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CHARACTERIZATION OF THE NEWLY-FORMED INTERNUCLEOTIDE BOND OF IN VITRO SPLICED mRNAs

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SUMMARY: Precursor RNAs were synthesized in vitro from a plasmid in which the early region 2 (E2) of adenovirus 2 is fused to an efficient bacteriophage promoter (Salmonella phage 6). The RNAs were purified and utilized as substrates for in vitro splicing in the presence of nuclear extracts prepared from MOPC-315 mouse myeloma cells. We have shown previously (Goldenberg, C.J., PNAS, August, in press, 1984) that in vitro splicing in those extracts was accurate at the nucleotide level. We now show that: i) the new internucleotide bond at the splice junction generated in vitro is a 3',5'-phosphodiester bond; and ii) the phosphate that forms the splice between the exons is derived from the pre-mRNA.

The mechanisms for the cleavage-ligation events of pre-tRNAs have been proposed for yeast (1,2), wheat germ (3-5), HeLa cells (6), and also for Tetrahymena pre-rRNAs (7,8). Almost no information is available regarding the biochemical mechanism of pre-mRNA splicing. Recently, we reported that in vitro synthesized adenovirus 2 (Ad2) early region 2 (E2) precursor mRNA from a plasmid in which the E2 gene is fused to an efficient bacteriophage promoter (Salmonella phage 6) was accurately and efficiently spliced by nuclear extracts prepared from MOPC-315 mouse myeloma cells (9).

In this report, we have investigated the nature of the newly formed bond between the exons in the in vitro spliced RNAs. We conclude that the bond is a typical 3',5'-phosphodiester and that the phosphate that forms the splice between the exons is derived from the pre-mRNA.

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MATERIALS AND METHODS: The 3.3 KB RNA was synthesized *in vitro* in the presence of $\alpha[^{32}P]$GTP and incubated in the presence of a nuclear extract prepared from MOPC-315 mouse myeloma cells as described (9,10). The RNAs were glyoxalated and subjected to electrophoresis on 1.4% agarose gels. The 1.5 KB RNA was eluted from the agarose gels as described (11) and digested with 10 µg of RNase A in 2 µl of TE buffer (10 mM Tris-HCl pH 7.4, 1 mM EDTA) at 37°C for 2 hr. The digest was then loaded on a 20% polyacrylamide sequencing gel. The individual 13-mer oligonucleotide was eluted from the gel as described (12) and digested with 6 units of RNase-T_1 in 5 µl of TE buffer at 37°C for 1 hr. The products were analyzed on polyethylenlimine-cellulose (PEIC) plates as described (13). Each individual spot was eluted from the plate (14) and digested in 10 µl of 50 mM ammonium acetate, pH 4.5, with RNase-T_2 at 37°C for 4 hr. The products were analyzed in PEIC plates as described (14).

RESULTS AND DISCUSSION

Figure 1 shows the expected RNA sizes for the 3.3 KB pre-RNA incubated in the presence of a nuclear extract. The structure of the new internucleotide bond between the 3'-exon and the second RNA leader (see Figure 1) in the spliced 1.5 KB RNA was investigated. The *in vitro* spliced 1.5 KB RNA was purified as described in Figure 2, digested with RNase A, and the products analyzed on a sequencing gel (Figure 2B). Based on the nucleotide sequence of the E2-SP6 gene, we conclude that RNase A digestion of the 1.5 KB RNA would generate a 13-mer unique oligonucleotide

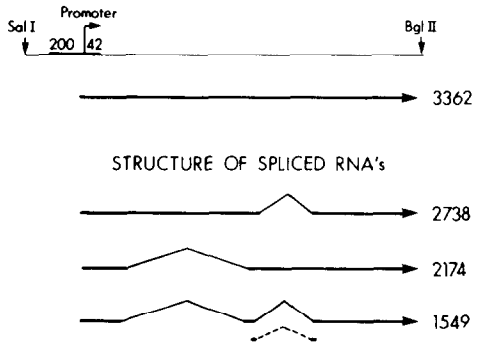

Figure 1. *In vitro* RNA synthesized from the E2-SP6 DNA and structure of the *in vitro* spliced RNAs. The plasmid DNA was linearized with SalI and subsequently with BglII. The 3.3 KB RNA contains the signals for splicing of two introns. Removal of one of the introns by *in vitro* splicing generates a 2738 or 2174 RNA. The removal of both introns generates a 1549 RNA. The solid lanes with the dashed caret symbol below the 1549 RNA represent the RNase A oligonucleotide at the splice junction, identified in the nucleotide sequence of the E2-SP6 gene, analyzed in this study.

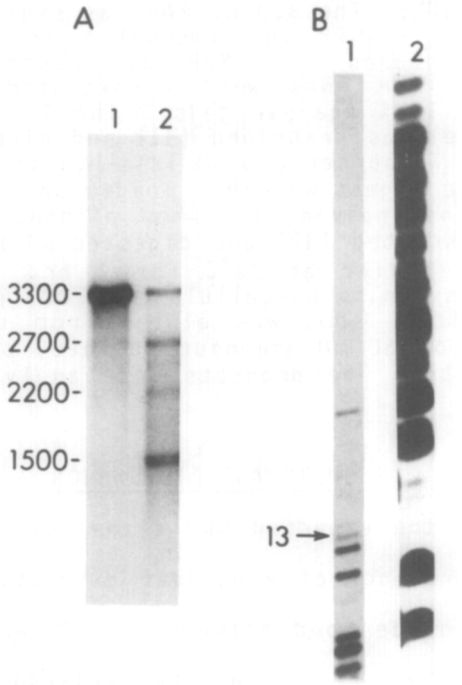

Figure 2. Pancreatic RNase digestion of the 1.5 KB RNA spliced in vitro. The ^{32}P-labeled pre-RNAs (720 pmoles of the completed 3.4 KB RNA, 7 x 10^6 cpm in 300 µl reaction volume) were incubated for 20 min at 30°C in the presence of a nuclear extract prepared from MOPC-315 mouse myeloma cells (protein concentration 4.86 mg/ml of reaction). After the reaction was terminated, the RNAs were extracted, denatured with glyoxal, and subjected to electrophoresis in a 1.4% agarose gel. The 1.5 KB RNA band was excised from the gel and eluted as described (11). The RNA was then digested with pancreatic RNase as described in Materials and Methods. The products of digestion were analyzed on 20% polyacrylamide sequencing gels. The 13-mer band was eluted from the sequencing gel as described (12). ^{32}P-labeled RNAs of a known sequence digested with RNase A were run in parallel (data not shown). A) In vitro splicing of the SP6-E2 RNAs. Lane 1: In vitro transcribed RNAs (10,000 cpm) from the SH_2 plasmid DNA linearized with SalI and BglII. Lane 2: In vitro transcribed RNAs (3.3 KB) after incubation with the nuclear extract (10,000 cpm). B) Pancreatic RNase digestion of the 1.5 KB RNA. The products of digestion were applied to a 85 cm x 17.5 cm x 0.04 cm sequencing gel. Lane 1: Represents the upper 42 cm of the gel. Lane 2: Represents the lower 43 cm of the gel.

which spans the splice junction between the 3'-exon and the second leader (see Figures 1 and 4A). The splice junction sequences in the 1.5 KB RNA were aligned according to the "GT-AG" rule (15).

The 13-mer oligonucleotide was eluted from the sequencing gel, digested with RNase-T_1, and the products analyzed by two-dimensional chromatography (Figure 3A). The RNase-T_1 digestion of

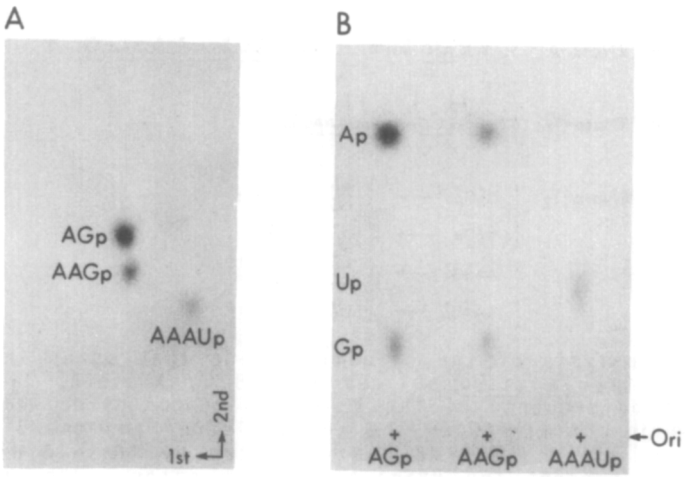

Figure 3. RNase T_1 and T_2 analysis of the oligonucleotide which spans the splice junction in the 1.5 KB *in vitro* spliced RNA. The 13-mer oligonucleotide in the 1.5 KB RNA RNase A digestion product was isolated as described in Figure 2. A) The oligonucleotide was digested with RNase-T_1 as described in Materials and Methods and the products analyzed by two-dimensional chromatography. B) Each individual spot of the plate shown in Panel A was eluted from the plate, digested with RNase-T_2, and the products analyzed by PEIC chromatography as described in Materials and Methods.

the 13-mer oligonucleotide produced only three spots with the expected migration of AGp, AAGp, and AAAUp. This result strongly indicates that the newly formed bond in the spliced 1.5 KB is a typical 3',5'-phosphodiester bond. The fact that the nucleotide bond at the splice junction in the 1.5 KB RNA was RNase-T_1 sensitive rules out the possibility of a 2'-phosphate (as in yeast tRNAs) or other modifications in the 2'-position (methylation). This result is in agreement with another report (16). Each one of the three generated RNase-T_1 spots (Figure 3A) was eluted from the PEIC plate and digested with RNase-T_2. The products were analyzed by chromatography on PEIC plates (Figure 3B). The AG spot produced Ap and Gp in a molar ratio of 2:1. This result was expected if the phosphate group at the splice junction is derived from the original precursor RNA molecule rather than from nucleotides present during the *in vitro* reaction. The RNase-T_2 digestion products of the AAG oligonucleotide generated, as expected,

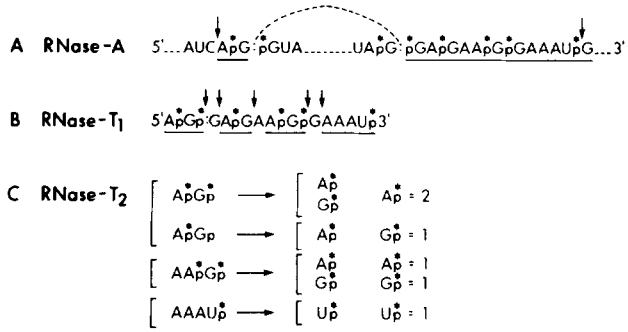

Figure 4. Analysis of the oligonucleotide that spans the splice junction in the in vitro spliced 1.5 KB by RNase-A, T_1 and T_2. A) Nucleotide sequence of the 13-mer oligonucleotide which spans the splice junction between the second leader and the 3'-exon of the in vitro spliced 1.5 KB RNA generated by RNase-A digestion. The arrows represent the RNase-A cleavage sites. The underlined nucleotides are the 13-mer oligonucleotides which span the splice junction in the 1.5 KB RNA. The dashed caret symbol represents the splice junction between the two exons. The asterisks represent the ^{32}p-labeled phosphates. B) The 13-mer oligonucleotide with the RNase-T_1 cleavage sites (represented by arrows). C) The RNase-T_1 oligonucleotides after digestion with RNase-T_2. The numbers represent the molar ratios found in the experiment of Figure 3.

equal molar amounts of Ap and Gp. The AAAU oligonucleotide produced as expected only Up (Figures 3B and 4C). The experiments described in this paper indicate that the newly in vitro generated internucleotide bond at the splice junction is a 3'-5' phosphodiester bond and that the phosphate that forms the splice junction between the exons is derived from the precursor RNA.

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