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Functional group substitutions of the branchpoint adenosine in a nuclear pre-mRNA and a group II intron

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ABSTRACT

Splicing of nuclear mRNA precursors (pre-mRNAs) takes place in the spliceosome, a large and complex ribonucleoprotein. Nuclear pre-mRNA splicing and group II intron self-splicing occur by a chemically identical pathway involving recognition of a specific branchpoint adenosine and nucleophilic activation of its 2′-hydroxyl group. The chemical similarity between these two splicing reactions, as well as other considerations, have suggested that the catalytic core of the spliceosome and group II introns may be related. Here we test this hypothesis by analyzing splicing and RNA branch formation of a pre-mRNA and a group II intron in which the branchpoint adenosine was substituted with purine base analogues. We find that replacement of the branchpoint adenosine with either of two modified adenosine analogues or guanosine leads to remarkably similar patterns of splicing and RNA branch formation in the two systems.

Keywords: branchpoint mapping; group II intron splicing; modified nucleotides; pre-mRNA splicing; spliceosome

INTRODUCTION

The removal of intervening sequences (introns) from both nuclear and group II intron pre-mRNAs is mediated by two sequential in-line *trans*-esterification reactions. The first step of splicing is initiated by a nucleophilic attack of the 2′-hydroxyl group of a specific intronic adenosine (the branchpoint adenosine) at the 5′ terminus of the intron to generate a lariat intermediate containing a 2′-5′ phosphodiester bond. This step is followed by attack of the 3′ hydroxyl group of the 5′ exon on the phosphodiester linkage at the 3′ splice site, resulting in exon ligation and release of the intron in a lariat configuration (reviewed in Lambowitz & Belfort, 1993; Moore et al., 1993; Pyle, 1993; Madhani & Guthrie, 1994; Ares & Weiser, 1995; Michel & Ferat, 1995).

Nuclear pre-mRNA splicing is catalyzed by a dynamic ribonucleoprotein, the spliceosome. Formation of the spliceosome is a complex process that involves the stepwise assembly of four (U1, U2, U5, and U4/U6) small ribonucleoproteins (snRNPs), as well as many non-snRNP protein splicing factors (Moore et al., 1993; Madhani & Guthrie, 1994; Ares & Weiser, 1995; and references therein). The similarity in the mechanism of group II intron auto-catalytic excision and nuclear pre-mRNA splicing has suggested that the catalytic cores of these two enzymes may be functionally related (Sharp, 1985; Cech, 1986; Jacquier, 1990; Weiner, 1993). According to this hypothesis, the snRNAs and not the proteins, form the active site of the spliceosome. Whether the aforementioned similarities between pre-mRNA splicing and group II self-splicing indeed reflect a functional, perhaps ancestral relationship, or are due to "chemical determinism" (Weiner, 1993) remains an open question.

In this report, we have compared the ability of the two splicing reactions to process RNA substrates in which the branchpoint adenosine, a substrate for enzyme recognition, bears substitutions of specific functional groups. Our results provide a new line of evidence for the functional relatedness between group II intron and nuclear pre-mRNA splicing.

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RESULTS

Experimental design

For both nuclear pre-mRNAs and group II introns, the identity of the nucleotide at which RNA branch formation occurs can affect both steps of splicing (Newman et al., 1985; Hornig et al., 1986; Freyer et al., 1987; Query et al., 1996; Liu et al., 1997). To understand the role of the functional groups involved in selection and activation of the branchpoint adenosine, well-characterized, model nuclear pre-mRNA (MINX; Zillmann et al., 1988) and group II intron (aI5γ; Jarrell et al., 1988) substrates were constructed in which the branchpoint adenosine was modified by substitution with purine riboside (removal of the 6-NH_2 group), 7-deazaadenosine (replacement of the N^7 group by C-H), or guanosine (substitution of 6-NH_2, N-1, and C2-H by 6-oxo, N-1H, and C2-NH_2, respectively) (see Fig. 1A,B). The consequences of these substitutions for RNA splicing and branch formation in both nuclear pre-mRNA and group II introns were then analyzed. To facilitate comparison, reaction conditions were selected for group II introns that favored self-splicing via lariat formation (Jarrell et al., 1988). In principle, a particular substitution could: (1) have no effect on the ability of that residue to function as a nucleophile, or (2) be completely or partially inhibitory. In addition, these effects may or may not be accompanied by RNA branch formation at alternative positions ("cryptic RNA branch formation").

Functional group substitutions of the branchpoint adenosine in nuclear pre-mRNAs

Figure 2 shows the results of in vitro splicing (Krainer et al., 1984; Gaur et al., 1995) of nuclear pre-mRNAs bearing modified branchpoint nucleotides. Replacement of the branchpoint adenosine by purine riboside or 7-deazaadenosine reduced the rate of the first step by 8% and 30%, and the second step by 51% and 67%, respectively. Substitution of the branchpoint adenosine by guanosine was significantly more deleterious, decreasing the rate of the first step by ~20-fold (see Table 1). These results are in general agreement with other pre-mRNA splicing studies in which mutation or modification of the branchpoint adenosine was analyzed (Newman et al., 1985; Hornig et al., 1986; Freyer et al., 1987; Query et al., 1996). Furthermore, and as discussed below, with the guanosine-substituted RNA, the presence of additional lariat RNA species was evident.

Modification of the branchpoint adenosine can lead to cryptic RNA branch formation (Ruskin et al., 1985; Hornig et al., 1986; Freyer et al., 1987; Reed & Maniatis, 1988; Query et al., 1994). To determine whether these substitutions activated cryptic branchpoints, lariat RNAs were isolated by denaturing PAGE, digested to completion with nuclease P1, and the nuclease P1 products were separated by two-dimensional thin-layer chromatography (TLC) (Konarska et al., 1985). Nuclease P1 hydrolyzes RNA to 5′ mononucleotides, but does not cleave within a branch trinucleotide (Reilly et al., 1989). Figure 3 shows, as expected, that the wild-type lariat RNA yielded a single nuclease P1-resistant product, indicative of a single RNA branch. Lariat RNAs containing purine riboside (Fig. 3, purine riboside) or 7-deazaadenosine (Fig. 3, 7-deazaadenosine) resulted in two nuclease P1-resistant products. Because only a single phosphate residue is radiolabeled (N215), the presence of two nuclease P1-resistant products indicates RNA branches at both G214 and N215 (see scheme

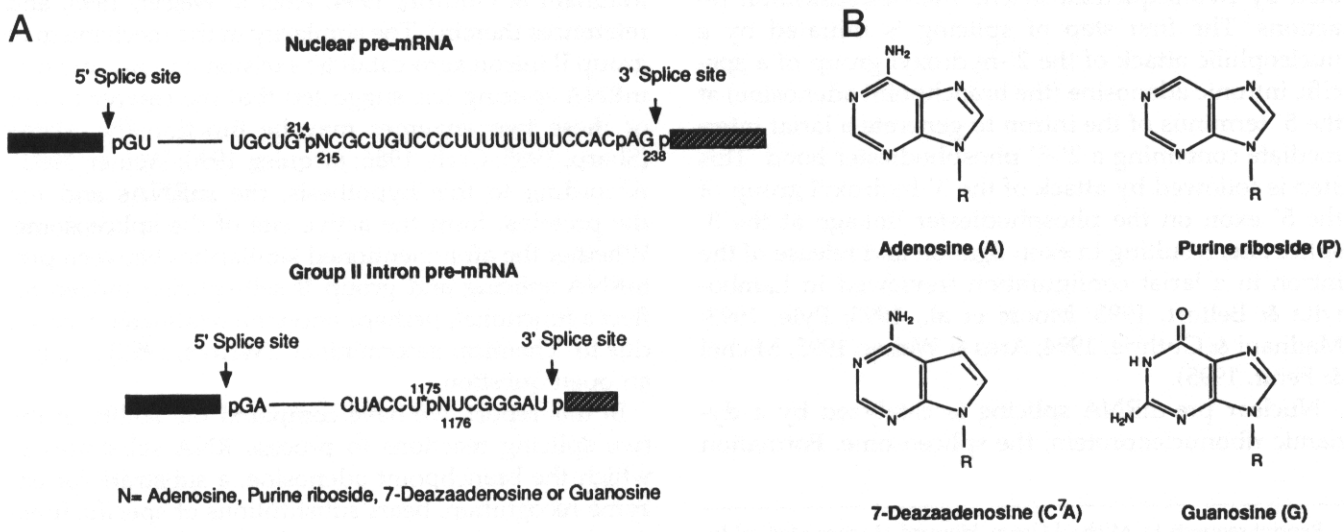

FIGURE 1. Construction of site-specific modified pre-mRNA substrates. **A:** Scheme for site-specific insertion of base analogues into the nuclear pre-mRNA and group II intron self-splicing substrate. **B:** Structures of the purine base analogues. R, ribose.

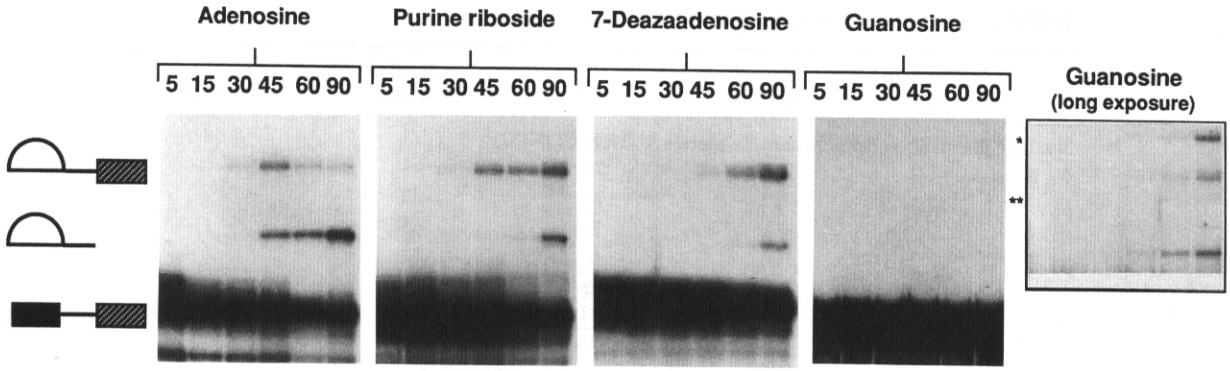

FIGURE 2. Splicing of nuclear pre-mRNA substrates. The nucleotide at the normal position of RNA branch formation and time (min) is indicated above. A schematic representation of the RNA product is indicated at the left. Because the radiolabel is present in the intron, the spliced product and first exon are not visualized. Splicing intermediates of the pre-mRNA substrate containing the guanosine substitution are shown in two different exposures: (*) and (**), lariat intermediate and product, respectively, resulting from use of a distant cryptic branchpoint.

in Fig. 3). Each nuclease P1-resistant product was isolated by elution with water, concentrated by butanol extraction (Cathala & Brunel, 1990), and further characterized by treatment with alkaline phosphatase; whether or not inorganic phosphate was released by this treatment enabled us to determine whether a particular nuclease P1-resistant product contained an RNA branch at position G214 or N215. These results (data not shown) permitted the assignment of branch nucleotides shown in Figure 3.

Lariat RNAs containing the guanosine substitution gave rise to two nuclease P1-resistant products and 5′-[^{32}P]-GMP (Fig. 3). 5′-[^{32}P]-GMP can be generated only if RNA branch formation occurred at a position other than G214 or N215. Thus, guanosine substitution results in formation of cryptic RNA branches both adjacent to, and distant from, the normal position of RNA branch formation (N215).

Functional group substitutions of the branchpoint adenosine in group II introns

The self-splicing reactions of group II intron substrates bearing nucleotide analogues at the branchpoint adenosine are shown in Figure 4. Under the reaction conditions we used, the rate-limiting step is formation of the lariat intermediate, which is converted rapidly to the excised lariat RNA product (Jacquier & Jacquesson-Breuleux, 1991). Our results indicate that substitution of the branchpoint adenosine by purine riboside or 7-deazaadenosine reduced the rate of splicing by 14% and 37%, respectively (Fig. 4; see Table 1). Significantly, as with nuclear pre-mRNA splicing, guanosine substitution drastically decreased the rate of lariat formation (compare adenosine with guanosine in Fig. 4; see Table 1).

Although cryptic RNA branch formation in nuclear pre-mRNAs is well documented (Ruskin et al., 1985; Hornig et al., 1986; Freyer et al., 1987; Reed & Maniatis, 1988; Query et al., 1994), cryptic RNA branch formation in a group II intron has been described only once (Schmelzer & Schweyen, 1986). We tested for this possibility as described above. Nuclease P1 analysis (Fig. 5) indicated formation of a single branch trinucleotide for wild-type pre-mRNA, whereas pre-mRNAs containing purine riboside (Fig. 5, purine riboside) or 7-deazaadenosine (Fig. 5, 7-deazaadenosine) generated two nuclease P1-resistant products. These results indicate that, for wild-type RNA, branch formation occurred only at adenosine (N1176), whereas in pre-mRNAs containing purine riboside or 7-deazaadenosine, the 5′ adjacent uridine (U1175) was also used (see scheme in Fig. 5). Nuclease P1-resistant products were further characterized by alkaline phosphatase treatment (data not shown) to permit assignment of the branched nucleotide (Fig. 5). As with nuclear pre-mRNA, guanosine substitution gave rise to two nuclease P1-resistant products, as well as 5′-[^{32}P]-GMP, indicating that RNA branch formation occurred at the normal, adjacent, and a distant position(s). To rule out the possibility that the 5′-[^{32}P]-GMP was due to contamination of the purified lariat with pre-mRNA, a zero-minute sample was analyzed in parallel. The absence of 5′-[^{32}P]-GMP in this sample (data not shown) confirmed that the 5′-[^{32}P]-GMP was derived from the lariat as a result of a cryptic RNA branch.

Mapping the distant cryptic RNA branch in the guanosine-substituted group II intron

We next mapped the position of the distant cryptic RNA branch in the guanosine-substituted group II intron. The excised lariat generated from this unlabeled RNA was purified on a 5% denaturing polyacrylamide gel, 3′ end-labeled (Krupp, 1991) with [5′-^{32}P]pCp and RNA ligase (Fig. 6A), subjected to limited alkaline hydrolysis (Krupp, 1991), and the reaction products separated on a 25% denaturing polyacrylamide gel. Because the phosphodiester bond immediately 3′ to

TABLE 1. Relative rates of splicing and RNA branch formation.[a]

N	First step yield	Second step yield	% Position N	% Position 5′ cryptic	% Position distant cryptic
Nuclear pre-mRNA Branchpoint Sequence 5′-UGCUGNC-3′					
A	1.0	1.0	100	—	—
P	0.92	0.49	63/35	36/65	—
C^7A	0.69	0.33	61/26	38/73	—
G	0.05	0.11	11/18	1.0/1.5	88/81
Group II intron pre-mRNA Branchpoint Sequence 5′-UACCUNU-3′					
A	1.0	—	100	—	—
P	0.86	—	71	29	—
C^7A	0.63	—	82	18	—
G	0.04	—	50	16	33

[a]Rates were calculated independently of the specific site of RNA branch formation. The relative amount of RNA in each band was expressed as a percentage of the total obtained by summing the values of lariat intermediate (LI), lariat product (L), and pre-mRNA (P) at that time point. Rates were calculated in the linear range and were normalized to the wild-type substrate. For nuclear pre-mRNA, the rates of first and second steps were calculated as:

$$\text{First step} = \frac{[LI] + [L]}{[LI] + [L] + [P]},$$

$$\text{Second step} = \frac{[L]}{[LI] + [L]}.$$

For the group II intron, the 5′ splice site cleavage is the rate-limiting step and it is assumed that the amount of lariat product at a particular time point represents the rate of splicing and is calculated using the formula:

$$\text{Rate of splicing} = \frac{[L]}{[L] + [P]}.$$

The percentage of RNA branch formation at position N was determined as the ratio of $^{/G}_{pNpC}$ to $^{/G}_{pGpN}$ + 5′-NMP in nuclear pre-mRNA and $^{/G}_{pNpU}$ to $^{/G}_{pUpN}$ + 5′-NMP in the group II intron. Values were calculated by densitometric analysis using a Pharmacia LKB Ultroscan XL densitometer. The numerator and denominator in nuclear pre-mRNA indicate the percentage position N used in lariat intermediate and lariat product, respectively.

the 2′-5′ linkage will not be hydrolyzed, the expected result is a ladder of short digestion products from the 3′ tail of the lariat, followed by a gap, and then resumption of a ladder of longer digestion products (Schmelzer & Schweyen, 1986; Reilly et al., 1989).

As shown in Figure 6B (lane 2), partial alkaline hydrolysis of the wild-type lariat intron resulted in six oligonucleotide species followed by a gap, as expected for the short 3′ linear tail of a lariat intron (Schmelzer & Schweyen, 1986; Reilly et al., 1989). The presence of six oligonucleotide bands in the wild-type lariat intron confirmed that an RNA branch formed at adenosine 1176 (see Fig. 6A). Partial alkaline hydrolysis of the guanosine-substituted lariat intron (guanosine 1176) resulted in nine oligonucleotide bands followed by a gap (Fig. 6B, lane 4), mapping a distant cryptic RNA branch to cytosine 1173.

DISCUSSION

An important and unresolved issue in nuclear pre-mRNA splicing is the relationship between the spliceosome and the self-splicing group II ribozyme. The well-established similarities in the chemistry of nuclear pre-mRNA splicing and group II intron self-splicing have raised the possibility that the two enzymes may be related. Here we have modified the branchpoint adenosine in a nuclear pre-mRNA and a group II intron and analyzed the effect of these modifications on splicing and RNA branch formation. We have demonstrated that substitution of the branchpoint adenosine by purine riboside, 7-deazaadenosine, or guanosine affected nuclear pre-mRNA splicing and group II intron self-splicing in a remarkably similar manner (summarized in Table 1). Removal of either the 6-NH_2 group (purine riboside) or the N^7-nitrogen (7-deazaadenosine)

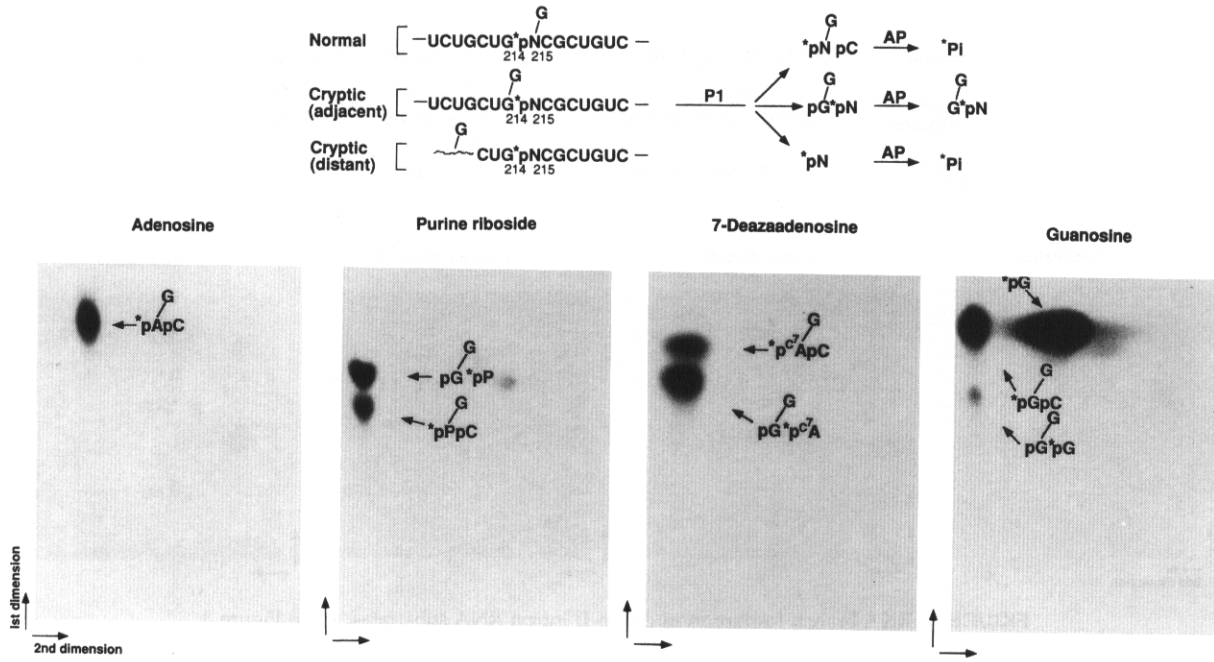

FIGURE 3. RNA branch formation in nuclear pre-mRNA substrates. Upper: Scheme for the analysis of RNA branch formation. A single labeled phosphorus (*) was placed 5′ to the normal branchpoint adenosine (N). P1, nuclease P1; AP, alkaline phosphatase. Lower: Analysis of nuclease P1-digestion products by two-dimensional TLC.

had a modest effect, reducing the rate of splicing by 10–40% in both systems. Substitution by guanosine dramatically reduced splicing of both the nuclear pre-mRNA and group II intron.

Our results allow us to draw some general conclusions about how the branchpoint adenosine is recognized. Both the modest effect on splicing rate, and the analysis of cryptic RNA branch formation (discussed below), indicate that the 6-NH_2 group, a potential hydrogen bond donor, and the N^7-nitrogen, a potential hydrogen bond acceptor, participate in recognition of the branchpoint adenosine. However, removal of these groups did not abolish RNA branch formation at this position. Thus, recognition of the branchpoint adenosine is likely achieved through multiple interactions, no single one of which is essential. The adenosine to guanosine substitution can result in loss of up to two hydrogen bonds, at the N-1 and 6-NH_2 positions (Fig. 1B), and, because the orientation of potential hydrogen bonds also changes, electrostatic clash may occur. In addition, the 2-NH_2 group of guanosine could cause steric hinderance. Taken together, these considerations likely explain why guanosine substitution is the most deleterious of the variants tested.

The effects on splicing rates were mirrored by cryptic RNA branch formation. In both systems, substitu-

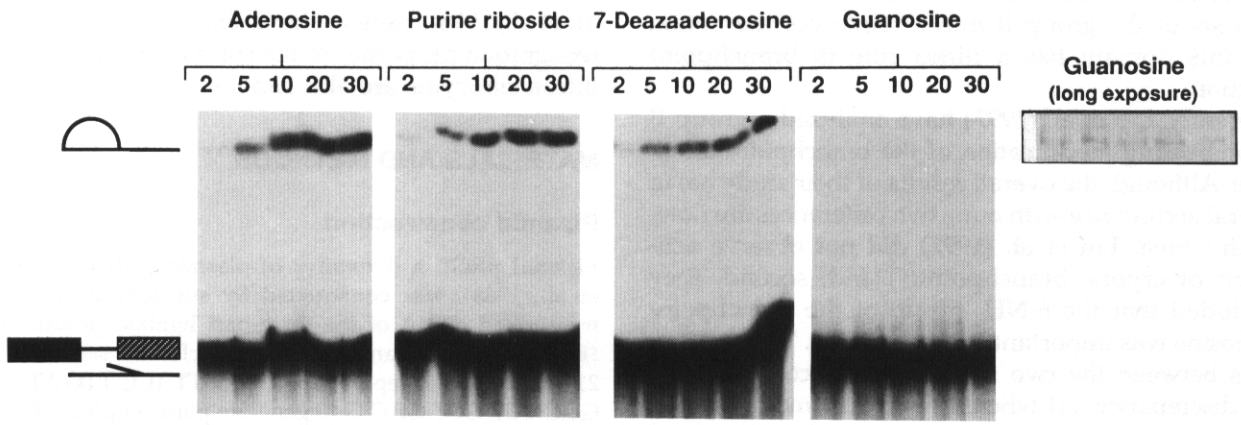

FIGURE 4. Splicing of group II intron RNA substrates. As in Figure 2. The positions of lariat product, precursor, and broken lariat RNA species are indicated schematically. Linear excised intron, which is generally generated via the hydrolysis, could not be resolved from the broken lariat.

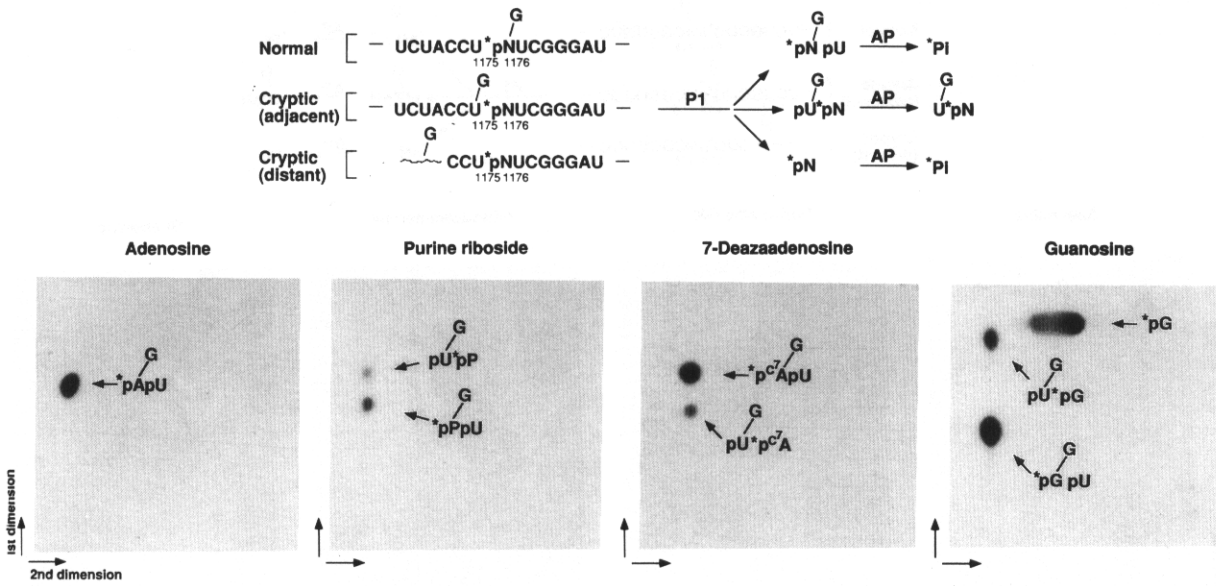

FIGURE 5. RNA branch formation in group II intron RNA substrates. As in Figure 3.

tion with purine riboside or 7-deazaadenosine resulted in RNA branch formation at the 5′ adjacent position. The loss of a hydrogen bonding interaction due to removal of the 6-NH_2 or N^7-nitrogen may have decreased the fidelity of the enzyme, leading to activation at the adjacent nucleotide for nucleophilic attack. Substitution with guanosine resulted in cryptic RNA branch formation predominantly at a distant site. Most likely, the loss of hydrogen bonds, and/or the interfering effects described above, virtually eliminated recognition of the guanosine. Our finding that RNA branch formation also occurred at cytosine (C1173) and uridine (U1175) further strengthens the functional similarities between the two splicing reactions; several studies have shown that RNA branch formation can occur at cytosines and uridines in nuclear pre-mRNAs (Hornig et al., 1986; Adema et al., 1988, 1990; Hartmuth & Barta, 1988; Query et al., 1996). The fact that cryptic RNA branch formation occurred within domain six of the group II intron reinforces the notion that this domain has a direct role in branchpoint selection.

Recently, Liu et al. (1997) have analyzed a group II intron-bearing modification of the branchpoint adenosine. Although the overall results of their study are in general accordance with ours, two differences are noteworthy. First, Liu et al. (1997) did not observe activation of cryptic branchpoint(s) and, second, they concluded that the 6-NH_2 group of the branchpoint adenosine was important for recognition. Three differences between the two studies may account for the first discrepancy: (1) whereas we performed nuclease analysis of isolated lariats, Liu et al. (1997) analyzed the unfractionated reaction mixture; (2) the two studies used different systems for TLC; and (3) the RNA substrates used in the two studies had different size exons, which could influence branchpoint selection. Finally, we note that Liu et al. (1997) did report low levels of cryptic branchpoint activation in a *trans*-splicing reaction. With regard to the second difference, the role of the 6-NH_2 group, the conclusion of Liu et al. (1997) was based upon a *trans*-splicing reaction, whereas our conclusion was based upon *cis*-splicing.

Well-established similarities between pre-mRNA splicing and group II intron auto-catalysis include identical chemical pathways and stereochemistry (Moore & Sharp, 1993; Padgett et al., 1994). In addition, potentially analogous secondary structural features of group II introns and spliceosomal pre-mRNA/snRNAs, including a bulged branchpoint adenosine (Query et al., 1994), have been proposed (Michel et al., 1989; Madhani & Guthrie, 1992; Steitz & Steitz, 1993; Chanfreau & Jacquier, 1994; Nilsen, 1994; Peebles et al., 1995; Yu et al., 1995). Here we have shown that the substrate recognition properties of the spliceosome and group II intron ribozyme are also similar.

MATERIALS AND METHODS

Plasmid construction

Plasmid pRG1, a derivative of plasmid pMINX (Zillmann et al., 1988), was constructed by subcloning of reverse-transcribed cDNA of the three-part ligation product RNA (1–205), (206–215), and (216–283). Nucleotide sequence 191–217 of MINX was replaced by GAT GTT TCC TTG ATG ATG GGC TCT GCT GAC G to generate plasmid pRG1. Plasmid pRG1 encodes the following nucleotide sequence: 5′-GAAUACACGGAAUUCGAGCUCGCCCACUCUUGGAU CGGAAACCCGUCGGCCUCCGAACG/GUAAGAGCCUA

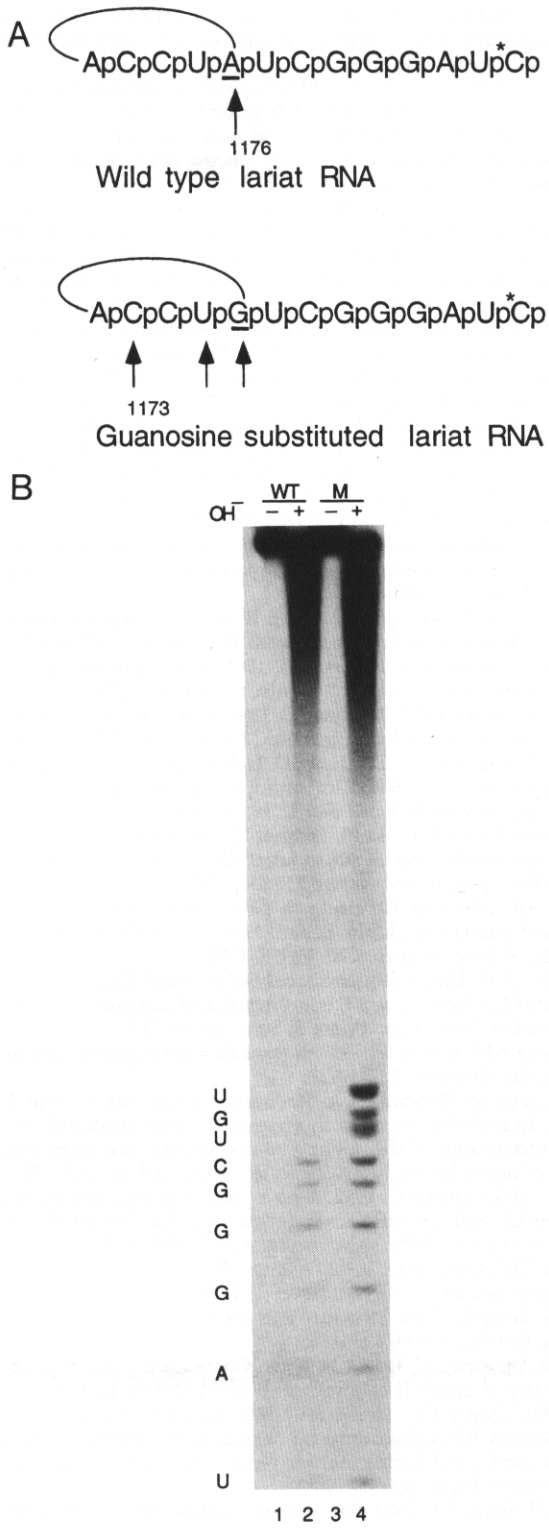

FIGURE 6. Mapping the distant cryptic RNA branch in a guanosine-substituted group II intron. **A:** Schematic representation of wild-type and guanosine-substituted lariat intron. * represents ^{32}P-labeled phosphate generated by 3′-end labeling of the lariat intron by [5′-^{32}P]pCp. The position at which RNA branch formation normally occurs is underlined. Arrows indicate nucleotides at which RNA branching occurs. **B:** Partial alkaline hydrolysis of 3′ end-labeled lariat intron. Lanes 1 and 2, wild-type lariat intron not subjected to or subjected to alkali hydrolysis, respectively; lanes 3 and 4, guanosine-substituted lariat intron not subjected to or subjected to alkali hydrolysis, respectively.

GCAUGUAGAACUGGUUACCUGCAGCCCAAGCUUGCU
GCACGUCUAGGGCGCAGUAGUCCAGGAUGUUUCCUU
GAUGAUGGGCUCUGCUGACGCUGUCCCUUUUUUUU
CCACAG/CUCGCGGUUGAGGACAAACUCUUCGCGG
UCUUUCCAGUGGGGAUC-3′. Slashes indicate 5′ and 3′ splice sites, and *A* indicates the preferred branchpoint. Plasmid pJD20 (Jarrell et al., 1988) is the template for wild-type pre-mRNA containing aI5γ, the fifth intron of the gene for subunit I of yeast mitochondrial cytochrome oxidase.

Preparation of pre-mRNA substrates

The nuclear pre-mRNA substrate was prepared (Moore & Sharp, 1992) by ligating three segments [RNA (1–214), RNA (215–236), and RNA (237–283)] containing a single substitution at the normal branchpoint adenosine (designated N215, see Fig. 1A). For nuclear pre-mRNA substrates, the nucleotide numbering of MINX has been followed. RNAs (1–214) and (237–283) initiated with m^{7}G (5′)ppp(5′) G, cap analogue (NEB), and ApG dinucleotide (Sigma), respectively, were transcribed with a PCR-amplified DNA template generated from plasmid pRG1. RNA (215–236) containing adenosine, purine riboside, 7-deazaadenosine, or guanosine at the 5′ terminus was chemically synthesized on an Applied Biosystems 392 DNA synthesizer as described earlier (Fu & McLaughlin, 1992; Fu et al., 1993). The four common nucleoside phosphoramidites were obtained commercially (Glen Research). Purine riboside and 7-deazaadenosine phosphoramidites were synthesized according to reported methods (Fu & McLaughlin, 1992; Fu et al., 1993) and were characterized by ^{1}H and ^{31}P NMR spectroscopy. A 949-nt long group II intron pre-mRNA consisting of 70 nt of exon 1 and 879 nt of intron terminating before the branchpoint adenosine was transcribed with T7 RNA polymerase (BRL) from a DNA template generated by PCR amplification of plasmid pJD20 (Jarrell et al., 1988). This RNA was ligated (Moore & Sharp, 1992) to a 24 mer synthetic oligoribonucleotide containing adenosine, purine riboside, 7-deazaadenosine, or guanosine at the 5′ terminus (see Fig. 1A).

The integrity of the 3′-5′ phosphodiester linkage and the presence of modified nucleotide in the ligated pre-mRNA substrate was determined by P1 and T2 nucleases digestion (data not shown) followed by two-dimensional TLC analysis (Konarska et al., 1985).

Splicing and analyses of RNA branch formation

High specific activity pre-mRNAs, labeled site-specifically 5′ to the branchpoint adenosine, were incubated under standard splicing conditions in HeLa nuclear extract as described (Krainer et al., 1984; Gaur et al., 1995). The products of the splicing reactions were separated and purified on a 13% polyacrylamide (29:1)/8 M urea gels run in 1× TBE buffer.

The isolated pre-mRNA, lariat intermediate, and lariat product were digested to completion with nuclease P1 (0.4 μg; US Biochemicals) in 4 μL of 30 mM sodium acetate, pH 5.2, 0.1 mg/mL yeast tRNA for 1 h at 37 °C. Digestion products were separated (Konarska et al., 1985) by two-dimensional TLC on cellulose plates (Macherey-Nagel) using solvent A (isobutyric acid/concentrated NH_4OH/

H_2O; 577:38:385) in the first dimension and solvent B (*t*-butanol/concentrated HCl/H_2O; 14:3:3) in the second dimension. For the guanosine-substituted substrate, solvent C (0.5 N NH_4OH/isobutyric acid; 5:3) was used for separation in the first dimension. The branch trinucleotide was isolated and digested with calf intestine alkaline phosphatase (0.4 U, Boehringer) for 3 h at 37 °C in 4 μL of 50 mM Tris-HCl, pH 8.5, 0.1 mM EDTA.

Group II intron self-splicing reactions (Koch et al., 1992) were performed for various times in 0.5 M $(NH_4)_2SO_4$, 0.1 M $MgCl_2$, and 40 mM Tris-HCl, pH 7.4, at 45 °C. Reactions were stopped by adding loading dye, and the products were analyzed on a 5% polyacrylamide (29:1)/8 M urea gels. Nuclease P1 digestion and the separation of the reaction products was performed as described above. Solvent A and B were used for separation in the first and second dimensions, respectively.

3′-End labeling of the lariat RNA and partial alkaline hydrolysis

Approximately 100 pmol of unlabeled pre-mRNA generated by in vitro transcription was incubated under self-splicing conditions and the lariat intron was purified on a 5% denaturing polyacrylamide gel. The purified lariat RNA was incubated at 4 °C overnight in 21-μL reaction volume containing 7 μL 3× RNA ligase buffer (30% DMSO; 150 mM HEPES, pH 8.0; 10 mM DTT; 60 mM $MgCl_2$; 15 μM ATP; 30 μg/mL BSA), 1 μL RNA ligase (10 u/μL; Boehringer-Mannheim), 1 μL RNasin (Promega), and 20–30 μCi [5′-^{32}P]pCp. [5′-^{32}P]pCp was prepared essentially as described (Krupp, 1991). After 3′ end-labeling, the RNA was gel purified, ethanol precipitated in the presence of 5 μg carrier tRNA, and incubated with 4 μL hydrolysis buffer (60 μL 9 M urea, 25 μL water, 4.5 μL 1 N NaOH, and 1 μL 1% tracking dyes) for 2 min at 80 °C. Reaction products were analyzed on a denaturing 25% polyacrylamide gel.

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