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Nucleotide sequence and newly formed phosphodiester bond of spontaneously ligated satellite tobacco ringspot virus RNA

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ABSTRACT

The satellite RNA of tobacco ringspot virus (STobRV RNA) replicates and becomes encapsidated in association with tobacco ringspot virus. Previous results show that the infected tissue produces multimeric STobRV RNAs of both polarities. RNA that is complementary to encapsidated STobRV RNA, designated as having the (-) polarity, cleaves autolytically at a specific ApG bond. Purified autolysis products spontaneously join in a non-enyzymic reaction. We report characteristics of this RNA ligation reaction: the terminal groups that react, the type of bond in the newly formed junction and the nucleotide sequence of the joined RNA. The nucleotide sequence of the ligated RNA shows that joining of the reacting RNAs restored an ApG bond. The junction ApG has a 3'-to-5' phosphodiester bond. Thus the net ligation reaction of STobRV (-)RNA is the precise reversal of autolysis. We discuss this new type of RNA ligation reaction and its implications for the formation of multimeric STobRV RNAs during replication.

INTRODUCTION

Small satellite RNAs (1,2) of plant viruses depend on a specific virus to support satellite RNA increase and provide coat protein for encapsidation. They have no extensive nucleotide sequence homology with the supporting virus genomic RNAs. However, they may alter drastically the virus yield and the severity of symptoms. The satellite RNA of tobacco ringspot virus (STobRV RNA) propagates in the presence of tobacco ringspot virus (TobRV) and acts effectively as a parasite of TobRV by reducing its accumulation and ameliorating the symptoms that TobRV alone induces (3). There is no evidence for a translation product of STobRV RNA (4,5), and the details of the RNA replication cycle are unknown.

Encapsidated STobRV RNA, arbitrarily designated as having the (+)polarity, is mainly of the 359 nucleotide residue "monomeric" form (5). However, encapsidated STobRV (+)RNA includes small amounts of repetitive sequence, multimeric forms (6). Double-stranded RNA from tissues that were infected with TobRV and STobRV RNA released, upon denaturation, monomeric and, in lesser amounts, multimeric STobRV RNAs of both polarities. Infected

tissue, but not virus particles, also is a source of circular forms (7,8) of STobRV RNA.

Prody et al. (9) observed non-enyzmic, autolytic processing of the dimeric and trimeric STobRV (+)RNA at a specific CpA bond to produce biologically active monomeric RNA. To more conveniently study the chemical reactions of multimeric STobRV RNAs, Gerlach et al. (10) prepared circularly permuted, dimeric copies of the STobRV RNA sequence and inserted them in both orientations relative to a bacteriophage SP6 promoter. Transcription of the dimeric (+) orientation construction generated RNA with two CpA (residues 359 and 1) autolytic cleavage junctions. As expected, the transcript autolytically processed at both junctions to generate monomeric STobRV (+)RNA and two bordering RNA fragments. The biological activity (10) of the monomeric STobRV (+)RNA confirmed the fidelity of the cloned sequence.

Buzayan et al. (11) and Gerlach et al. (10) showed that STobRV (-)RNA from in vitro transcription also autolytically cleaves. However, the cleavage site in STobRV (-)RNA (Figure 1A) is between residues 49 and 48, numbered according to the STobRV (+)RNA sequence, rather than between residues 359 and 1 (5,9). Cleavage occurs at only one of the 22 ApG bonds in the STobRV (-)RNA sequence and yields a guanylate 5'-hydroxyl and an adenylate 2':3'-cyclic phosphodiester as the new terminal groups (11). We designate the autolysis product derived from the 5' portion of a transcript as RNA P, since it is proximal to the promoter. RNA D is derived from the promoter distal portion of the transcript (Figure 1B,C), and M is a monomeric STobRV RNA sequence. The cleavage of the primary transcript occurred during the transcription reaction, implying that the magnesium ions and spermidine in the reaction stimulated specific cleavage of the STobRV (-)RNA transcript just as they stimulate autolytic processing of dimeric and trimeric STobRV (+)RNA from virus particles (9).

The products of the STobRV (-)RNA autolysis reaction exhibited apparent ligation reactions (11). When incubated in a buffered solution of magnesium ions and spermidine, P and D formed species P-D, and M formed as the major product a circular molecule, cM. We presumed that such a reverse reaction involves an attack of the 5'-hydroxyl group upon a 2':3'-cyclic phosphodiester bond to form a 3'-to-5' phosphodiester bond, but the newly formed junction was not characterized. We report here the results of tests that were designed to determine whether these joining reactions are true ligations, by isolating and characterizing portions of the RNA molecule at and near the site of the expected junction.

MATERIALS AND METHODS

Plasmids and plasmid transcription

Construction, linearization and transcription of plasmids and electrophoretic purification of RNA were as described (5,10-12). Initiation of transcription at the bacteriophage SP6 promoter of SmaI-linearized pSP641 causes the GTP-initiated synthesis of the primary transcript, designated P_{199}-M-D_{165}. Subscripts denote the number of nucleotide residues in the P and D portions of the transcript that correspond to STobRV (-)RNA. P_{199}-M-D_{165} is expected to have, in the 5'-to-3' direction, a 34 nucleotide residue leader, a 723 residue circularly permuted, dimeric STobRV (-)RNA sequence and, at the 3'-end, 2 additional nucleotide residues derived from the plasmid multiple cloning site. Complete autolysis of the primary transcript generates, in addition to monomeric STobRV (-)RNA, P_{199} and D_{165}, as indicated in Figure 1B. Plasmid p231J130 has a permuted monomeric STobRV (-)RNA inserted next to a bacteriophage T7 promoter. The 392 residue primary transcript of BamHI-linearized p231J130, P_{231}-D_{130}, is expected to have a GTP-initiated 24 nucleotide residue leader and 7 non-STobRV RNA residues at the 3'-end (Figure 1C).

A solution of pSP641 or p231J130, linearized with the indicated restriction endonuclease, was extracted with a 1:1 mixture of water-saturated phenol and chloroform, and the DNA was precipitated by addition of ethanol. One hr incubations were respectively with the RNA polymerase of bacteriophage SP6 (New England Biolabs; 1 unit/µl final concentration, 40°) or of bacteriophage T7 (U.S. Biochemicals; 2 units/µl, 37°) at a template concentration of approximately 50 µg/ml in 40 mM Tris-HCl, pH 7.5, 20 mM NaCl, 6 mM $MgCl_2$, 2 mM spermidine-HCl, 10 mM dithiothreitol (DTT), 500 M of each rNTP and 1 unit/µl ribonuclease inhibitor RNasin (Promega Biotech). There was no indication of premature termination among the transcripts catalyzed by either polymerase. Depending on the extent of labeling desired, reaction mixtures contained up to 1 µCi/µl [α-^{32}P]GTP. RNAs were recovered by ethanol precipitation after phenol/chloroform extraction. Dissolved nucleic acids were combined with an equal volume of formamide dye mixture (95% formamide, 10 mM sodium EDTA, pH 8.0, 0.2 mg/ml each of bromophenyl blue and xylene cyanol FF) and heated to 80° for 30 sec before separation by preparative electrophoresis through 0.5 mm thick, 40 cm long 6.5% polyacrylamide gels in TBE buffer (90 mM Tris, 90 mM boric acid, 2.5 mM EDTA) and 7 M urea. RNAs were recovered by soaking of excised gel zones that had been located by autoradiography or staining (11-13).

Non-enzymic ligation reactions

Electrophoretically purified monomeric [^{32}P]STobRV (-)RNA was incubated at an RNA concentration of about 2 mg/ml in 40 mM Tris-HCl, pH 7.5, 20 mM NaCl, 6 mM $MgCl_2$, 2 mM spermidine-HCl, at room temperature for 35 minutes to produce a mixture of about 50 to 60% linear monomer and 40 to 50% cyclic monomer (cM, Figure 1B; 11). The mixture was used directly for analyses or was resolved by electrophoresis. RNA cM was eluted from the gel as described in the previous section. A mixture of [^{32}P]P_{199} and unlabeled D_{130} was incubated under the same conditions at a total concentration of 2 to 2.5 mg/ml to give a partial conversion to [^{32}P]P_{199}-D_{130} according to the reaction shown in Figure 1C.

cDNA synthesis from templates of ligated RNA

Hybridization of an oligodeoxyribonucleotide to a mixture of P_{199}-D_{165} and its P and D starting materials, and to a mixture of cM and M, was as described in the legend to Figure 4 of Buzayan et al. (11). Synthetic oligodeoxyribonucleotide d349-15(+) is [5'-^{32}P]dGATACCCTGTCACCGGATGTGCTTTC. It corresponds in sequence to residues 349 through 359 and then 1 through 15 of STobRV (+)RNA (i.e., to the (+) strand junction region). Transcription and isolation of the cDNA products primed by d349-15(+) were as described (11), and nucleotide sequences were determined by partial chemical cleavage (5,14).

Protection and recovery of the junction region

Oligodeoxyribonucleotide d35-68(+) (Figure 1A) was obtained from the University of California at Davis Protein Structure Laboratory. It is complementary to a less common form of the STobRV (+)RNA sequence that has a uridylate rather than a guanylate residue at position 54 (5). The less common sequence is in RNA M (Figure 1B), but d35-68(+) is mismatched to P_{199}-D_{130} at position 54 (Figure 1AC).

To a 25 µl solution of reactants and ligation products [^{32}P]P-D or [^{32}P]cM we added sodium acetate to 0.1 M and three volumes of ethanol. The collected and dried precipitate was dissolved in 60 µl of 100 mM Tris, 84 mM HCl, 330 mM sodium acetate, pH approximately 7.5, containing 10 µg of electrophoretically purified d35-68(+). The solution was heated to 90° for 2 min and then transferred to a 60° water bath. The bath was cooled to 35° over a period of 2 hr. The DNA-RNA hybrids were digested at room temperature with 11 units/µl final concentration of ribonuclease T1 (six-year-old Sigma R-8251) in this same solution. Digestion reactions were stopped by adding an equal volume of formamide dye mixture and heating to 80° for 30 sec. Electrophoresis was through 0.5 x 160 x 400 mm long 12% polyacrylamide gel in

TBE buffer and 7 M urea at a constant power of 35 watts. Protected oligoribonucleotides were recovered by soaking gel pieces (12).

Partial nucleotide sequence analysis of RNA fragments

An electrophoretically purified oligoribonucleotide, derived from 10 to 30 min digestions of protected RNA with ribonuclease T1 and of apparent size 39 nucleotide residues, was incubated with 50 μCi of 3000 Ci/mmol [γ-32P]ATP and 12 units of bacteriophage T4 polynucleotide kinase (Pharmacia) in 20 μl of 20 mM Tris-HCl, pH 9.0, 10 mM $MgCl_2$, 5% glycerol, 10 mM DTT at 37° for 30 min. After purification by electrophoresis through 20% polyacrylamide gel in 7 M urea, the oligoribonucleotide was partially digested by incubation in separate reactions with ribonuclease T1, with base (15) and with ribonuclease U2 (16). Fragments were resolved by electrophoresis through 20% polyacrylamide gel in 7 M urea.

Analysis of ApG

Unfractionated reaction mixtures, electrophoretically purified P-D or cM or protected oligoribonucleotide was digested with ribonucleases and phosphatase to recover ApG. In a typical digestion about 50 ng of protected oligoribonucleotide was incubated for 2 hr at 37° in 30 μl of 0.05 M collidine, 0.025 M acetic acid, 0.5 mM EDTA, (pH approximately 7.4) with 40 units of ribonuclease T1 and 9 units of pancreatic ribonuclease A. The solution was brought to pH 8.2 by the addition of 10μl of 0.2 M NH_4OH and was incubated with 2 units of calf intestinal alkaline phosphatase for 45 min at 37°. The sample was vacuum dried for 2 hours without heating, dissolved in 5 μl of water and combined with 2 ug each of (2'→5')ApG and (3'→5')ApG (Sigma Chemical Co.). The sample was spotted on a 9cm long cellulose thin layer (CTL) plate (Eastman #13255). The plate was rinsed with methanol for 30 sec, dried and chromatographed in acetic acid: water:isopropanol, 1:100:100. In this solvent system (2'→5')ApG and (3'→5')ApG co-chromatograph but inorganic phosphate moves with the solvent front. Alternatively, the material was chromatographed (one or two dimensions) on CTL in 500:1, methanol:acetic acid (to remove inorganic phosphate) or in 70:28:2, tert-butanol:water:acetic acid to remove most of the adenosine phosphates.

The spot containing ApG, located by ultraviolet absorbance and radioactivity, was cut out and eluted in 0.6 ml water over a period of 30 min with shaking. The vaccuum-dried sample was dissolved in 5 μl water and 1 ug each of (2'→5')ApG and (3'→5')ApG standards were added. The two isomers separated during chromatography on a 9 cm polyethyleneimine (PEI) CTL in 0.5 M KH_2PO_4.

(2'→5')ApG and (3'→5')ApG also were distinguished by the susceptibility of the latter to ribonucleases T2 and U2. ApG recovered from CTL chromatography was digested for 1 hr at 37° with 1 unit of ribonuclease T2 in 5 µl of 0.1M ammonium acetate, 1mM EDTA, adjusted to pH 4.5 with acetic acid. The ribonuclease T2 was removed by phenol extraction, and the supernatant was dried under vacuum. The sample was dissolved in 3 µl water and chromatographed on PEI CTL as above. To demonstrate the specificity of ribonuclease U2, 2 µg of each ApG isomer were separately incubated with 4.4 units of ribonuclease U2 at 55° for 40 min in 7 M urea, 20 mM sodium citrate, 1 mM EDTA, pH 3.5. The digest was chromatographed on a CTL in t-butanol:water:concentrated HCl, 70:15:15.

RESULTS

Intact, monomeric STobRV (-)RNA and fragments of it were obtained from autolytic processing reactions (11) of the transcripts of two plasmids, plasmid pSP641 (Figure 1B) and plasmid p231J130 (Figure 1C).

Terminal groups and reaction conditions for ligation

The ligation of electrophoretically purified P and D occurred readily in buffered solution of spermidine and magnesium ions but not in buffered EDTA solution (Figure 2, lanes 1 and 2). When concentrations of P and D were increased to 3.3X the concentration used for lane 2, the radioactivity of the P-D zone increased from 18% to 27% of the total in all three zones, showing that the bimolecular reaction is concentration dependent. In another experiment, spermidine and magnesium ions were tested separately in the same buffer for their effects on the yield of P-D from electrophoretically purified P and D. After 1 hr at 25°, P-D was 5% of the total RNA in the reaction mixture that contained 6 mM $MgCl_2$. P-D was 12% of the RNA after incubation in buffered 2 mM spermidine-HCl solution and 14% of the RNA in the three zones when both cations were present. In a solution of both cations the yield was reduced to 2.4% when the solution was incubated at 0°, for 1 hr.

Neither the phosphatase treatment nor the subsequent 5'-phosphorylation of P prevented the reaction of P with D (Figure 2, lanes 3,4). Phosphatase treatment of D did not interfere with its reaction with P (Figure 2, lane 3). However, when D was 5'-phosphorylated it failed to react with P to form P-D (Figure 2, lane 5). These are the expected results if reactive P is terminated with a phosphatase-resistant, 2':3'-cyclic phosphodiester group that is attacked by the terminal 5'-hydroxyl group of D in the non-enzymic ligation reaction.

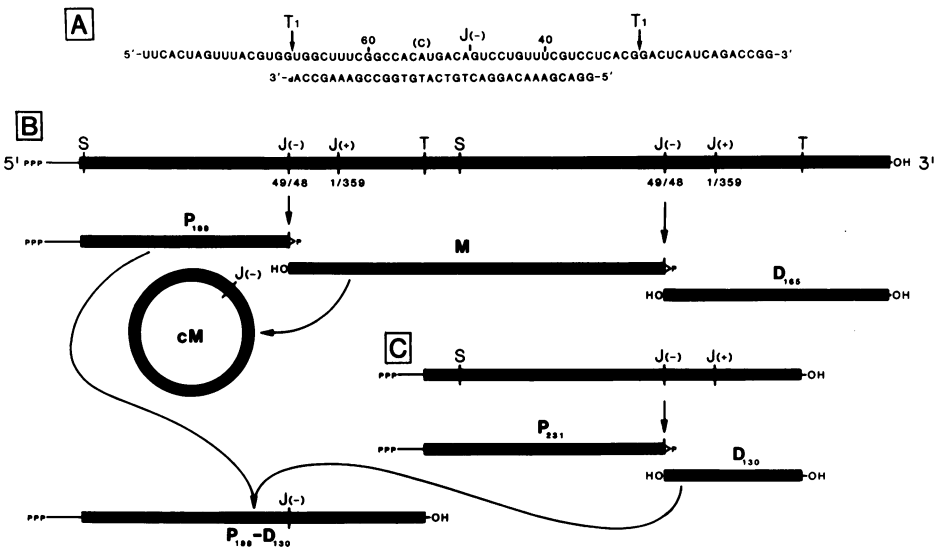

Figure 1. Autolysis and ligation reactions of STobRV (-)RNA. Sequences are numbered according to the STobRV (+)RNA sequence. A. The top line displays the autolytic junction, J(-), region of STobRV (-)RNA. Two variants of the sequence (5) at position 54 are indicated. The second line shows the nucleotide sequence of oligodeoxyribonucleotide d35-68(+). Arrows above the RNA sequence demarcate the 39-residue region of STobRV (-)RNA that is expected to be protected from digestion by ribonuclease T1 by d35-68(+). B. A diagram of the primary transcript of SmaI-linearized plasmid pSP641 (11), that has a circularly permuted dimeric STobRV (-)RNA sequence. J(+) is the corresponding junction of STobRV (+)RNA, between residues 1 and 359. Sites in the template for restriction endonucleases Sau3AI (S, between residues 244 and 243) and TaqI (T, between residues 278 and 277) are indicated. Straight arrows show autolytic cleavage reactions that generate monomeric STobRV(-)RNA and the two bordering RNA fragments, D_{165} and P_{199}. The RNA terminal triphosphate (ppp), cyclic phosphodiester (>p) and hydroxyl groups are marked. C. The primary transcript of BamHI-linearized plasmid p231J130, that has a circularly permuted monomeric STobRV RNA insert. The single J(-) site generates two RNA fragments, P_{231} and D_{130}. Curved arrows define the two non-enzymic ligation reactions that were used to produce RNAs cM and P_{199}-D_{130}.

Retention of nucleotide sequences during ligation

P-D survived heating in denaturing aqueous solvents (11). These results imply covalent bond formation and that P-D and cM should be able to serve as templates for reverse transcriptase. We compared the nucleotide sequence of the junction region of two forms of P_{231}-D_{130}: the uncleaved primary transcript and the product of the non-enzymic ligation of P_{231} and D_{130}. Each was hybridized to primer d349-15(+), which was extended by the action of reverse transcriptase. Nucleotide sequence analyses of the transcripts showed

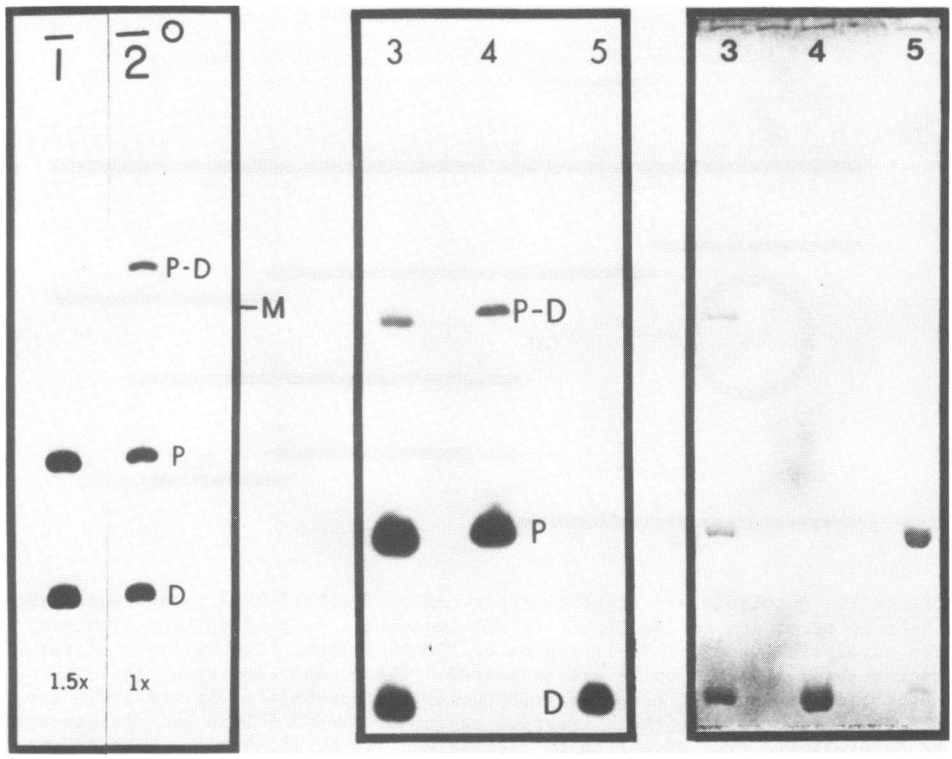

Figure 2. Influence of terminal groups on the spontaneous ligation of autolytically derived RNA fragments P_{199} and D_{165}. RNA fragments were incubated for 1 hr at room temperature and pH 7.5 in 10 µl volumes of 40 mM Tris-HCl, 20 mM NaCl, 5 mM Na_2EDTA (lane 1) or in 40 mM Tris-HCl, 20 mM NaCl, 6 mM $MgCl_2$, 2 mM spermidine hydrochloride (lane 2). The RzA concentration for the lane 1 sample was 1.5X that for lane 2, but equal amounts of RNA were applied to the gel. One other lane separated lanes 1 and 2zin the original gel. The origin of the electrophoresis gel is marked O. Lanes 3-5: autoradiography (central panel) and toluidine blue O staininz (right hand panel) revealed the effects of 5'-phosphorylation on the sponzaneous ligation of P and D RNA fragments. The RNAs were incubated as for lane 2. Lane 3: 0.4 µg each of [5'-^{32}P]P and [5'-^{32}P]D and 5 µg each of P and D. Lane 4: 0.4 µg [5'-^{32}P]P and 10 µg D. Lane 5: 0.4 µg [5'-^{32}P]D and 10 µg P.

that the uncleaved RNA and the ligation product have the same sequence. The same primer was extended on a cM template; nucleotide sequence analysis of the transcript demonstrated that the circularization of M to cM also had the sequence expected from a simple ligation reaction.

P_{199} labeled by incorporation of [α-^{32}P]GTP and unlabeled D_{130} were mixed under ligation conditions. The reaction products were hybridized to the 34 nucleotide residue deoxyoligoribonucleotide d35-68(+) (Figure 1A). Hybrid

also was prepared from a mixture of cM and M, labeled with [α-32P]GTP. We digested the DNA/RNA hybrid with ribonuclease T1 under high salt conditions. The expectation from the nucleotide sequence of STobRV (-)RNA is that the largest oligoribonucleotide to survive digestion of protected P-D or cM under these conditions (17) should be a 39-residue oligoribonucleotide that includes the junction ApG (Figure 1A). The catalogue of ribonuclease T1-resistant oligoribonucleotides deduced from the nucleotide sequence of (unprotected) P-D or cM has one each of a 21-mer, a 17-mer and a 13-mer. An oligoribonucleotide with the expected mobility of a 39-mer survived the separate digestions of P-D and cM, each hybridized to d35-68(+). It was the largest such resistant oligoribonucleotide and was not detected on the autoradiograms of similar digests of unprotected RNAs.

The presumed 39-mer was labeled by the action of polynucleotide kinase. Partial digestions of the labeled 39-mer derived from P_{199}-D_{130} with ribonucleases T1 and U2, and subsequent analysis of the products by gel electrophoresis, revealed the nucleotide sequence 5'-GGYYAYYYGAYAGYYYYGYYYYG-3' (junction dinucleoside phosphate underlined). The same sequence, with one nucleotide substitution, was obtained for the 39-mer from cM: 5'-GGYYAYAYGAYAGYYYYGYYYYG-3'. These sequences are consistent with the nucleotide sequences of the cDNA clones (5) from which the RNAs were transcribed and can only be derived from one location in STobRV (-)RNA: the region of the J(-) junction. The other ApG bonds that are closest to the junction are residues 21-20 and 79-78, which should not be protected by d35-68(+).

Non-enzymic ligation results in a 3'→5' phosphodiester bond

The results described in the preceding paragraph show that the ApG bond of the ligated junction, as it is recovered in the 39-mer oligoribonucleotide, is susceptible to cleavage by ribonuclease U2 and hence is 3'-to-5' (18). Our sample of ribonuclease U2 failed to hydrolyze the (2'→5')ApG standard. However, the recovery of the 39-mer was not quantitative. Thus the possibility remained that some of the ApG actually is 2'-to-5'. A 2'-to-5' bond at the junction of some of the cM or P-D molecules might have escaped detection if it sufficiently distorted the DNA-RNA hybrid, after protection with d35-68(+), to allow ribonuclease T1 digestion even under high salt conditions. Also, the junction phosphodiester bond is known to be especially reactive, so that a 2'-to-3' phosphate shift is conceivable.

A mixture of non-protected M and cM, labeled with [α-32P]GTP, was digested the guanylate-specific ribonuclease T1, the pyrimidine-specific

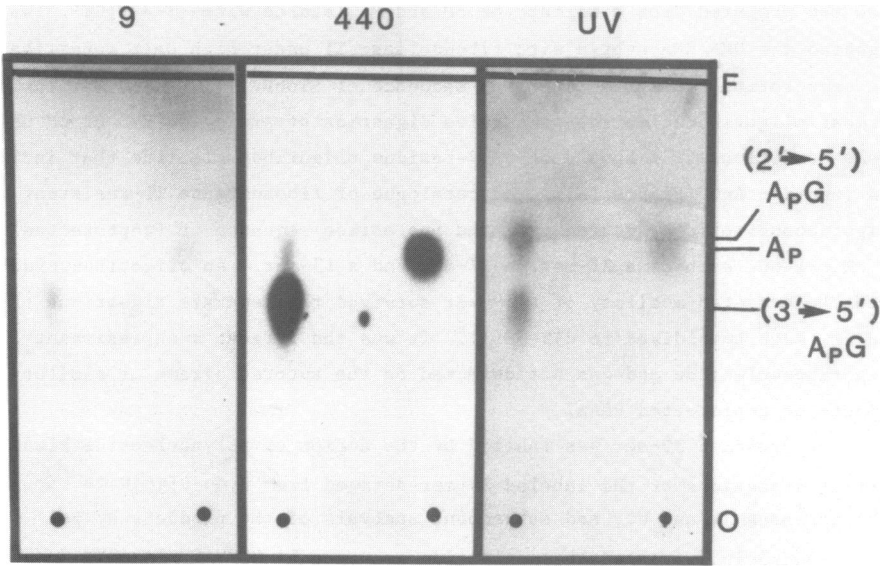

Figure 3. Release of (3'→5')ApG after ribonuclease digestion of cM. A mixture of cM and M derived from a non-enyzmic ligation reaction, was digested with ribonucleases T1 and A and phosphatase. Inorganic phosphate was removed by a preliminary chromatography step. Products were analyzed by PEI CTL chromatography both before (left lane of each panel) and after (right lane of each panel) digestion with ribonuclease T2. Autoradiograms of the thin layer were exposed for 9 min (left hand panel) and 440 min (central panel). Standards, with mobilities indicated on the right, were detected by quenching of fluorescence (right hand panel).

pancreatic ribonuclease A, and by calf intestinal alkaline phosphatase. The digest should yield the dinucleoside phosphate ApG not only from the presumed junction in the ligated RNA but also from each of the remaining ApG sequences in STobRV (-)RNA. The products were analzyed for the 3'-to-5' and 2'-to-5' forms of ApG (Figure 3). The greater mobility of (2'→5')ApG on the PEI CTL is anticipated because moving the N-1 of the adenine ring closer to the negatively charged phosphate allows it to be protonated more readily. The ApG from cM was digested readily by ribonuclease T2, whereas ribonuclease T2 did not digest (2'→5')ApG, as expected (18).

The junction ApG should represent about 5% of the ApGs from cM, which in turn is about half of the cM and M mixture. Comparisons of autoradiograms for 50-fold differences in exposure time (Figure 3) showed that less than 2% of the ApG could have been detected had that proportion of the ApG migrated with (2'→5')ApG or resisted digestion catalyzed by ribonuclease T2. A small

amount of material was detected that migrated slightly more rapidly than (3'→5')ApG but less rapidly than (2'→5')ApG on the PEI CTL. This material co-migrated with adenosine-2':3'-cyclic phosphate and Ap, the former being an expected product of the digestion of linear M by ribonucleases A and T1 (5,11). The results are consistent with all or nearly all of the ApG in cM being 3'-to-5'-linked.

Similar results were obtained with P_{199}-D_{130} (non-enzymically ligated from [^{32}P]P_{199} and unlabeled D_{130}, reducing the expected number of labeled ApGs to 13). The dinucleoside phosphate ApG also was isolated from the protected 39-mer of cM and of P_{199}-D_{130}. Complete digestion by ribonucleases T1 and A and subsequently with calf intestinal alkaline phosphatase gave a product that comigrated with (3'→5')ApG. No detectable product comigrated with (2'→5')ApG or resisted digestion by ribonuclease T2.

DISCUSSION

Characteristics of the ligation reaction

The sizes of the STobRV (-)RNAs P and D, which are products of the autolysis reaction and are able to ligate, show that neither the cleavage nor the joining reactions require a full monomeric length of STobRV RNA sequence on both sides of the J(-) junction, as shown by the efficient joining of P_{199} to D_{130}. The acceptance of spontaneously ligated RNA as template by reverse transcriptase shows that no branch (19) or 2'-phosphorylation (20) is present at the junction after ligation. Nucleotide sequence analyses of cDNA transcripts and of the oligodeoxyribonucleotide-protected, junction-containing RNA fragment show that there is no gain or loss of a nucleotide or nucleoside residue during ligation, since the nucleotide sequences of the reactants anticipate the nucletide sequence of the product. The reversibility of the autolysis and ligation reactions (11) also is consistent with this conclusion.

The dinucleoside phosphate ApG, whether isolated from protected junction fragment or in a mixture representing ApG sequences from various parts of STobRV (-)RNA, had, according to its chromatographic mobility and its susceptibility to ribonucleases, a 3'-to-5' phosphodiester bond. Hence a 3'-to -5' phosphodiester bond forms between adenylate and guanylate residues during the non-enzymic ligation reaction of STobRV (-)RNA. The bond forms during the attack of the 5'-hydroxyl group of a terminal guanylate residue of monomeric RNA or RNA fragment on the 2':3'-cyclic phosphodiester of a terminal adenylate residue of monomeric RNA or fragment, leading to the circular or linear ligation product. Thus the net ligation reaction must be the reverse of the autolysis of the original 3'-to-5', ApG phosphodiester bond formed

during polymerization of the polyribonucleotide chain. Another chemical reaction, the direct polymerization of adenosine-2':3'-cyclic phosphate, also favors formation of a 3'-to-5' phosphodiester bond (21).

Phosphodiester bond formation during ligation of P and D apparently is not directly catalyzed either by magnesium ions or by spermidine, since either can be omitted individually without preventing P-D formation. Probably magnesium ions or, more effectively, protonated spermidine ions, stabilize conformations of the P and D RNAs that favor the ligation reaction. How a particular phosphodiester bond is activated for autolysis, and how the 5'-hydroxyl group of D and M can so readily attack the 2':3'-cyclic phosphodiester group of P and M, are unknown. Presumably, a particular RNA conformation must favor a trigonal bipyramid conformation about the reactive phosphorus atom (22). The great difference in the autolysis and ligation reactions of STobRV (+)RNA (9), which greatly favors autolysis, and of STobRV (-)RNA, which exhibits a significant ligation reaction, shows how strongly the nucleotide sequence can influence RNA reactivity.

Comparison with other RNA ligation reactions

We have documented here the first example of a spontaneous, non-enzymic ligation reaction of a naturally occurring RNA sequence in which a 2':3'-cyclic phosphodiester is attacked by a 5'-hydroxyl group. A similar, but enzyme-catalyzed, reaction is the ligation of tRNA half-molecules by extracts from HeLa cells (23). RNA with a 2':3'-cyclic phosphodiester terminal group also is the substrate for wheat germ RNA ligase (24). However, the other reactant has a 5'-phosphoryl group, and the product has a junction with a 3'→5'-phosphodiester bond and a 2'-phosphoryl group, which also is characteristic of the circular satellite RNAs of velvet tobacco mottle virus and Solanum nodiflorum mottle virus (20). The 2'-phosophoryl at the junction is documented for yeast (25) and plant (26) tRNA splicing, as well. Usher and McHale (27) reported the non-enzymic ligation of oligoadenylate with terminal 5'-hydroxyl and 2':3'-cyclic phosphodiester groups. However, unlike the ligation reaction reported here, the oligoadenylate-coupling reaction was polyuridylate template-dependent and the phosphodiester bonds formed were predominantly 2'-to-5'.

Other enzymic and non-enzymic RNA ligation reactions, including those associated with splicing reactions, have, as reactants or intermediates, RNA molecules with 3'-hydroxyl and 5'-phosphoryl groups (bacteriophage T4 RNA ligase (28); self-splicing Group I introns (29); self-splicing Group II introns (30,31); nuclear mRNA precursor splicing (32,33)).

Possible significance of ligation for STobRV RNA replication

The facility with which STobRV (-)RNA fragments P and D combine, and with which the monomeric RNA circularizes, raises the possibility that spontaneous ligation reactions of STobRV (-)RNA have roles in the replication or origin of STobRV RNA, or both. Since cM seems to consist entirely of 3'-to-5' phosphodiester bonds and lacks 2'-phosphoryl groups, it is a good candidate template for the rolling circle transcription that frequently has been postulated to explain the multimeric forms of satellite and viroid RNAs (6,34-37). Buzayan et al. (11) found that cM served as template for reverse transcriptase in vitro, and transcription proceeded across the ligation junction. In fact, transcription in the presence of actinomycin D generated what are apparently very high molecular weight RNAs that remained near the gel well after electrophoresis on a 6.5% polyacrylamide gel in 7 M urea, showing that the structure of cM did not interfere with the processive (38) movement of reverse transcriptase.

Such a circular template is a logical source for the multimeric STobRV (+)RNA that has been detected both in TobRV virus-like particles and in the double-stranded RNA from infected tissue (6). Certainly, spontaneous ligation to form cM reduces the complexity of any replication model for STobRV RNA that incorporates cM. Circular forms of STobRV (+)RNA were detected in infected tissue (8), and Sogo and Schneider (7) found circular double-stranded forms by electron microscopy. The characteristics of in vivo STobRV (-)RNA, with regard to the termini of linear forms and the presence of circles, are under investigation.

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