Reactivity of modified ribose moieties of guanosine: new cleavage reactions mediated by the IVS of *Tetrahymena* precursor rRNA

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ABSTRACT

An RNA molecule consisting of the 5'exon and intervening sequence (IVS) of *Tetrahymena* precursor rRNA was oxidized with sodium periodate to convert the ribose moiety of the 3' terminal guanosine into a dialdehyde form. The modified RNA undergoes a specific cleavage reaction at the 5' splice site, but has no apparent cyclization activity. This novel reaction mediated by the IVS RNA is pH dependent over the range 6.5-8.5 and leaves a 5' phosphate and a 3'-OH at the newly created termini. The dialdehyde form of monomer guanosine is also capable of causing a specific cleavage reaction at the 5' splice site although the nucleotide is not covalently attached to the IVS RNA in the final product. These and other findings described in this report suggest that the cis diol of the intact ribose moiety of guanosine is not an absolute requirement for the IVS-mediated reactions.

INTRODUCTION

The intervening sequence (IVS) of *Tetrahymena* precursor rRNA is capable of mediating self-splicing *in vitro* and has been shown to be an RNA catalyst (1,2). The splicing reaction proceeds via two consecutive transesterification reactions. In the first reaction, monomer guanosine attacks the 5' splice site to cleave off the 5'exon, leaving the guanosine covalently attached to the 5' end of an RNA consisting of the IVS and 3'exon. In the second reaction, the free 5'exon joins to the 3'exon, completing exon ligation. Several other self-splicing reactions of this type have been reported for precursor RNAs which contain group I introns (3,4). In the case of the *Tetrahymena* system, the excised linear IVS RNA is capable of mediating a variety of other reactions, including cyclization (5), reverse cyclization (6), intermolecular ligation (7), specific hydrolysis (8), nucleotidyl transfer (2) and dephosphorylation (9). It has been proposed that these reactions and the self-splicing reaction proceed through a common reaction mechanism, although the details of this mechanism are not known (7).

The requirements for specific functional groups of the guanosine involved in the first stage of splicing have been well characterized (10). However,

it is not clear whether the ribose moiety of guanosine is also essential for this reaction. In the intramolecular reaction of an RNA consisting of the 5'exon attached to the IVS (5'exon•IVS RNA), the guanosine residue at the 3' end (G₄₁₄) (11) acts as a nucleophile in the cleavage of phosphodiester bonds located after a sequence of pyrimidines at and downstream from the 5' splice site. This reaction results in the production of circular molecules. It can be regarded as equivalent to the first stage of splicing since both reactions involve nucleophilic attack by a guanosine residue (7). Information regarding the reactivity of G₄₁₄ at its ribose moiety should be helpful in understanding the mechanism of the self-splicing reaction.

In this report, we investigated reactions in which G₄₁₄ or monomeric guanosine had been chemically modified at their ribose moieties, and found varying degrees of reactivity. We conclude that, although the IVS RNA of *Tetrahymena* is designed to react with guanosine, the 2',3'-cis diol of the ribose moiety is not an absolute requirement for reactivity of the nucleophile.

MATERIALS AND METHODS

A. Materials

T7 RNA polymerase and T4 polynucleotide kinase were purchased from U.S. Biochemical Corp., T4 RNA ligase and restriction enzymes from New England Biolabs, and calf intestinal phosphatase from Boehringer Mannheim. All radiolabeled nucleotides were from ICN Radiochemicals. Sodium periodate, periodate-oxidized nucleotide 5'-triphosphates, semicarbazide, aniline, and NaBH₄ were obtained from Sigma. pGNH₂ (3' deoxy, 3'-amino guanosine 5' monophosphate) and pG̃ (9-(1,3-hydroxy-2-propoxy)-methyl guanine) were gifts from L.E. Orgel and W.S. Zielinski.

B. Preparation of Precursor rRNA

Precursor rRNA was prepared by transcription *in vitro* of the plasmid pT7TT1A3 which had been truncated with EcoRI endonuclease (12). This yields a 549 nucleotide RNA consisting of a 48 nucleotide 5'exon, a 413 nucleotide intervening sequence, and an 88 nucleotide 3'exon. The 1 ml transcription mixture, which contained 30 µg of plasmid, 4000 U of T7 RNA polymerase, 500 µCi of [3H]UTP or [α³²P]ATP, 500 µM each of the NTPs, 6 mM MgCl₂, 2 mM spermidine, 10 mM DTT, and 40 mM Tris•HCl (pH 7.5), was incubated at 37°C for 2 h. The RNA was separated by electrophoresis in a 5% polyacrylamide/8M urea gel, eluted, purified by chromatography on Sephadex G50-150 and ethanol precipitated.

C. Hydrolysis of Precursor rRNA

Precursor rRNA was incubated in 10 mM MgCl₂, 200 mM NaCl, 50 mM HEPES (pH 8.5) at 42°C for 2 h. The major hydrolysis products are the 3'exon and an RNA molecule consisting of the 5'exon attached to the intervening sequence. The latter, which we refer to as 5'exon•IVS RNA, was separated by electrophoresis, eluted and purified as described above.

D. Modification of the 3' end of 5'exon•IVS RNA

1) Sodium periodate treatment of 5'exon•IVS RNA (13). Approximately 10-20 pmol of 5'exon•IVS RNA was incubated in a 30 µl reaction volume with 0.25 mg of NaIO₄ at 25°C for 1 h in the dark. 10 µl of 10% ethylene glycol was added to stop the reaction and the mixture was diluted tenfold with water. NaOAc (pH 5.2) was added to a final concentration of 0.25 M, and the RNA was precipitated with 2.5 volumes of ethanol. The product (5'exon•IVS=0 RNA) was purified by chromatography on Sephadex G50-150 and ethanol precipitation. Its purity was confirmed by attempting to ligate [³²P]p•Cp onto its 3' end (14) and finding no detectable ligation.

2) Sodium borohydride treatment of 5'exon•IVS=0 RNA. 5-10 pmol of 5'exon•IVS=0 RNA was dissolved in 300 µl of KH₂PO₄/Na₂HPO₄ (pH 7.0). 100 µl of 0.1 M NaBH₄ was added, and the mixture was incubated at 0°C for 4 h in the dark (15). The reaction was stopped by addition of 20 µl of acetic acid. The RNA product was precipitated in 0.25 M NaOAc (pH 5.2)/ethanol and purified by chromatography on Sephadex G50-150.

3) Semicarbazide treatment of 5'exon•IVS=0 RNA. 5-10 pmol of 5'exon•IVS=0 was dissolved in 250 µl of 0.25 M NaOAc (pH 5.2), a 1000-fold molar excess of semicarbazide was added, and the solution was incubated at 25°C for 1 h (15). The RNA was purified by ethanol precipitation and chromatography on Sephadex G50-150.

4) Aniline treatment of 5'exon•IVS=0 RNA. 5-10 pmol of 5'exon•IVS=0 RNA was dissolved in 50 µl of aniline solution (PhNH₂:H₂O, 1:33; pH adjusted to 5.2 with HCl) and incubated at 45°C for 30 min (13). 150 µl of water was added and the solution was adjusted to 0.25 M with NaOAc (pH 5.2). The RNA was purified by ethanol precipitation and chromatography on Sephadex G50-150.

All of the 3' end-modified RNAs described above were further purified by electrophoresis in a 5% polyacrylamide/8M urea gel. The RNAs were recovered from the gel as described for RNA transcription.

E. Gel Electrophoresis and Fluorography

Electrophoresis was performed using either 1.0 x Tris-borate buffer (100 mM Tris, 83 mM boric acid, 1 mM Na₂EDTA) or 0.4 x Tris-borate buffer.

The latter buffer decreases the electrophoretic mobility of circular RNAs (16). For fluorography, the gels were soaked in 1 M sodium salicylate for 30 min. and dried.

RESULTS

Reactions of 5'exon•IVS=0 RNA

The specific hydrolysis reaction at the 3' splice site of the precursor rRNA produces the free 3'exon and an RNA molecule (461 nucleotides) which consists of the 5'exon joined to the intervening sequence (7). The terminal residue of uniformly labeled [32P]5'exon•IVS RNA (G414) was oxidized with NaIO4 to convert the 2',3'- hydroxyl groups into the dialdehyde form. The resulting RNA, which we term 5'exon•IVS=0, was examined side by side with

Figure 1: Reactions of 5'exon•IVS and 5'exon•IVS=0 RNAs. (a) The uniformly labeled [³²P]5'exon•IVS RNA was incubated in 2 mM (lane a) or 10 mM (lane b) MgCl₂, 50 mM HEPES (pH 8.0) at 50°C for 7 min; 5 mM (lane c) or 25 mM (lane d) MgCl₂, 200 mM NaCl, 50 mM HEPES (pH 7.5) at 42°C for 1.0 h.; 2 mM MgCl₂, 50 mM HEPES (pH 7.5) (lane e) or 50 mM CHES (pH 9.0) (lane f) at 30°C for 1 h; and under standard splicing conditions, 5 mM MgCl₂, 100 mM (NH₄)₂SO₄, 50 mM HEPES (pH 7.5) at 30°C for 1 h with 100 μM GTP (lane g). (b) The uniformly labeled [³²P] 5'exon•IVS=0 RNA was incubated under the same conditions as described in (a). The reaction mixtures for (a) and (b) were subjected to electrophoresis in a 5% polyacrylamide/8M urea gel run in 0.4 x Tris-borate buffer. Autoradiograms are shown.

uniformly labeled [³²P]5'exon•IVS RNA under a variety of conditions as shown in Figure 1. 5'exon•IVS RNA is largely converted to two circular forms; C^o IVS RNA obtained from the attack by G₄₁₄ at the 5' splice site and C IVS RNA obtained from the attack by G₄₁₄ at the cyclization site (located between the 14th and 15th nucleotide in the IVS. See abbreviations in ref. 11). In a

Figure 2: Production of 5' exon•14 and/or 5' exon from 5' exon•IVS RNA and 5' exon•IVS=O RNA. The uniformly labeled [³²P] 5' exon•IVS=O (lanes a to c) and 5' exon•IVS (lanes d to f) RNAs were incubated in 2 mM (lanes a and d), 10 mM (lanes b and e), or 25 mM (lanes c and f) MgCl₂, 50 mM HEPES (pH 8.0) at 50°C for 7 min. The reaction mixtures were electrophoresed in a 10% polyacrylamide/8M urea gel run in 1.0 x Tris-borate buffer, the autoradiogram of which is shown. The bands at the origin in lanes d to f correspond to the RNAs produced upon 5' exon•IVS cyclization (C° IVS, C IVS).

reaction that increases proportionally with increasing pH, C IVS RNA is hydrolyzed at the cyclization junction to give a linear molecule (399 nucleotides) (L-15 IVS RNA), as described previously (8). In the reaction with 5' exon•IVS=O RNA, two linear IVS RNAs were obtained, but no circular

Figure 3: Mg²⁺ requirement for 5' exon•IVS=0 and 5' exon•IVS RNAs. Both 5' end labeled [³²P] RNAs were incubated in 0 to 5.0 mM MgCl₂, 50 mM HEPPS (pH 8.0) at 50°C for 7 min. Electrophoresis was run in a 10% polyacrylamide/8M urea gel with 1.0 x Tris-borate buffer, an autoradiogram of which is shown.

molecule was detected. The products corresponded in size to L IVS and L-15 IVS RNA in a 5% polyacrylamide denaturing gel. These results are compatible with two possible interpretations for the reaction mechanism involving 5' exon•IVS=0 RNA. Either 5' exon•IVS=0 RNA is hydrolyzed directly at the 5' splice site and at the cyclization site in the IVS, or the reaction proceeds to the linear products via highly labile circular intermediates.

To identify small RNA fragments produced in the reactions of 5' exon•IVS=0 RNA and 5' exon•IVS RNA, portions of the reaction mixtures were electro-

phoresed in a 10% polyacrylamide denaturing gel (Figure 2). In the reactions of 5'exon•IVS RNA, the free 5'exon (48 nucleotides) and the 5'exon conjoined with the first 14 nucleotides of the IVS (62 nucleotides) (5'exon•14) were identified as the 5' half cleavage products resulting from cyclization at the 5' splice site and at the cyclization site in the IVS, respectively. Formation of the two RNAs was found to be dependent on MgCl₂ concentration as has been previously reported (7).

In the reactions of 5'exon•IVS=0 RNA, the major 5' half cleavage product was an RNA whose size corresponded to that of the free 5'exon. Only a very small amount of an RNA corresponding in size to 5'exon•14 was detected. Both RNAs were found to comigrate in a 20% polyacrylamide sequencing gel with authentic markers and the 3' ends of both molecules were shown to have a free 2',3'-OH by ligation with [³²P]p•Cp (data not shown) (14). Since the major 3' half cleavage product was found to be an RNA whose size corresponds to that of L-15 IVS RNA and because the major 5' half cleavage product corresponds to an RNA equal in size to 5'exon (Figure 2, lane a to c), it is plausible that formation of the L-15 IVS RNA is largely due to two sequential reactions: promotion of cleavage at the 5' splice site yielding IVS RNA, followed by promotion of cleavage at the cyclization site in the IVS yielding L-15 IVS RNA.

The reactions of 5'exon•IVS=0 RNA were studied at pH 8.0 and a range of MgCl₂ concentrations (Figure 3). It was found that, as in other IVS mediated reactions, there is a requirement for MgCl₂ (10). In one such reaction, 5'exon•IVS and 5'exon•IVS=0 were incubated with GTP in a reaction analogous to the first stage of splicing. Comparing their reactivities, we found that 5'exon•IVS=0 is predominantly converted to an RNA whose size corresponds to L IVS RNA (Figure 1b, lane g), while 5'exon•IVS yields only trace amounts of L IVS RNA and C^o IVS RNA (Figure 1a, lane g). The results indicate that the reactive site in 5'exon•IVS=0 RNA is more susceptible to attack by GTP than the comparable sites in 5'exon•IVS RNA which is presumably due to the unmodified G₄₁₄ in the 5'exon•IVS RNA.

pH dependence of the reactions of 5'exon•IVS=0 RNA

The pH dependence of the reactions of 5'exon•IVS RNA and 5'exon•IVS=0 RNA was examined under standard cyclization conditions (8). 5' end-labeled [³²P]5'exon•IVS=0 RNA or [³²P]5'exon•IVS RNA was incubated at 42°C and pH 6.5, 7.5 or 8.5, and the radioactivity appearing in the 5' half cleavage products was quantified. As shown in Figure 4, there was only a weak dependence on pH in the cyclization reactions of 5'exon•IVS RNA over the

Figure 4: pH dependence of 5' exon-IVS and 5' exon-IVS=0 RNAs. The 5' end labeled [^{32}P] 5' exon-IVS and [^{32}P] 5' exon-IVS=0 RNAs were incubated in 10 mM MgCl_2 , 200 mM NaCl , 50 mM HEPES (pH 6.5, 7.5, or 8.5) at 42°C and for the times indicated. The reaction was stopped by freezing in a dry ice/acetone bath and adding 2 μl of 0.05 M Na_2EDTA . The fraction reacted was quantified by measuring the radioactivity of the 5' half cleavage products.

range of 6.5 to 8.5. However, the rate of cleavage of 5' exon-IVS=0 RNA increased proportionately with increasing pH, though the degree of the acceleration was not linearly proportional to the increase in hydroxyl ion concentration.

In an attempt to identify any possible kinetic intermediates of the 5' exon-IVS=0 self-reaction, uniformly labeled [^{32}P] 5' exon-IVS=0 was incubated at 42°C and pH 7.5, 8.5, or 9.5 for time points ranging from 30 seconds to 1 hour (data not shown). No circular products, however, were detected.

Reactions with other 3' end-modified 5' exon-IVS RNAs

5' exon-IVS=0 RNA was further modified at the 3' terminal ribose moiety, either by reduction with NaBH_4 to produce an RNA which we term 5' exon-IVS-OH RNA or by reaction with semicarbazide to produce an RNA which we term 5' exon-IVS-N=NR RNA (Figure 9a) (15). The effect of these modifications was studied by incubating the RNAs under standard splicing conditions

Figure 5: Reactions of 3' end-modified 5' exon•IVS RNAs. The uniformly labeled [³²P] 5' exon•IVS, 5' exon•IVS-OH, 5' exon•IVS=O, and 5' exon•IVS-N=NR RNAs were incubated either in the standard splicing buffer, 5 mM MgCl₂, 100 mM (NH₄)₂SO₄, 50 mM HEPPS (pH 7.5) at 30°C for 1.5 h, or in 10 mM MgCl₂, 50 mM HEPPS (pH 8.0) at 50°C for 7 min. In the standard splicing reaction, the RNA was incubated without cofactor, with 200 μM GTP, or with 200 μM GTP=O. The reaction mixtures were electrophoresed in a 5% polyacrylamide/8M urea gel run in 0.4 x Tris-borate buffer. An autoradiogram is shown.

(pH 7.5, 30°C) or in the presence of 10 mM MgCl₂ at pH 8.0 and 50°C. The reaction mixtures were electrophoresed in a 5% polyacrylamide denaturing gel as shown in Figure 5 (lanes a, c, d, g, h, j, k and n). The newly-prepared RNAs were all much less reactive than 5' exon•IVS=O RNA or 5' exon•IVS RNA. Small amounts of circular IVS RNAs were observed in the reactions with 5' exon•IVS-OH RNA. This proves that the dihydroxyl of the reduced ribose moiety at the 3' terminus of the IVS is still reactive even when the pentose ring is not intact. Linear IVS RNAs were also produced from the incubation of 5' exon•IVS-OH RNA. This result is presumably due to the specific hydrolysis of the circular RNAs (7), although direct hydrolysis of the

Figure 6: Reactions of 5'exon•IVS=0 and 5'exon•IVS-1 RNAs under splicing conditions. The uniformly labeled $[^3\text{H}]$ 5'exon•IVS=0 and $[^3\text{H}]$ 5'exon•IVS-1 RNAs were incubated in 10 mM MgCl_2 , 100 mM $(\text{NH}_4)_2\text{SO}_4$, 50 mM HEPES (pH 7.5) and in the presence of no cofactor, 200 μM GTP, or 200 μM GTP=O. The reaction was carried out at either 30°C for 1.5 h or at 42°C for 1.0 h and the mixtures were subsequently analyzed by electrophoresis in a 5% polyacrylamide/8M urea gel run in 0.4 x Tris-borate buffer. A fluorogram of the gel is shown. The band appearing above both 5'exon•IVS=0 and 5'exon•IVS-1 RNAs was co-purified with the 5'exon•IVS RNA during transcription. The contribution of this minor RNA fragment was negligible as compared with the reaction products of the main starting material.

starting material could also have resulted in these products. In the reactions of 5'exon•IVS-N=NR RNA, small amounts of linear RNAs were the only products observed. Since the extent of the reaction was so minimal, we cannot be certain whether this reaction involved the semicarbazide or was due to residual 5'exon•IVS=0 RNA.

Reactions with monomer guanosine derivatives modified at the ribose moiety

The reactivity of G₄₁₄ in the cyclization reaction of the IVS can be regarded as equivalent to that of monomer guanosine which is required for the first step of the self-splicing reaction (7). This implies that monomer guanosine, if oxidized in the same manner as described above, would still be capable of promoting cleavage at the 5' splice site and/or the cyclization site in the IVS.

In a preliminary experiment, GTP or oxidized GTP (GTP=O) was incubated with the uniformly labeled $[^{32}\text{P}]$ 5'exon•IVS RNA derivatives under standard splicing conditions for 1.5 hrs. The resulting reaction mixtures were analyzed by electrophoresis in a 5% polyacrylamide denaturing gel as shown in Figure 5 (lanes b, e, f, i, l and m). GTP promoted cleavage at the 5' splice site of 5'exon•IVS-OH RNA, 5'exon•IVS=0 RNA, and 5'exon•IVS-N=NR RNA to

produce the appropriate linear IVS products. The reactivity of GTP was considerably lower in the reaction involving 5'exon•IVS RNA. GTP=0 did not exhibit detectable reactivity with any of the four RNA substrates under the conditions employed.

5'exon•IVS=0 RNA and a 5'exon•IVS RNA derivative which lacks the terminal guanosine residue (5'exon•IVS-1 RNA) were incubated side by side with either GTP or GTP=0 under splicing conditions with 10 mM MgCl₂ (Figure 6). At 30°C, GTP reacted efficiently with both substrates to give RNAs whose sizes correspond to L IVS RNA and L-15 IVS RNA. In this case, 5'exon•IVS-1 RNA was cleaved to a greater extent than 5'exon•IVS=0 RNA. This suggests that the oxidized G₄₁₄ of 5'exon•IVS=0 RNA can still act as a competitive inhibitor of GTP, thus supporting the hypothesis that the guanosine base of G₄₁₄ and GTP binds to the same position in the active site of IVS RNA (7). At 42°C, the reaction of either 5'exon•IVS=0 or 5'exon•IVS-1 with GTP produced a large amount of RNA equal in size to L-15 IVS RNA. Although the reaction with 5'exon•IVS-1 RNA and GTP=0 was nearly undetectable at 30°C, at 42°C the reaction led to the production of an RNA equal in size to L IVS RNA. However, when ATP=0, CTP=0, or UTP=0 was incubated with 5'exon•IVS-1 RNA under similar conditions, no reaction was observed (data not shown). This demonstrates the requirement of a guanine base in order for the reaction to proceed.

Figure 7: Sequence analysis of the GTP and GTP=0 addition products of 5'exon•IVS-1 and the linear IVS product of 5'exon•IVS=0. Uniformly labeled [³H] 5'exon•IVS=0 RNA was incubated in 10 mM MgCl₂, 100 mM (NH₄)₂SO₄, 50 mM HEPES (pH 7.5) at 42°C for 1 h. In a parallel treatment, two reaction mixtures of uniformly labeled [³H]UTP 5'exon•IVS-1 RNA were incubated under the same conditions but with the cofactor GTP or GTP=0 (200 μM). The 5' end of the above linear IVS products were subsequently treated with alkaline phosphatase, labeled with [γ³²P]ATP and T4 polynucleotide kinase, loaded onto a 5% polyacrylamide/8M urea gel, cut and eluted from the gel, and purified by ethanol precipitation and chromatography on Sephadex G50-150. The resultant [³²P]5' end-labeled RNAs were treated to a limited extent with OR- and RNase T1 as shown. The superscript ' corresponds to the treatment of the [³²P]5' end-labeled product derived from incubating 5'exon•IVS-1 RNA with GTP; '', to the treatment of the [³²P]5' end-labeled product derived from incubating 5'exon•IVS-1 RNA with GTP=0; and ''', to the treatment of the [³²P]5' end-labeled product derived from incubating 5'exon•IVS=0. In lane g the production of the G•IVS sequence can be read to a minor extent, which is presumably due to the GTP contamination in GTP=0. NT denotes untreated starting RNA. N denotes unknown nucleotides derived from the sequencing of a minor RNA contaminant co-purified with the L IVS band. Electrophoresis was run in a 20% polyacrylamide/8M urea gel with 1.0 x Tris-borate buffer, an autoradiogram of which is shown.

The reactivity of other monomeric guanosine derivatives which had been modified at their ribose moieties was investigated. 5'exon•IVS-1 RNA was incubated with either 3' deoxy, 3'-amino guanosine 5' monophosphate (pGNH₂) (18) or 9-(1,3-hydroxy-2-propoxy)-methyl guanine (pG̃) (19) under the splicing conditions at either 30°C or 42°C, and the resulting mixtures were analyzed in a 5% polyacrylamide denaturing gel. Both guanosine derivatives showed no reactivity (Figure 8 and 9a).

Sequence analysis of the 5' end of linear IVS RNA products

The enzymatic sequence analysis technique was used to examine the 5' end of linear IVS RNAs produced in the reaction of 5'exon•IVS-1 RNA with either GTP or GTP=0, and in the reaction of 5'exon•IVS=0 RNA with no cofactor (17). The linear IVS RNA products were treated with alkaline phosphatase and then 5' end-labeled with [γ ³²P]ATP and T4 polynucleotide kinase. The labeled RNAs were partially digested with RNase T1, and subsequently electrophoresed in a 20% polyacrylamide sequencing gel (Figure 7). Since the reaction with GTP is identical to the first stage of the splicing reaction, one would expect to find an extra guanosine at the 5' end of the resulting IVS RNA (1). Indeed this was the case. Thus GTP acts in the same manner to cleave 5'exon•IVS-1 RNA, thereby producing 5'exon and L IVS. However, in the reaction of 5'exon•IVS-1 RNA with GTP=0 and the reaction of 5'exon•IVS=0 RNA, the sequence at the 5' end of the linear products was identical to that of the IVS without the added guanosine. This confirms that oxidized guanosines acting either as a monomer or as the 3' terminal residue of the IVS have the same reactivity, promoting cleavage at the 5' splice site without becoming stably linked to the 5' end of the IVS RNA.

DISCUSSION

Mechanism of the cleavage reaction of 5'exon•IVS=0 RNA

Specific cleavage reactions of 5'exon•IVS=0 RNA seem to proceed sequentially; first at the 5' splice site and then at the cyclization site in the IVS. No circular RNAs were detected under the conditions we employed. In contrast, when 5'exon•IVS RNA is incubated under the same conditions, it is converted to circular RNAs and to the linear forms of the IVS. In the cyclization reactions, 5'exon•IVS RNA undergoes nucleophilic attack by 3'-OH at its terminal guanosine at either the 5' splice site or at the cyclization site in the IVS (7). The circular RNAs are subsequently linearized by specific hydrolysis of the phosphodiester bonds at the cyclization junctions. The fact that circular RNAs were not detected in the reactions involving

5'exon•IVS=0 RNA suggests that the two cyclization sites were directly hydrolyzed by free hydroxyl ions to produce the linear products. The newly created termini have a 5' phosphate and 2',3'-OH, as is characteristic of the specific hydrolysis reaction mediated by the IVS RNA (8). Specific hydrolysis of C IVS RNA is known to be a first order reaction with respect to hydroxide ion concentration over the pH range 6.5-9.0. The rate of cyclization, however, is independent of pH over the range 7.5-9.0. The time course of the reaction involving 5'exon•IVS=0 RNA resembles that of specific hydrolysis rather than cyclization since it increases with pH over the range 6.5-8.5 (Figure 4). Therefore this result supports the direct hydrolysis mechanism.

Nonetheless, it is still possible that this reaction involves two steps; first, a pH-independent cyclization reaction that produces a labile circular intermediate, and then a rapid hydrolysis reaction that generates the linear products. The oxidized nucleotides exist in two hydrated forms in aqueous solution (20), one of which closely resembles the conformation of unmodified guanosine (Figure 9b). By mimicking the 3'-terminal guanosine of the IVS, oxidized guanosine could attack the phosphodiester bond at the 5' splice site or at the cyclization site in the IVS, thus forming a circular molecule. This circular RNA would undergo rapid hydrolysis since the unconventional

Figure 8: Reactions of 5'exon•IVS-1 with monomer guanosine derivatives. Uniformly labeled [³²P] 5'exon•IVS-1 RNA was incubated in either 2 mM (lanes a to c) or 10 mM (lanes d to m) MgCl₂, 100 mM (NH₄)₂SO₄, 50 mM HEPES (pH 7.5) at 30°C for 1.5 h (lanes a to h) or at 42°C for 1.0 h (lanes i to m) with no cofactor, 200 μM GTP, 200 μM GTP=O, 200 μM pG_{NH₂}, or 200 μM pG⁻. NT denotes untreated starting RNA. The reaction mixtures were subjected to electrophoresis in a 5% polyacrylamide/8M urea gel run in 0.4 x Tris-borate buffer. An autoradiogram is shown.

Figure 9: The modifications of guanosine at its ribose moiety. (a) Schematic representations of the modified ribose moieties of either terminal or monomer guanosine derivatives. G-OH represents the oxidized and reduced guanosine; G_{NH_2} represents the semicarbazide; and, $\tilde{G}$ represents the 9-(1,3-hydroxy-2 propoxy)-methyl guanine. (b) A possible reaction mechanism of the cleavage reaction by $G=O$. The hydrated forms of $G=O$ and the reaction intermediate are illustrated. R and R' represent the IVS and the 5'exon, respectively.

phosphodiester bond at the cyclization junction consists of a highly labile hemiacetal. The pH dependence of the reaction would be a reflection of the equilibrium between the dialdehyde form and the hydrated forms of the modified terminal guanosine. An increase in pH would favor the hydrated form, thereby facilitating cleavage. At present, we have no conclusive evidence to support either the direct hydrolysis or labile intermediate hypothesis.

Figure 10: The secondary structure at the 5' end of the IVS. (a) The equilibrium between the interaction of IGS and the 3' end of the 5'exon and the one of IGS and the three uridines preceding the cyclization site in the IVS sequence is illustrated. (b) The sequential reaction of the 5'exon·IVS=0 RNA is schematically presented on the basis of the interactions between IGS and the pyrimidines preceding the reaction sites. It is to be noted that the three pyrimidines (UUU) preceding the cyclization site have been suggested to interact with an alternative AGG sequence in the IGS (22). This alternative sequence is located two nucleotides downstream from the AGG sequence shown in the figure.

Selection of the reaction sites

5'exon·IVS=0 RNA was cleaved in a sequential manner at the 5' splice site and the cyclization site in the IVS independent of MgCl_2 concentration. In contrast, choice of the reaction site in the cyclization reaction of 5'exon·IVS RNA is dependent on MgCl_2 concentration. At low MgCl_2 concentrations the cyclization site located in the IVS is preferred, while at high MgCl_2 concentrations the 5' splice site becomes the more favorable site for cyclization (7). It is thought (6, 7) that the IVS RNA promotes cyclization on the basis of the local structure of the cyclization site

aligned with a region of the molecule termed the "internal guide sequence" (IGS) (3, 21, 22) (Figure 10a). The mechanism of cyclization site selection cannot be explained by simply comparing the two relevant local structures (7). Since the duplex involving the IGS and the 3' end of the 5'exon is likely to be more stable than that involving the IGS and the three uridines upstream from the cyclization site, one would expect that the 5' splice site is preferred under low $MgCl_2$ conditions. This is simply not the case. In the reactions of 5'exon•IVS=0 RNA, however, the 5' splice site is preferentially cleaved irrespective of $MgCl_2$ concentration (Figure 10b). We believe that the difference in behavior of 5'exon•IVS and 5'exon•IVS=0 is related to differential binding of normal guanosine versus oxidized guanosine to the IVS. This hypothesis could explain the fact that GTP=0 promotes cleavage predominantly at the 5' splice site of 5'exon•IVS-1 RNA, while GTP cleaves both at the 5' splice site and at the cyclization site in the IVS (Figure 8).

Reactions of other guanosine analogues modified at the ribose moiety

GTP=0 promotes the cleavage of 5'exon•IVS=0 RNA or 5'exon•IVS-1 RNA. The reactions are directed specifically at the 5' splice site as is the case in the corresponding reaction of 5'exon•IVS=0 RNA incubated by itself. In the reaction of 5'exon•IVS with GTP=0, GTP=0 does not become attached to the 5' end of the IVS RNA. The reactivity of GTP=0 therefore appears to be equivalent to that of the terminal residue of 5'exon•IVS=0 RNA. This is interpreted as new evidence in support of the equivalence between the first stage of the self-splicing reaction and the cyclization reactions.

pGNH₂ did not show any reactivity with 5'exon•IVS-1 RNA, despite the fact that the amino group of pGNH₂ is a much better nucleophile than the 2',3'-cis diol of guanosine (18). These results, combined with the fact that dGTP does not act as a cofactor in the splicing reaction, strongly suggest that the 2'-OH group is used to coordinate nucleophilic attack by the 3'-OH in a unique manner (10). When 5'exon•IVS RNA was reduced with NaBH₄, the resulting dihydroxyl-terminated RNA was still capable of undergoing cyclization (Figure 5), albeit at a much lower efficiency than that of 5'exon•IVS RNA. We take this as evidence that the 2'-OH of 5'exon•IVS-OH RNA is still capable of coordinating attack by the 3'-OH. p $\tilde{G}$, however, showed no reactivity with 5'exon•IVS-1 RNA, despite the fact that it contains two OH groups. We believe that this is due to differences in the specific location of the hydroxyl groups relative to the binding site for the guanine base.

In summary, we conclude that although the *Tetrahymena* IVS RNA is designed

to recognize a guanosine with a 2',3'-cis diol, hydroxyl groups that are stereochemically equivalent to those of the diol are still eligible for cleavage reactions.

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11. Abbreviations: IVS, the intervening sequence (413 nucleotides); L IVS, the linear intervening sequence with a guanosine covalently attached to its 5' end (414 nucleotides); G414, the terminal guanosine at the 3' end of the intervening sequence. (This numbering system assigns the covalently attached guanosine as nucleotide number 1); L-15 IVS, a linear form of the intervening sequence, missing the first 15 nucleotides of the L IVS.
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