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Group II intron RNA-catalyzed recombination of RNA *in vitro*

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ABSTRACT

We report the first evidence for a novel reaction mediated by the self-splicing yeast mitochondrial group II intron bI1; the site-specific recombination of RNA molecules *in vitro*. Upon incubation of the intron lariat with two different RNAs, each harbouring a short sequence complementary to exon binding site 1 (EBS1) of the intron, novel recombined RNAs are formed. As a result of this intron-mediated shuffling of gene segments, the 5′ part of RNA1 is ligated to the 3′ part of RNA2 and, reciprocally, the 5′ part of RNA2 to the 3′ part of RNA1. Sequence analysis of the recombinant junction shows that the site of recombination is precisely located 3′ to intron binding site 1 (IBS1). The hypothesized mechanism of recombination involves exchange of RNA 5′ parts after the first step of a reverse splicing reaction. The possible role of this mechanism *in vivo* and during prebiotic evolution is discussed.

INTRODUCTION

Some group I and group II introns can be autocatalytically excised from a preRNA *in vitro* in the absence of proteins. In both systems, self-splicing proceeds by a two-step mechanism, both steps occuring by *trans*-esterification (1−4). The first, attack by the 3′ OH group of an external guanosine (for group I) or the 2′ OH group of the branch adenosine (for group II) at the 5′ splice site, generates a free 5′ exon. In the second reaction step, the 3′ OH group of this freed exon attacks the 3′ splice site, generating ligated exons and the excised intron (shown for group II introns in Fig. 1a).

Ample evidence has now accumulated showing that autocatalytic RNAs are not only involved in self-splicing and self-cleavage but can also catalyze reactions on exogenous RNAs. Thus, ribonucleic acids can act as true biological catalysts; these activities include endonucleolytic cleavages (e.g. the 'hammerhead' motifs of plant viroid RNAs; the M1 catalytic RNA subunit of Ribonuclease P) and template-directed ligation of oligonucleotides mediated by the *Tetrahymena* rRNA group I intron, i.e. the *Tetrahymena* ribozyme can work like a replicase (5−12). Furthermore, as recently could be shown for the *Tetrahymena* intron and for group II intron bI1 from yeast mitochondria, introns can integrate into foreign RNAs by reversal of the self-splicing reaction (13, 14). The mechanism of the group II intron reverse self-splicing reaction is shown in Fig. 1b. In the first step of the reverse reaction the attack of the 3′ OH group of the intron 3′ terminus at the junction site of the ligated exons yields a splicing intermediate, the intron-3′ exon lariat, and the free 5′ exon. In the second step, the 5′ exon which is still bound to the lariat via the IBS1/EBS1 base pairing can attack the 5′−2′ phosphodiester bond of the branch. This *trans*-esterification step leads to reconstitution of the original precursor. The analogous reaction of the intron with a foreign RNA harbouring an IBS1 motif results in site-specific integration downstream of the IBS1 sequence (14).

Thus, intron movement occurs not only on the DNA-level with the aid of intron-encoded endonucleases by site-specific recombination (shown for some group I introns, 15−20), but introns of both groups have the potential capability of transposing to new locations at the RNA-level. Whereas the unidirectional movement of group I intron DNA ('homing') requires rather elaborate sequences (18 nt) to be recognized and cleaved by intron-encoded endonucleases, recognition sequences for transposition of intron-RNAs are less complex. A short sequence of 6 nt which is complementary to the EBS1 sequence (for group II introns) or IGS (internal guide sequence of the group I introns) obviously seems to be sufficient (13, 14).

In this work we report the first characterization of a novel reaction of a group II intron, the reciprocal recombination of RNAs *in vitro*. The demonstration that intron RNAs are not only able to transpose and to replicate, but also can catalyse recombinations between exogenous RNAs supports the theory of a prebiotic RNA world.

MATERIALS AND METHODS

Materials

Restriction enzymes, Klenow polymerase, Taq DNA polymerase, T4 DNA ligase, polynucleotide kinase, AMV reverse transcriptase, RNA ligase, T7- and T3 RNA polymerase were purchased from Pharmacia. α-^{35}S-UTP,^{32}P-pCp and γ-^{32}P-ATP (2000−3000 Ci/mM) were obtained from Amersham.

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Preparation of RNA

Transcripts — uniformly labelled with ^{35}S-UTP — were synthesized by *in vitro* transcription with T3 or T7 RNA polymerase. Transcription assays were carried out in a 20–200 μl reaction containing 5–50 μg of template DNA, 50–500 units of the enzyme, 40 mM Tris-HCl (pH 7.5), 6 mM $MgCl_2$, 10 mM dithiothreitol, 4 mM spermidine, 500 μM of each ribonucleoside triphosphate and 20 μCi ^{35}S-UTP for 2 h at 37°C. Following transcription, preRNAs were electrophoresed on 5% polyacrylamide 8M-urea gels, autoradiographed, extracted from the gel and purified. As a template for synthesis of full-length bI1 pre RNA, plasmid BS/bI1Δ+6, digested with EcoRI, was used. PreRNA from this plasmid contains the entire autocatalytic group II intron bI1, 16 nt of bE1 with IBS1, and 238 nt from bE2. For synthesis of bI1 intron lariats and RNA1 (bE1–bE2), this preRNA was subjected to a self-splicing reaction and after electrophoretic separation of the products lariats and ligated exons were eluted from the gel. RNA2 was prepared by *in vitro* transcription of BglII digested plasmid BSC$^-$/1700FF which harbours the yeast nuclear *CBS2* gene (21).

In vitro splicing

In vitro splicing was performed in 20 μl buffer S (40 mM Tris-SO_4 pH 7.5, 60 mM $MgSO_4$, 2 mM spermidine and 500 mM $(NH_4)_2SO_4$) at 45°C for 30 min. The reaction was stopped by ethanol precipitation. The resulting pellet was washed with 70% ethanol before being dried under vacuum. For reverse splicing reactions, intron RNA was mixed with equimolar amounts of end-labelled RNA1 and RNA2 (>0.5 μM) and incubated in buffer S. The concentration of $MgSO_4$ in buffer S was increased to 240 mM and incubation was at 30°C for various times. The reactions were stopped by ethanol precipitation and products were electrophoresed on denaturing 5% polyacrylamide gels.

End-labelling of RNA

For 5′ end-labelling, substrate RNAs were treated with calf intestinal phosphatase, subsequently extracted with phenol/chloroform and ethanol precipitated. Dephosphorylated RNAs were 5′ end-labelled with T4 polynucleotide kinase and γ-^{32}P-ATP.

For 3′ end-labelling, transcripts were incubated in the presence of 10 μCi of ^{32}pCp with 10 units of RNA ligase according to England and Uhlenbeck (22).

Primer extension analysis

For primer extension analysis, RNAs isolated from polyacrylamide gels were co-precipitated with the 5′-^{32}P labelled oligonucleotides. cDNA synthesis with AMV reverse transcriptase was performed in the presence (sequence reactions) or absence (continuous cDNA synthesis) of dideoxynucleotides.

PCR

Supposed recombined RNAs were eluted from polyacrylamide gels and reverse transcribed with AMV reverse transcriptase in the presence of primers corresponding to the 3′ exon portion (primers are described in the text). cDNAs were incubated in a 100 μl PCR reaction mixture containing 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.1% gelatin, 200 μM of each deoxynucleoside triphosphate, 20 pmol of each primer, and 2.5 units of Taq DNA polymerase. Before addition of the enzyme and deoxynucleotides, the mixture was denaturated for 5 min at 94°C. The cycling conditions were as follows: 92°C for 1 min, 50°C for 1 min, and 72°C for 5 min for 30 cycles. PCRs were performed in a Perkin-Elmer/Cetus DNA thermocycler and reaction products were electrophoresed in 2% agarose. Desired DNA fragments were eluted from the gel, blunt-end ligated into a SmaI-digested BlueScript KS + vector (Stratagene) and sequenced from both ends.

RESULTS

As a typical intermediate of both the forward and reverse self-splicing reaction of group II introns, the intron-3′ exon lariat intermediate is observed which is charged with the free 5′ exon via the IBS/EBS pairing (Fig. 1a and 1b, step 2). Theoretically, rapid discharge and re-charge of 5′ exons before the next reaction step could lead to a new combination of 5′ exons and 3′ exons, i.e. site-specific intermolecular recombination at the RNA level. This would depend on whether the re-bound 5′ exon is derived from the original or a different RNA.

To test this hypothesis experimentally, we mixed bI1 intron lariats in equimolar amounts with two different RNA species, each containing the bI1-IBS1 motif GACAGA and incubated the mixture at 30°C for various times. RNA1 (254 nt) represents ligated exons bE1–bE2 generated by the autocatalytic splicing reaction of a bI1 containing preRNA (harbouring 16 nt of bE1 with IBS1, and 238 nt from bE2; IBS1 is located 11–16 nt downstream from the 5′terminus). This RNA was labelled either at its 5′- or 3′ end. RNA2 (625 nt) represents part of the *CBS2*

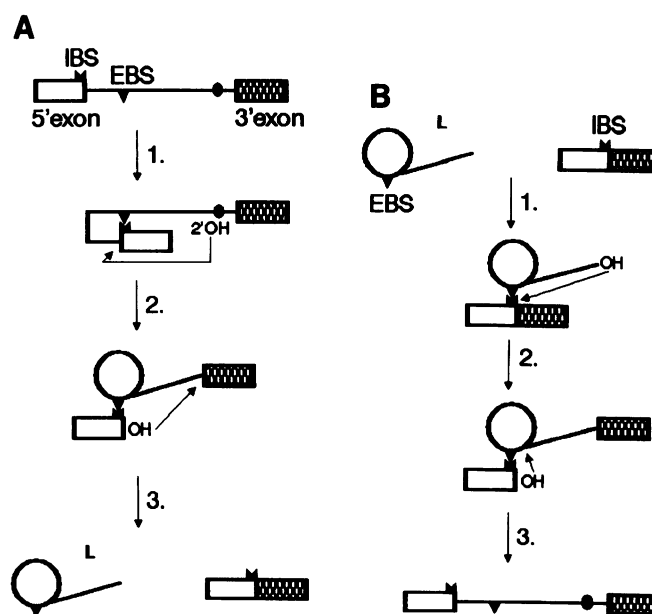

Figure 1. Schematic diagram showing the proposed mechanisms of the group II intron reaction (A) and the reverse reaction (B). *A*. The self-splicing reaction is initiated by binding of the intron site(s) (IBS) of the 5′ exon to the exon binding site(s) (EBS) of the intron (35) and nucleophilic attack by the branch 2′ OH group at the 5′ splice site *(1)*. The 5′ end of the intron is joined to the branchpoint nucleotide via a 2′–5′ phosphodiester bond *(2)*. The 3′ terminal OH-group of the 5′ exon attacks the 3′ junction; this *trans*-esterification leads to exon ligation and liberation of the intron lariat (L) *(3)*. *B*. The first step of the reverse reaction is initiated by binding of ligated exons to the lariat via EBS/IBS base pairing *(1)*. Nucleophilic attack by the 3′ terminal OH-group of the intron at the ligation junction yields the intron-3′ exon intermediate and the free 5′ exon *(2)*. In the last step, the 3′ OH group of the 5′ exon attacks the 5′–2′ phosphodiester bond of the branch and opens the lariat structure; this *trans*-esterification reaction results in reconstitution of the original precursor *(3)*.

antisense transcript with a cryptic IBS1 sequence located 169−174 nt downstream from its 5′ end. For *CBS2* RNA, bI1 intron insertion via this IBS1 element was recently demonstrated (14).

Fig. 2 shows the time course of the reaction of the bI1 intron lariat with RNA1 and RNA2. After 2 hours of incubation, reaction products typical of the reverse reaction were observed. The RNA with the lowest electrophoretic mobility should correspond to the intron-3′ exon lariat, reflecting covalent transfer of the 3′ portion of RNA1 to the 3′ end of the lariat (= first step of the reverse reaction) (Fig. 2, right panel). Additional products are the reconstituted pre RNA bE1−bI1−bE2 (P_2, 1026 nt) and a 5′ end-labelled RNA (left panel) corresponding to the 5′ exon-intron, which has been characterized previously (14, 23). Besides these intermediates and end products of the reverse reaction, additional new RNAs were generated. From intron-mediated recombination between both RNA1 and RNA2—with the postulated site of recombination downstream of IBS1—two novel RNA species (besides the original RNAs) are expected: RNA3 (467 nt), with the 5′ part of RNA1 and the 3′ part of RNA2, and conversely, RNA4 (412 nt) with the 5′ part of RNA2 and the 3′ part of RNA1 (cf. Fig. 2, lower part).

Based on its size, the 5′ end-labelled species of about 470 nt could correspond to the novel RNA3, i.e. a recombined product which consists of the 5′ part of RNA1 and the 3′ part of RNA2. Accordingly, this RNA species is not observed in the reaction

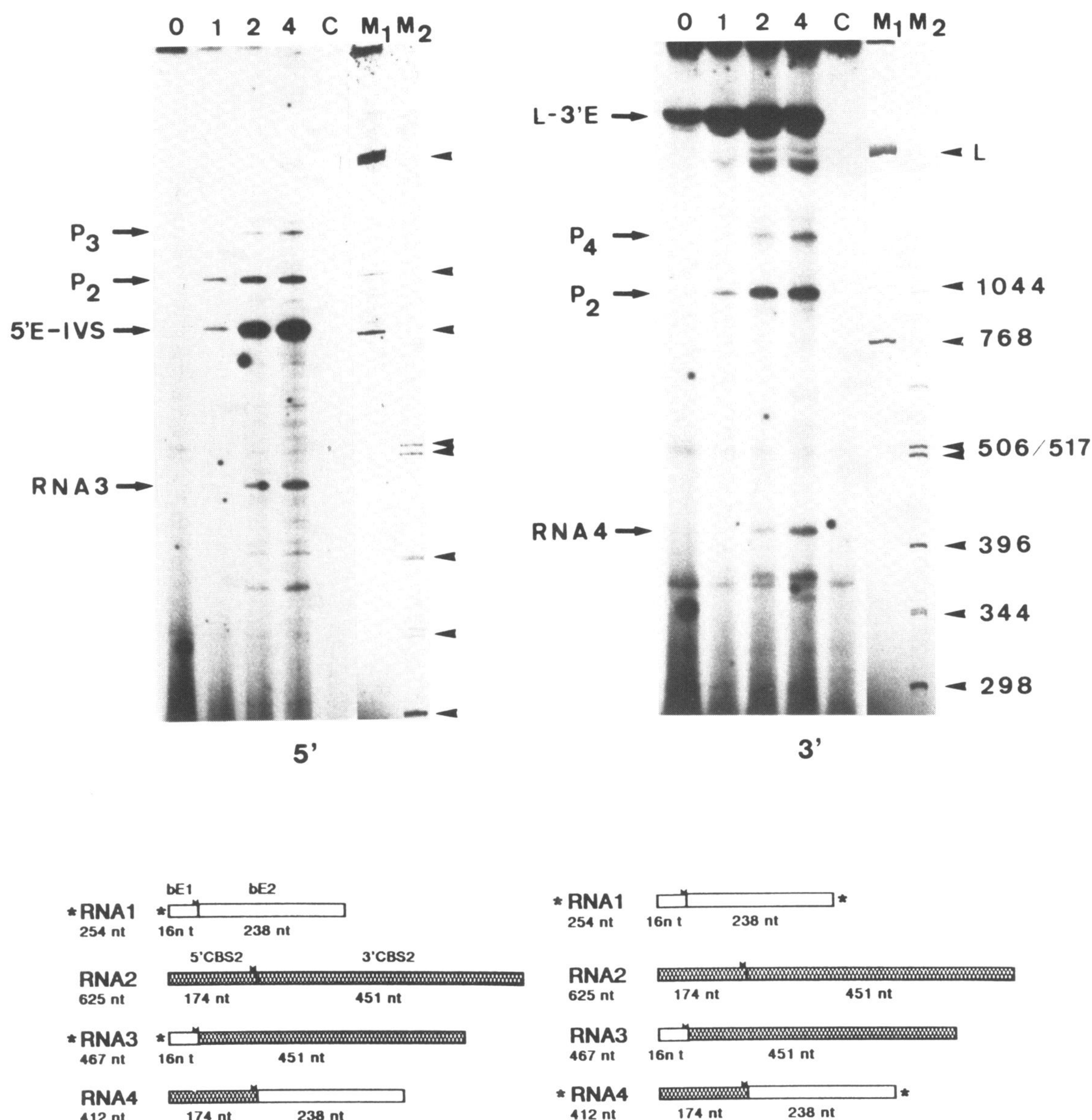

Figure 2. Incubation of the bI1 intron lariat with two different RNAs leads to the formation of novel RNAs. Reaction of 1 μM unlabelled bI1 intron lariat with 1 μM either 5′ end-labelled (left panel) or 3′ end-labelled (right panel) RNA1, and cold RNA2. Incubations were at a constant temperature of 30°C for 0, 1, 2, or 4 h (lanes 0−4, respectively) as described previously (14). M_1: bI1-lariat (L), preRNA (1044 nt) and linear intron RNA (768 nt), M_2: end labelled HinfI×EcoRI-fragments of pBR322. L-3′E: intron-3′ exon lariat, P_2, P_3, P_4: reconstituted preRNAs, 5′E-IVS: 5′ exon-linear intron. RNA3 (476 nt) and RNA4 (412 nt) are the presumed recombined RNAs. C: Incubation of RNA1 and RNA2 for 4 hours without lariat demonstrates that formation of novel RNAs is dependent on the presence of the bI1 lariat. Samples were separated on 5% polyacrylamide-8M urea gels; subsequently, gels were dried and autoradiographed. Due to its size, RNA1 (254 nt) is not visible on the autoradiogram. Composition and sizes of original and expected recombined RNAs are drawn schematically at the bottom of the figure. Asterisks denote the 5′-end 3′ end-label, respectively.

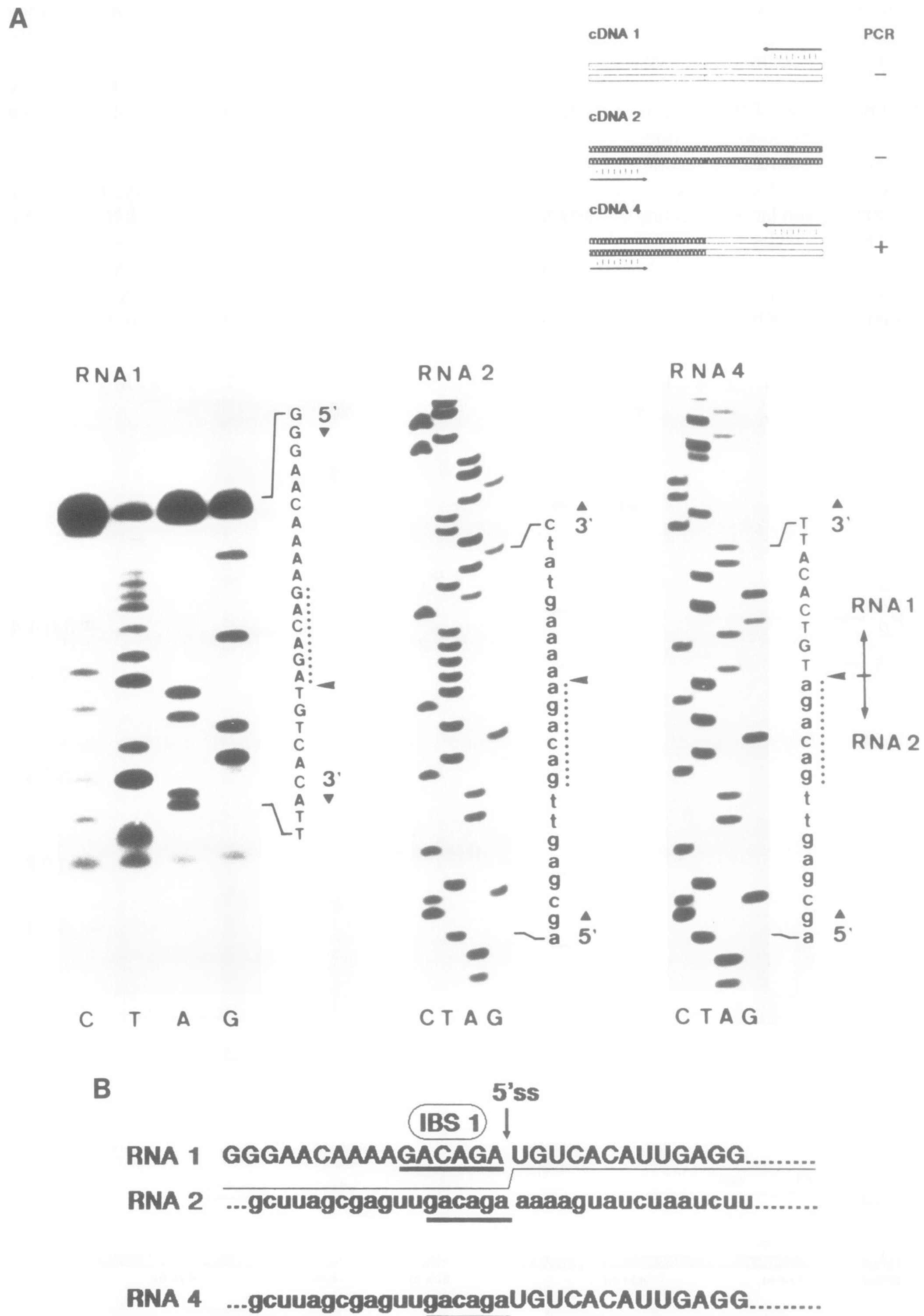

Figure 3. Product characterization. Analysis of a putative product of recombination, RNA4. *(A)*. The RNA4 band was eluted from the gel and used as template in a reverse transcriptase reaction with an oligonucleotide complementary to the 3′ part of RNA1 (GGCGATATTTATATTACC, located 12−29 nt downstream of the IBS1 motif). After addition of the second primer, which is homologous to the 5′ portion of RNA2 (GGTCTCCTTAACTGC, located 127−141 nt downstream from the 5′ terminus), cDNAs were PCR-amplified. The strategy used which allows only amplification of recombined products is diagrammed schematically. After re-cloning, inserts were sequenced with the 5′ exon primer. For comparison, sequences of original RNA1 (obtained by primer extension) and RNA2 (obtained by sequencing the plasmid DNA by the chain termination reaction) are shown. Lettering on the right sides of the autoradiograms corresponds to the sequence complementary to the cDNA, thus corresponding to the RNA sequence. lanes are labelled according to the dideoxynucleotide used in the sequence reactions. Arrowheads denote the positions where recombinations occurred. The dotted line marks IBS1 (GACAGA). RNA1 sequences are in capital letters, RNA2 sequences are in small letters. *(B)*. Sequences of the original RNAs and recombined RNA4 flanking the site of recombination (= 5′ splice site, denoted by the arrow). The IBS1 sequence which is assumed to be involved in recombination is underlined.

with 3′ end-labelled RNA1. Besides this RNA species (and products of the reverse reaction as mentioned above, P_2 and 5′ E-IVS) an additional weak signal of an RNA with a high molecular weight is clearly detectable, which could also represent a reconstituted preRNA. The size of this product is approximately as expected for bE1−bI1−3′CBS2 preRNA (1228 nt), i.e. a recombined pre RNA (P_3).

Upon incubation of the lariat with 3′ end-labelled RNA1 and cold RNA2 under identical conditions, a 3′ end-labelled RNA with an approximate length of 410 nt was observed. This could indicate the formation of RNA4, i.e. recombination between the 5′ part of RNA2 and the 3′ exon of RNA1. It will be shown that this is indeed the case. In addition, part of the label is transferred to an array of slowly migrating RNAs, which have not been further characterized. Whereas two of these might reflect the formation of the original preRNA (P_2) and the intron-3′ exon lariat, P_4 could correspond to a recombined preRNA 5′ CBS2−bI1−bE2 (1180 nt).

To verify its composition and the site of recombination, RNA4 was eluted from the gel and subjected to reverse transcription with a primer complementary to the 3′ portion of RNA1 downstream of the IBS1 motif. Following reverse transcription, cDNA was amplified by the polymerase chain reaction with Taq DNA polymerase. The strategy used only allowed amplification of cDNA from recombined RNAs (schematically shown in Figure 3a). The cDNA fragment was flanked by two oligonucleotides, homologous to the 5′ part of RNA2 and complementary to the 3′ part of RNA1. After enzymatic amplification, products were electrophoresed and fragments with expected sizes were blunt end ligated into the SmaI of a BlueScript vector and sequenced. Results shown in Figure 3 reveal that RNA4 actually is the product of an intron-mediated recombination. It is composed of the 5′ portion of RNA2 ending with the GACAGA motif and the 3′ portion of RNA1 downstream of the GACAGA sequence, i.e. the site of recombination is immediately 3′ to IBS1.

Recombination of RNAs does not require a lariat structure of the intron. As shown in Fig. 4, incubation of the linear bI1 intron with 5′ end-labelled RNA1 and unlabelled RNA2 under the same reaction conditions as described in Fig. 2 also leads to formation of novel RNAs; efficiency is similar to the lariat-mediated reaction. One of these new species with a 5′ end-label could correspond, due to its size, to RNA3. Thus, we assume that the reaction of the linear intron can lead to the same recombinants as described for the lariat-mediated process.

These results are consistent with the current knowledge of the mechanism of the group II intron reverse self-splicing reaction and transposition at the RNA level. A model for intron-dependent RNA recombination is presented in Figure 5. In the first step of recombination, RNA1 and RNA2 interact with lariats via the IBS1/EBS1 base pairing. As intermediates of the first step of a reverse reaction, two products are generated: the intron 3′ exon (RNA1) lariat with the 5′ exon from RNA1 still bound, and the intron 3′ exon (RNA2) lariat with bound 5′ exon from RNA2 (step 2). After exchange of the 5′ exons (step 3), the immediate forward reaction would yield recombined RNA3 and RNA4 (step 4). This pathway does not require the complete reversal of the self-splicing reaction, but only the reverse and forward 3′ cleavage. This presumption is confirmed by results obtained with the linear form of the intron, which is not able to perform the second step of the reverse reaction (14). Alternatively, after 5′ exon exchange the second step of a reverse reaction could yield reconstituted preRNAs with exons from different partners (step 4a). The subsequent forward reaction would also result in the production of recombined RNAs (step 4b/4). Presumably, both reaction pathways may coexist under *in vitro* conditions.

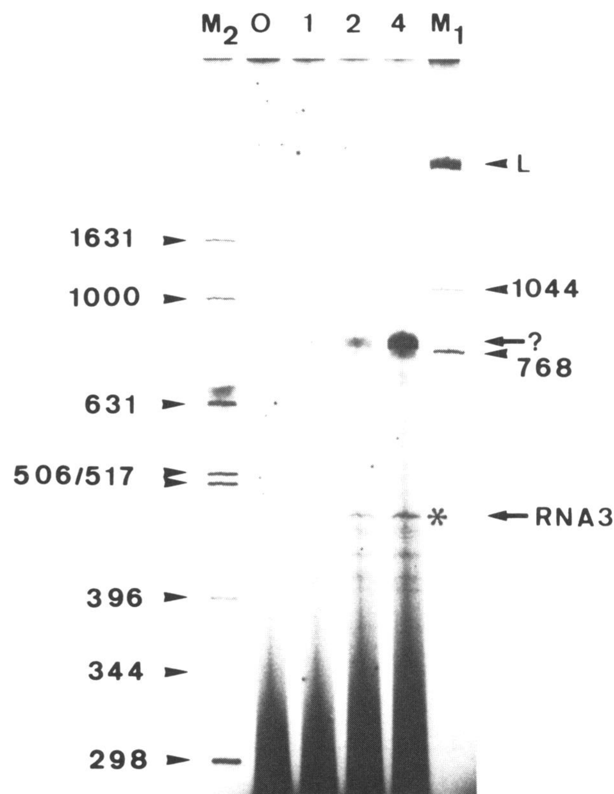

Figure 4. Demonstration that the linear intron also can carry out recombination. Reaction of 1 μM unlabelled linear bI1 intron with 1 μM 5′ end-labelled RNA1 and cold RNA2. Incubation conditions were as described in Fig. 2. M_1: bI1-lariat (L), preRNA (1044 nt) and linear intron RNA (768 nt), M_2: end-labelled HinfI×EcoRI-fragments of pBR322. Arrows denote the proposed recombined RNA3 (*) and new slow migrating RNAs (?) which so far have not been further characterized.

As shown in Fig. 6, both original RNA1 and RNA2 can form stem and loop structures with moderate stability involving the IBS1 motif. Possibly, these structural obstacles could explain the low efficiency of the recombination reaction. This explanation would be in accordance with the observations of Woodson and Cech (13) who could show that the inefficiency of the *Tetrahymena* intron reverse splicing reaction obviously might be due to a rather stable secondary structure in the ligated exons.

DISCUSSION

For group I introns from bacteriophage T4 it was shown that they can mediate exon shuffling at the DNA level by site-specific recombination between homologous regions of two different group I introns (24), thus providing experimental data for support of the exon shuffling hypothesis (25). The recombination reaction leads to formation of a new hybrid intron which separates exons from different genes and which is still functional, thus ensuring that splicing and exon ligation is accurate.

The findings presented here demonstrate, for the first time, that catalytic intron RNAs can also create new combinations of genetic elements at the RNA level by reciprocal recombination *in vitro*. Theoretically, rearrangements between RNA molecules

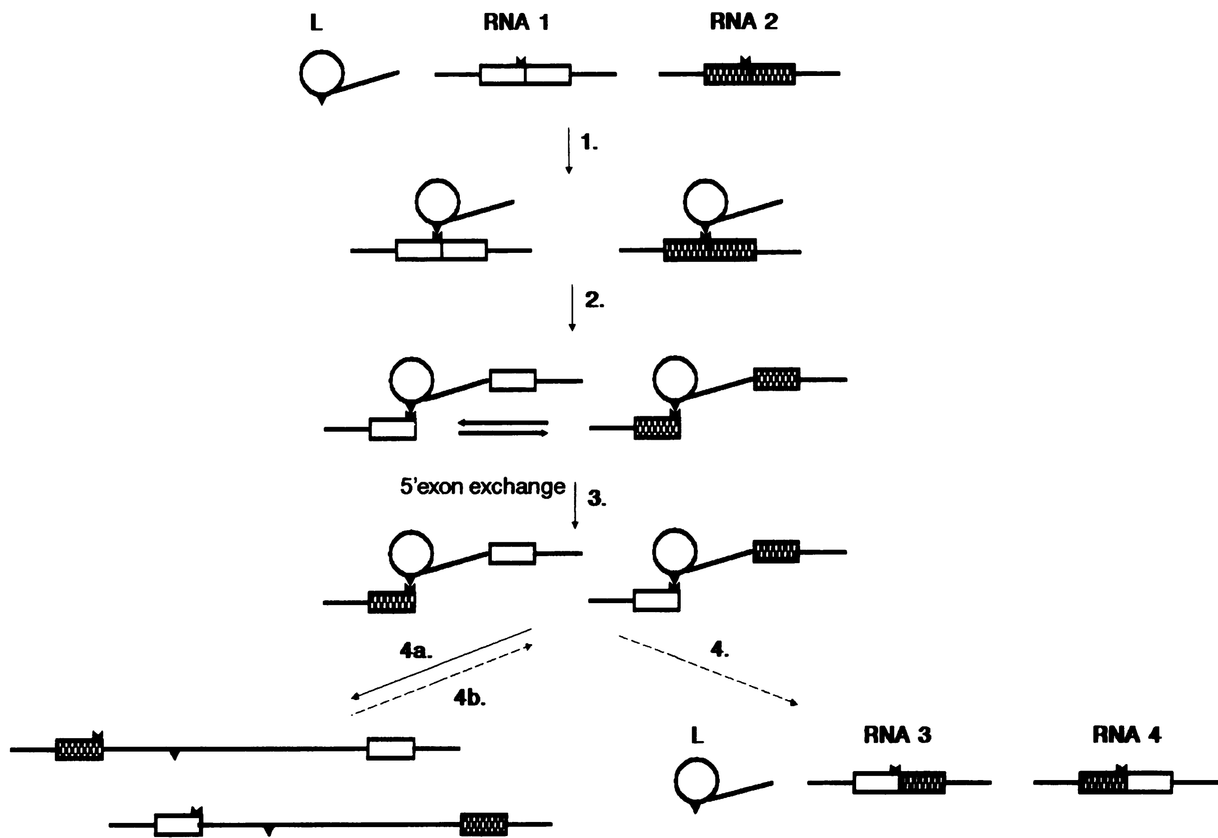

Figure 5. Proposed mechanism of intron-mediated recombination of RNAs. The first step of recombination might be initiated by binding of the intron lariat (L) via an EBS1-element to the IBS1-elements of RNA1 and RNA2 *(1)*. Nucleophilic attack by the 3′ end of the intron at the activated site (immediately downstream of IBS1) would yield the intron-'3′ exon' lariat intermediate via *trans*-esterification, and the free 5′ exon *(2)*. In the third step, the '5′ exon' dissociates and the ribozyme is re-charged with a different '5′ exon' *(3)*. 3′ cleavage and ligation of a forward reaction yields recombined RNA3 and RNA4 *(4)*. Alternatively, while complete reversal of the reaction yields recombined pre RNAs *(4a)*, the forward reaction of these preRNAs also results in formation of recombined RNA3 and RNA4 *(4b)*.

could be accomplished by several mechanisms and the existence of different modes of recombination *in vivo* has been convincingly demonstrated. First, poliovirus can recombine by a copy choice mechanism, in which the virus replicase switches the template strand during synthesis of the negative RNA strand (26). Second, a *trans*-splicing reaction, which involves ligation of the 5′ exon from a spliced leader transcript to pre-mRNAs carrying a 3′ splice site, has been described for trypanosomes and *Caenorhabditis elegans* (27–30). Third, for several viral systems, initiation of mRNA synthesis by the use of RNA primers has been observed (see ref. 31, for review).

An additional mechanism, which can be invoked to explain RNA recombination, would propose a breaking and rejoining reaction. Our results indicate that catalytic group II intron RNA actually can rearrange exogenous RNA segments by this mechanism at least *in vitro*, i.e. by site-specific cleavage and religation of RNA fragments via a *trans*-esterification reaction. The observations that, in principle, RNAs might be capable of i) maintaining genetic information by RNA catalysed replication (10) and ii) introducing genetic variation by recombinational events between RNA elements, supports the view that prebiotic catalytic intron RNAs should not merely be considered as mobile parasites which, at best, did not hamper gene expression, but might have played an important role in early evolution.

It is worth noting that the 5′ exon exchange does not require shifts of temperature to facilitate the melting of the IBS1/EBS1 helix and the re-charging of the ribozyme with a different 5′ RNA unit. This, it seems reasonable to suppose that the 5′ exon exchange reaction can occur not only in the reaction vessel but also under less artificial conditions *in vivo* and the intriguing question arises as to whether this mechanism could have any significance in contemporary cell organelles. Provided that the frequency of recombination is high enough one could expect this reaction occasionally to result in the generation of novel functional RNAs offering a selective advantage. This incidental benefit would of course require reverse transcription of the short-lived RNA and recombination of the cDNA copy with the genome for overcoming its transient nature. In fact, several findings suggest the occurrence of reverse transcription in mitochondria although so far direct experimental evidence is lacking (33; for review). In most cases, intron-mediated rearrangements would probably create nonfunctional RNAs. A correct recombination between different exons, i.e. an exon shuffling like event, would require strict homology of IBS-sequences among all 5′ exons flanking the group II introns. However, among all introns of this group sequenced so far, exon binding sites (and corresponding intron binding sites of 5′ exons) are not conserved (34) and this divergence of exon/intron binding sites would exclude correct recombinations between authentic 5′ exons and 3′ exons.

Nevertheless, these arrangements do not contradict an important role of this mechanism when life was still based on RNA before the origin of protein synthesis and protein-catalysed reaction. It is conceivable that—according to our model—progenitors of group II introns could have assembled appropriate

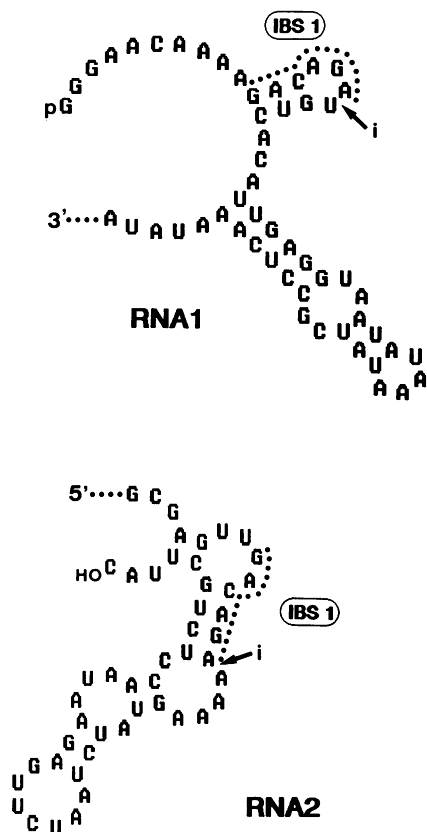

Figure 6. Secondary structures of RNA1 and RNA2. Sequences of original RNA1 and RNA2 flanking the supposed site of recombination (i) with potential stem-loop structures which could hamper the recombination reaction are shown. IBS1 sequences are marked by dotted lines.

RNA pieces in a more or less random fashion without the need of a hundred percent precision. This process not only could have lead to the generation of new but also more complex RNA molecules with novel functions and evolutionary selection of advantageous combinations.

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