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Self-splicing of the mobile group II intron of the filamentous fungus *Podospora anserina* (COI I1) *in vitro*

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The first intron of the mitochondrial gene coding for cytochrome oxidase subunit I (COI I1) of *Podospora anserina* can undergo self-splicing *in vitro* at high concentrations of NH_4Cl or KCl. Under these conditions cleavage at the 5′ splice junction takes place without branch formation probably via hydrolysis by water or OH^- and the intron is released in a linear form. *In vitro* transcripts that contain mutated introns with large deletions in nonconserved domain IV comprising >50% of the intronic sequence display a more efficient splicing reaction and, surprisingly, 5′ cleavage via transesterification and lariat formation is re-established to a low degree under NH_4Cl. In contrast to the self-splicing group II introns aI5γ and bI1 from yeast mitochondria cleavage at the 3′ splice site of the *Podospora* intron is reduced and cleavage by hydrolysis *in trans* (i.e. exon reopening) is almost completely suppressed. Both observations could be interpreted as a result of unfavourable spatial conformations of the intron that (i) lead to a steric hindrance of the 5′ exon to attack the 3′ splice site *in cis* and (ii) block intron-dependent cleavage reaction of the ligated exons *in trans*. Alternatively, the possibility that a weak overall interaction of the postulated exon- with the corresponding intron-binding sites (EBS–IBS pairings) is responsible for the remarkable differences to the self-splicing reaction of other group II introns is discussed.

Key words: COI gene/group II intron/mitochondria/*Podospora anserina*/self-splicing

Introduction

Group II introns represent one of two unrelated intron groups that have been shown to exist in the mosaic genes of organelles from fungi, algae and plants, as well as (for group I) in nuclear encoded rRNA genes of protists and genes of bacteriophage T4 (group I introns: reviewed by Cech, 1988; group II introns: reviewed by Michel *et al.*, 1989). The members of each group share distinct conserved sequence elements and a very similar potential secondary structure (Davies *et al.*, 1982; Michel *et al.*, 1982; Michel and Dujon, 1983; Schmelzer *et al.*, 1983; Waring and Davies, 1984; Burke *et al.*, 1987).

The consensus secondary structure model of group II introns, well established now by a comparative analysis of all the sequencing data available, is characterized by a typical central core comprising ~30–40 nucleotides (nt) and six radiating key base-paired regions (domains I–VI), which bring the 5′ and 3′ splice junctions in close proximity to each other (see Figure 1A). Moreover, the extensive analysis of >70 group II introns has revealed some specific differences in both the primary sequence and some potential helices, especially in subdomains of domain I, that allow the division of group II introns into subgroups A and B (Michel *et al.*, 1989).

One of the introns arranged in subgroup IIA is the well-characterized first intron of the gene encoding the cytochrome oxidase subunit I (COI I1) localized on the mitochondrial genome of the ascomycete *Podospora anserina* (Osiewacz and Esser, 1984; Cummings *et al.*, 1985; Kück *et al.*, 1985). The COI I1 represents the only group II intron described so far, which can exist in two different molecular states in mitochondria: during the course of senescence of the fungus the complete intronic sequence accumulates as a circular plasmid in mono- and multimeric forms (p1 DNA or α sen DNA) (Stahl *et al.*, 1978, 1980; Cummings *et al.*, 1979; Belcour *et al.*, 1981; Kück *et al.*, 1981; recently reviewed by Kück, 1989). Due to this unusual feature the COI I1 can also be considered as a mobile intron (Osiewacz and Esser, 1984; Esser, 1985). As in all known fungal subgroup IIA introns the mobile intron contains a very long open reading frame (ORF), which is mostly looped out of the core structure by the nonconserved domain IV. The intronic ORF shows high homologies to the ORF of a yeast group II intron RNA maturase essential for splicing of this intron *in vivo* (Carignani *et al.*, 1983) and possesses a putative coding capacity for a reverse transcriptase-like enzyme (Michel and Lang, 1985). Furthermore the involvement of the intron-encoded protein in some recombination events on the mitochondrial DNA in senescing mycelia of *Podospora* has been discussed (Schulte *et al.*, 1988). As predicted from the RNA splicing mechanism of other group II introns (see below) the *Podospora* COI I1 could be shown to excise as a lariat from the COI precursor *in vivo* (Schmidt *et al.*, 1987).

Most of what we know about the removal of group II introns from pre-mRNAs comes from the study of *Saccharomyces cerevisiae* subgroup IIB intron aI5γ, the fifth intron in the cytochrome oxidase subunit I gene, and bI1, the first intron in the cytochrome *b* gene. Both mitochondrial introns are the first ones of this group that have been reported to catalyse RNA splicing *in vitro* in the absence of any protein (Peebles *et al.*, 1986; Schmelzer and Schweyen, 1986; van der Veen *et al.*, 1986). Recently an additional self-splicing group II intron in the mitochondrial large subunit ribosomal RNA (LSUrRNA) gene of the green alga *Scenedesmus obliquus* could be identified (Kück *et al.*, 1990).

Self-splicing activity of yeast subgroup IIB introns *in vitro* has been shown to occur via a two-step transesterification

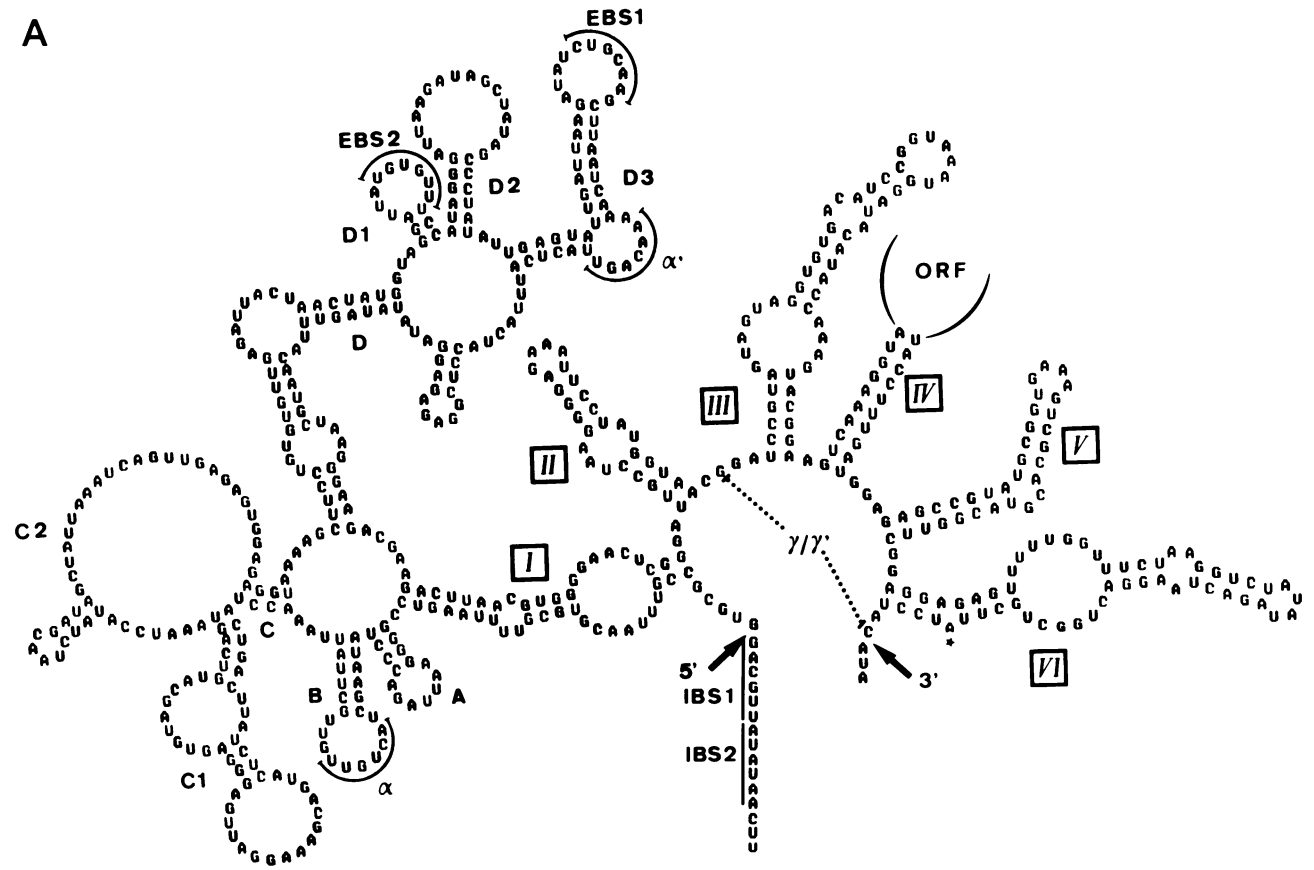

Fig. 1. Secondary structure model of *Podospora* intron COI I1 (A) and schematic representation of the 5′ part of the COI gene and T7 templates (B). (**A**) The complete sequence of COI I1 was taken from Osiewacz and Esser (1984) and refolded according to the consensus secondary structure model of subgroup IIA introns proposed by Michel *et al.* (1989). Domains of the core structure are numbered I–VI. Domain I is composed of different subdomains A, B, C and D, and two of these are subdivided further (C1, C2, D1–D3). Intron and exon bindings sites (IBS and EBS), as well as long-range intron internal base pairings α and α', γ and γ' are marked by curved or straight lines. The branchpoint adenosine is marked by an asterisk. The nomenclature of stem–loop structures and three-dimensional pairings follows that of Michel and Jacquier (1987) and Michel *et al.* (1989). (**B**) A short fragment of the 95 kb mitochondrial DNA of *P.anserina* that comprises the first exon (E1), the first intron (I1) and the second exon (E2) of the COI gene is shown at the top. Black boxes indicate exon sequences (Cummings *et al.*, 1985; Kück *et al.*, 1985), and the open box represents the intronic ORF in phase with the upstream exon (Osiewacz and Esser, 1984). Restriction sites of enzymes used for cloning and run-off transcription are marked by arrowheads (Sa, *Sac*I; D, *Dra*I; B, *Bam*HI; A, *Acc*I; H, *Hin*dII; S, *Sal*I; Bg, *Bgl*II; Sp, *Sph*I). The 3059 bp *Sac*I–*Sph*I fragment and the 2781 bp *Dra*I–*Sph*I fragment were recloned from subclones of *Podospora* mtDNA into vectors pSPT18^{TM} and pT7T3 18U^{TM}, respectively (plasmids pTP7 and pTP7/3 I1). Plasmids pTP7ΔHindII and pTP7ΔAccI are derived from pTP7 by deletion of intron internal *Hin*dII (1341 bp) and *Acc*I (1446 bp) fragments, respectively. Due to the cloning strategy all constructs contain a 542 bp fragment of pBR322 downstream of the 36 bp sequence of the second exon (see Materials and methods for details). The single *Pst*I site (P) of both cloning vectors downstream of the pBR322 fragment is also used to generate run-off precursors. The open triangle represents the T7 promoter. Dashed lines indicate vector sequences and long arrows *Sph*I and *Pst*I run-off transcripts.

pathway: the first step is cleavage at the 5′ splice junction leading to separate 5′ exon and formation of the intron lariat−3′ exon with the 5′ end of the intron covalently joined to a conserved adenosine residue near the 3′ end of the intron via a 2′,5′-phosphodiester bond. In the second step the 3′ OH of the 5′ exon attacks the phosphodiester bond at the 3′ splice site of the intron lariat−3′ exon intermediate resulting in exon−exon ligation and release of the intron lariat.

Under reaction conditions using high concentrations of KCl or NH_4Cl branch point dependent 5′ cleavage as well as lariat formation are replaced to a high degree by cleavage without transesterification (Jarrell *et al.*, 1988a; Bachl and Schmelzer, 1990). The 5′ splice junction is presumably cut by site-specific hydrolysis after nucleophilic attack by water or free OH^- to release 5′ exon and a linear intron−3′ exon intermediate (Jacquier and Rosbash, 1986). The first step is followed by a normal transesterification reaction initiated by the attack of the 5′ exon on the phosphodiester bond at the 3′ junction which yields a linear intron and ligated exons. Subsequently the exon ligation product can be reopened by hydrolysis in an intron-dependent *trans*-reaction releasing separate 5′ and 3′ exon (Jarrell *et al.*, 1988a).

In this report we present evidence that the *Podospora* COI I1 possesses self-splicing activity under high salt conditions *in vitro*. The required reaction conditions and the array of splicing products and intermediates are compared with those of yeast introns aI5γ and bI1. Differences in the self-splicing reactions are discussed in view of the structural variations between subgroup IIA and subgroup IIB introns.

Results

Self-splicing of complete COI I1

In order to investigate splicing of the COI I1 *in vitro*, different DNA templates were cloned behind the T7 promoter of vectors pSPT18 and pT7T3 18U, respectively (for details of the construction see Materials and methods). As can be seen from Figure 1B, plasmids pTP7 and pTP7/3 I1 contain all of the first exon (160 bp), the 2539 bp intron and 36 bp of the second exon of the COI gene, but differ in the length of sequences preceding the first exon.

Precursor RNAs with a length of 3601 nt containing a 484 nt 5′ exon, the entire intron and a 581 nt 3′ exon were generated by transcription of *Pst*I-digested pTP7 *in vitro* in the presence of [^{35}S]UTP (Figure 1B). After purification of the pre-RNA from 3.5% polyacrylamide−8 M urea gels self-splicing was tested under different salt conditions *in vitro*.

Under low salt conditions (e.g. 10 mM Tris−HCl, pH 7.0, 10 mM $MgCl_2$, 2 mM spermidine) which lead to autocatalytic excision of yeast mitochondrial subgroup IIB introns aI5γ and bI1 (Peebles *et al.*, 1986; Schmelzer and Schweyen, 1986; van der Veen *et al.*, 1986), the *Podospora* COI I1 does not display any detectable self-splicing activity even after 4 h of incubation (Figure 2, left panel, lanes 1−5). Only a nonspecific degradation of the precursor RNA can be observed. However, under high salt conditions (in the presence of 1.25 M NH_4Cl) two major RNA species appear, corresponding in length to specific splicing products, that is the ligated exons (1065 nt) and the separate 5′ exon (484 nt) (Figure 2, right panel, lanes 1−5). Formation of the characteristic product of group II intron splicing, the

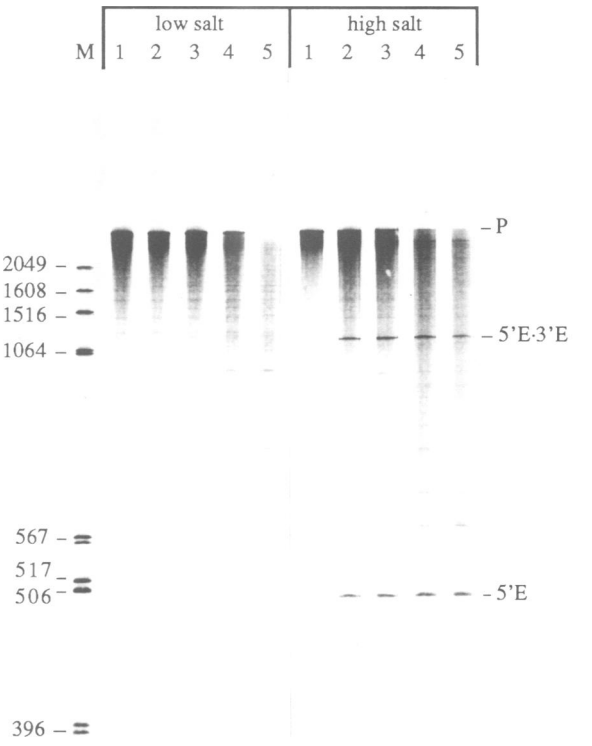

Fig. 2. Time course of self-splicing reactions of pre-RNAs containing complete COI I1 under low salt and high salt conditions. pTP7 *Pst*I run-off transcripts uniformly labelled with [^{35}S]UTP were incubated at 45°C for various times (0, 30, 60, 120 and 240 min, lanes 1−5, respectively) in reaction buffer containing 10 mM Tris−HCl, pH 7.0, 10 mM $MgCl_2$, 2 mM spermidine (low salt, left panel) or 40 mM Tris−HCl, pH 7.0, 60 mM $MgCl_2$, 2 mM spermidine, 1.25 M NH_4Cl (high salt, right panel) and separated on a 5% polyacrylamide−8 M urea gel. As a size marker (M) denatured 3′ end-labelled fragments of pMC1403 (Casabadan *et al.*, 1980) digested with *Hin*fI were used. Abbreviations: P: precursor, 5′E·3′E: ligated exons, 5′E: free 5′ exon.

intron lariat, which migrates more slowly than the precursor on denaturing polyacrylamide gels, could not be detected in any experiment even after prolonged exposure of the autoradiograms. The absence of lariat formation and the occurrence of putative free 5′ exon indicates that, when NH_4Cl is provided, the first step of the splicing reaction is exclusively initiated by 5′ cleavage without transesterification. For self-splicing of subgroup IIB introns aI5γ and bI1 from yeast it is well known that high concentrations of KCl or NH_4Cl increase the rate of reaction. Under these conditions the 5′ junction is predominantly cleaved by site-specific hydrolysis, i.e. by water or OH^- (Jacquier and Rosbash, 1986) to release the 5′ exon and a linear intron−3′ exon intermediate. In the second step the 5′ exon attacks the phosphodiester bond at the 3′ junction to yield a linear intron and ligated exons (Jarrell *et al.*, 1988a; Bachl and Schmelzer, 1990).

Thus, if the 1065 nt and the 484 nt RNA are true products of the autocatalytic reaction of COI I1, the linear intron and the linear intron−3′ exon intermediate should also be found. This is indeed the case when a shortened form of the intron is used for splicing (see below). Due to the large size of both the linear intron (2539 nt) and linear intron−3′ exon (3120 nt) these products are not distinctly separable from the precursor RNA (3601 nt) under the conditions described

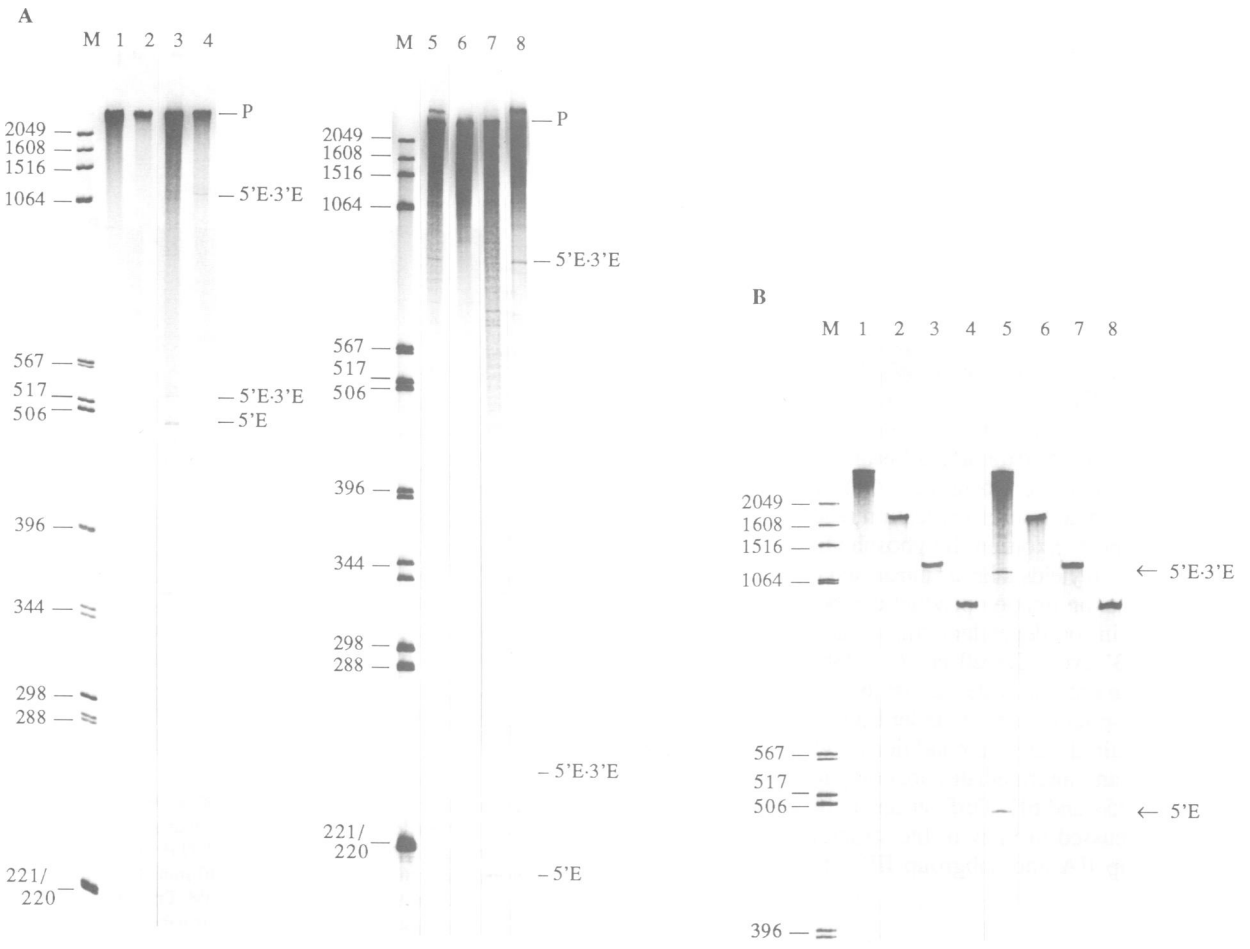

Fig. 3. Self-splicing reaction of COI I1 pre-RNAs transcribed from DNA templates with 5′ and 3′ exon sequences of various size (A) and effect of 3′ end truncation of the intron within domains III and IV on 5′ cleavage (B). (**A**) Radioactive transcripts were generated on pTP7 *Sph*I, pTP7 *Pst*I, pTP7/3 I1 *Sph*I and pTP7/3 I1 *Pst*I templates and separated on 5% denaturing polyacrylamide gels before (**lanes 1, 2, 5** and **6**, respectively) and after incubation (**lanes 3, 4, 7** and **8**) for 4 h under high salt conditions as already described in the legend to Figure 2. Abbreviations: M: pMC1403 digested with *Hin*fI, P: precursor, 5′E·3′E: ligated exons, 5′E: free 5′ exon. (**B**) Plasmid pTP7 was truncated with *Pst*I, *Bgl*II, *Sal*I and *Bam*HI, respectively. Labelled precursor RNAs of pTP7 *Pst*I (3601 nt), *Bgl*II (1869 nt), *Sal*I (1120 nt) and *Bam*HI (889 nt) were separated on a 5% denaturing polyacrylamide gel before (**lanes 1–4**, respectively) and after (**lanes 5–8**) incubation under high salt conditions. The positions of the ligated exons and the free 5′ exon of the pTP7 *Pst*I reaction are marked by arrows. Assay conditions, marker (M) and indication of splicing products as in (A).

(see legend to Figure 2), even when 3.5% polyacrylamide gels were employed (not shown). Possibly the linear intron could be related to the faint band migrating close to the pre-RNA (Figure 2, right panel, lanes 4 and 5).

In order to confirm that the two visible major reaction products derived from pTP7 *Pst*I RNA are the free 5′ exon and ligated exons, respectively, we next analysed self-splicing of transcripts derived from DNA templates with different 5′ and 3′ exon sequences. Restriction of the pTP7 construct with *Sph*I (see Figure 1B) should lead to a mobility shift indicative of the 3′ exon. As can be seen from Figure 3A (lanes 3 and 4), the 1065 nt RNA in reactions with *Pst*I precursors is shifted to a 520 nt RNA in reactions with *Sph*I precursors, which is consistent with its being the product of exon–exon ligation. To identify splicing products containing the 5′ exon, plasmid pTP7/3 I1 linearized at the *Pst*I and the *Sph*I sites, respectively, was used as a template (Figure 1B). In this construct a 278 bp fragment of the 484 bp 5′ exon of pTP7 has been deleted. Results of *in vitro* splicing are shown in Figure 3A (lanes 7 and 8). As expected for the free 5′ exon product, the 484 nt RNA is displaced by a 206 nt RNA, whereas the 5′/3′ exon ligation product is correctly shifted to a 787 nt RNA in *Pst*I transcripts and to a 242 nt RNA in *Sph*I transcripts. For the self-splicing intron aI5γ in yeast mitochondria, Jarrell *et al.* (1988b) could demonstrate in a *trans*-splicing reaction, that the highly conserved domain V of group II introns is absolutely necessary for cleavage at the 5′ splice site by transesterification or hydrolysis. Therefore, as further proof that the 484 nt RNA in reacted pTP7 run-off transcripts is a specific 5′ cleavage product, DNA templates truncated at different positions within domains III and IV of COI I1 were used for splicing (see Figure 1B). Restriction of pTP7 with *Bam*HI (intron positions 405 and 501) leads to formation of precursors lacking complete domains IV, V and VI, while run-off RNAs transcribed from templates truncated at the single *Sal*I (intron position 636) and *Bgl*II sites (intron position 1385) lack domains V and VI. Results of a 4 h incubation of shortened precursor RNAs in the presence of NH_4Cl are shown in Figure 3B (lanes 6–8). In all reactions with pTP7 *Bam*HI, *Sal*I and *Bgl*II run-offs no specific splicing products are detectable indicating that 5′ cleavage is suppressed.

Considering these observations together we suppose that

the 484 nt and the 1065 nt RNA are the separate 5′ exon and the ligated exons, respectively, indicating that in all probability the mobile intron of *P.anserina* can undergo self-splicing *in vitro* under high salt conditions.

Self-splicing of COI I1 with large deletions in the intronic ORF

To get a better separation of COI I1 splicing products on acrylamide gels and to facilitate further investigations we decided to construct DNA templates with a shortened intron. The most promising candidate for a partial deletion of group II introns that keeps self-splicing activity unchanged seemed to be domain IV, due to its extreme size variation and lack of conserved sequences (Michel *et al.*, 1989). Furthermore, Jarrell *et al.* (1988b) could show that substitution of complete domain IV by 13 nt of a polylinker sequence did not have any influence on the splicing efficiency of yeast intron aI5γ. In contrast to the small self-splicing subgroup IIB introns of *S.cerevisiae* the 2539 nt mobile intron of *P.anserina* possesses a long ORF, which covers the first 2366 nt and is mostly looped out of the core structure by domain IV (intron positions 461–2444, see also Figure 1A).

Plasmid pTP7ΔHindII (Figure 1B) is a derivative of pTP7 in which the 1341 bp internal *Hin*dII fragment, comprising about two-thirds of domain IV (intron positions 636–1977), has been removed. Following standard *in vitro* transcription, a 2263 nt pre-RNA of pTP7ΔHindII restricted at the *Pst*I site was synthesized, having 5′ and 3′ exons identical to pTP7 *Pst*I run-off transcripts and a 1198 nt intron. This transcript was reacted under high salt conditions in the presence of 1.25 M NH_4Cl. As can be seen from Figure 4A, pre-RNA lacking most of domain IV seems to be more reactive over a time course of 4 h than the wild-type RNA. As expected, the putative 5′ exon and exon–exon ligation products are the same as in reactions with full-length intron precursor RNAs. Moreover, presumed intron-containing products, which are not separable from precursors including the entire intron (see above), are now visible, i.e. the linear intron (1198 nt) and the linear intron–3′ exon intermediate (1779 nt). Surprisingly, an additional faint RNA band appears after an incubation period of 30 min, migrating above the pre-RNA, as is characteristic of group II intron lariats. These findings suggest that in contrast to the full-length wild-type intron the shortened intron has not only the capacity for 5′ cleavage by hydrolysis but also, to a lower degree, by transesterification. Obviously in reactions with NH_4Cl at 1.25 M, a minor portion of molecules is able to fold into a conformation that facilitates branch point-dependent 5′ cleavage and lariat formation. As estimated by scintillation counting, ~8–10% of the spliced intron seems to arise in a lariat form.

In order to identify intron-containing products (linear intron, linear intron–3′ exon and intron lariat) a second construct with a deletion in domain IV was derived from pTP7. In plasmid pTP7ΔAccI an internal *Acc*I fragment of 1446 bp (intron positions 636–2082) is lacking, resulting in a 1093 bp intron that is 105 bp shorter than pTP7ΔHindII (Figure 1B). Figure 4B shows the time course of self-splicing of the 2158 nt pre-RNA of pTP7ΔAccI *Pst*I under high salt conditions already described. The transcripts remained reactive with almost the same kinetics as pTP7ΔHindII *Pst*I run-offs. Supposed exon-containing products migrate as in pTP7ΔHindII reactions, while RNAs corresponding in size

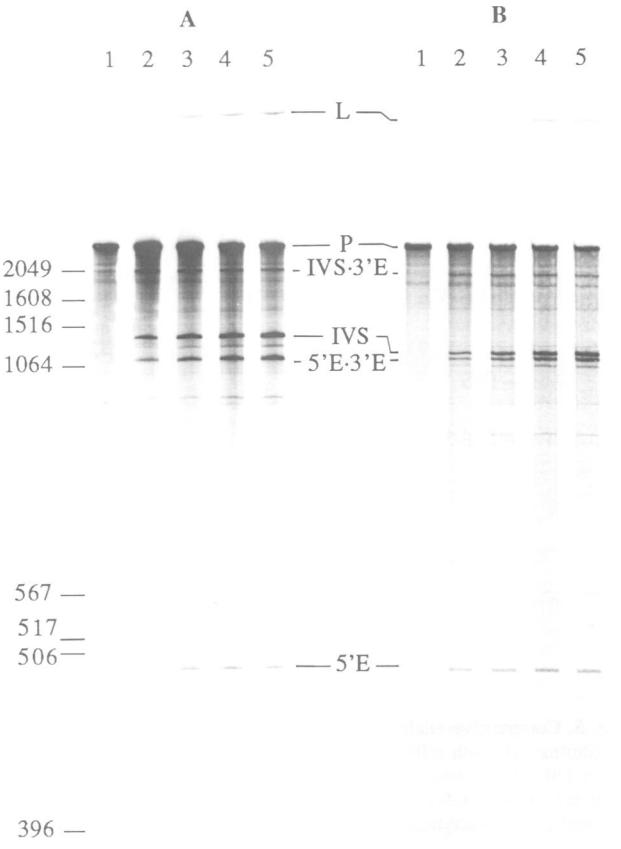

Fig. 4. Time course of self-splicing reactions of COI I1 pre-RNAs containing a mutated intron with a 1341 nt (A) and a 1446 nt deletion (B) in domain IV. Assay conditions for splicing under high salt and gel separation of ^{35}S-labelled transcripts of pTP7ΔHindII *Pst*I (**A**) and pTP7ΔAccI *Pst*I (**B**) were the same as described in Figure 2. Incubation times were 0, 30, 60, 120 and 240 min (**lanes 1–5,** respectively). Abbreviations: L: intron lariat, P: precursor, IVS·3′E: linear intron with 3′ exon, IVS: linear intron, 5′E·3′E: ligated exons, 5′E: free 5′ exon.

to intron-containing products show, as expected, an increased electrophoretic mobility.

The structure of the slow migrating intron containing RNA was confirmed by separation of self-splicing products of pTP7ΔHindII *Pst*I run-offs on denaturing gels with different polyacrylamide concentrations (Figure 5). In 3.5%, 4% and 5% polyacrylamide–8 M urea gels the migration of the pre-RNA and the linear reaction products is decreased relative to each other while the distance between the slow migrating RNA and the precursor is increased. This high retardation of a specific RNA species depending on the pore size of the polyacrylamide matrix is characteristic of circular and lariat-shaped molecules (Bruce and Uhlenbeck, 1978; Sänger *et al.*, 1979; Domdey *et al.*, 1984; Grabowski *et al.*, 1984; Ruskin *et al.*, 1984).

Characterization of 5′ and 3′ cleavage sites

Next we attempted to show correct splicing at the 5′ and 3′ junction employing gel-purified products of a preparative self-splicing reaction of pTP7ΔHindII *Pst*I run-offs as templates for a primer extension analysis with reverse transcriptase.

An oligonucleotide complementary to intron nucleotides 28–43 (numbering from the 5′ splice site) could be hybridized to the 1198 nt and the 1779 nt splicing product.

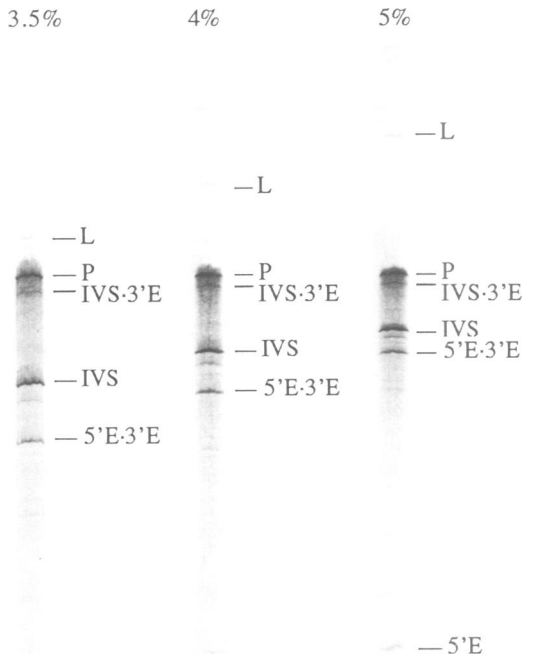

Fig. 5. Comparative analysis of COI I1 self-splicing products on denaturing gels with different acrylamide concentrations. Products from a 3 h self-splicing reaction of labelled pTP7ΔHindII *Pst*I pre-RNAs in high salt (assay conditions see Figure 2) were separated using 3.5%, 4% and 5% polyacrylamide−8 M urea gels (ratio of cross-linking was 30:1). Abbreviations for individual RNA bands are the same as in Figure 4.

After extension of the primer by reverse transcription both newly synthesized cDNAs show a strong stop exactly at the 5′ end of the intron, when compared with a double-stranded DNA sequencing reaction with plasmid pTP7ΔHindII using the same intron primer (Figure 6A, lanes 1 and 2). This result indicates that the 1189 nt and the 1779 nt RNA species might be the linear intron and the linear intron−3′ exon intermediate, respectively.

Detection of a correct 3′ cleavage by reverse transcription starting from a specific 3′ exon primer is more complicated. In contrast to the self-splicing reaction of yeast subgroup IIB introns aI5γ and bI1, a product corresponding in length to the separate 3′ exon (e.g. a 581 nt RNA in *Pst*I run-offs, see Figure 1B) is only detectable in trace amounts, and only in a small number of experiments (not shown). The free 3′ exon has been shown to be the result of a specific reopening reaction of the ligated exons by the intronic RNA *in trans*, when high concentrations of KCl or NH_4Cl are provided (Jarrell *et al.*, 1988a). Therefore, exon reopening seems to be almost totally blocked in reactions with the *Podospora* COI I1.

Thus, the 1065 nt RNA of the pTP7ΔHindII *Pst*I splicing reaction had been used not only to demonstrate the correct 5′−3′ exon ligation product but also to show precise cleavage at the 3′ junction. Upon purification from a polyacrylamide gel, this RNA was sequenced directly with reverse transcriptase primed by an oligonucleotide complementary to nucleotides 19−33 of the 3′ exon (numbering from the 3′ splice site). As can be seen in Figure 6B (right panel) the synthesized cDNA reads from the 3′ exon into the 5′ exon, which confirms correct 3′ cleavage and exon−exon joining. (In addition, a weak sequence across the intron−3′ exon junction is visible, which is probably

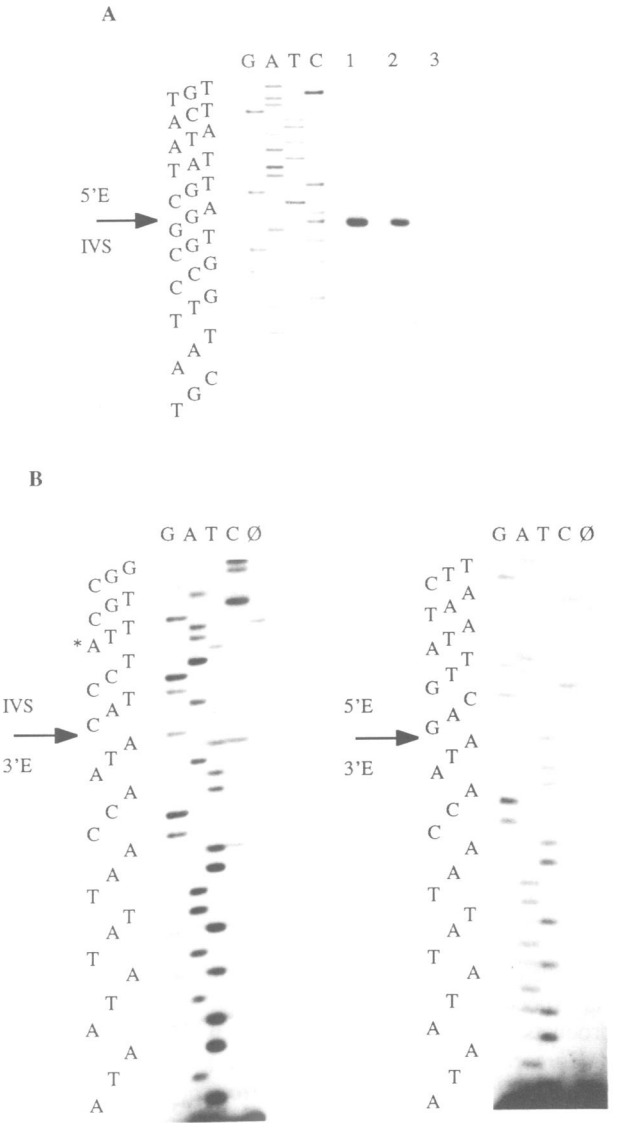

Fig. 6. Demonstration of the correct 5′ and 3′ cleavage and characterization of COI I1 self-splicing products by primer extension analysis. ^{35}S-Labelled pTP7ΔHindII *Pst*I precursor and the putative splicing products were isolated from a polyacrylamide gel and purified. Oligonucleotides used as primers (see text) were 5′ end-labelled with [γ-^{32}P]ATP and polynucleotide kinase and gel-purified. Reactions were separated on 8% polyacrylamide−8 M urea gels. (**A**) To verify correct 5′ cleavage the 1198 nt RNA (**lane 1**), the 1779 nt RNA (**lane 2**) and, as a control, the precursor (**lane 3**) were used as templates in a primer extension reaction with the intron primer. An appropriate size marker was obtained by double strand sequencing of plasmid pTP7ΔHindII using the same primer. Letters at the top of each sequencing ladder refer to the corresponding dideoxynucleotide, while lettering on the left side of the gel indicates the sequence complementary to the cDNA which corresponds to the RNA sequence. The 5′ junction site is marked by an arrow. (**B**) The 3′ exon primer was used to sequence the 1779 nt RNA (**left panel**) and the 1065 nt RNA (**right panel**) directly by primer extension with reverse transcriptase in the presence of dideoxynucleotides. Sequencing ladders are marked as in (A). To identify nonspecific stops a primer extension in the absence of dideoxynucleotides starting from the same primer is shown for each template (lanes ϕ). The junction sites of the linear intron−3′ exon (IVS·3′E) and the ligated exons (5′E·3′E) are indicated by arrows. The predicted branchpoint adenosine is marked by an asterisk.

caused by an unavoidable contamination of the gel-purified RNA with products from nonspecific hydrolysis of the pre-RNA.)

Figure 6B (left panel) displays the cDNA sequence ladder of reverse transcription of the 1779 nt RNA hybridized also with the 3′ exon primer. Compared with the sequencing products of the exon−exon ligation, the cDNA sequence clearly extends from the 3′ exon into the intron. This result provides strong evidence for the 1779 nt reaction product being the linear intron−3′ exon intermediate. In addition, the presumed linear structure of this splicing product resulting from specific 5′ cleavage by hydrolysis is supported by the observation that no stop to primer extension at the hypothetical branchpoint is visible, which should be located 7 nt upstream from the 3′ splice site (van der Veen *et al.*, 1986; Michel *et al.*, 1989; see also Figure 1A).

Variation of reaction conditions

In a first attempt to find some additional characteristic features of the autocatalytic excision of subgroup IIA introns, we next analysed the effect of incubation temperatures and different salts at high concentrations on self-splicing of COI I1.

Pre-RNA of pTP7ΔHindII linearized with *Pst*I was incubated in NH_4Cl buffer for 2 h at temperatures ranging from 25°C to 60°C (Figure 7A). The highest self-splicing efficiency with regard to both transesterification and hydrolysis was observed at temperatures near 45°C, as has already been described for *in vitro* splicing of subgroup IIB introns from yeast under low salt conditions (Peebles *et al.*, 1986; Schmelzer and Schweyen, 1986). In contrast to these low salt reactions, NH_4Cl mediated splicing of shortened COI I1 displays a broader optimum, which seems to be in perfect agreement with $(NH_4)_2SO_4$- or KCl-containing reactions of yeast intron aI5γ (Peebles *et al.*, 1987). The reaction rates at 40°C, 50°C and 55°C are barely reduced when compared with 45°C and low concentrations of splicing products are evident even at 30°C. Above 55°C self-splicing is suppressed almost completely and a large portion of the pre-RNA is lost by nonspecific degradation.

Next we examined the reactivity of pTP7ΔHindII *Pst*I transcripts at 45°C in the presence of NH_4Cl, KCl and $(NH_4)_2SO_4$ at 500 mM. Addition of 500 mM NH_4Cl yields the same splicing efficiency and product pattern over a 3 h time course as that already observed at 1.25 M NH_4Cl (Figure 7B, left panel), suggesting a lower dependence from added salt than initially presumed. When 500 mM KCl is provided, the splicing reaction is more efficient, especially over the first 30 min (Figure 7B, middle panel). If the incubation time is longer than 60 min a higher rate of nonspecific hydrolysis occurs. The array of major self-splicing products of the KCl reaction does not alter with the exception that the lariat form of the intron is conspicuously absent. Only after prolonged exposure of the autoradiogram are trace amounts of RNA comigrating with the lariat RNA of the NH_4Cl reaction detectable (not shown). Interestingly, in added KCl one of the new faint bands corresponds in length to separate 3′ exon (581 nt), which probably originates from a somewhat stronger exon reopening than in NH_4Cl reactions. Nevertheless, one of the most important differences in self-splicing of subgroup IIA and IIB introns seems to be the almost complete absence of the exon reopening reaction of subgroup IIA introns in the presence of NH_4Cl or KCl. As can be seen from Figure 7B (right panel), the rate of self-splicing reaction is largely decreased by incubation in a buffer containing $(NH_4)_2SO_4$.

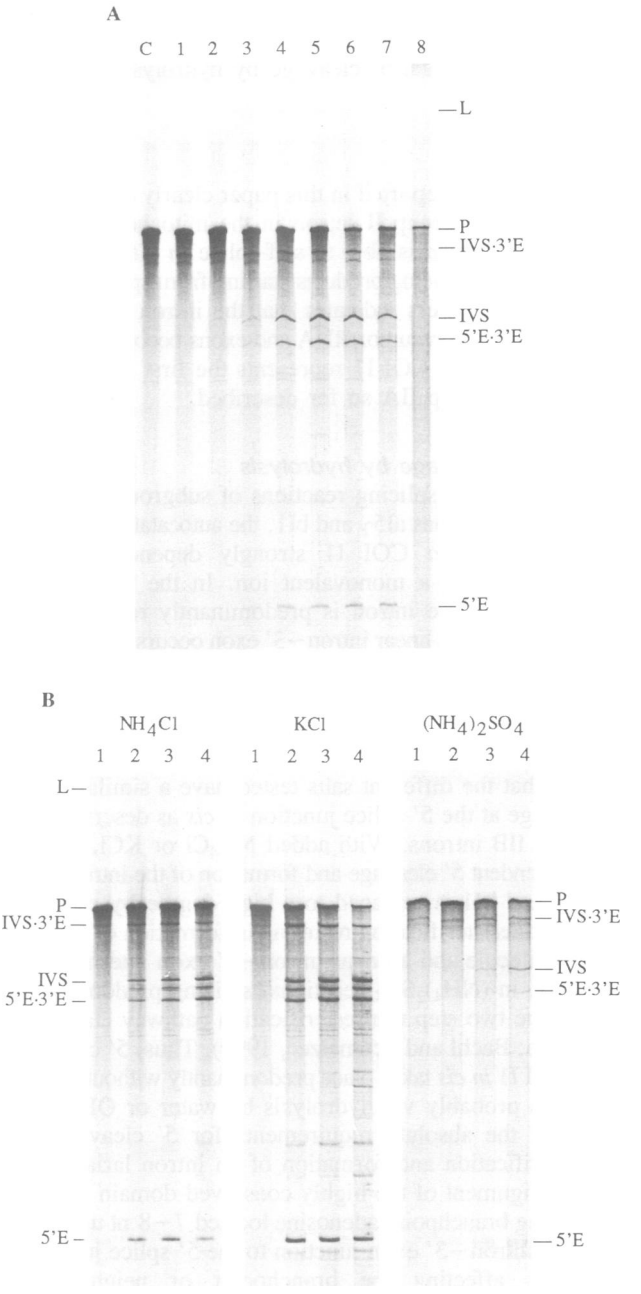

Fig. 7. Self-splicing of COI I1 pre-RNAs under altered reaction conditions. ^{35}S-labelled pre-RNAs obtained from pTP7ΔHindII restricted with *Pst*I were incubated as described below. Gel separation, marker and abbreviations of RNA bands are the same as in Figures 2 and 4 respectively. (**A**) Transcripts were reacted for 2 h at 25, 30, 35, 40, 45, 50, 55 and 60°C (**lanes 1−8**, respectively) under the high salt conditions described in Figure 2. C: Control lane with unincubated pre-RNA. (**B**) Transcripts were incubated for 0, 30, 60 and 180 min (**lanes 1−4**) at 45°C in high salt buffers containing a 500 mM concentration of NH_4Cl (**left panel**), KCl (**middle panel**) or $(NH_4)_2SO_4$ (**right panel**).

The splicing products resemble those of the reactions with added NH_4Cl and KCl and, as for the KCl reaction, the intron lariat is only visible after prolonged exposure (not shown). In contrast to NH_4Cl and KCl, $(NH_4)_2SO_4$ could be shown to accelerate self-splicing of yeast subgroup IIB introns only via the transesterification pathway (Jarrell *et al.*, 1988a). Therefore, the low amount of splicing products

of the shortened COI I1 in $(NH_4)_2SO_4$-containing reactions might be due to the almost complete lack of transesterification and a residual 5′ cleavage by hydrolysis.

Discussion

The experiments reported in this paper clearly demonstrate that the mobile group II intron in the mitochondrial COI gene of *P.anserina* is able to self-splice *in vitro*. Reverse transcription of splicing products starting from specific intron and 3′ exon primers indicates that the intron is precisely excised from the precursor RNA and exons become correctly ligated. Therefore COI I1 represents the first self-splicing intron of subgroup IIA so far described.

Efficient 5′ cleavage by hydrolysis

In contrast to the splicing reactions of subgroup IIB yeast mitochondrial introns aI5γ and bI1, the autocatalytic reaction of the *Podospora* COI I1 strongly depends on high concentrations of a monovalent ion. In the presence of NH_4Cl or KCl the intron is predominantly released in a linear form and the linear intron−3′ exon occurs as a splicing intermediate. The linear intron products are also present when $(NH_4)_2SO_4$ is provided for the reaction, however, the splicing efficiency is largely reduced.

Apart from the strong dependence on salt, these data indicate that the different salts tested have a similar effect on cleavage at the 5′ splice junction *in cis* as described for subgroup IIB introns. With added NH_4Cl or KCl, branch point-dependent 5′ cleavage and formation of the intron lariat of aI5γ and bI1 is replaced to a high degree by cleavage without transesterification resulting in formation of a linear intron molecule and a linear intron−3′ exon intermediate. However, in $(NH_4)_2SO_4$ reactions, splicing predominantly follows the two step transesterification pathway (Jarrell *et al.*, 1988a; Bachl and Schmelzer, 1990). Thus, 5′ cleavage of the COI I1 *in cis* takes place predominantly without branch formation probably via hydrolysis by water or OH^-.

One of the absolute requirements for 5′ cleavage via transesterification and formation of an intron lariat is the precise alignment of the highly conserved domain VI with the bulging branchpoint adenosine located 7−8 nt upstream from the intron−3′ exon junction to the 5′ splice junction. Mutations affecting the branchpoint or neighbouring sequences of introns aI5γ and bI1 allow only less efficient splicing by 5′ hydrolysis *in cis* and the intron is released in a linear form (Schmelzer and Müller, 1987; van der Veen *et al.*, 1987b). In addition, complete deletion of domain VI of aI5γ eliminates branch formation *in trans*, while site specific cleavage at the 5′ junction occurs efficiently (Jarrell *et al.*, 1988b; Altura *et al.*, 1989). Obviously most of the *Podospora* COI I1 molecules are not capable of folding into a spatial structure *in vitro*, that allows correct alignment of the branchpoint 2′ OH nucleophil to the 5′ cleavage site thereby precluding hydrolysis by water or OH^-.

Transesterification at the 5′ splice site of COI I1 might be influenced also by an unusual peripheral structure of domain VI (see Figure 1A). For yeast intron bI1 it could be shown that alteration of an internal loop of domain VI distal from the branchpoint adenosine reduces efficient lariat formation (Schmelzer and Schweyen, 1986).

Altura *et al.* (1989) could demonstrate in *trans*-splicing reactions of intron aI5γ that the presence of a large portion of the 5′ part of domain I is also necessary for formation of branched molecules. Thus, the striking differences in the secondary structure of domain I (especially in subdomain ID) of subgroup IIA and IIB introns could be responsible for the almost complete absence of 5′ cleavage by transesterification in COI I1 splicing under reaction conditions described for *in vitro* splicing of subgroup IIB introns.

Furthermore, we could demonstrate that by deletion of a large part of domain IV 5′ cleavage via transesterification and lariat formation can be re-established to a low degree under NH_4Cl. Although domain IV lacks any conserved sequences (Michel *et al.*, 1989) and only a residual short stem−loop seems to be necessary for self-splicing *in vitro* (Jarrell *et al.*, 1988b), the enormous size of domain IV of COI I1, which contains most of the intronic ORF and involves 78% (!) of the intron sequence, could influence the active conformation of the intron *in vitro*. 5′ cleavage by transesterification and lariat formation could be inhibited by (i) mutual recognition and binding of domain IV sequences to subregions of domains I and VI or to sequences of the core structure that lead to a steric hindrance of the exact alignment of domains I and VI, (ii) incorrect base pairings of internal domain IV sequences that probably affect the folding of stem IV close to the central wheel of the core structure. A removal of domain IV leaving only a short hairpin that allows formation of the consensus secondary structure should clarify its participation in COI I1 self-splicing.

In contrast to the splicing reaction *in vitro*, the COI I1 is efficiently released in a lariat form *in vivo* (Schmidt *et al.*, 1987). Correct folding of the intron into a three-dimensional structure leading to precise 5′ cleavage by transesterification and branch formation at physiological temperatures might be realized in mitochondria of *P.anserina* by (i) a specific ionic environment that differs from the more or less nonphysiological *in vitro* salt conditions, (ii) participation of nuclear encoded protein(s) or a protein encoded by the intronic ORF (RNA-maturase), and (iii) attachment of ribosomes to the intronic ORF during translation.

Reduced 3′ cleavage by transesterification

One of the characteristic features of the COI I1 self-splicing reaction at high NH_4Cl or KCl concentrations is the accumulation of the separate 5′ exon and the linear intron−3′ exon intermediate, while a molecule corresponding in length to the free 3′ exon could only be detected as a very faint band in a few KCl incubations. From this observation we conclude that the second step of the *cis* reaction—cleavage at the 3′ splice site via transesterification resulting in exon−exon ligation and release of the intron—seems to be markedly reduced in comparison with 5′ cleavage. Furthermore, the intron catalysed exon reopening *in trans* observed at high KCl concentrations for aI5γ and bI1 is almost completely absent from the COI I1 reactions.

One attractive hypothesis that could explain a diminished cleavage at the 3′ junction arises from the study of domain II mutants of yeast self-splicing subgroup IIB introns. Although this helix lacks any conserved regions and varies in size in a wide range among all group II introns (Michel *et al.*, 1989), replacing the 102 nt domain II of intron bI1 by a small 18 nt stable hairpin structure results in an accumulation of the intron−3′ exon lariat and the linear

intron products in added KCl and an almost complete suppression of transesterification at the 3′ splice site in $(NH_4)_2SO_4$ reactions (Bachl and Schmelzer, 1990). For intron aI5γ, Kwakman *et al.* (1989) could show that splicing at the 3′ junction is reduced under low salt conditions when most of the 164 nt domain II is removed leaving a single stable stem−loop with the identical size of 18 nt. As for the mutated yeast introns the short form of domain II of *Podospora* COI I1, which comprises only 29 nt (see Figure 1A), could be responsible for a diminished cut at the 3′ splice site.

A more speculative interpretation is based on the perception that in a two step splicing reaction after 5′ cleavage, the 5′ exon has to remain attached to the intron−3′ exon in an intermediate splicing complex by non-covalent bonds (Peebles *et al.*, 1986; Schmelzer and Schweyen, 1986; van der Veen *et al.*, 1986, 1987a). The first clear evidence for an interaction between the 5′ exon and the intron was provided by splicing reactions of aI5γ *in trans* (Jacquier and Rosbash, 1986). From the investigation of mutated constructs including the same intron, Jacquier and Michel (1987) could show that the two reaction intermediates of group II intron splicing are held together by a mutual recognition of specific 5′ exon and intron sequences (EBS1−IBS1 and EBS2−IBS2 pairing) (see also Figure 1A).

A comparison of the IBS−EBS interactions of self-splicing group II introns indicates that the proposed non-covalent bonds of the *Podospora* COI I1 to its 5′ exon are significantly weaker than for aI5γ and bI1. The resulting helices give rise to free energy increments for Watson−Crick and GU base pairs that are only ~65−75% of those for the two yeast mitochondrial introns. In particular the IBS2−EBS2 interaction of the *Podospora* intron, which includes four AU and two GU pairs, seems to be very unstable and implies that it is responsible for a premature release of the 5′ exon from the intron before the 3′ OH nucleophil of the 5′ exon is in a precise position to attack the 3′ splice junction.

Inhibition of cleavage by hydrolysis in trans (=exon reopening)

The weak interactions of the 5′ exon with the intron after hydrolytic cleavage of the 5′ junction *in cis* (resulting predominantly from a very unstable IBS2−EBS2 pairing) could also account for the virtually complete absence of specific cleavage of the ligated exons by hydrolysis *in trans* (=exon reopening). This hypothesis seems to be in good agreement with the aforementioned experiments of Jacquier and Michel (1987) who found that the mutational alteration of the IBS2−EBS2 pairing blocks self-splicing of intron aI5γ *in trans*. The same intron could be shown to cut at cryptic splice sites immediately downstream of an IBS1-like motif *in cis* and *in trans* even when both authentic IBS sequences are present. Deletions of the original IBS2 sequence markedly enhances cleavage of surrogate splice sites *in trans*, which points to the importance of the IBS2−EBS2 interaction for exon−intron recognition (Müller *et al.*, 1988). Moreover, yeast intron bI1 has been reported to integrate into the ligated exons and even into a foreign RNA by reversal of the self-splicing reaction. The integration succeeds downstream of an IBS1 sequence in the absence of an IBS2 motif, but is more efficient in the presence of IBS2 or at a decreased incubation temperature of 30°C which compensates for the absence of IBS2 (Mörl and Schmelzer, 1990). Presupposing an analogous mechanism for these intermolecular reactions and exon reopening one can conclude that the requirement for precise 5′ exon recognition by the intron *in trans* have to be more stringent than *in cis* and a stronger overall interaction of the IBS−EBS pairings is necessary for exon reopening mediated by the *Podospora* COI I1.

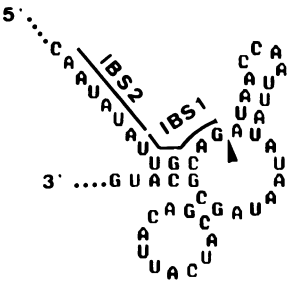

Fig. 8. Hypothetical secondary structure of COI exon sequences encompassing IBS1 and IBS2 after removal of the first intron (I1). The exon−exon junction site is marked by an arrow and IBS sequences are indicated by lines.

The inability of COI I1 to cut the exon−exon ligation product at the junction site could also be explained by a conformational alteration of the free intron, regardless of the observation that it seems to be unimportant whether the intron is present in a true linear or lariat form (Jarrell *et al.*, 1988a). A change in the three-dimensional intron structure after completion of the splicing reaction might facilitate a rapid diffusion of the ligated exons from the intron and reassociation of the intron−exon complex is suppressed. Alternatively, binding of the spliced exons to the intron could be allowed, but cleavage at the ligation junction is eliminated.

Finally, there is also an indication that a conformational change of the ligated exons might prevent exon reopening. Woodson and Cech (1989) could demonstrate that a reverse self-splicing reaction of the *Tetrahymena* rRNA group I intron succeeds when employing short oligonucleotides that consist of the exon ligation junction but fails when the mature rRNA is used. They proposed that the inhibition is most likely the result of a folding of those 5′ exon sequences into a conserved hairpin of the mature rRNA, that have to base pair with the appropriate recognition sequence of the intron (i.e. the internal guide sequence). As can be seen from Figure 8, the exon−exon ligation product of the COI I1 splicing reaction is also foldable into a secondary structure that traps most of the IBS1 sequence and might prevent the non-covalent binding required for exon reopening.

Materials and methods

Recombinant plasmids

Plasmids used as templates for *in vitro* transcription are schematically drawn in Figure 1B and were constructed as follows. A 1104 bp *Sac*I−*Sal*I fragment from plasmid pKP402 (Kück *et al.*, 1981) and a 1939 bp *Sal*I−*Sph*I fragment from plasmid pOP1 (Osiewacz and Esser, 1984) were cloned together into vector pBR322 (plasmid Hy11/314). Plasmid pTP7 contains a 3585 bp *Sac*I−*Cla*I fragment of Hy11/314 recloned in the *Sac*I and *Acc*I sites of the polylinker of vector pSPT18^{TM} (Pharmacia). This fragment harbours a 308 bp fragment of *P.anserina* mtDNA preceding the COI gene, the 160 bp first exon, the 2539 bp first intron and 36 bp of the second exon of the COI gene plus a 542 bp *Sph*I−*Cla*I fragment of vector pBR322. In plasmid pTP7/3 I1 a 283 bp *Sac*I−*Dra*I fragment of the 308 bp upstream of the COI sequences was eliminated. Therefore, a 821 bp *Dra*I−*Sal*I fragment of Hy11/314 including the first exon and the 5′ part

of the intron was recloned in the *Sma*I and *Sal*I sites of the polylinker of vector pT7T3 18U^{TM} (Pharmacia) and a 2484 bp *Sal*I–*Pst*I fragment of pTP7 including the 3′ part of the intron and 36 bp of the second exon was inserted. Plasmids pTP7ΔHindII and pTP7ΔAccI were derived from pTP7 by deletion of a 1341 bp *Hin*dII and a 1446 bp *Acc*I intronic fragment respectively.

Preparation of transcripts and self-splicing of pre-RNAs

Plasmids (see above and Figure 1B) were restricted to completion with *Sph*I and *Pst*I, respectively, and the resulting 3′ protruding ends were trimmed by incubation with T4 DNA polymerase for 15 min at 37°C in the presence of deoxyribonucleoside triphosphates according to Maniatis *et al.* (1982). Uniformly labelled transcripts were generated from linearized plasmids in 20 μl reactions containing 2–5 μg DNA, 40 mM Tris–HCl, pH 8.0, 8 mM $MgCl_2$, 5 mM DTT, 2 mM spermidine, 25 mM NaCl, 25 U RNase inhibitor, 500 μM of each ribonucleoside triphosphate, 20 μCi [α-^{35}S]UTP and 40–60 units T7 RNA polymerase for 2 h at 37°C. Upon separation on 3.5% or 5% polyacrylamide–8 M urea gels and autoradiography, full-length precursor RNAs were gel-extracted and purified as described by Frendewey and Keller (1985). *In vitro* splicing assays with purified pre-RNA were carried out at 45°C in a volume of 20 μl in low salt buffer containing 10 mM Tris–HCl, pH 7.0, 10 mM $MgCl_2$, 2 mM spermidine or in high salt buffer containing 40 mM Tris–HCl, pH 7.0, 60 mM $MgCl_2$, 2 mM spermidine and 500 mM NH_4Cl, 1.25 M NH_4Cl or 500 mM KCl. In a few experiments a high salt buffer containing 40 mM Tris–SO_4 pH 7.0, 60 mM $MgSO_4$, 2 mM spermidine and 500 mM $(NH_4)_2SO_4$ was used. The reactions were stopped by chilling on ice and ethanol precipitation. The resulting pellets were rinsed with 70% ethanol and taken to dryness under vacuum. After dissolving the pellets in water an equal volume of gel loading buffer was added and products were analysed on 5% polyacrylamide–8 M urea gels (ratio of cross-linking 30:1).

Primer extension analysis and dideoxy sequencing

The 16mer oligonucleotide 5′-GGGTCTAATTCCCCGG-3′ and the 15mer 5′-GCGCTGTAATGATGC-3′ hybridizing to the 5′ end of the intron and the 3′ exon, respectively, were used as primers. These oligonucleotides were 5′ end-labelled with T4 polynucleotide kinase and [γ-^{32}P]ATP following the method of Maxam and Gilbert (1980).

Individual RNAs isolated from polyacrylamide gels were coprecipitated with 5′ end-labelled oligonucleotides and redissolved in 500 mM NH_4OAc, 0.1 mM EDTA and 0.1% SDS. After incubation for 2 min at 60°C and 10 min at 25°C the solution was ethanol precipitated and the pellet was rinsed with 70% ethanol and dried under vacuum. The pellet was dissolved in 50 mM Tris–HCl, pH 7.9, 7.5 mM $MgCl_2$, 10 mM DTT and 50 μM of each deoxyribonucleoside triphosphate. For sequence analysis cDNA synthesis with reverse transcriptase was performed in the presence and, for characterization of specific stops, in the absence of dideoxynucleotides according to the method of Tabak *et al.* (1984). The products were analysed on 8% polyacrylamide–8 M urea gels (ratio of cross-linking 19:1).

For sequencing of double-stranded plasmid DNA with unlabelled intron and exon primer a ^{T7}Sequencing^{TM} Kit (Pharmacia) was used as recommended by the manufacturer.

Enzymes and nucleotides

T4 DNA polymerase, T7 DNA polymerase, M-MuLV reverse transcriptase, unlabelled ribonucleoside triphosphates and dideoxyribonucleoside triphosphates were purchased from Pharmacia and unlabelled deoxyribonucleoside triphosphates from BRL and Pharmacia. RNase inhibitor, [α-^{35}S]UTP (400 Ci/mmol), [α-^{35}S]dATP (600 Ci/mmol) and [γ-^{32}P]ATP (5000 Ci/mmol) were obtained from Amersham, T7 RNA polymerase and T4 polynucleotide kinase from Boehringer and restriction enzymes from Boehringer, BRL and Pharmacia, respectively.

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