An RNA conformational change between the two chemical steps of group II self-splicing

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As for nuclear pre-mRNA introns, the splicing pathway of group II self-splicing introns proceeds by two successive transesterifications involving substrates with different chemical configurations. These two reactions have been proposed to be catalysed by two active sites, or alternatively by a single active site rearranging its components to accommodate the successive substrates. Here we show that the structural elements specific for the second splicing step are clustered in peripheral structures of domains II and VI. We show that these structures are not required for catalysis of the second chemical step but, instead, take part in a conformational change that occurs between the two catalytic steps. This rearrangement involves the formation of a tertiary contact between part of domain II and a GNRA tetraloop at the tip of domain VI. The fact that domain VI, which carries the branched structure, is involved in this structural rearrangement and the fact that modifications affecting the structures involved have almost no effect when splicing proceeds without branch formation, suggest that the conformational change results in the displacement of the first-step product out of the active site. These observations give further support to the existence of a single active site in group II introns.

Keywords: group II introns/modification interference/ribozymes/RNA structure/splicing

Introduction

Group II self-splicing introns are found in organelles and bacteria and show conserved secondary structures composed of six structural domains (Michel et al., 1989; Michel and Ferat, 1995). Their excision proceeds by two distinct chemical steps in a pathway similar to nuclear pre-mRNA splicing. Each of these steps is a transesterification reaction, but the nucleophile for the first step is the 2' hydroxyl of a conserved adenosine, while the nucleophile for the second transesterification is the 3' hydroxyl of a non-conserved nucleotide (Figure 1A). The sequences of the splice junctions are also different. These observations have led to the proposals that these reactions are catalysed by two distinct active sites (Moore and Sharp, 1993) or alternatively, by a single active site undergoing some structural rearrangement to accommodate the different substrates (Steitz and Steitz, 1993). Consistent with the single active site model, we have recently shown that several components required for catalysis of both steps are clustered in the highly conserved structural domain V (Chanfreau and Jacquier, 1994). We have now surveyed the entire intron for structures that might be involved exclusively in the second step of splicing. The results obtained show that the nucleotides whose modification specifically affects the second step of splicing are mainly located among peripheral structures of domains II and VI. We show that these positions are not involved in catalysis but that they are instead involved in a conformational change occurring between the two chemical steps. One of the structures involved in this conformational change is located in domain VI which carries the branched structure. Because the major phenotype of the mutants disrupting this structure is the enhancement of the first step (branch formation) reversal, and since the modifications affecting these structures have very little effect when splicing occurs without branch formation, it is likely that this structural rearrangement is involved in displacement of the first-step branched product out of the active site.

Results

Identification of nucleotides specifically involved in the second step of splicing by modification interference

To define elements of group II introns important for each splicing step, we have undertaken a modification interference analysis of group II self-splicing with diethyl pyrocarbonate (DEPC) which modifies purines (Conway and Wickens, 1989). By comparing the pattern of modified nucleotides in reacted versus unreacted populations of molecules, modification interference allows in a single experiment the mapping of positions important for a given reaction. To adapt the modification interference technique to group II self-splicing, we have designed a multiple subunit group II self-splicing intron. The yeast mitochondrial group II intron Sc.cox1/5 was divided into two or three subunits, by deleting part of a non-essential helix within domain I (helix D2B) and part of the non-essential domain IV. In each subunit, we replaced these structures by long GC-rich complementary sequences (‘G-C clamps’; see Materials and methods). Figure 1B illustrates the different constructions used to reconstitute a group II intron from different subunits. Under annealing conditions, the G-C clamps hybridize to reconstitute a full-length precursor, which can be gel purified under semi-denaturing conditions. Such multiple subunit ribozymes self-splice efficiently, as shown in Figure 1C. Thus, each subunit can be independently modified and end-labelled at the end of
the G-C clamps, allowing the independent modification interference analysis of each subunit.

In order to look specifically for first or second splicing-step defects, two types of transcript with different 5' exons were used. In the splicing of Δ52 transcripts, the second splicing step is very fast, and only the rate-limiting first step can be monitored. Transcripts with shortened 5' exon (Δ13 transcripts) lack one of the two exon 1-intron interactions. It follows that the intermediate complex is unstable and its premature dissociation competes with the second splicing step. The ratio between the dissociated lariat intermediate and the excised lariat intron, which remains constant during the reaction, provides a sensitive assay for the second splicing-step efficiency (Figure 1A; Jacquier and Michel, 1990).

Figure 2 shows examples of gels from which the functional map was established. They correspond to the regions of the ribozyme which exhibited the most significant second-step inhibitions. Modifications at positions in domains II and VI strongly interfered with the second step of splicing without impairing the first step (compare, in panels A and C, the patterns in lanes 5 and 6 for step two with the patterns in lanes 2 and 3 for step one). Figure 3 summarizes the functional map of purines for almost the entire intron. The sequence of the intron is shown (Figure 3A) with the quantification by PhosphorImager scanning of the inhibitory effects on the first step (Figure 3B) and on the second step (Figure 3C). The ratio between the signal in the precursor population and the lariat intron population in the Δ52 construct was used to monitor the inhibitory effects of modification of the purines on the first step (P/IL). The ratio between the signal in the lariat intermediate population and the lariat intron population (IL-E2/IL) in the Δ13 constructs reflected the inhibitory...
effect of modifications of nucleotides on the second step. Because some modifications strongly inhibited the first step (P/IL > 5), we were not able to monitor accurately the effects of DEPC modification for the second step at all positions. Such positions are indicated by dots, as are the pyrimidines which were not analysed (they are not modified by DEPC). Figure 3D summarizes the purines whose modification specifically inhibits the second splicing step, without impairing the first step.

Nucleotides whose modifications strongly affect the first step were found in domain V, in the bottom stem of domain III and in domain I. In domain I, severe defects were observed for nucleotides in stems I, A, B and C, in stems D and D and in EBS1. In contrast with domain V, the interference of modifications in domain I did not strictly correlate with the sequence conservation. For example, strong inhibitions observed in stem I mapped to a region which is not conserved, neither in sequence nor in length (Michel et al., 1989; see Discussion). The most severe second-step-specific defects were observed for some non-conserved positions in domains II and VI, and at nucleotides G6, A587 and A589 (Figure 3D). Nucleotides in regions of domain I involved in some tertiary interactions (EBS1, α, β) were also found to be important (Figure 3C), but these effects were not specific to the second step.

**An interaction between the tetraloop of domain VI and a helix of domain II is required for a conformational change between the two catalytic steps**

To further analyse the role of second step-specific structures, mutations were introduced at positions showing the strongest inhibitions upon DEPC modification. Nucleotide A587 (γ) had already been analysed in detail and was shown to be involved in 3′ splice site recognition (Jacquier and Michel, 1990). Substitutions of nucleotides G6 and A589 affected both splicing steps, in contrast with the modification analysis (data not shown). Therefore we focused our analysis on domains II and VI (Figure 4A). Mutation of the GUAA domain VI tetraloop to UUCG (L6 mutant) reduced the second-step efficiency but did not impair the first step (Figure 4B). Deletion of the peripheral structures of domain II (ΔI mutant) or mutations (D2ACC and D2GGU mutants) disrupting two consecutive G:C base pairs in domain II dramatically reduced the second-step efficiency without reducing the first-step rate (Figure 4B). Combination of the D2ACC and D2GGU mutations (D2GC mutant), which restored the potential base pairing, partially rescued the second step, but to a level significantly lower than the wild-type (Figure 4B). This result suggested that this stem could be involved in a tertiary interaction requiring a specific sequence. Recent
studies showed that GUAA tetraloops are able to interact directly with two consecutive G:C base pairs (Jaeger et al., 1994; Pley et al., 1994; Costa and Michel, 1995). Therefore the GUAA tetraloop of domain VI was a good candidate for interacting directly with the double G:C in domain II.

While a GAAA tetraloop can interact with two consecutive G:C base pairs (Jaeger et al., 1994; Pley et al., 1994; Costa and Michel, 1995), it was also shown to interact strongly with a specific 11-nucleotide structural motif CCUAAG...UAUGG (Costa and Michel, 1995). In contrast, this motif does not interact efficiently with either GUAA or GUGA loops, providing a sensitive genetic assay for this interaction. In the context of the wild-type GUAA tetraloop, introduction of the motif in domain II significantly reduced the second-step efficiency (D2Δ-Motif in Figure 4B). The single GaAA mutation in domain VI tetraloop showed no significant effect in this assay but, as expected, could suppress the second-step defect induced by the motif. This suppression was specific since another GNRA loop (GUgA) did not rescue the second step.

These results provided genetic evidence for an interaction between domain II and the tetraloop of domain VI (η−η'; Figure 3A). This interaction is required after the first splicing step. The relatively mild effects of the mutations on the second step, together with the fact that the sequences involved are not generally conserved in group II introns, strongly suggested that this interaction is not directly involved in catalysis, but is rather implicated in a conformational change occurring between the two transesterifications. In this hypothesis, the lariat intron–exon 2 intermediate would exist in two distinct conformations, one specific for each chemical step. After the first step, the structural rearrangement would mediate the transition from a conformation competent for the first transesterification to a conformation specific for the second transesterification. According to this model, disruption of the η−η' interaction would enrich the lariat intermediate population in molecules in the first-step-specific conformation, and this should facilitate the reversal of the first splicing step.

We tested this prediction by incubating purified lariat intermediate molecules with exon 1 molecules. Since, in this assay, the second splicing step is very fast and interferes with analysis of the first-step reversal (Jacquier and Jacquesson-Breuleux, 1991; Chin and Pyle, 1995), we blocked the second transesterification by incorporating Rp phosphorothioates at the 3’ splice site (Padgett et al., 1994; see Figure 5A and B). This modification is expected to induce a minimal effect on the ribozyme structure and is likely to inhibit the second splicing step at the catalytic stage (Padgett et al., 1994). Under these conditions, domain II and VI mutants efficiently induced the reversal of the first step, while the wild-type performed this reaction very inefficiently (Figure 5C and D), providing evidence for a kinetic step occurring between the two chemical steps. In particular, introduction of the D2GC mutant or of the motif, which showed only modest effects on the second step (r2 = 0.32 and 0.4 respectively; see Figure 4B) now dramatically increased the level of first-step reversal (Figure 5C and D), consistent with the disruption of the η−η' interaction being part of the rate limiting step in this assay. The GUAA to GaAA mutation only slightly increased the rate of first-step reversal, consistent with the observation that GAAA loops can interact with two consecutive G:C base pairs (Pley et al., 1994). However, the combination of this loop sequence with the motif was able to suppress completely the strong phenotype induced by the latter (Figure 5C and D). Thus, this first-step reversal assay provided additional evidence for the η−η' interaction and its role in the conformational change.

**Discussion**

*A functional map of the structures of a group II self-splicing intron*

Our initial goal was to investigate the involvement of the different structures of group II introns in each of the two splicing steps. In order to do so as comprehensively and as extensively as possible, we chose to use the modification interference technique. To this purpose, we have divided the intron into several parts to reconstitute an active precursor by inserting long G-C rich complementary sequences. Several strategies were previously used to reconstitute active group II ribozymes from separated subunits (Jarrell et al., 1988; Franzen et al., 1993; Pyle and Green, 1994), but our approach is the only one in which the different subunits are strongly linked to the substrate, a requirement for the modification interference study, in which reacted molecules must be purified from unreacted ones. This strategy should be of general use in the structure–function relationship studies of large RNA molecules, in which precursor and products of a reaction can be separated.

Using this method, we have identified a set of structures exclusively involved in the second step of splicing. In contrast, the strategy we used could not provide evidence that structures essential for the first step of splicing are not essential for the second step, since in the assay, molecules blocked by modifications before the first step cannot be analysed for the second step. We have previously described a trans-splicing assay using linear intermediate molecules which should allow such an analysis (Chanfreau and Jacquier, 1994), but this approach has not yet been used systematically. Furthermore, the assays used for the interference analysis performed in this study did not allow the distinction between effects of modifications on RNA folding or on catalysis. The comparison of the functional map for the first step with the sequence conservation (compare Figure 3B and A) allows some hypotheses to be made. As nucleotides directly involved in the catalytic centre are expected to be highly conserved, positions located in poorly conserved regions are unlikely to be part of the active site. For example, among the strongest effects observed on the first step, some are located on nucleotides of stem I(3) which is conserved neither in sequence nor in length (Figure 3A; Michel et al., 1989). Thus, these modifications are likely to inhibit formation of the stem at the bottom of domain I which could be critical for the stability of domain I or the folding pathway of the ribozyme. Likewise, some unconserved nucleotides in domain I are important for both splicing steps. Some of them are involved in previously described tertiary interactions [interaction EBS1–IBS1 (Jacquier and Michel, 1987); interaction α′–α′ (Harris-Kerr et al., 1993)]. It is likely that modifications at some of these positions disrupt
tertiary contacts which are required for proper folding and stability of the ribozyme structure. Alternatively it is interesting to note that domain V nucleotides are the only very conserved nucleotides whose modification strongly inhibits the first step. In contrast, some highly conserved nucleotides exhibit a modest or no effect upon modification. One of the most striking examples of such a discrepancy is given by the universal G between domains II and III. It is possible that such nucleotides are conserved because of their interaction with other conserved nucleotides but that these interactions are not rate-limiting in the \textit{in vitro} assay used in this study. In conclusion, the purine nucleotides most important for catalysis appear to be all clustered in domain V (see also Chanfreau and Jacquier, 1994).

\textbf{A structural rearrangement between the two chemical steps of group II self-splicing}

We have identified a new kinetic step occurring between the two transesterification reactions of group II introns.
This additional kinetic step corresponds to a structural rearrangement involving a new interaction (η—η'). In a recent report, Chin and Pyle characterized the kinetics of the forward and reverse branching reactions (Chin and Pyle, 1995). To do so, they used a bimolecular system in which the functional ribozyme is reconstituted from two separate pieces of RNA. They found the first-step reversal to be an efficient reaction and proposed that the overall

![Second Step Map](image1)

**Fig. 3.** Sequence of *Sc.coxl5* intron and quantification of interference by DEPC modification on the two splicing steps. (A) Sequence, secondary structure and tertiary interactions of group II intron *Sc.coxl5*. Characteristic structural elements (domains and names of the stems) are as in Michel et al. (1989). Conserved nucleotides in subgroup IIB are circled. Invariant nucleotides in subgroup IIB are in black circles (Michel et al., 1989). (B) Quantification of modification effects on the first step. Quantitations were done with a PhosphorImager. For each position, values are the ratios of band intensity in the precursor over band intensity in the lariat intron (P/IL ratio) for the Δ52 constructs. The small points indicate non-tested nucleotides: pyrimidine nucleotides (not tested since not modified by DEPC), purine nucleotides downstream of the branchpoint (shifted in migration after branch formation), exonic nucleotides and nucleotides in the G-C clamps. (C) Quantification of modification effects on the second step. For each purine nucleotide, values are the ratios of band intensity in the dissociated lariat intermediate over band intensity in the lariat intron (ILE2/IL ratio) for the Δ13 constructs. Small points represent positions not tested: as in (B) and, in addition, the nucleotides whose modification strongly affected the first step (ratio ≥ 4 in Figure 3B). (D) Summary of modifications specifically affecting the second step. Only the modification effects which are specific for the second step are reported. Hence, the empty circles represent purines whose modification either does not affect the second step or affects both steps.
The self-splicing reaction is driven forward by the very efficient second step. However, our results indicate that, in a wild-type transcript, with all sequences in cis (in contrast to the transcripts used by Chin and Pyle), a conformational change also contributes to drive the splicing reaction forward. Indeed, when the second step is blocked by incorporation of a phosphothioate at the 3' junction, reversal of the first step is slower by an order of magnitude for the wild-type molecules, compared with mutants specifically affecting the second step conformation (mutants disrupting the η-η'; see Figure 5B). Interestingly, the kinetics observed by Chin and Pyle for the first-step reversal could be separated into two phases. In the first phase, ~19% of the transcripts reacted very rapidly, while the remaining population reacted with a rate smaller by two orders of magnitude. Chin and Pyle propose that the slow rate corresponds to 'the rate of conformational change from an inactive state to a state that is capable of reverse branching'. The observations we have made allow a more precise hypothesis to be made: the slow rate would correspond to the conversion from the step two-specific conformation toward the step one-specific conformation.

The precise role of this conformational change is still hypothetical. However, it is noteworthy that this structural rearrangement involves domain VI which carries the branched G1 (see Figure 6). Moreover, modification of the second-step-specific sequences in domain II (compare, in Figure 2, lanes 2 and 3 of panel B with lanes 5 and 6 of panel A) and domain VI [compare lanes 5 and 6 in Figure 2C with Figure 2A in Chanfreau and Jacquier (1994)] has very little effect when the second step is performed with a linear intermediate instead of the normal lariat intermediate, strongly suggesting that this rearrangement is involved in handling the branched product. Finally, mutations affecting the conformational change induce reversal of the first step, consistent with the first-step products being stalled in the active site. Thus, this rearrangement most likely mediates the displacement of the first-step branched product out of the active site (Figure 6). It is also interesting to note that mutants disrupting the η-η' interaction reproducibly increased the rate of the first splicing step (Figures 4B and 5D). In our model, formation of η-η' prior to the first step would result in displacing the 2'OH nucleophile away from the 5' splice

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**Fig. 4.** Splicing efficiencies of mutants in domains II and VI. (A) Summary of the mutations introduced in domains II and VI. Mutant sequences are in lower case. The D2GC mutant corresponds to the double D2GGU/D2ACC mutant. ΔII stands for the deletion of the peripheral structures of domain II (double arrows) which were replaced by a SalI site. (B) First step apparent constants (k1) in monomolecular reactions and second-step efficiencies relative to wild-type measured in a Δ13 construct (r2). k1 values were measured as in Chanfreau and Jacquier (1993) with 3' labelled Δ52 transcripts in LS-Mg buffer (40 mM Tris–HCl pH 7.5, 100 mM MgCl2, 0.05% Na-dodecyl-sulfate). 3' labelling of Δ52 transcripts was performed as described in Materials and methods using the 'split' technique. r2 values are the second-step relative efficiencies measured as the ratio: (ΔLE2/ΔL-ΔLE2/ΔL)mutant/(ΔLE2/ΔL)wild-type determined in Δ13 transcripts (Jacquier and Michel, 1990) in HS buffer. D2Δ (WT*) was chosen as the wild-type reference for calculation of r2 values for all D2Δ derived mutants (lower half of the table). r2 of D2Δ/WT = 0.8. All values are the means of triplicates. Standard deviations did not exceed 15% of the mean.
Fig. 5. Cis- and trans-splicing reactions of wild-type and 3' splice-site phosphorothioate substituted substrates. (A) RNA fragments used to reconstitute 3' splice-site phosphorothioate substituted transcripts using template-directed ligation (Moore and Sharp, 1992). The 3' fragment was transcribed either with adenosine triphosphate or with Sp phosphorothioate adenosine triphosphate, leading to a fully adenosine phosphorothioate substituted fragment. In addition to the adenosine at the 3' splice site, another intronic nucleotide (A887) is also Rp phosphorothioate substituted, but we have previously observed that this modification does not interfere with splicing (Chanfreau and Jacquier, 1994). The radioactive symbol indicates the single 5' label of the 3' fragment. (B) Products of a cis-splicing reaction in wild-type and phosphorothioate-substituted substrates. The reconstituted precursors were incubated at 45°C in HS buffer for the times indicated and splicing products were separated by 4% PAGE. Because of the single intron internal label, the ligated exons are not labelled. No products migrating faster than the precursor were detectable on the portion of the gel which is not shown. (C) Products of a trans-splicing reaction with phosphorothioate-substituted wild-type and domains II and VI mutant molecules. Lariat intermediates were gel-purified from preparative splicing reactions of reconstituted phosphorothioate-substituted transcripts. They were preincubated for 10 min at 45°C in LS-Mg buffer and incubated at 15 nM in the presence of a saturating concentration of exon 1 molecules (10 μM; the apparent $K_m$ for the trans-splicing reaction was estimated to be 1 μM; data not shown). The products shown on the gel result from a 15 min incubation after addition of exon 1. Splicing products were fractionated on a 4% sequencing gel. IL-E2 is the lariat intron-exon 2 intermediate. P is the precursor resulting from the reversal of the first splicing step. Gel-purified P product was amplified by reverse transcription followed by polymerase chain reaction. The sequence of 12 independent clones across the 5' junction was found to be identical to the wild-type (data not shown). (D) Time-course and first-step reversal ($k$-1) kinetic constants of the tran-splicing reaction for phosphorothioate substituted wild-type and mutant molecules. Experimental conditions are as in (B). The fraction of lariat intermediate converted into precursor (first-step reversal) is plotted against time. The curves represent the best fit of the data assuming a simple model:

$$\text{IL-E2} + \text{E1} \xrightarrow[k-1]{k'1} \text{P}$$

P, precursor; IL-E2, lariat intermediate; E1, exon 1. The values are the kinetic parameters corresponding to the fitted curves. Values within brackets stand for an estimated standard deviation which estimates the fit between the curve and the data.

site. Thus, establishment of this interaction prior to the first chemical step would be detrimental to its efficiency. This model is also fully compatible with the observation that all the catalytic site components identified thus far are common to both catalytic steps (Chanfreau and Jacquier, 1994). Indeed, the existence of a single active site in group II introns infers the occurrence of a conformational change that substitutes the successive substrates in the catalytic site (Steitz and Steitz, 1993).

All these observations imply that the new kinetic step that we have identified is an intrinsic and general feature of the group II splicing pathway. Nevertheless, the η-η′
interaction involves poorly conserved structures (Michel et al., 1989) located in peripheral regions of domains II and domain VI. Only seven or other members of subgroup IIB potentially include the specific η–η′ interaction (Michel et al., 1989; Kuck et al., 1990; Ferat and Michel, 1993; Fontaine et al., 1995). In other introns, the conformational change could be mediated by equivalent but non-identical interactions between domain VI and domain II. For example, in peripheral parts of group I introns, pseudoknots and loop-helix structures can substitute for each other to stabilize the core structures (Jaeger et al., 1994). Other non-equivalent structures could also mediate the displacement of the branched product out of the active site. Indeed, using similar trans-splicing assays with the Sc.cytb1 intron, Müller et al. observed that a previously identified interaction between the branched G1 and the 3' splice site (Chanfreau and Jacquier, 1993) is involved in a similar structural rearrangement taking place between the first and the second transsterification steps (M.W.Müller, personal communication). In addition, proteins may also play a role in this step in vivo.

Finally, it is interesting to compare the occurrence of a conformational change in group II self-splicing with events taking place during pre-mRNA splicing between the two chemical steps. A conformational change occurs in the spliceosome between the two splicing steps which results in the protection of the 3' splice site (Schwer and Guthrie, 1992). Therefore, this conformational change probably promotes the positioning of the 3' splice site, which is the step two-specific substrate, into active site structures.

The equivalent is found in group II introns where several step two-specific RNA interactions involving the 3' splice site take place, most likely only after the first step (Jacquier and Michel, 1990; Jacquier and Jacquesson-Breuleux, 1991; Chanfreau and Jacquier, 1993). No rearrangement involving the branch site structure after the first step of splicing in the spliceosome has yet been identified that would be equivalent to the conformational change identified here. However, several proteins are associated with the branch region at several stages of the splicing reaction (MacMillan et al., 1994 and references therein). Thus, it is possible that catalytic functions in the spliceosome remained associated with RNA components, while most of the structural changes are now mediated by protein factors.

Materials and methods

Plasmids and in vitro transcription templates

G-C clamps were inserted into group II intron sequences using standard site-directed mutagenesis and molecular cloning techniques. Site-directed mutagenesis was performed using the "gun" procedure (Kunkel, 1985). The plasmids used for in vitro transcription were the following. Wild-type precursor was obtained by transcription of Δ52 plasmid (Jacquier and Michel, 1987). Plasmids B52XC and SK+CX used to generate Δ52XC and CX subunits respectively, have been described (Chanfreau and Jacquier, 1994). Δ13 XC subunit was obtained by SP6 transcription of BamHI-digested Δ13XC plasmid which yields transcripts identical to B52XC except that exon 1 sequences are shortened to the sequence of Δ13 precursor (Jacquier and Michel, 1987).

E1-N C(Δ52 or Δ13 transcripts) were encoded by BamHI-digested Δ52NC and Δ13NC plasmids, which are derivatives of Δ52 and Δ13 plasmids (Jacquier and Michel, 1987). These plasmids yield transcripts composed of vector-derived sequences: GAATACACAGTGCAT, followed by nucleotides -52 or -13 to 228 of Sc.cytb1 precursor, followed by the 5' side of the G-C clamp: 5'-CTAGAGCCGCGC- CCCCCCCGCGCGCGCGCGCGCGCGCGCGCGCGCGCG. The CN subunit was obtained from EcoRI-digested SK+NC plasmid, which yields a transcript composed of the 5' side of the G-C clamp C (the anti-parallel sequence of the G-C clamp C 5' side) followed by nucleotides 303–887 of the intron and the first 187 nucleotides of the 3' exon. E1-NK (Δ52 or Δ13 transcripts) were encoded by BamHI-digested Δ52NK and Δ13NK plasmids respectively, which differ from the Δ52NC and Δ13NC plasmids by the sequence in the G-C clamp K 5' side : 5'-GGCGCGCCTCG- ACCGCCCGCCCGGCGCAGCGCGGGCGCGCG that replaces the G-C clamp C. The KNXC subunit was used to bridge E1-NK and CX subunits was obtained by T7 transcription of BamHI-digested SK+KNXC plasmid. This subunit is composed of vector-derived sequences: 5'-GATC, followed by the 3' side of the G-C clamp K (the anti-parallel sequence of the G-C clamp K 5' side), nucleotides 303–681 of the intron and by the G-C clamp C 5' side (see sequence above). The E1 RNA was transcribed with SP6 polymerase from an FokI digested PCR product giving rise to an exon 1 molecule identical to exon 1 of Δ52 (Jacquier and Jacquesson-Breuleux, 1991).

RNAs for the ligation giving rise to the 3' splice site phosphorothioate transcripts were obtained as follows. The 5' molecule was obtained by SP6 transcription of a BsmAI-digested Δ13-887G plasmid. The 887T to G mutation creates a BsmAI site that can be used to generate a truncated precursor molecule ending at nucleotide 883. The 3' molecule was obtained by T7 transcription of an EcoRI-digested PCR fragment which generates nucleotides 884–887 of the intron followed by the first 187 nucleotides of exon 2.

RNA synthesis and labelling

RNAs were synthesized by in vitro transcription of linearized plasmids using SP6 or T7 RNA polymerase. In vitro transcription was performed at 37°C (T7 Pol.) or 40°C (SP6 Pol.) in 40 mM Tris-HCl pH 7.5, 6 mM MgCl2, 2 mM spermidine, 12.5 mM DTT, 12.5 mM NaCl. Nucleotide concentration was 1 mM, except for RNAs to be end-labelled (see below). After transcription, samples were treated with DNase I, extracted with phenol/chloroform and ethanol-precipitated. The pellet was washed with 80% ethanol, dried and resuspended in water.

RNAs to be 5' end-labelled were synthesized with 1 mM ATP, UTP, CTP, 0.2 mM GTP and 1 mM guanosine, ensuring a high rate of
incorporation of guanosine at the beginning of the transcript (Waring, 1989). After gel-purification, RNAs were 3' end-labelled with T4 polynucleotide kinase under standard conditions. 

RNAs to be 3' end-labelled were synthesized with 1 mM ATP, 1 mM GTP, 0.2 mM CTP, 0.2 mM UTP (T7 transcripts) or in standard conditions (SP6 transcripts). 3' end-labelling was performed using the 'split' technique (Hauser et al., 1990). After gel-purification, 1-5 pmol of RNA was denatured 2 min at 95°C in the presence of a 5-fold excess of oligo(dG)-oligo(dC) mixture 5'-GTTTTTTTTGATCCGGCGG-GGGGCCC-3' whose 3' end is complementary to the 3' end of E1-NC and KNXc transcripts. Annealing buffer was added to a final concentration of Tris–HCl pH 7.5 10 mM; NaCl 50 mM; EDTA 1 mM and the samples were allowed to cool slowly to 30°C. RNA was 3'-end labelled by incubating the sample 15 min at 37°C with 5 pmol of [α-32P]dATP in a 5 μl volume of 37°C incubation buffer (50 mM Tris–HCl pH 7.5, 1.25 mM MgCl2, 20 mM NaCl and 5 mM dithiothreitol). After labelling, the transcripts were phenol/chloroform extracted and ethanol-precipitated.

RNA ligation experiments were performed as described (Moore and Sharp, 1992). The oligonucleotide used to bridge the two half-molecules for RNA ligation was 5'-CCATTTGATAATCATAGTATCCCCGATGTAGTACCTTATAC. 

Modification interference
Modification of RNA by DEPC was performed as described (Conway and Wickens, 1989; Chanfreau and Jacquier, 1994) to ensure less than one modification per transcript. Annealing of the RNA subunits was performed by denaturing 2 min at 95°C in water and slow cooling to 40°C in Tris–HCl pH 7.5 10 mM, NaCl 50 mM and EDTA 1 mM. Annealed molecules were semi-denatured in 1x loading buffer containing 25% w/v urea, 20 mM EDTA, 0.05% xylene cyanol, and 0.05% bromophenol blue) 5 min at 37°C and were gel purified on a 50% urea–4% polyacrylamide gel. Gel purified reconstituted precursor RNAs were incubated under high-salt splicing conditions and unreacted precursor, lariat intermediate and lariat intron were gel purified under semi-denaturing conditions. The resulting purified RNAs were cleaved with aniline, precipitated as described (Conway and Wickens, 1989) and lyophilized overnight. Pellets were dissolved in 98% formamide, 20 mM EDTA, 0.05% xylene cyanol, 0.05% bromophenol blue, denatured 2 min at 95°C and loaded on 5% or 6% sequencing gels. Dried gels were scanned with a PhosphorImager (Molecular Dynamics) and exposed for autoradiography without intensifying screens.

Splicing reactions
Gel-purified RNAs were incubated at 45°C in high salt buffer [HS: 40 mM Tris–HCl pH 7.5, 100 mM MgCl2, 0.5 M (NH4)2SO4, low salt magnesium buffer (LS-Mg: 40 mM Tris–HCl pH 7.5, 100 mM MgCl2, 0.01 M Na2-ascorbate, 1 μM Na2-dodecylsulfate) ] at concentration of 5 μg/ml and 2 mM spermidine (LS-Sp: 40 mM Tris–HCl pH 7.5, 5 mM MgCl2 and 2 mM spermidine; Jacquier and Michel, 1987). During trans-splicing experiments, lariat intermediates and exon 1 molecules were precipitated at 90°C in 30% ethanol. The resulting precipitates were resuspended in 10 mM Tris–HCl pH 7.5, 100 mM NaCl and 20 mM DTT. The reaction was performed at 55°C in 20 μl using RT-HGico Superscript reverse transcriptase (100 units) in 50 mM Tris–HCl pH 8.3, 40 mM KCl, 1 mM dithiothreitol, 6 mM MgCl2, 200 μM dNTPs. RNA was degraded using RNase A and RNase D and RNAs were phenol/chloroform-extracted and ethanol-precipitated. PCR was performed by 30 rounds of amplification (94°C, 15 s; 55°C, 1 min; 72°C, 1 min) using primer PCR52 (5'-GGCCGATCAGCGTCTT-CAATTGATGAGC which is specific for exon 1 and primer NPCR2 which is specific for the 3' junction (5'-GGAATTCTAGATCCGATG- GGTACCTTACAAATTTGTT)), BamHI and EcoRI sites located on primers were used to clone PCR products.

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