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Sequence Requirements in Different Steps of the Pre-mRNA Splicing Reaction: Analysis by the RNA Modification-Exclusion Technique

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The stepwise assembly of splicing complexes and the subsequent splicing reaction were analyzed by the RNA modification-exclusion technique, which generates the equivalent of a complete set of point mutations in a single reaction. We found that although the sequences surrounding the 5′ splice site, the branch point, and the 3′ splice site, including the 3′ AG, were required for presplicing complex formation, modified nucleotides at these positions were not completely excluded. The same sequences were required for splicing complex formation; however, modified nucleotides in these sequences were excluded to a much greater extent.

Spliceosome assembly occurs in a stepwise manner in nuclear extracts from mammalian cells. During the splicing reaction, two intermediate complexes are formed. Complex H (for heterogeneous; 17) results from the non-sequence-specific binding of proteins to the RNA immediately after it is introduced into a nuclear extract. The second intermediate, the A complex (the 35S or presplicing complex), has been shown to be a true splicing intermediate (2). The spliceosome (the 50S or B complex) appears after 10 to 15 min and gradually replaces the presplicing complex. Spliceosomes can form only on pre-mRNAs that contain all three splicing signals: the 5′ splice site, the branch point, and the 3′ splice site. They may contain either the unprocessed pre-mRNA or the RNA intermediates of splicing (the 5′ exon plus the intervening sequence 3 [IVS] 3′ exon lariat).

Although the pre-mRNA sequences necessary for correct splicing are known (12, 20, 34, 38), the sequential requirements for individual nucleotides during the formation of the A and B complexes have not been fully elucidated. We have used the modification-exclusion technique to determine the pattern of nucleotide usage during presplicing and splicing complex formation (7, 29). In this technique, an average of one nucleotide per RNA molecule is first modified chemically. After modification, the RNA is introduced into a standard in vitro splicing reaction; subsequently, RNA that has been either incorporated into splicing complexes or successfully processed is separated from the unprocessed precursor by gel electrophoresis. Nucleotides necessary for pre-mRNA splicing are not recognized by the splicing machinery if they are modified; therefore, pre-mRNAs containing these modified nucleotides are excluded from the splicing process. The processed and unprocessed RNAs are then chemically cleaved at the modified nucleotides, and the products are compared on denaturing polyacrylamide gels. Cleaved RNA fragments present in the sequencing ladder of the unprocessed RNA but absent from the processed RNA are modified at a nucleotide that is necessary for splicing complex formation or for splicing. The information obtained by this analysis is thus analogous to that obtained from a complete series of point mutations made in the RNA substrate.

The pre-mRNA used as a model substrate in these experiments was an SP6 transcript derived from the adenovirus type 2 major late (AdML) transcription unit IVS 2 (6; Fig. 1). The basic method used for analysis of the splicing reaction was as follows. Nonradioactive substrate RNA was synthesized with SP6 polymerase as described by Humphrey et al. (15) and 3′ end labeled with [^{32}P]pCp and RNA ligase according to the procedure of Conway and Wickens (7). The RNA was isolated on a 6% polyacrylamide gel and eluted as described previously (9). The radiolabeled pre-mRNA molecules were then modified (7, 29) by chemical sequencing procedures (21, 22). For all modification reactions, 4×10^6 dpm of end-labeled RNA was used. For purine modifications, the reaction was chilled on ice for 5 to 10 min before the addition of 0.5 μl of diethyl pyrocarbonate (DEPC; Sigma Chemical Co.). After vortexing, the sample was incubated at 90°C for 30 s. Pyrimidine modifications were done as described by Conway and Wickens (7) except that the reaction was incubated on ice for 15 min. After modification, the RNA was incubated in a 200-μl splicing reaction with HeLa cell nuclear extract (8) for 50 min under standard conditions.

For isolation of RNA contained in the A and B complexes, one half of each splicing reaction was stopped by the addition of 5 μl of a 10-mg/ml heparin (Sigma) solution and loaded on a 4% nondenaturing polyacrylamide gel (6, 10). The gels were electrophoresed at 17 V/cm until the xylene cylanol migrated off the bottom. The RNA was then electroblotted onto an NA45 DEAE membrane (Schleicher & Schuell, Inc.; 23) in running buffer containing 0.1% sodium dodecyl sulfate for 4 h at 150 mA. The membrane was exposed to X-ray film, and the bands containing A-complex and B-complex RNAs were excised. The RNA was eluted off the membrane as described previously (23).

For isolation of lariat intermediate and spliced product, the other half of the splicing reaction (100 μl) was incubated with 400 μl PK buffer (0.2 M Tris hydrochloride [pH 7.5], 0.3 M NaCl, 25 mM EDTA, 2% sodium dodecyl sulfate) and 80 μg of proteinase K (Boehringer Mannheim Biochemicals) for 30 min at 30°C, extracted with an equal volume of phenol-chloroform-isoamyl alcohol, and precipitated with ethanol. To debranch the lariat intermediate, the RNA was dissolved

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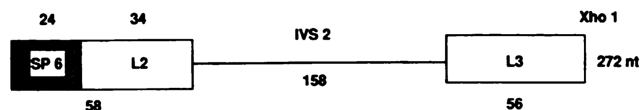

FIG. 1. AdML IVS 2. An IVS 2-containing substrate was used instead of our standard splicing substrate (RNA1;9) containing AdML IVS 1 because the 3′ splice site-polypyrimidine tract of IVS 1 contains eight consecutive uridine residues. SP6 RNA polymerase "stutters" during transcription of this polyuridine stretch, which results in the formation of heterogeneous transcripts containing variable numbers of pyrimidines (16). Such molecules cannot be used in sequencing experiments. pSP IVS2 was constructed by ligating the *ScaI*/*Eco*RI fragment from pSP 1-2 (6) to pSP64 cut with *Hin*dII and *Eco*RI. For RNA synthesis, pSP IVS2 was cut with *Xho*I. This template directs the synthesis of a 272-nucleotide (nt) transcript containing a 58-nt exon I (24 nt of pSP64 and 31 nt of AdML leader 2 (L2), a shortened 158-nt IVS (a 2,303-nt *Bgl*I-*Xma*III fragment was removed from AdML IVS 2 to construct pSP 1-2), and a 56-nt exon 2.

in 10 μl of H_2O, and 15 μl of HS500 (which contains the debranching enzyme; 18, 27) was added. The reaction was incubated at 30°C for 20 min and stopped by the addition of PK buffer, followed by incubation with 20 μg of proteinase K as described above. The RNA from the A and B complexes and the debranched RNA were electrophoresed on an 8% denaturing polyacrylamide gel, the gel was exposed to film, and the RNAs were eluted as described previously (10). After cleavage with aniline (7), the RNAs were electrophoresed on 8 and 12% thin denaturing polyacrylamide gels (7, 21, 29).

Pre-mRNAs containing modified nucleotides at the 5′ splice site, ACG|GUAAGA, the branch point, CGCUAAC, and the 3′ splice site, AUUGUUGUGUAG|GUAC (underlined nucleotides are those affected; branch point adenosine is double underlined), were underrepresented in the A complex (Fig. 2A and B, lanes 3 and 7). The same nucleotides involved in A-complex formation were involved in B-complex formation, but they were more strongly excluded from the B complex than from the A complex (Fig. 2A and B, lanes 4 and 8; Fig. 3). In the B complex, the excluded nucleotides at the 5′ splice site, the branch point, and the 3′ splice site were, respectively, ACG|GUAAGA, CGCUAACG, and AUUGUUGUGUAG|GUAC (bold nucleotides excluded to a greater extent). A quantitative summary of the sequencing gel data obtained from the various steps of the splicing reaction is presented in Fig. 3. For this purpose, densitometer scans were taken on two different exposures of the same gel. For each lane, the intensity of the bands representing modifications at noncritical nucleotides was measured; the average value for each modified nucleotide (A, G, C, and U) was calculated and given the arbitrary value of 100%. This value was determined for each lane, since the lanes differed in the amount of radioactivity they contained. The intensity of each band representing excluded nucleotides was then calculated relative to this value.

For the debranched lariat intermediate, it was not possible to analyze the nucleotides at the 5′ splice site because they were obscured by the strong signal of the unmodified RNA (Fig. 2A and B, lanes 5 and 9). Modified nucleotides at the branch point and the 3′ splice site were excluded to a lesser extent than in the B complex. There are two possible explanations for this result. First, the background may have been higher in the lariat intermediate because of the isolation technique. The debranched lariat intermediate migrated faster than the pre-mRNA on the denaturing polyacrylamide gel and therefore may have been contaminated by degradation products of the pre-mRNA. However, in other experiments in which this contamination was not possible, we also observed more RNAs containing modified nucleotides at the branch point and 3′ splice site in the lariat intermediate than in the B complex (data not shown). High background may also result from degradation of the lariat intermediate itself during the isolation procedure. An alternative explanation is that RNAs which have modifications at important nucleotides may occasionally be included in the A and B complexes and even cleaved at the 5′ splice site but totally prevented from being cleaved at the 3′ splice site. These RNAs would then accumulate as lariat intermediates. Consistent with this explanation, some mutants in the branch point consensus sequence allow cleavage at the 5′ splice site and lariat intermediate formation but prevent cleavage at the 3′ splice site (11, 14, 31).

In addition to the intermediates of the splicing reaction, the spliced product was isolated and assayed (Fig. 2C). If the relative intensities of bands in lane 2 (A and G modified) are compared, the RNAs cleaved at modified guanosines immediately surrounding the splice site (the last base of exon 1 and the first base of exon 2) were lower in intensity than the RNAs cleaved at modified guanosines at other positions. In lane 3 (C and U modified), the intensities of the bands were similar throughout the gel, indicating that none of the pyrimidines in the exons were required for splicing. Therefore, the last nucleotide of exon 1 and the first nucleotide of exon 2 (Fig. 2C and 3) appeared to be the only nucleotides in the exons that were required for either formation of the splicing complex or the splicing reaction.

Our results may be summarized as follows. For splicing, we observe a requirement for five and possibly six nucleotides at the 5′ splice site (5′-ACG|GUAAGA-3′; required nucleotides are in bold face; it was not possible to determine whether the fifth nucleotide of the IVS [G] was affected because of the strong signal of the sixth nucleotide [A], which was not excluded). The sequence at the 5′ end of U1 small nuclear RNA (snRNA) is 3′-GUCCAUUCAUA-5′ (the bold nucleotides would base pair with the bold nucleotides at the 5′ splice site; 37). The requirement for specific bases for the binding of factors to the RNA is weaker in the A complex than in the B complex, especially at the 5′ splice site (~30 to 40% versus ~80 to 90% exclusion, respectively). In fact, the exclusion from the A complex of nucleotides modified at the 5′ splice site is sometimes difficult to detect (Fig. 2). The observation that the nucleotides at the 5′ splice site are required to a greater extent in the B complex than in the A complex agrees with results from RNase T_1 protection experiments (4).

The results that we obtained concerning the role of the 5′ splice site in A-complex formation are in contrast with those obtained by the same and other methods to analyze the splicing of yeast pre-mRNAs in vitro. Rymond and Rosbash (29), using the modification-exclusion technique, observed that nucleotides modified at the 5′ splice site were excluded (~90%) from complex III, the first complex observed during yeast spliceosome assembly. Therefore, there seems to be less of a requirement for sequences at the 5′ splice site during A-complex formation in the mammalian splicing system. Indeed, in mammalian but not yeast nuclear extracts, A complexes will form on an RNA lacking a 5′ splice site if the RNA contains a branch point and a 3′ splice site (3, 5, 23).

The branch point consensus sequence is UNYURAC; six of these seven nucleotides (5′-CGCUAAC-3′; branch point is

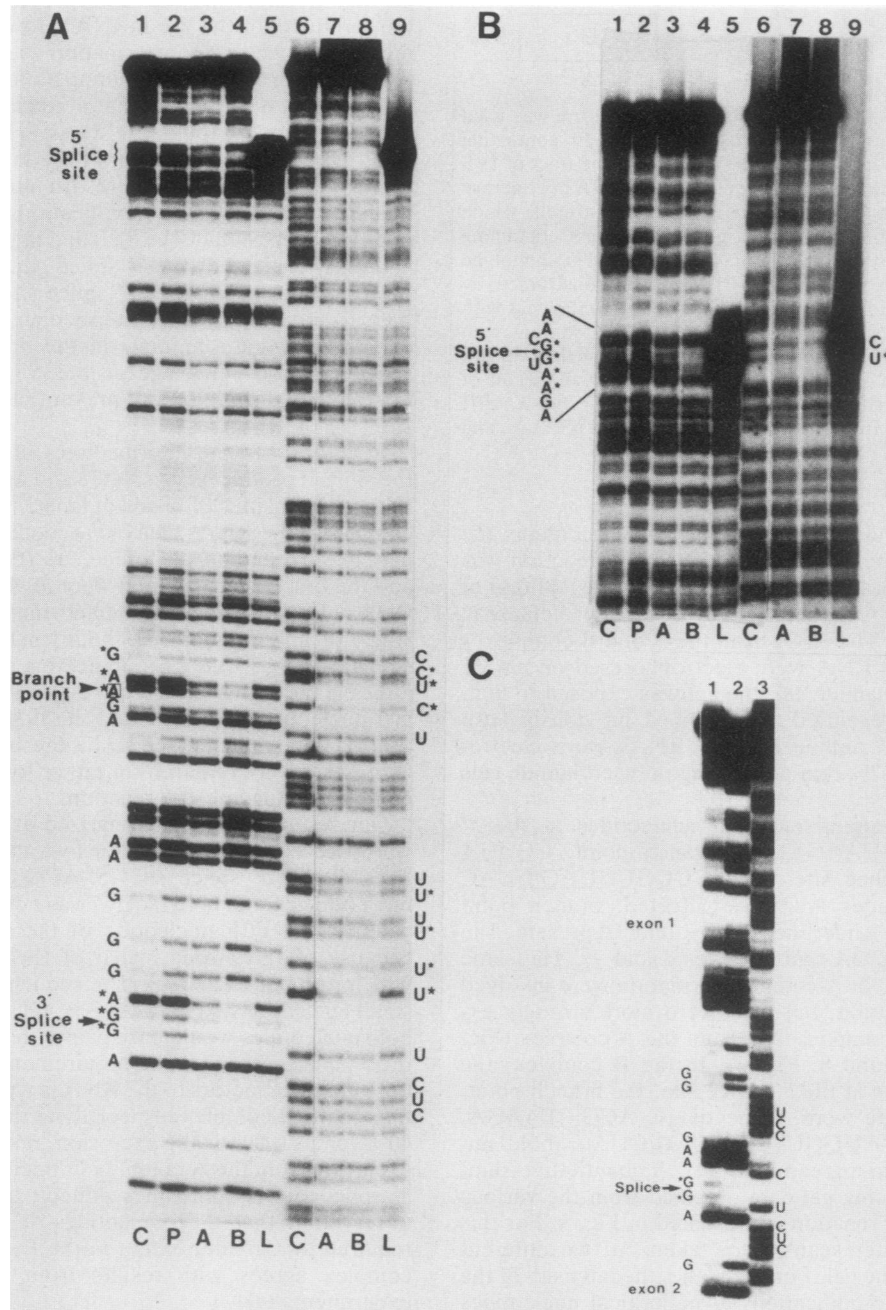

FIG. 2. Modification-exclusion analysis of spliceosome formation and splicing. The DEPC-modified (A+G) and hydrazine-modified (C+U) RNAs were incubated in a splicing reaction under standard conditions. RNA was isolated from splicing complexes and from reaction intermediates and products, cleaved with aniline, and separated by gel electrophoresis as described in the text. (A) 8% sequencing gel. Lanes: 1 to 5, DEPC-modified RNA (A+G); 6 to 9, hydrazine-modified RNA (C+U); 1 and 6, control RNA (C), which was modified and cleaved without intermediate steps; 2 to 5 and 7 to 9, modified RNAs incubated in nuclear extract, purified as described above, and cleaved; 2, precursor RNA isolated after incubation in nuclear extract (P). Bands generated by cleavage at adenosines are more intense than those cleaved at guanosines (lanes 1 and 2) because adenosines react approximately four times more efficiently with DEPC than do guanosines. Guanosine residues from RNAs that have been treated with DEPC and subsequently processed in a nuclear extract are more susceptible to cleavage by aniline than are residues from unprocessed RNAs (compare the guanosines in lanes 1 and 2). The explanation for this phenomenon is unknown; however, other authors have made the same observations (7, 29). Other lanes: 3 and 7, A-complex RNA (A); 4 and 8, B-complex RNA (B); 5 and 9, debranched lariat intermediate (L). (B) 12% sequencing gel. Lanes are as in panel A. Modified nucleotides excluded from splicing complexes or the lariat intermediate are marked with asterisks (larger asterisks in panel A mark those nucleotides excluded to the greatest extent). The 5′ and 3′ splice sites and the branch point are indicated. (C) Modification-exclusion analysis of spliced product. Lanes: 1, spliced product isolated and subsequently modified with DEPC (A+G) as a control; 2, DEPC-modified (A+G) RNA incubated under splicing conditions and isolated from a denaturing polyacrylamide gel; 3, hydrazine-modified (C+U) RNA incubated under splicing conditions and isolated from a denaturing polyacrylamide gel. Excluded nucleotides are marked with asterisks. Exon 1, exon 2, and the location of the splice are indicated.

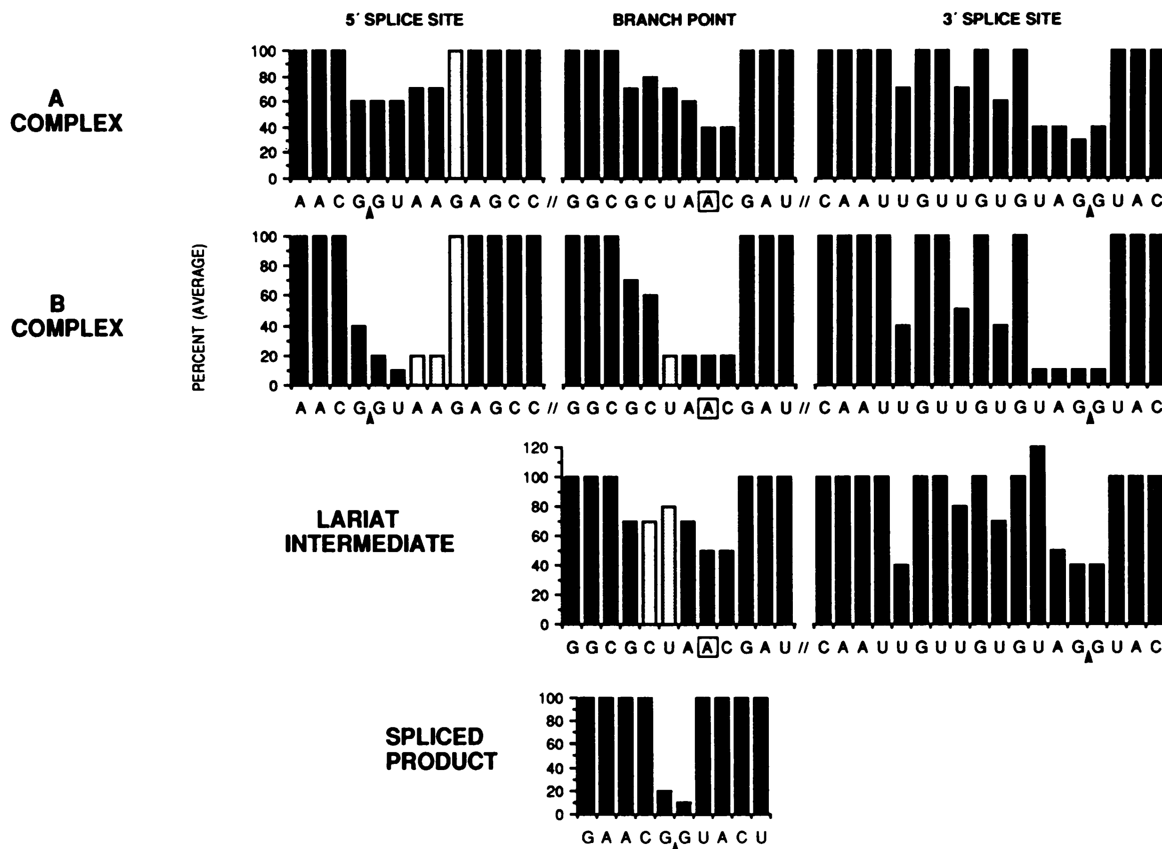

FIG. 3. Summary of the modification-exclusion data, showing a schematic representation of the exclusion of specific modified nucleotides from the A and B complexes, the lariat intermediate, and the spliced product. The graphs for the branch point and 3′ splice site represent scanning densitometry readings from the gels presented in Fig. 2. The graphs for the 5′ splice site represent an average of several different experiments, including the one presented in Fig. 2B. The lightly shaded bars represent nucleotide positions for which no accurate figure could be determined because neighboring bands on the gel were too intense or adjacent bands were not separable. The arrows indicate either the location of the 5′ or 3′ splice site or the location of the splice. The boxed A represents the branch point adenosine.

double underlined) are required for the formation of both A and B complexes on the AdML IVS 2 substrate. The underlined nucleotides in the branch point sequence could base pair with the nucleotides in U2 snRNA, 3′-AUGAU-G-5′ (the bold nucleotides would base pair exactly [with one G · U match]; the gap represents the bulged branch point adenosine; 19, 34, 38). Our results are in agreement with those of other experiments performed to determine the branch point nucleotides required for the splicing reaction (14, 26, 36).

It is somewhat surprising that we observe a requirement for individual nucleotides within the polypyrimidine stretch at the 3′ splice site. This requirement might be because the polypyrimidine stretch of AdML IVS 2 is very purine rich: normally there are at least 11 pyrimidine nucleotides directly upstream of the 3′ splice site, whereas in IVS 2 5 of the first 11 and 6 of the first 16 nucleotides are purines. Therefore, in an RNA substrate with a normal polypyrimidine stretch, modification of any individual nucleotide might have considerably less effect than that observed for AdML IVS 2. The pyrimidines closest to the 3′ splice site are excluded to the greatest extent when modified, whereas the pyrimidines further upstream are not affected at all. The binding of U2 snRNP to the branch site may take up a certain length of RNA, and therefore the region close to the branch point could be unavailable for the binding of other factors.

When any nucleotide in the sequence UAG|G at the 3′ splice site is modified, the RNA is excluded both from the A complex (~60 to 70%) and from the B complex (~90%). Indeed, 3′ AG mutations in certain transcripts are known to prevent cleavage at the 5′ splice site (1). However, in other transcripts the 3′ AG is not required for spliceosome formation or cleavage at the 5′ splice site (9, 25, 28, 32). Consistent with previous results for yeast cells (13), Rymond and Rosbash (29), using the modification-exclusion method, observed that modifications in the 3′ AG do not prevent spliceosome formation or cleavage at the 5′ splice site. Recently, Smith et al. (32), using α-tropomyosin as a model substrate, proposed that in mammalian systems as well, only the branch point sequences and the polypyrimidine tract of the 3′ splice site are required for complex formation and subsequent cleavage at the 5′ splice site. Our results do not support this hypothesis; they demonstrate that the AG dinucleotide is required for spliceosome formation on AdML IVS 2 transcripts. Reed (24) found that if the polypyrimidine tract was extensive and included few purines (as in α-tropomyosin), the 3′ AG was not required for cleavage at the 5′ splice site. However, if a pre-mRNA had a poor polypyrimidine tract (as in AdML IVS 2), the 3′ AG became necessary for splicing. These observations may account for the differences between our results and the hypothesis of Smith et al. (32).

We have analyzed the specific sequence requirements for the stepwise assembly of the spliceosome. Our results are consistent with those obtained by other methods (summarized in references 13 and 33; 34, 38). Our data also exclude the requirement for additional specific sequences in either the intron or the exons. The 5′ splice site and the branch point sequences that could base pair with U1 and U2 snRNA, respectively, were the ones that were excluded from complex formation and splicing when modified. Although the same sequences are required for A- and B-complex assembly, these sequences are excluded to a much greater extent from the B complex than from the A complex. This finding suggests that the association of the splicing factors with the RNA may occur by more than one type of interaction. For example, a weak protein-RNA binding could occur initially, followed by stronger RNA-RNA base pairing later during the assembly process. For the binding of U1 snRNP to the 5′ splice site, there is some evidence that RNA-RNA interactions, although necessary, are not sufficient for splice site recognition to occur (4, 30, 31, 35). In addition, the analysis of complementary mutations between U2 snRNA and the branch point sequences suggests that base pairing is not the sole determinant in branch point recognition (34, 38). The modification-exclusion technique has allowed us to determine the specific nucleotides required for each step of spliceosome assembly and the splicing reaction.

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