his3*	D201S	601–2	GA	**TC**	201	GAT (D)	**TCT (S)**
ST36	*MATα*, *his3*	V221F	661	G	**T**	221	GTT (V)	**TTT (F)**
CST14	*MATα*, *his3*, *kar1-1*	I250N	749	T	**A**	250	ATT (I)	**AAT (N)**

[a] All mutants were obtained as described in Materials and methods. For other explanations see the legend to Table 1

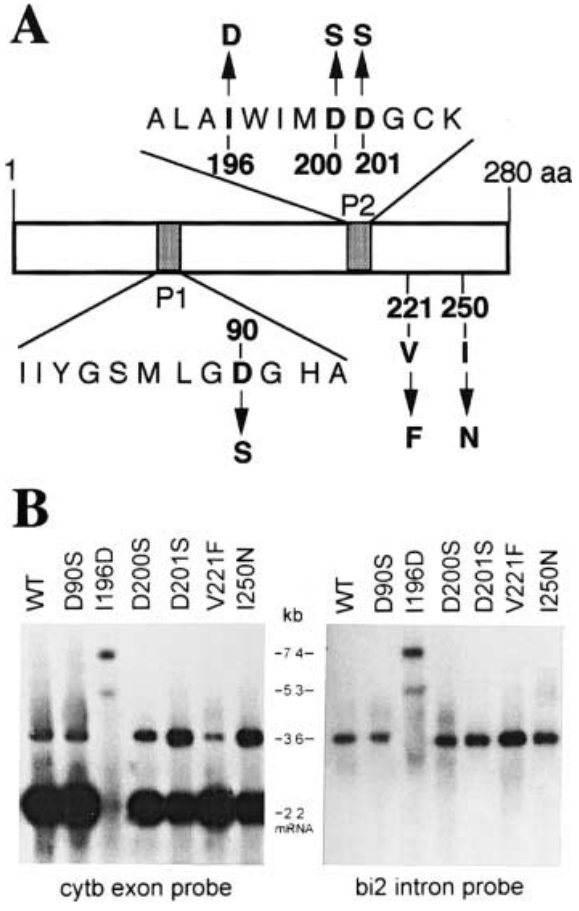
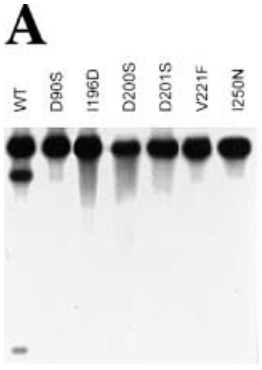


Fig. 2A, B Effects of directed mutations introduced into the *S. capensis* bi2 intron on maturase activity. **A** The diagram shows the *S. capensis* bi2 intron ORF. The locations and sequences of the P1 and P2 dodecapeptide motifs are given. The positions (*numbers in bold*) and nature (*in bold*) of six directed mutations are indicated. **B** Northern analysis of mutant strains. WT indicates the total mtRNA extracted from the wild-type *S. capensis* strain CST32. For other explanations see the legend to Fig. 1B



B

Mutation	Transmission of markers (%)			
	O^S	E^S	$bi2^+$	$ai1^+$
D90S	37	35	35	40
I196D			46	47
D200S	51	49	47	46
D201S	30	36	37	40
V221F	42	35	42	40
I250N	42	35	68	40
WT	39	49	**98**	39

Fig. 3A, B Effect of directed mutations introduced into the *S. capensis* bi2 intron on the I-*Sca*I endonuclease activity and intron mobility. **A** In vitro assay of the endonuclease activity. The linearized substrate was incubated with mitochondrial extracts from the *S. capensis* wild-type strain (WT) and six mutant strains carrying the indicated mutations. The endonuclease assays were carried out as described in Materials and methods. **B** Transmission of the modified bi2 introns to the intronless *S. cerevisiae cyt b* gene. All donor strains carrying modified bi2 introns (listed in Table 2) were crossed with the recipient strain CKYL2 (*MAT***a**, *leu1*, *kar1-1*), which has an intronless mitochondrial genome (Szczepanek et al. 1994). The wild-type strain CST32 was used as a control. Quantitative analysis of the progeny from each cross was performed as described in Materials and methods. Data for transmission of the donor markers O^S, E^S, $bi2^+$ and $ai1^+$ are given

a non-polar fashion (Lazowska et al. 1994). Since transmission of these markers cannot be measured when the strains have a respiration-deficient phenotype (mutant CST21, Table 2), we used as an additional non-polar marker the non-mobile first intron of the *coxI* gene from *S. capensis* (Szczepanek et al. 1994). The results shown in Fig. 3B clearly indicate that the transmission of the modified bi2 intron present in all mutant strains, except the mutant with the I250N mutation, is the same as that of the non-polar markers carried by the same donor genome. Thus, the five introduced mutations completely abolish the I-*Sca*I activity and mobility in crosses (Fig. 3A, B). The transmission of the bi2 intron carrying the I250N mutation is intermediate (68%) between those observed for the non-polar markers carried by the same donor genome (on average 39%) and for the mobile intron (98%) (Fig. 3B). This result indicates that the mutation I250N does not abolish but does significantly diminish the homing activity. Nevertheless, it has not been possible to detect the I-*Sca*I activity in vitro.

Taken together, the results presented above demonstrate that all strains carrying any of the different D → S, V221F or I250N mutations show no or reduced homing activity while retaining the splicing function. The mutation I196D located in the P2 motif completely destroys both activities of the protein and none of the introduced mutations has been found to affect only the maturase activity.

Discussion

This work has focused on a mutational study of the second intron of the *cyt b* gene that has allowed us to identify critical base substitutions leading to a splicing deficiency and to the loss of homing function. Analysis of the non-directed and directed mutations located in the protein encoded by the bi2 intron ORF shows that the integrity of the conserved P1 and P2 motifs is necessary for splicing and homing activities. Importantly, we have established that the region located near the P2 motif in the C-terminal part of the protein is involved in both functions of this protein.

The residues essential for maturase activity have been identified by analysing a set of mutants selected in vivo that have mutations located in the *S. cerevisiae* bi2 maturase and a set of constructed mutations introduced into the *S. capensis* bi2-encoded bifunctional protein. As we have shown previously, these two proteins differ by only four amino acid replacements, and introduction into the *S. cerevisiae* sequence of any of the four residues or different combinations of them does not affect the structural specificity needed to facilitate RNA catalysis (Szczepanek and Lazowska 1996). The decisive in vivo data show that the conserved P1 motif is essential for maturase activity. Two independent mutants have been shown to carry the same mutation (G85D) that replaces the first highly conserved glycine of the P1 motif by aspartic acid. Interestingly the same mutation (P1-G4D) introduced into the protein encoded by the ai4 intron does not affect the splicing function (Henke et al. 1995). Another mutational change that causes a very strong splicing deficiency is the replacement of histidine, which is the penultimate residue in the P1 motif of bi2, by proline (H92P, Fig. 2A). The position occupied by histidine, in contrast to the almost invariable glycine residue (at the fourth position in the P1), is variable and is mostly occupied by polar and hydrophobic residues but never by proline (Dalgaard et al. 1997). Importantly, replacement of isoleucine at the fourth position in the P2 motif by aspartic acid, as in one of the constructed mutants, blocks maturase function. These data clearly show that the integrity of the two motifs is essential for maturase activity. The involvement of both conserved motifs in the splicing function has been previously demonstrated in the case of the bi4 maturase. Substitution of the conserved aspartic acid at the seventh position in P1 (D7S, D7N and D7R) or the conserved glycine at the tenth position in the P2 motif (G10D) is detrimental to maturase activity (Anziano et al. 1982; Lamb et al. 1983; Perea et al. 1990). In contrast, the activity of the latent maturase encoded by the ai4 intron of the *cox1* gene (see Introduction) only requires the integrity of the P2 motif. Mutational analysis of this protein has shown that substitution of the conserved glycines at the fourth and tenth positions in the P2 motif by aspartic acid residues blocks maturase activity, whereas the equivalent substitutions in the P1 motif inhibit homing function (Henke et al. 1995). Although it is clear that the residues located in the conserved motifs identified previously by mutation, and in this work, must be involved in maturase activity, their exact roles (and even the exact roles of the conserved motifs) in splicing function are not known. Recently, maturase-dependent splicing in vitro has been demonstrated for the maturase encoded by the intron (AnCOB) of the *A. nidulans cyt b* gene, which is homologous to the *S. cerevisiae* intron bi3. It has been shown that the intron AnCOB which self-splices in the presence of a high concentration of Mg^{2+} requires a maturase to stabilize the tertiary structure of the RNA and to activate splicing when Mg^{2+} concentrations are low (Ho et al. 1997; Ho and Waring 1999). The splicing reaction of the yeast mitochondrial maturase-encoding group I introns, which do not self-splice in vitro (Gampel and Tzagoloff 1987; Ho et al. 1997; our unpublished results), appears to be more complex, since at least one and perhaps more nucleus-encoded protein factors are needed, together with the maturase, to promote the correct folding of the intron RNA into a catalytically active conformation (reviewed by Cech 1990; Lambowitz et al. 1999). Thus far, an in vitro system for the splicing of maturase-encoding yeast group I introns has not been developed and the mode of action of the maturase remains to be determined.

We have recently reported the in vitro characterization of the I-*Sca*I endonuclease activity using a truncated form (p28bi2) of the protein expressed in *E. coli*, and found that the properties associated with its activity are very similar to those described for other LAGLIDADG endonucleases. This protein catalyses a double-strand cleavage of the DNA downstream from the intron insertion site to give 4-nt long 3′-overhangs, and recognizes an asymmetrical 16–19 bp sequence spanning the intron insertion site (Monteilhet et al. 2000). The results of the in vivo mutational analysis of the P1-P2 motifs in the *S. capensis* bi2-encoded bifunctional protein clearly indicate that, as for maturase function, the integrity of both motifs is required for homing activity. All directed mutations located either in the P1 (D90S) or P2 (I196D, D200S and D201S) motifs completely abolished both intron mobility in crosses and I-*Sca*I endonuclease activity. The recent determination of the crystal structures of three endonucleases, I-*Cre*I (Heath et al. 1997) PI-*Sce*I (Duan et al. 1997) and I-*Dmo*I (Silva et al. 1999), shows that the conserved motifs play a central role in the structure and catalytic activity of these enzymes. The two α-helical LAGLIDADG motifs are packed in parallel to form the domain interface and provide amino acid residues to closely spaced active sites. Although the biochemical and structural studies identified a number of residues involved in the activity of, or substrate binding by, LAGLIDADG endonucleases, only the two acidic residues (either an aspartic or a glutamic acid) are conserved in all three structures. Each of these acidic residues, located at the end of the LAGLIDADG motifs, coordinates a divalent metal ion in two closely packed active sites which are suitably

positioned to cleave across the DNA minor groove (Jurica and Stoddard 1999; Silva et al. 1999). Given that the replacement of each aspartic acid residue in either conserved motif of the I-*Sca*I endonuclease inactives homing function, we are tempted to speculate that these residues are involved in the catalytic centers. Importantly, the splicing activity is not affected by the same mutations, suggesting that these aspartic acid residues are not involved in specific interaction with the RNA substrate. In contrast, the replacement of isoleucine by aspartic acid (mutation I196D) at the fourth position in the P2 motif of the bifunctional protein inactivates both activities.

Another significant result of this and previous studies of mutations that affect the functions of the bi2-encoded protein is the finding that the region located outside the conserved motifs is crucial for gain or loss of homing function and for the maintenance of maturase activity. This was established by the observation that the acquisition of endonuclease activity by the *S. cerevisiae* bi2-maturase requires the simultaneous replacement of two threonine residues at positions 212 and 239 by alanine and histidine, respectively, whereas the single substitutions (either alanine or histidine) can not activate this function (Szczepanek and Lazowska 1996). This may suggest that these two non-adjacent residues (A212 and H239) located in the vicinity of the P2 motif could be catalytic residues or be involved in the DNA binding site. The crystal structure of I-*Cre*I and phylogenetic data on related homodimeric endonucleases indicate that the residues Q47 and R70, located outside the LAGLIDADG motifs, contribute to the I-*Cre*I active site (Heath et al. 1997; Seligman et al. 1997; Turmel et al. 1997). The two missense mutations (V221F and I250N) located in close proximity to positions 212 and 239, observed first in the mutant selected in vivo (G1909) and later introduced into the bifunctional protein, are detrimental to both activities (Lazowska et al. 1980; Szczepanek and Lazowska 1996). In the present work we have provided evidence that neither of the individual mutations affects intron splicing; in contrast, each of them inhibits the homing activity to some extent. The V221F mutation completely abolished intron homing, while the second (I250N) significantly diminished it. This important result, which shows that only the occurrence of two non-adjacent mutations leads to the loss of maturase function, would suggest that these amino acids, are closely spaced in the folded protein and might be important for protein structure and/or maturase function. Additional support for the involvement of the C-terminal region proximal to the P2 motif in maturase activity was provided by the finding that two mutations, S236Y and V252M, identified as being responsible for the splicing defect of the mutant W401, selected in vivo, are also close to the sites of the G1909 mutations.

Although much progress has been made concerning the molecular mechanisms of homing and the structure and function of the LAGLIDADG endonucleases, the LAGLIDADG maturases are less well characterized. It is clear that the *S. capensis* protein, which inhibits both enzymatic activities, is an attractive model for further investigation of the relationship between maturase and endonuclease functions.

Acknowledgments We thank L. Sperling and C. Monteilhet for critical reading of the manuscript, D. Menay for the synthesis of oligonucleotides. T.S. was the recipient of a postdoctoral fellowship from the CNRS within the framework of the Jumelage Franco-Polonais. K.J. is a recipient of a doctoral fellowship from the Tunisian government.

References

Anziano PQ, Hanson DK, Mahler HR, Perlman PS (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, 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, Jacq C (1987) Efficient splicing of two yeast mitochondrial introns controlled by a nuclear encoded maturase. EMBO J 6: 1085–1091

Bechmann H, Haid A, Schweyen RJ, Mathews S, Kaudewitz F (1981) Expression of the "split gene" *COB* in yeast mtDNA. Translation of intervening sequences in mutant strains. J Biol Chem 256: 3525–3531

Belfort M, Roberts RJ (1997) Homing endonucleases: keeping the house in order. Nucleic Acids Res 25: 3379–3388

Carignani G, Netter P, Bergantino E, Robineau S (1986) Expression of the mitochondrial split gene coding for the subunit I of cytochrome oxidase in *S. cerevisiae*: RNA splicing pathway. Curr Genet 11: 55–63

Cech TR (1990) Self-splicing of group I introns. Annu Rev Biochem 59: 543–568

Dalgaard JZ, Klar AJ, Moser MJ, Holley WR, Chatterjee A, Mian IS (1997) Statistical modeling and analysis of the LAGLIDADG family of site-specific endonucleases and identification of an intein that encodes a site-specific endonuclease of the HNH family. Nucleic Acids Res 25: 4626–4638

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

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

Duan X, Gimble FS, Quiocho FA (1997) Crystal structure of PI-*Sce*I endonuclease with protein splicing activity. Cell 89: 555–564

Dujardin G, Jacq C, Slonimski PP (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

Gampel A, Tzagoloff A (1987) In vitro splicing of the terminal intervening sequence of *Saccharomyces cerevisiae* cytochrome *b* pre-mRNA. Mol Cell Biol 7: 2545–2551

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

Heath PJ, Stephens KM, Monnat RJ, Stoddard BL (1997) The structure of I-*Cre*I, a group I intron-encoded homing endonuclease. Nat Struct Biol 4: 468–476

Henke RM, Butow RA, Perlman PS (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

Ho Y, Waring RB (1999) The maturase encoded by a group I intron from *Aspergillus nidulans* stabilizes RNA tertiary structure and promotes rapid splicing. J Mol Biol 292: 987–1001

Ho Y, Kim SJ, Waring RB (1997) A protein encoded by a group I intron in *Aspergillus nidulans* directly assists RNA splicing and is a DNA endonuclease. Proc Natl Acad Sci USA 94: 8994–8999

Hodges RA, Perler FB, Noren J, Jack WE (1992) Protein splicing removes intervening sequences in an archea DNA polymerase. Nucleic Acids Res 20: 6153–6157

Jurica MS, Stoddard BL (1999) Homing endonucleases: structure, function and evolution. Cell Mol Life Sci 55: 1304–1326

Labouesse M, Netter P, 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 CJ, Dujardin G, Slonimski PP (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 MR, Anziano PQ, Glaus KR, Hanson DK, Klapper HJ, Perlman PS, Mahler HR (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 AM, Belfort M (1993) Introns as mobile genetic elements. Annu Rev Biochem 62: 587–622

Lambowitz AM, Caprara MG, Zimmerly S, Perlman PS (1999) Group I and group II ribozymes as RNPs: clues to the past and guides to the future. In: Gesteland RF, Atkins JF, Cech TR (eds) The RNA world II. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., pp 451–485

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

Lazowska J, Claisse M, Gargouri A, Kotylak Z, Spyridakis A, Slonimski PP (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, 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. CR Acad Sci Paris 315: 37–41

Lazowska J, Meunier B, 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

Monteilhet C, Dziadkowiec D, Szczepanek T, Lazowska J (2000) Purification and characterization of the DNA cleavage and recognition site of I-*Sca*I mitochondrial group I intron encoded endonuclease produced in *Escherichia coli*. Nucleic Acids Res 28: 1245–1251

Mulero JJ, Fox TD (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, Jacq C (1990) Function of intron-encoded proteins from yeast mitochondrial genome. RNA maturase and DNA endonuclease. In: Quagliariello E, Papa S, Palmieri F, Saccone C (eds) Structure, function and biogenesis of energy transfer systems. Elsevier, Amsterdam, pp 205–208

Schäfer B, Wilde B, Massardo DR, Manna F, Del Giudice L, Wolf K (1994) A mitochondrial group-I intron in fission yeast encodes a maturase and is mobile in crosses. Curr Genet 25: 336–341

Seligman LM, Stephens KM, Savage JH, Monnat RJ Jr (1997) Genetic analysis of the *Chlamydomonas reinhardtii* I-*Cre*I mobile intron homing system in *Escherichia coli*. Genetics 147: 1653–1664

Silva GH, Dalgaard JZ, Belfort M, Van Roey P (1999) Crystal structure of the thermostable archaeal intron-encoded endonuclease I-*Dmo*I. J Mol Biol 286: 1123–1136

Szczepanek T, Lazowska J (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. EMBO J 15: 3758–3767

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

Turmel M, Otis C, Coté V, Lemieux C (1997) Evolutionarily conserved and functionally important residues in the I-*Ceu*I homing endonuclease. Nucleic Acids Res 25: 2610–2619

Weiss-Brümmer B, Rödel G, Schweyen RJ, 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 JM, Saldanha RJ, Butow RA, Perlman PS (1989) A latent intron-encoded maturase is also an endonuclease needed for intron mobility. Cell 56: 421–430