An RNA Processing Activity That Debranches RNA Lariats

Barbara Ruskin and Michael R. Green

Abstract. *The excised introns of pre-messenger RNA's (pre-mRNA's) and intron-containing splicing intermediates are in a lariat configuration in which the 5′ end of the intron is covalently joined by a 2′,5′-phosphodiester bond to a specific adenosine residue near the 3′ end of the intron. A 2′,5′-phosphodiesterase activity in HeLa cell extracts has been detected that debranches RNA lariats, converting them to linear RNA molecules by specific cleavage of the 2′,5′-phosphodiester bond. This lariat debranching activity is distinct from previously reported 2′,5′-phosphodiesterases with regard to its biochemical and substrate requirements as well as its stringent cleavage specificity. The debranching activity is observed only if the RNA lariats generated during in vitro processing are deproteinized and added back to the extract. These results suggest that during the normal in vitro splicing reaction the 2′,5′-phosphodiester bond of RNA lariats is protected from cleavage by the lariat debranching activity.*

The primary transcripts of most eukaryotic structural genes (pre-messenger RNA's or pre-mRNA's) contain intervening sequences that are removed by RNA splicing. To study the mechanisms of pre-mRNA splicing, we have characterized the intermediates and products generated during processing of a simple SP6/β-globin pre-mRNA substrate (*1*) in an efficient in vitro splicing system (*2*). The excised intron (IVS1) of the SP6/β-globin pre-mRNA and a probable splicing intermediate, containing IVS1 and exon 2, are in a lariat configuration in which the 5′ end of IVS1 is joined to an adenosine residue located 37 nucleotides (nt) upstream from the 3′ end of the IVS1 (*1*). On the basis of the nuclease resistance of this linkage, label-transfer experiments, and the original study of RNA branches in nuclear RNA (*3*), we proposed that the 5′-terminal guanosine of IVS1 is joined to the adenosine residue by a 2′,5′-phosphodiester linkage (*1*). The human β-globin RNA lariats exhibit several unusual properties, which include an anomalous electrophoretic mobility (*1*), a primer extension block at the position of the RNA branch (*1, 2*), and the presence of a nuclease-resistant component (*1*). In addition to human β-globin pre-mRNA processing, lariat structures have also been detected during adenovirus pre-mRNA splicing in vitro (*4, 5*) and in vivo for rabbit β-globin (*6*) and yeast pre-mRNA's (*7, 8*). It is likely that the excision of introns as lariat structures is a universal, intrinsic feature of the pre-mRNA splicing mechanism.

We previously reported that in the in vitro extract the excised IVS1 (143 RNA) is slowly converted to another discrete RNA product (130 RNA) that on the basis of its anomalous electrophoretic mobility also contains an unusual structural component (*1*). However, we did not detect a simple linear form of IVS1, and similarly the IVS1–exon 2 RNA species (380 RNA) is predominantly or exclusively in a lariat configuration. These results imply that during pre-mRNA splicing (i) cleavage at the 5′ splice site and lariat formation are either coupled reactions or 2′,5′-phosphodiester bond formation rapidly follows cleavage at the 5′ splice site and (ii) the 2′,5′-phosphodiester bond of the RNA lariats is stable in the crude nuclear extract.

Barbara Ruskin is a graduate student and Michael R. Green is an assistant professor in the Department of Biochemistry and Molecular Biology, Harvard University, Cambridge, Massachusetts.

We now describe the detection and preliminary characterization of an enzymatic activity that converts RNA lariats to linear molecules by specific cleavage of the 2′,5′-phosphodiester bond. The biochemical and substrate requirements and cleavage specificity of this activity indicate that it results from a novel RNA processing enzyme. In addition, we definitively establish the structure of the minor form of the excised intron, the 130 RNA species. As is expected from its anomalous electrophoretic mobility (*1*), the 130 RNA species is also in the form of a lariat. These results have implications with regard to the mechanism of pre-mRNA splicing and the structure (or structures) of the RNA processing products generated during in vitro processing.

An enzymatic activity that debranches RNA lariats. We have previously characterized the major RNA species generated during splicing of a truncated SP6/β-globin pre-mRNA substrate in crude extracts prepared from HeLa cell nuclei (*1*). The 380 and 143 RNA's are the predominant IVS1-containing RNA species and accumulate in the form of a lariat during the course of the in vitro splicing reaction. These lariat structures are stable on prolonged incubation in the extract although the 143 RNA is slowly converted to an RNA species with an electrophoretic mobility, based on DNA markers, of approximately 130 nt. Like the 143 RNA species, the 130 RNA species has an abnormal electrophoretic mobility on a two-dimensional denaturing gel system, indicating that it is not a simple linear RNA molecule (*1*).

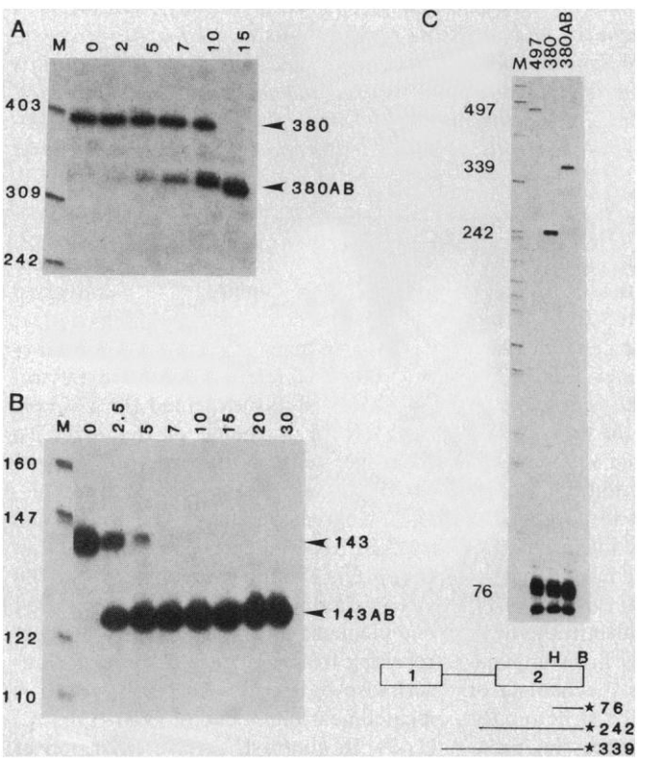
The 380 and 143 RNA species were purified by gel electrophoresis and used as substrates to search for additional RNA processing activities. When the purified, deproteinized 380 RNA is "added back" to the nuclear extract or to the cytoplasmic S100 fraction and incubated under the same biochemical conditions used in the splicing reaction, it is rapidly and quantitatively converted to a discrete RNA species (380 add back; 380AB) with an electrophoretic mobility of 339 nt (Fig. 1A). This is the electrophoretic mobility expected for a simple linear RNA molecule of this size. To determine whether the 380AB RNA species is in fact a simple linear molecule, primer extension analysis of the 380 and 380AB RNA species was carried out (Fig. 1C). Primer extension of the 380 RNA produces a 242-nt ^{32}P-labeled complementary DNA (cDNA) product resulting from blockage of reverse transcriptase by the RNA branch, which is located near the 3′ end of IVS1 (Fig. 1C) (*1, 2*). In contrast, primer extension of the 380AB RNA produces a single ^{32}P-labeled cDNA whose size is consistent with the 339-nt primer extension product expected for a simple linear RNA with a 5′ end at position 1 of IVS1 (Fig. 1C). These results suggest that the 380AB RNA species is a linear

molecule that lacks an RNA branch.

When the excised IVS1 (143 RNA) is purified by gel elecrophoresis and added back to the extract it is also quantitatively converted to a discrete RNA species (143AB) with an electrophoretic mobility of 130 nt, consistent with a simple linear structure (Fig. 1B). To determine whether the 143AB RNA was a linear RNA, resulting from specific cleavage of the 2′,5′-phosphodiester bond, the 143AB RNA was digested by ribonuclease (RNase) T1 and the oligonucletides were fractionated by high-voltage paper electrophoresis and homochromotography (fingerprint) (Fig. 2). The RNase T1 fingerprint of the 143 RNA has been described (*1*). Significantly, RNase T1 fragment 12 (ACUCUCUCUG), which is located 28 to 37 nt upstream from the 3′ splice site, is shifted relative to the same unmodified RNase T1 fragment derived from an unprocessed in vitro transcript. The altered mobility of RNase T1 fragment 12 is due to a guanosine residue, originating from the 5′ end of IVS1, that is joined to the adenosine residue by a 2′,5′-phosphodiester bond (*1*). When the fingerprints of the 143 and 143AB RNA species are compared, it is clear that the position of RNase T1 fragment 12 from the 143AB RNA is altered (Fig. 2, A and B). The position of RNase T1 fragment 12 from the 143AB RNA is that expected for the unmodified RNase T1 fragment [see figure 5A in reference (*1*)].

To confirm that RNase T1 fragment 12 from the 143AB RNA is in fact unmodified and contains a free 2′-hydroxyl group, this fragment was purified and digested to completion with RNase A (Fig. 3). The unmodified ^{32}P-UTP–labeled RNase T1 fragment 12 (ACUCUCUCUG) yields two ^{32}P-labeled products, Cp and ApCp, upon secondary digestion with RNase A (*1*). When RNase T1 fragment 12 derived from a ^{32}P-UTP–labeled 143 RNA species is digested with RNase A, the product ApCp is absent and is replaced by a new RNase A digestion product (X) (Fig. 3) (*1*). The radically altered mobility of this RNase A digestion product is due to the fact that the adenosine residue of the ApCp dinucleotide is linked by a 2′,5′-phosphodiester bond to the guanosine residue from the 5′ end of IVS1 (*1*). In contrast, when RNase T1 fragment 12 from the ^{32}P-UTP–labeled 143AB RNA is digested by RNase A, the two resultant ^{32}P-labeled products are Cp and ApCp, as expected for the unmodified fragment (Fig. 3) (*1*). Finally, in the 143 RNA species, the 2′,5′-phosphodiester bond of the adenosine renders the 3′,5′-phosphodiester bond of ApC in RNase T1 fragment 12 resistant to cleavage by RNase T2 (*1*). In contrast, purified ^{32}P-CTP–labeled RNase T1 fragment 12 from the 143AB RNA is completely digested by RNase T2 (*9*), indicating that the adenosine contains an unmodified 2′-hydroxyl group. Taken together these results demonstrate that the lariat debranching activity has specifically hydrolyzed the 2′,5′-phosphodiester bond of the 143 RNA species generating an adenosine residue that contains an unmodified 2′-hydroxyl group.

If the lariat debranching activity converts RNA lariats to linear RNA's by cleavage at the 2′ side of the 2′,5′-phosphodiester bond, the resultant linear RNA will contain a 5′-terminal monophosphate (as shown below). Therefore, RNase T1 digestion of the linear RNA should produce pGp. The RNase T1 fingerprint of the 143AB RNA contains an additional spot (labeled pGp) compared to the fingerprint of the 143 RNA species



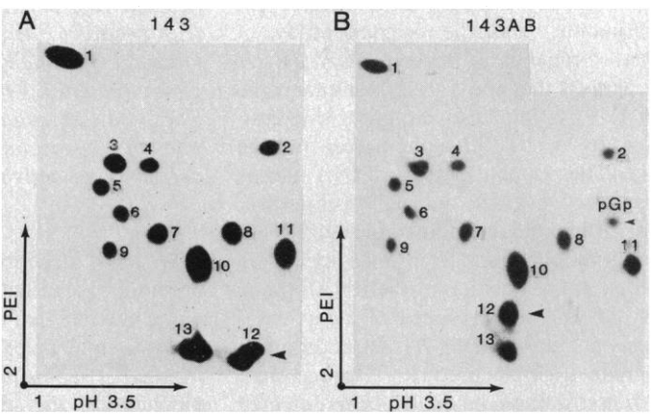


Fig. 1 (left). An enzymatic activity that debranches RNA lariats. Purified ^{32}P-labeled 380 (A) or 143 (B) RNA species (*1*) were added to a HeLa cell nuclear extract (*20*) under the standard conditions for in vitro splicing (*1*, *2*). At the time (minutes) indicated at the top of each lane, the RNA was purified and analyzed on a denaturing 5 percent polyacrylamide gel. M indicates ^{32}P-labeled markers of Msp I–digested pBR322 DNA. The names of the various RNA species are indicated on the right. The sizes (nt) of the ^{32}P-DNA markers are indicated on the left. (C) Primer extension analysis of the 380 and 380AB RNA species. Primer extensions were conducted as described (*1*, *2*). The purified 380 and 380AB RNA species (A) were subjected to primer extension analysis with the use of the 76-nt Bam HI–Hae III fragment [positions +405 to +481 of the β-globin gene (*21*)], which was 5′ end-labeled with ^{32}P at the Bam HI site. The sizes of the primer extension products are indicated on the left. M, ^{32}P-labeled markers of Msp I–digested pBR322 DNA. A diagram of the primer extension products is shown below the autoradiogram. Boxes indicate exons; lines indicate introns; 1 and 2 indicate exons 1 and 2; H indicates the Hae III site and B, the Bam HI site. The position of the ^{32}P end label is indicated by a star. Fig. 2 (right). RNase T1 fingerprint analysis of the 143 and 143AB RNA species. The purified 143 (A) and 143AB (B) RNA species, labeled with ^{32}P-UTP, were digested with RNase T1 and fingerprinted as described (*1*). The directions of the first dimension, high-voltage paper electrophoresis at *p*H 3.5, and second dimension, homochromatography on PEI plates, are indicated by arrows. The composition of the numbered oligonucleotides has been described (*1*). Spot 12, which contains the branched adenosine (*1*), is indicated by an arrow. This spot often appears as a doublet, possibly because in some molecules one of the two 3′ guanosines is not cleaved to completion with RNase T1 and therefore contains a 2′,3′ cyclic phosphate (*22*). In the 143AB fingerprint, the product pGp is indicated.

(Fig. 2, A and B). When this new RNase T1 digestion product is purified and analyzed by thin-layer chromatography it comigrates with a pGp marker. Furthermore, when a pGp marker is subjected to the two-dimensional fingerprint analysis it is found at the identical position of this new spot. Taken together these results indicate that the lariat debranching activity converts RNA lariats to linear molecules by a single endonucleolytic cleavage at the 2′ side of the 2′,5′-phosphodiester bond.

Biochemical requirements of the lariat debranching activity. The lariat debranching activity was originally detected under the same biochemical conditions that are used for in vitro splicing, which include mono- and divalent cations and adenosine triphosphate (ATP) (*2*). The biochemical requirements for the lariat debranching activity were determined by the conversion of the ^{32}P-labeled 380 RNA to the 380AB RNA as monitored by denaturing gel electrophoresis (Fig. 1B). These results are summarized in Table 1. Conversion of the RNA lariats to linear molecules requires the addition of the nuclear extract (NE) or the cytoplasmic fraction (S100); under these standard conditions the RNA lariat structure is stable on incubation in the buffer alone or in heat-inactivated extracts. Surprisingly, debranching activity does not require the addition of monovalent cations, divalent cations, or ATP. However, it is difficult to rule out that a low concentration of monovalent cations is present in the extract and required for lariat debranching. High concentrations of monovalent cations (400 m*M*) inhibit debranching (more than 95 percent). The activity is maintained in the presence of 50 m*M* EDTA or 50 m*M* EGTA, indicating the absence of a divalent cation requirement or the presence of an enzyme-bound divalent cation. To rule out the possibility that residual ATP in the extract is required for lariat debranching, we made an effort to completely remove ATP from the extract. The nuclear extract contains a nucleotide phosphorylase activity that depletes the ATP pool in the absence of creatine phosphate (*2*). Preincubation of the extract at 30°C in the absence of creatine phosphate completely depletes the ATP pool as determined by assaying the disappearance of [α-^{32}P]ATP by thin-layer chromatography (*2*). However, this depletion has no effect on the subsequent assay for lariat debranching (*9*). Furthermore, ATP analogs with nonhydrolyzable β-γ or α-β bonds do not inhibit debranching (*9*). Treatment of the extract with micrococcal nuclease prior to addition of the RNA substrate has no apparent effect on lariat debranching, while proteinase K digestion completely abolishes the activity. Furthermore, under the standard conditions, the debranching activity is irreversibly inactivated at temperatures above 52°C. These results suggest that the debranching enzyme is composed of protein and lacks an essential nucleic acid component. Treatment of the extract with *N*-ethylmaleimide (NEM) does not inhibit lariat debranching, suggesting that an activated sulfhydryl group is not required for catalysis. We have also found that the debranching activity chromatographs as a single component over several ion-exchange columns (*10*).

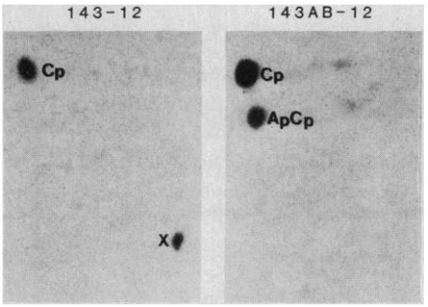


Fig. 3. RNase A secondary digestion analysis of spot 12 from the 143 and 143AB RNA species. The 143 and 143AB RNA species labeled with [^{32}P]UTP were digested with RNase T1 and fingerprinted. Spot 12 was purified, digested with RNase A, and the products fractionated by two-dimensional thin-layer chromatography (*1, 23*). The sequences of the RNase A digestion products are indicated. The RNase A–resistant component is designated by an X.

Substrate requirements of the lariat debranching activity. The lariat debranching enzyme displays an unusually restricted cleavage specificity; in the RNA lariats only the single 2′,5′-phosphodiester bond and no 3′,5′-phosphodiester bonds are hydrolyzed. This restricted cleavage specificity, along with the biochemical requirements described above, distinguishes the debranching activity from the previously reported 2′,5′-phosphodiesterases (*11–13*). To determine the essential components of an active substrate for the lariat debranching enzyme, several substrates that contain 2′,5′-phosphodiester bonds were tested in the crude extract. Since under the conditions of the standard debranching reaction all other reported 2′,5′-phosphodiesterases are inactive (*11–13*), we assume that cleavage of the 2′,5′-phosphodiester bond of these RNA substrates is due to the lariat debranching enzyme. We first asked whether the 2′,5′-phosphodiester bond of a substrate that contains a single nucleotide RNA branch is cleaved by the lariat debranching activity. Digestion of the 143 RNA species with nuclease P1 produces a nuclease P1–resistant trinucleotide core consisting of the branched adenosine residue in a 2′,5′-phosphodiester bond to the 5′-terminal guanosine of IVS1, and in a 3′,5′-phosphodiester linkage to the adjacent cytosine residue of IVS1 (Fig. 4A) (*1, 3*). Treatment of the [^{32}P]GTP-labeled trinucleotide core with snake venom phosphodiesterase, a nonspecific 2′,5′-phosphodiesterase, results in complete hydrolysis of the 2′,5′-phosphodiester bond producing [^{32}P]5′-GMP (Fig. 4A). When the trinucleotide core is incubated in the S100 extract, a discrete [^{32}P]5′-GMP product is detected, although most of the trinucleotide substrate remains intact (Fig. 4A). This result suggests that an RNA containing a single nucleotide RNA branch is an active substrate for the lariat debranching enzyme. The failure of the debranching reaction to go to completion is most likely due to the high molar amount of the [^{32}P]GTP-labeled

Table 1. Biochemical requirements of the lariat debranching activity. Debranching reactions were set up under the specified conditions. The conversion of 1 to 2 fmol of purified ^{32}P-labeled 380 RNA species to the 380AB RNA species was monitored by denaturing polyacrylamide gel electrophoresis (Fig. 1). Buffer D (*20*) is 20 m*M* Hepes (*p*H 8.0), 20 percent glycerol, 100 m*M* KCl, 0.5 m*M* dithiothreitol (DTT); the symbols + or − refer to whether (+) or not (−) more than 95 percent of the RNA lariat is converted to a linear RNA molecule. The specifications include: nuclear or cytoplasmic extract, 20 percent (by volume); ATP, 500 μ*M*; Mg^{2+}, 3.2 m*M*; *N*-ethylmaleimide, 10 m*M*. Extracts were treated with micrococcal nuclease and subsequently the micrococcal nuclease was inhibited by the addition of EGTA before the addition of the RNA substrate (*24*). Based on the results in this table, the standard debranching reaction, which was used throughout the remainder of this work, was in 25 μl of buffer D containing 20 percent (by volume) S100 and 8 m*M* EDTA. Incubation was at 30°C for 30 minutes.

Reaction components	Activity
Buffer D, ATP, Mg	−
Buffer D, NE, ATP, Mg	+
Buffer D, S100, ATP, Mg	+
Buffer D, S100	+
Buffer D, S100, 50 m*M* EDTA	+
Buffer D, S100, 50 m*M* EGTA	+
Buffer D, S100, 400 m*M* KCl	−
Buffer D, S100 (NEM)	+
Buffer D, S100 (micrococcal nuclease)	+
Buffer D, S100 (proteinase K)*	−
Buffer D, S100†	−

*4 μg at 45°C for 15 minutes. †55°C for 10 minutes.

trinucleotide core that is required for detection in this experiment (see legend to Fig. 4A). It is also possible that a single nucleotide RNA branch is a less efficient substrate than an intact lariat and is therefore cleaved at a reduced rate.

Next, we tested whether 2′,5′-oligo(A), the standard substrate used for assaying 2′,5′-phosphodiesterases (*11–13*), is cleaved by the lariat debranching enzyme. This substrate consists of three adenosine residues joined by 2′,5′-phosphodiester bonds and entirely lacks 3′,5′-phosphodiester bonds (Fig. 4B). As shown in Fig. 4B, 5′-^{32}P-end-labeled 2′,5′-oligo(A) is cleaved by snake venom phosphodiesterase releasing [^{32}P]5′-AMP. In contrast, [^{32}P]5′-AMP is not detectable after addition of this substrate to the S100 extract. Furthermore, addition of 0.5 m*M* 2′,5′-oligo(A) to the standard debranching reaction does not inhibit conversion of the 380 to the 380AB RNA species. Thus, the lariat debranching enzyme is not a nonspecific 2′,5′-phosphodiesterase. These results indicate that either (i) the lariat debranching enzyme is specific for the nucleotides involved in the 2′,5′-phosphodiester linkage [that is, the adenosine-(2′,5′)-guanosine linkage but not the adenosine-(2′,5′)-adenosine linkage is active], or (ii) the minimal active substrate is a branched nucleotide, which contains both a 2′,5′- and a 3′,5′-phosphodiester bond (*9*).

To determine whether the nucleotide composition of the 2′,5′-phosphodiester bond is required for lariat debranching activity, we tested whether RNA lariat that contains an adenosine-(2′,5′)-adenosine linkage is an active substrate. When the GT at the 5′ end of the intron of the yeast ribosomal protein L29 gene (CYH2) is changed to AT, cleavage at position 1 of the intron occurs and an RNA lariat is formed that contains an adenosine-(2′,5′)-adenosine linkage (*14*) (Fig. 4C, diagram). This ^{32}P-labeled RNA lariat was added to the S100 extract and as a control a normal yeast CYH2 RNA lariat, containing an adenosine-(2′,5′)-guanosine linkage, was also tested. As shown in Fig. 4C, incubation of both RNA lariats in the HeLa cell extract results in a radical increase in their electrophoretic mobilities. Following enzymatic debranching, the electrophoretic mobilities of the RNA's are 810 nt, consistent with their size and indicating that they are linear RNA molecules. Thus, the 2′,5′-phosphodiester bonds of both the adenosine-(2′,5′)-guanosine and adenosine-(2′,5′)-adenosine lariats are completely hydrolyzed, indicating the lack of a stringent requirement for the nucleotide composition of the 2′,5′-phosphodiester linkage. Taken together these results suggest that the minimal substrate for the lariat debranching enzyme is a branched nucleotide that contains both a 2′,5′- and 3′,5′-phosphodiester bond. However, confirmation of this conclusion will require the analysis of additional RNA substrates with the purified or partially purified debranching enzyme.

Structure of the 130 RNA species. During the normal in vitro processing reaction of the SP6/β-globin pre-mRNA, the 143 RNA species is not debranched but rather is slowly converted to another discrete RNA species (130 RNA) that also has an anomalous electrophoretic mobility (*1*). To determine whether the 130 RNA species is also in a lariat config-

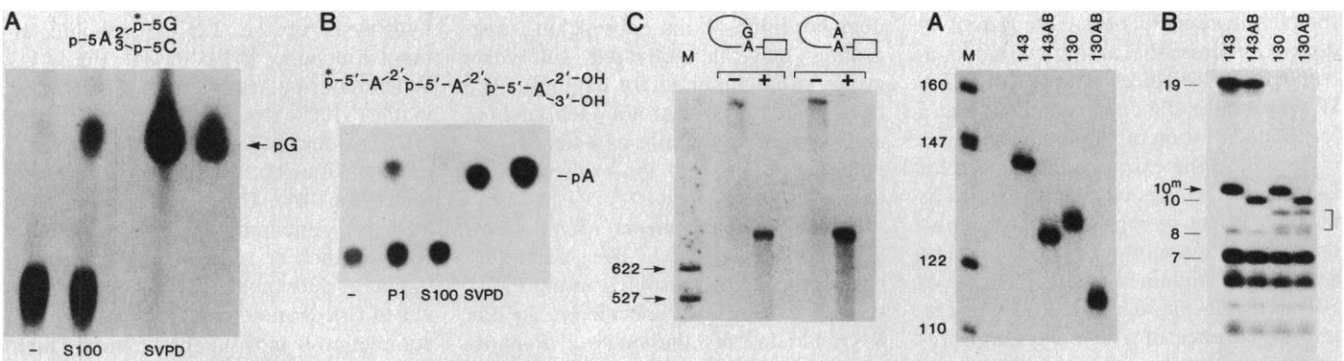


Fig. 4 (left). Substrate specificity of the lariat debranching activity. The structures of the various substrates is shown above each autoradiogram. The position of the ^{32}P label is indicated by a star. These ^{32}P-labeled RNA substrates were incubated in the S100 fraction under the standard conditions for enzymatic debranching (legend to Table 1). Snake venom phosphodiesterase digestions were in 50 m*M* tris-HCl (*p*H 8.0), 5 m*M* $MgCl_2$, and snake venom phosphodiesterase (20 to 200 μg/ml) at 37°C for 30 minutes. (A) A [^{32}P]GTP-labeled 143 RNA species was digested with nuclease P1 and the nuclease-resistant trinucleotide was purified on a 25 percent nondenaturing polyacrylamide gel. Approximately 50 fmol of this ^{32}P-labeled RNA substrate was treated as indicated at the bottom of each lane; the products were purified by adsorption to activated charcoal and fractionated on cellulose plates; the solvent was 0.1*M* sodium phosphate (*p*H 6.8), ammonium sulfate, *n*-propanol (100:60:2; volume:weight:volume) (*25*) as described (*1*). (−), No treatment; S100, standard enzymatic debranching reaction; SVPD, snake venom phosphodiesterase digestion; pG, 5′-GMP. (B) Oligoisoadenylate (Sigma) was 5′ end-labeled with [γ-^{32}P]ATP and polynucleotide kinase (Promega Biotec). The total reaction mixture was treated with nuclease P1 and the nuclease-resistant trinucleotide was purified on a 25 percent nondenaturing polyacrylamide gel. This ^{32}P-labeled substrate was treated as specified below each lane, and the digestion products were fractionated by thin-layer chromatography as in (A). The small amount of ^{32}P label that is sensitive to digestion with nuclease P1 indicates that some of the ^{32}P-ATP-label is in a 3′,5′-phosphodiester bond, due to a contaminant in the commercially available starting material. (−), No treatment; P1, nuclease P1 digestion; pA, 5′-AMP. (C) ^{32}P-labeled IVS1-exon 2 RNA species generated during in vitro processing of the normal and mutant yeast CYH2 pre-mRNA's (see text) were subjected to enzymatic debranching, and the products were fractionated on a 5 percent denaturing polyacrylamide gel. The nucleotide composition of the 2′,5′-phosphodiester linkage in the RNA lariats is indicated above the lanes. Boxes, exons; lines, introns; the symbols + and − indicate whether (+) or not (−) the substrates were incubated in the extract prior to electrophoresis. M, ^{32}P-labeled markers of Msp I–digested pBR322 DNA. Fig. 5 (right). Structural characterization of the 130 RNA species. (A) Electrophoretic fractionation of the human β-globin IVS1 RNA species. The purified ^{32}P-labeled RNA species indicated at the top of each lane were subjected to electrophoresis on a denaturing 5 percent polyacrylamide gel and detected by autoradiography. M, ^{32}P-labeled markers of Msp I–digested pBR322 DNA. (B) RNase T1 digestion analysis of the human β-globin IVS1 RNA species. The ^{32}P-labeled IVS1 RNA species indicated on the top of each lane were digested with RNase T1 with or without prior enzymatic debranching and the digestion products were fractionated on a denaturing 25 percent polyacrylamide gel. The sizes of some of the RNase T1 fragments are indicated. 10^m designates the modified 10 nt RNase T1 fragment 12.

uration, we added this purified RNA species to HeLa cell extracts. As shown in Fig. 5A, the 130 RNA was converted to a discrete RNA species (130AB) with an electrophoretic mobility of approximately 118 nt on a 5 percent denaturing polyacrylamide gel. In contrast to the intact 130 RNA species, the 130AB RNA displays an identical electrophoretic mobility on higher percentage polyacrylamide gels, suggesting that it is a simple linear RNA. The fact that the 130 RNA species is in a lariat configuration with an increased electrophoretic mobility compared to that of the 143 RNA species suggests that the 130 RNA is missing sequences from the linear 3′ end of IVS1.

The structure of the 130 RNA species was confirmed by RNase T1 digestion analysis. The 130, 130AB, 143, and 143AB RNA species were treated with RNase T1 and the digestion products were fractionated on a denaturing 20 percent polyacrylamide gel (Fig. 5B). The 130 and 130AB RNA species differ from the 143 and 143AB RNA's by the absence of a 19-nt RNase T1 fragment (Fig. 2, spot 13), derived from the 3′ end of IVS1 (*1*). Furthermore, the 130 and 130AB RNA species contain two additional RNase T1 fragments, approximately 9 nt in length. Secondary digestion of these new RNase T1 digestion products with RNase A indicates that they are both derived from the 5′ end of RNase T1 fragment 13 (*9*). The two new RNase T1 fragments differ from each other by at most one nucleotide and may only differ by the presence of a phosphate or hydroxyl group at their 3′ ends. Taken together these results indicate that the 130 RNA species is derived by a fairly precise removal of approximately 10 nt from the linear 3′ end of the 143 RNA. This conclusion is also supported by site-directed digestion of the 130 RNA species with RNase H and IVS1-specific synthetic oligonucleotides (*1, 9*).

An additional difference between the RNase T1 digestion products of the IVS1 RNA species is that the 10 nt fragment (Fig. 2, spot 12) from the 143AB and 130AB RNA species has an increased electrophoretic mobility compared to the same fragment derived from the 143 and 130 RNA species. The increased electrophoretic mobilities of the 143AB- and 130AB-derived fragments is due to the absence of the guanosine residue, which in the 143 and 130 lariat RNA's is linked to the adenosine residue of RNase T1 fragment 12 by a 2′,5′-phosphodiester bond (Fig. 2).

Discussion. Our results indicate that the excised IVS1 (143 RNA) can be further processed in vitro as summarized in Fig. 6. HeLa cell extracts contain an activity that debranches RNA lariats by specific cleavage of the 2′,5′-phosphodiester bond. This enzyme is distinct from the other known 2′,5′-phosphodiesterases with regard to its cleavage specificity as well as its biochemical and substrate requirements. The lariat debranching enzyme represents a novel RNA processing activity; the 129 normal 3′,5′-phosphodiester bonds in the 143 RNA are left intact while the single 2′,5′-phosphodiester bond is efficiently cleaved.

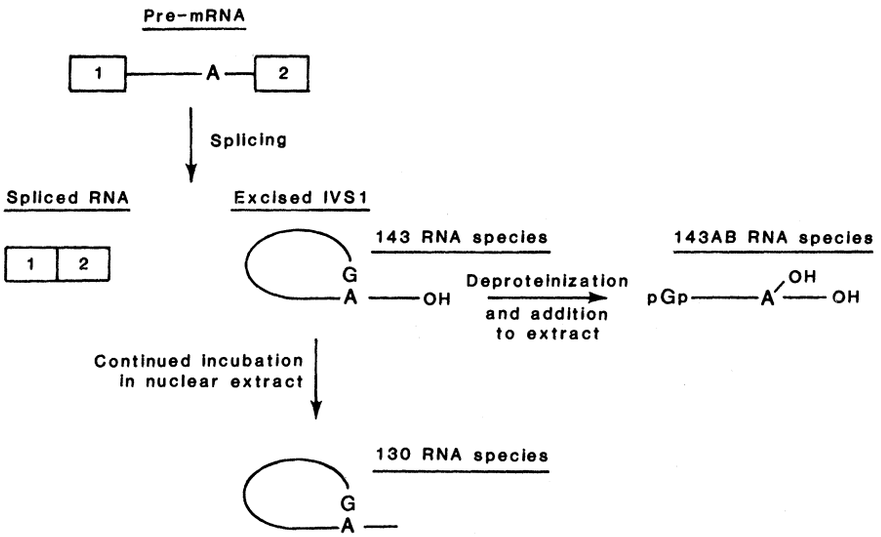


Fig. 6. Summary of the further in vitro processing reactions of the excised IVS1. The further processing pathways of the excised IVS1 in the in vitro extract are indicated.

What is the role of the lariat debranching enzyme in pre-mRNA processing? One possibility is that the lariat debranching enzyme has an essential function in the intron degradation pathway. Clearly a 2′,5′-phosphodiesterase activity is required for the complete degradation of excised introns, which are in a lariat configuration (*1, 4, 6–8*). Recently the intact excised intron, in a lariat structure, has been detected in vivo (*6–8*). Besides the intact lariat, a linear form of the intron, resulting from cleavage of the 2′,5′-phosphodiester bond, has also been detected in vivo for both yeast (*7*) and higher eukaryotes (*6*). It is possible that a lariat debranching activity mediates the in vivo conversion of the lariat form of an excised intron to its linear counterpart. An additional intron species detected in vivo is a lariat RNA lacking a portion of the 3′ linear end of the intron (*7, 8*). This in vivo RNA species is analogous to the in vitro–generated 130 RNA described in this article.

An intriguing possibility is that the lariat debranching reaction is catalyzed by an enzyme involved in the normal pre-mRNA splicing pathway. RNA lariat formation during pre-mRNA splicing requires a 2′,5′-RNA ligation reaction. Formation of the 2′,5′-phosphodiester bond and cleavage of the 3′,5′-phosphodiester bond at the 5′ splice site could be coupled reactions (*1*), which proceed by an isoenergetic transesterification mechanism [see (*15*) for an example and discussion of a transesterification mechanism]. The microscopic reversal of this putative transesterification reaction, is an enzyme-catalyzed cleavage of the lariat 2′,5′-phosphodiester bond, in which the 3′ hydroxyl group of the first exon (*1, 5, 9*) is used as the acceptor, regenerating the linear transcript. In the absence of the 3′-hydroxyl group of the exon acceptor, the enzyme might catalyze the phosphoryl transfer to the hydroxyl group of water, thus resulting in the linearization of the lariat.

Regardless of the biological role of the lariat debranching activity, the ability to specifically cleave the 2′,5′-phosphodiester bond of RNA lariats has proved extremely useful for characterizing the structure of pre-mRNA splicing intermediates and products. For example, the lariat debranching activity has been used (i) to confirm that primer-extension stops from in vivo RNA samples are due to RNA branches (*6, 16*), (ii) to aid in mapping the positions of branches in RNA lariats generated in vitro (*17, 18*), and (iii) to determine whether particular RNA species are in a lariat or circular configuration (*19*).

Significantly, the lariat debranching activity is not observed during a normal in vitro splicing time course (*1*) (Fig. 6).

Even after prolonged incubation of the splicing reaction at 30°C, the 2′,5′-phosphodiester bond of the RNA lariats (380, 143, and 130 RNA species) remain intact (*1, 9*). However, following deproteinization and addition of the RNA lariats to the extract, the debranching reaction occurs rapidly and quantitatively (Fig. 1). One explanation for these results is that during pre-mRNA processing in vitro, a factor binds to the branchpoint region and remains bound to the intron after excision. This putative factor could protect the 2′,5′-phosphodiester bond from cleavage by the lariat debranching activity. The specific association of a factor with the 143 RNA branchpoint may also be required for the production of the 130 RNA species. Conversion of the 143 RNA to the 130 RNA species may be mediated by a relatively nonspecific endo- or exonuclease that is restricted from further cleavage by a factor associated with the branchpoint region. Consistent with this possibility is the observation that when the deproteinized 143 RNA is added back to the HeLa extract the discrete 130 RNA species is never generated; prolonged incubation results only in random degradation of the 143 RNA (*9*) (Fig. 1). The identification and characterization of factors that are associated with specific regions of the RNA processing products remain to be determined.

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