The splicing of messenger RNA precursors (pre-mRNA) of eukaryotic cells involves the formation of a branched RNA intermediate known as a RNA lariat. This structure is formed in the first step of the reaction when a cleavage at the 5' splice site generates the 5' exon and a RNA species containing the intron and 3' exon in which the phosphate moiety at the 5' end of the intron is forming a 2'-5' phosphodiester bond with the 2'-hydroxyl moiety of a specific adenine residue near the 3' end of the intron forming a RNA branch with the following structure: $\text{pA}_5^2\text{pX}^3\text{pZ}_2$.

We have purified a debranching activity approximately 700-fold from the cytosolic fraction of HeLa cells. This activity catalyzes the hydrolysis of the 2'-5' phosphodiester bond of branched RNA structures yielding a 5'-phosphate end and a 2'-hydroxyl group at the branch attachment site. The activity possessed a sedimentation coefficient of 3.5 S. The reaction catalyzed by the purified fraction requires a divalent cation and is optimal at pH 7.0.

The purified activity can efficiently hydrolyze triester trinucleotide structures ($\text{p(A}^2\text{G}^3\text{P})$) prepared by digestion of RNA lariats with nuclease P1. In contrast, a 2'-phosphate monoester product ($\text{p(A}^2\text{G}^3\text{P})$), formed by the wheat germ RNA ligase, was not attacked.

Branch structures present in polyadenylated nuclear RNA were first discovered by Wallace and Edmonds (1), who proposed that these structures play a role in RNA processing. The development of cell-free extracts that accurately spliced pre-mRNAs in vitro (2-4) led to the identification of intermediates containing branched RNA (5, 6). These structures, called lariats, have been detected in vitro both in higher eukaryotes (7) and yeast cells (8, 9). A model for pre-mRNA splicing has been proposed that involves two discrete reactions (5, 10). In the first step, cleavage occurs at the 5' splice site, yielding the 5' exon with a 3'-hydroxyl end and an intron/exon 2 RNA species in which the 5'-phosphate end of the intron, generated by the cleavage reaction, is esterified to the 2'-hydroxyl moiety of a specific adenosine residue near the 3' end of the intron forming a RNA lariat structure. In the second step, cleavage occurs at the 3' splice site, the two exons are covalently linked to yield the mature mRNA, and the intron is released as a lariat structure.

The fate of excised intron lariat structures in the nucleus is unknown. The absence of RNA branches in cytoplasmic RNA and the low levels of branched RNA detected in the nucleus (1) suggest that these structures are rapidly degraded in the nucleus. A novel enzymatic activity that specifically hydrolyzes the 2'-5' phosphodiester bond of branched RNA has been described in extracts of HeLa cells (11). In this work, we present the partial purification and characterization of a RNA debranching activity from HeLa cells.

**EXPERIMENTAL PROCEDURES**

**Materials**—SP6 RNA polymerase was purchased from New England Biolabs; micrococcal nuclease, snake venom phosphodiesterase, and nuclease P1 were from Pharmacia LKB Biotechnology, and RNase T1 was from Bio-Rad Diagnostics. Purified wheat germ RNA ligase was prepared as described (14, 15). Vanadyl ribonucleoside complex was purchased from Bethesda Research Laboratories; (α-32P)GTP was from New England Nuclear. Blue dextran-Sepharose 4B (containing 5.8 mg of blue dextran/g of dry Sepharose) was prepared by coupling blue dextran to CNBr-activated Sepharose 4B as described by the manufacturer (Pharmacia). Preparation of Lariat Substrates—The substrates used to assay the debranching enzyme were RNA lariats formed during the in vitro splicing of a pre-mRNA transcript. The transcript (labeled with [α-32P]GTP, 50,000 cpm/pmol) was generated by the action of SP6 RNA polymerase on plasmid pKT1 DNA restricted with SacI (12, 21). This transcript contained sequences of the tripartite leader of the adenovirus-2 major late transcription unit (for sequence of the transcript see accompanying paper (23)). It included a 56-nucleotide long first exon (exon 1) followed by a shortened 86-nucleotide intron and the first 38 nucleotides of the second exon (exon 2, Fig. 1). Upon incubation with nuclear extract, the two exons are spliced together, and the intron is released as a lariat structure in which the 5' end is ligated through a 2'-5' phosphodiester bond to an A residue 24 nucleotides upstream from the 3' end of the intron (18; also see the accompanying paper (23)). The intron lariat structure contains a 63-nucleotide circular region and a 23-nucleotide linear portion (Fig. 1). In addition, an intron/exon 2 lariat was formed that contained a 61-nucleotide long linear portion including the 38 nucleotides of the exon 2. These two lariat species were gel purified as described below and were used in all experiments unless otherwise indicated.

Ten reaction mixtures (0.5 ml each) containing 20 mM Hepes' buffer (pH 7.6), 2 mM DTT, 5 mM MgCl2, 0.4 mM ATP, 20 mM creatine phosphate, 2% polyethylene glycol 8000, 14 pmol of pre-mRNA (3.5 X 106 cpm/mmol of molecules labeled with [α-32P]GTP), and 0.2 ml of nuclear extract (17 mg of protein/ml) prepared as described by Dignam et al. (13) were incubated for 60 min at 30 °C. Each reaction was stopped by the addition of 0.2 ml of a solution containing 0.2 M Tris-HCl buffer (pH 7.5), 25 mM EDTA, 0.3 M NaCl, and 2% SDS. The mixtures were pooled, extracted twice with phenol/chloroform, and twice with chloroform. The reaction products were ethanol-precipitated, resuspended in 120 μl of formamide, and electrophoresed on a 15% polyacrylamide-8 M urea sequencing gel (15 × 25 × 0.04 cm) with preparative 3-cm wide gels at 900 V for 12 h. After electrophoresis, the reaction products were visualized by autoradiography.

1. The abbreviations used are: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DT'T, dithiothreitol; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid.
radiography at 4°C, and the lariat species were excised and eluted by grinding the gel slice in 2 ml of buffer containing 20 mM Tris-HCl buffer (pH 7.5), 2 mM EDTA, 30 mM NaCl, 0.2 mM ammonium acetate, 0.2% SDS, and 20 μg/ml Escherichia coli tRNA. The mixture was frozen in dry ice for 10 min and then left at room temperature for 1-2 h. The actin bands removed by phenol/chloroform and chloroform extraction. The pellet was resuspended in 0.5 ml of 0.2 mM ammonium acetate and precipitated again with ethanol. This material was electrophoresed again as described above to separate contaminating RNA species from the lariat structures. The lariat bands were then eluted as described above and resuspended in 5 mM Hepes buffer (pH 6.8). This procedure yielded 300–900 fmol of lariat structures that included intron lariat, intron/exon 2 lariat, and three other shortened lariat species produced by degradation of the 3′ lariat pool.

Debranching Assay—Reaction mixtures (20 μl) containing 20 mM Hepes buffer (pH 7.0), 3 mM MgCl₂, 0.25 mg/ml bovine serum albumin, 1–5 fmol of lariat molecules (700–1200 cpm/fmol of molecules), and debranching enzyme were incubated for 30 min at 30°C. The reaction was stopped by the addition of 0.4 ml of a solution containing 20 mM Tris-HCl buffer (pH 7.5), 2 mM EDTA, 30 mM NaCl, 0.2 mM ammonium acetate, 0.2% SDS, and 50 μg/ml of E. coli tRNA. The mixture was extracted with phenol/chloroform and chloroform extraction. The pellet was resuspended in 0.5 ml of 0.2 mM ammonium acetate and precipitated again with ethanol. This material was electrophoresed again as described above to separate contaminating RNA species from the lariat structures. The lariat bands were then eluted as described above and resuspended in 5 mM Hepes buffer (pH 6.8). This procedure yielded 300–900 fmol of lariat structures that included intron lariat, intron/exon 2 lariat, and three other shortened lariat species produced by degradation of the 3′ lariat pool.

Debranching Enzyme Assay—The debranching assay was based on the anomalous electrophoretic mobility of RNA lariat structures in polyacrylamide gels (5, 6). Intron and intron/exon 2 lariat species were prepared as described under “Experimental Procedures.” The products of preparative splicing reactions (described in Fig. 1) were gel purified and incubated with debranching enzyme as described under “Experimental Procedures.” The products of the debranching reaction were analyzed by electrophoresis on 15% polyacrylamide–8 M urea gels. In this system, the intron/exon 2 and intron lariat species migrate with an apparent size of 358 and 248 nucleotides, respectively. Small differences in acrylamide and bisacrylamide concentrations resulted in variations in the migration of these species. After treatment with debranching enzyme, their mobility shifted to that expected of linear structures (124 and 86 nucleotides, respectively). The influence of increasing concentrations of S-100 extract on the cleavage of intron lariat is shown in Fig. 2.

Purification of RNA Debranching Enzyme—As shown in Table I, a debranching activity was purified approximately 700-fold. This activity was purified from the cytosolic fraction obtained in the preparation of nuclear extracts that support the splicing reaction in vitro (13). Debranching activity was readily detected in the nuclear fractions as well (data not shown).

The first step used in the purification procedure involved ammonium sulfate fractionation of the S-100 extract. The 25–40% ammonium sulfate fraction (AS25/40) contained only 10–20% of the activity. Debranching activity was also detected in the 40–60% ammonium sulfate fraction (AS40/60). We have used the AS25/40 fraction because the higher ammonium sulfate fractions contained a nucleolytic activity that gener-
FIG. 2. Influence of increasing amounts of S-100 extract in the debranching reaction. The intron lariat (5 fmol) was incubated under debranching conditions as described under “Experimental Procedures” for 30 min at 30°C in the presence of varying amounts of extract as indicated. The products of the reaction were separated by gel electrophoresis and quantified as described under “Experimental Procedures.” A, autoradiogram of reaction products separated on a 15% polyacrylamide-8 M urea gel. The structures of the RNA species are shown to the left. B, quantitation of the reaction products formed in A.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total protein</th>
<th>Total units</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>S-100</td>
<td>1,350</td>
<td>19,260</td>
<td>14</td>
</tr>
<tr>
<td>Ammonium sulfate (25-40%)</td>
<td>351</td>
<td>3,300</td>
<td>10</td>
</tr>
<tr>
<td>DEAE-cellulose</td>
<td>151</td>
<td>7,880</td>
<td>19</td>
</tr>
<tr>
<td>Bio-Rex 70</td>
<td>14.6</td>
<td>1,700</td>
<td>116</td>
</tr>
<tr>
<td>Bdx-1.0 M</td>
<td>0.20</td>
<td>405</td>
<td>2,025</td>
</tr>
<tr>
<td>Bdx-2.5 M</td>
<td>0.035</td>
<td>336</td>
<td>9,606</td>
</tr>
</tbody>
</table>

FIG. 3. Distribution of debranching activity after ammonium sulfate fractionation of S-100 extract. The intron lariat (2 fmol) was incubated as described under “Experimental Procedures” in the absence of enzyme (NO ENZ), with S-100 extract (8.8 μg of protein), with the AS25/40 fraction (11.7 μg of protein), or with the AS40/60 fraction (11.7 μg of protein). The structures of the reaction products are shown to the left of the autoradiogram.

FIG. 4. Glycerol gradient centrifugation of purified debranching enzyme. Purified debranching enzyme (Bdx-2.5 M fraction, 30,000 units) was layered onto a 19-ml 12.5-30% glycerol gradient containing 20 mM Hepes buffer (pH 7.3), 1 mM dithiothreitol, 3 mM MgCl₂, and 0.2 mM NaCl. An identical gradient was layered with a solution containing the following protein markers: catalase (11.2 S), aldolase (7.3 S), and cytochrome c (1.7 S). The gradients were centrifuged in a SW-41 rotor at 40,000 rpm for 41 h. Fractions were collected from the bottom by piercing the tube. Debranching activity in the applied material (LOAD) and the gradient fractions were assayed as described under “Experimental Procedures.” The numbers above the lanes correspond to the fraction number. The positions of the protein sedimentation markers are indicated by the arrows. NO ENZ, absence of enzymes.

FIG. 5. Effect of pH and divalent cations on the debranching reaction. A, the intron lariat (5 fmol) was incubated for 30 min at 30°C in reaction mixtures (20 μl) containing 3 mM MgCl₂, 0.25 mM/ml bovine serum albumin, 3 units of debranching enzyme, and 25 mM of one of the following buffers: sodium acetate buffer (pH 5.0, 5.5, or 6.0), Hepes buffer (pH 7.0, 7.5, or 8.0), Tris·HCl buffer (pH 8.5). B, the intron lariat (5 fmol) was incubated at 30°C for 30 min in reaction mixtures (20 μl) containing 20 mM Hepes buffer (pH 7.0), 0.25 mM/ml bovine serum albumin, 3 units of debranching enzyme, and varying concentrations of MgCl₂ or CaCl₂ as shown. Reaction products were quantified as described under “Experimental Procedures.”
The addition of 0.4 M NaCl or 0.4 M KCl completely inhibited the reaction (Table II), whereas the debranching activity was unaffected by 10 mM N'-ethylmaleimide, suggesting that sulfhydryl groups are not essential in the reaction. Vanadyl ribonucleoside, an inhibitor of RNase A that also inhibits RNA cyclase (19) and RNA ligase from HeLa cells (20) and in vitro splicing of pre-mRNA using HeLa cell nuclear extracts (4) or partially purified fractions (16), completely inhibited the debranching reaction at a concentration 0.5 mM (Table II).

Attempts to reverse the debranching reaction by incubating purified linear products with debranching enzyme in the presence or absence of ATP and/or GTP were unsuccessful (data not shown).

**Analysis of the Reaction Products**—The debranching reaction, using the intron lariat as a substrate, yielded a RNA species with the expected size for the linear intron (86 nucleotides, Fig. 6A). When the product of the reaction was purified and analyzed by primer extension using a 22-nucleotide long synthetic primer complementary to the linear portion of the intron lariat, an 86-nucleotide long extension product was obtained (Fig. 6B). No extension product should be obtained from the debranched product of the intron lariat. C, the products of the reaction were digested with nuclease P1, and the products were analyzed by thin layer chromatography on polyethyleneimine-cellulose plates using 0.5 M LiCl as solvent. The structure of the nuclease P1-resistant trinucleotide pA2'pGp3'pU and its migration were shown to the right of the autoradiogram. Ori., origin.

**TABLE II**

Influence of various additions on debranching activity

In experiment I, the intron/exon 2 lariat (7 fmol) was incubated with 6 units of debranching enzyme as described under “Experimental Procedures” or with various additions as indicated in the table. In experiment II, reactions were as in experiment I, with 6 fmol of intron/exon 2 lariat. Where indicated, vanadyl ribonucleoside was added, or the enzyme was pretreated with N'-ethylmaleimide (10 mM, 30 min at 4 °C), or with proteinase K (2 μg/ml of enzyme fraction) for 10 min at 30 °C.

<table>
<thead>
<tr>
<th>Additions</th>
<th>Linear product formed (fmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Experiment I</strong></td>
<td></td>
</tr>
<tr>
<td>Complete</td>
<td>6.0</td>
</tr>
<tr>
<td>Omit MgCl₂, add MnCl₂, 3 mM</td>
<td>5.2</td>
</tr>
<tr>
<td>Omit MgCl₂, add CaCl₂, 3 mM</td>
<td>5.6</td>
</tr>
<tr>
<td>EDTA, 10 mM</td>
<td>0.2</td>
</tr>
<tr>
<td>NaCl, 0.4 M</td>
<td>0.1</td>
</tr>
<tr>
<td>KCl, 0.4 M</td>
<td>0.1</td>
</tr>
<tr>
<td><strong>Experiment II</strong></td>
<td></td>
</tr>
<tr>
<td>Complete</td>
<td>5.0</td>
</tr>
<tr>
<td>Vanadyl ribonucleoside, 0.1 mM</td>
<td>1.1</td>
</tr>
<tr>
<td>Vanadyl ribonucleoside, 0.5 mM</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>N'-ethylmaleimide, 10 mM</td>
<td>5.1</td>
</tr>
<tr>
<td>Proteinase K</td>
<td>&lt;0.1</td>
</tr>
</tbody>
</table>

The digestion of the intact intron lariat yielded a RNA species that differed only in the nucleotide at which the branch was formed. A mutant pre-mRNA transcript that contained an A to G substitution precisely at the branch site sequence (23). The digestion of the mutant intron lariat was approximately one-half of the rate of debranching of the wild type intron/exon 2 lariat (21). A two-nucleotide long primer complementary to the linear portion of the intron lariat, an 86-nucleotide long extension product should be obtained from the debranched product of the intron lariat. C, the products of the reaction were digested with nuclease P1, and the products were analyzed by thin layer chromatography on polyethyleneimine-cellulose plates using 0.5 M LiCl as solvent. The structure of the nuclease P1-resistant trinucleotide pA2'pGp3'pU and its migration were shown to the right of the autoradiogram. Ori., origin.

**FIG. 6. Analysis of the products formed in the debranching reaction.** Purified intron lariat (5 fmol) was incubated in the absence (−) or presence (+) of debranching enzyme (25 units) as indicated under “Experimental Procedures.” The products of the reaction were analyzed in the following three ways. A, the products of the reaction were separated on a 15% polyacrylamide-8 M urea sequencing gel. B, the products of the reaction were analyzed by primer extension using a 22-nucleotide long primer complementary to the linear portion of the intron lariat. With such a primed template, no extension product was expected from the intact lariat, whereas an 86-nucleotide long extension product should be obtained from the debranched product of the intron lariat. C, the products of the reaction were digested with nuclease P1, and the products were analyzed by thin layer chromatography on polyethyleneimine-cellulose plates using 0.5 M LiCl as solvent. The structure of the nuclease P1-resistant trinucleotide pA2'pGp3'pU and its migration were shown to the right of the autoradiogram. Ori., origin.
the $^{32}$P-labeled $\text{pGp}$ residue at the branch site. Other minor bands observed in Fig. 8A, lane 2, were degradation products formed during the isolation of the T1 oligonucleotide that lack the terminal $^{32}$P-labeled $\text{pGp}$ residue at the 3' end. These bands were not labeled after debranching due to the removal of the labeled $\text{pGp}$ residue at the branch site.

The debranching reaction yields a 2'-OH group at the branch site. This was confirmed by the production of labeled $\text{pG}$ residue at the branch site. Other minor bands observed in Fig. 8B were not labeled after debranching due to the removal of the labeled $\text{pGp}$ residue at the branch site.

The cleavage of the branched trinucleotide, however, required higher levels of enzyme and longer incubation (1000 units, 2 h at 30 °C) than did RNase T1 oligonucleotides (Fig. 8A, 240 units for 30 min).

The question of whether the enzyme could act on a circular RNA that contained a single phosphomonoester group at the 2' position of one of its residues was examined. Such a substrate was prepared by circularizing [2'-3'-32P](Cp)11G$\rightarrow$lt) with wheat germ RNA ligase. This reaction produced a circular RNA molecule containing a single $^{32}$P-labeled phosphomonoester at the 2' position of the G residue (14). Incubation of this substrate with bacterial alkaline phosphatase resulted in the formation of labeled P, from the circular RNA (Fig. 8C, lane 2), whereas incubation with 150 units of debranching enzyme for 45 min at 30 °C yielded no detectable labeled P, indicating that debranching enzyme did not have phosphomonoesterase activity.

**DISCUSSION**

Purified debranching enzyme is a valuable reagent that can be used to detect branched RNA and aid in the determination of the RNA branch sequence among the products of RNA splicing.

An RNA debranching activity has been purified approximately 700-fold. The enzyme acts optimally at pH 7.0 and showed no significant difference in activity when the reaction was carried out at temperatures between 25 and 42 °C (data not shown). The debranching reaction was dependent on the addition of a divalent metal under the conditions specified in Table II. These results differ from those reported by Ruskin and Green (11), who used S-100 crude extracts. They showed that the debranching activity was unaffected by 50 mM EDTA. However, their reaction mixtures (25 µl) contained high concentrations of enzyme (20% by volume, approximately 500 units), and the reaction was carried out at pH 8.0. We have repeated their observations; debranching activity was readily detected using high concentrations of S-100 extract or purified enzyme (500 units in a 20-µl reaction mixture) under our assay conditions in the presence of 10 mM EDTA (data not shown).

Debranching enzyme can hydrolyze lariat structures, RNase T1-generated oligonucleotides with a 2'-5' branch consisting of a single $\text{pGp}$ residue, and nuclease P1-resistant branched trinucleotide. The release of labeled P after treatment of the nuclease P1-resistant branched trinucleotides with purified enzyme demonstrated that the reaction generated a 2'-hydroxyl group in the A residue at the branch site. The fact that no labeled P, was released after treatment of the circular RNA substrate (containing a single labeled phosphomonoester group at the 2' position of a G residue) with debranching enzyme shows that the debranching enzyme did not have phosphomonoesterase activity. These results suggest that the nuclease P1-resistant branched trinucleotide repre-

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**Fig. 7. Rate of hydrolysis of the A$^{2'3}$-pG and G$^{2'6}$-pG linkages in branch structures.** A reaction mixture (100 µl) containing 20 mM Hepes buffer (pH 7.0), 3 mM MgCl₂, 0.25 mg/ml bovine serum albumin, and 15 fmol of wild type intron/exon 2 lariat containing an A$^{2'3}$-pG branch (pKT.1), or 15 fmol of a mutant intron/exon 2 lariat containing a G$^{2'6}$-pG branch (pUS.A; see Ref. 23) was incubated with debranching enzyme (10 units) at 30 °C. At the indicated time, aliquots (20 µl) were removed, and the products were analyzed as described under "Experimental Procedures." A, autoradiography of products separated by electrophoresis on a 15% polyacrylamide-8 M urea gel. The structures of the RNA species are indicated to the right of the autoradiogram. B, the linear product was excised from the gel and quantified as described under "Experimental Procedures."

**Fig. 8. Debranching reaction with different substrates.** A, the RNase T1 oligonucleotide (14*-oligonucleotide) containing the branched structure indicated (prepared from wild type intron lariat as described in Ref. 23) was incubated as described under "Experimental Procedures" for 1 h at 30 °C in the absence (lane 2) or in the presence of debranching enzyme (240 units, lane 3). Lane 1 shows the RNase T1 digestion products of wild type pre-mRNA. B, the branched trinucleotide was prepared by nuclease P1 digestion of the wild type intron lariat as described in Ref. 23 and was incubated under standard debranching conditions for 2 h in the absence (lane 1) or in the presence (lane 2) of debranching enzyme (1000 units). The products of the reaction were resolved by thin-layer chromatography on polyethyleneimine-cellulose plates using 0.5 M LiCl as solvent. C, a circular RNA molecule containing a single $\text{G}$ residue with a phosphate substitution at the 2' position was prepared by the circularization of [2'-3'-32P](Cp)11G$\rightarrow$lt) with wheat germ RNA ligase as described under "Experimental Procedures." This substrate was incubated under debranching conditions for 45 min at 30 °C in the absence (lane 1) or presence (lane 3) of debranching enzyme (250 units) or with 0.07 unit of bacterial alkaline phosphatase (lane 2).
sent the minimal substrate that can be hydrolyzed by the debranching enzyme.

The rate of hydrolysis of the intron/exon 2 lariat containing a G\(^{\text{2t}}\)-5t linkage was 2-fold slower than the hydrolysis of lariats containing a A\(^{\text{2t}}\)-5t linkage. Rustin and Green (11) reported that S-100 extracts of HeLa cells hydrolyzed intron/exon 2 lariat structures containing a A\(^{\text{2t}}\)-5t linkage. We have not tested such a substrate with the purified enzyme. However, our results suggest that the rate of the debranching reaction may be affected by the sequence of the branch.

Substantial amounts of debranching activity were detected in nuclear extracts that supported RNA splicing, and in our partially purified splicing fractions Ia and Ib (Ref. [16] and data not shown), and in splicing fractions reported by Kramer and Keller (17). Whether the activities detected in the nuclear and cytosolic fractions correspond to the same enzyme is not known. The question whether the debranching enzyme plays any role in pre-mRNA splicing will remain unanswered until the splicing reaction can be reconstituted with fractions free of debranching activity. The release of the excised intron as a lariat structure in the second step of the splicing reaction seems too weak to account for the resistance of the pre-mRNA to the nuclease activity. The possibility that this protein(s) corresponds to one or more of the hnrNP core proteins is currently under investigation.

REFERENCES