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RNA MEDIATED FORMATION OF A PHOSPHOROTHIOATE DIESTER BOND

Jamal M. Buzayan*, Paul A. Feldstein*, George Bruening* and Fritz Eckstein+

* Department of Plant Pathology, College of Agricultural and Environmental Sciences, University of California, Davis, California 95616

+ Max-Planck-Institut für experimentelle Medizin, Abteilung Chemie, Hermann-Rein-Strasse 3, D-3400 Göttingen, West Germany

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Summary: Previous results showed that multimeric, tandemly sequence-repeated forms of satellite tobacco ringspot virus RNA of the encapsidated polarity (STobRV (+)RNA) autolytically process at a specific phosphodiester bond, the junction. Substituting a phosphorothioate diester bond for the STobRV (+)RNA junction drastically slowed autolytic processing. Here we show that for the complementary STobRV (-)RNA, in contrast, replacing sets of phosphodiester bonds with phosphorothioate diester bonds, even at the junction, did not greatly slow autolytic processing or spontaneous ligation, the usual reactions of the unmodified RNA. In the ligation reaction STobRV (-)RNA directed the formation of an ApG phosphorothioate diester bond.

STobRV RNA, like other plant virus satellite RNAs (1-5), replicates only in association with a specific supporting virus, TobRV. STobRV RNA reduces the TobRV titer and symptom severity and thus has potential as a practical antiviral agent (6). The nucleotide sequences of known STobRV RNA isolates (7) comprise 359 or 360 nucleotide residues (nt). Multimeric forms (8) of STobRV RNA have this "monomeric" sequence tandemly repeated, the repeats joined at a specific phosphodiester bond designated as the junction. Autolytic processing, at CpA junctions in the encapsidated, or (+), polarity sequence (9) and at the ApG junctions in the complementary, or (-), sequence (10,11), releases the respective monomeric RNAs.

"P-M-D" designates the transcript of a circularly permuted, dimeric STobRV RNA cDNA sequence from an appropriately linearized plasmid, with the hyphens representing the phosphodiester bond junctions. P-M-D autolytically processes into P, M, and D and its partial autolysis products P-M and M-D. The terminal groups generated during processing of STobRV (-)RNA sequences are a guanosine-5'-hydroxyl group and an adenylate-2':3'-cyclic phosphodiester (10). As reported (10), the reverse of the autolytic processing reaction of

Abbreviations: **STobRV RNA**, satellite tobacco ringspot virus RNA; **TobRV**, tobacco ringspot virus; **P**, promoter proximal region of transcript; **D**, promoter distal region of transcript; **M**, monomeric STobRV RNA; **cM**, circular monomeric STobRV RNA; **[αS]rNTP**, nucleoside-5'-O-(1-thiotriphosphate); **PAGE**, polyacrylamide gel electrophoresis.

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added directly to dried oligoribonucleotide. Analyses of products was by 20% PAGE and autoradiography.

Results

Effects of [αS]rNTPs on transcription and autolytic processing of transcripts: Transcription catalyzed by 3.6 U/μl bacteriophage T7 RNA polymerase gave little variation in the extent of P-M-D synthesis in 1 hr regardless of the set of rNTPs and [αS]rNTPs in the reaction mixtures. Substituting [αS]rGTP for rGTP or [αS]rCTP for rCTP reduced the extent of synthesis catalyzed by 1 U/μl bacteriophage SP6 RNA polymerase to 85% or less of the control value. Replacing rGTP with [αS]rGTP to modify the ApG junction and other NpG bonds did not decrease the autolytic processing of P-M-D as observed at the end of a 1 hr incubation (Fig. 1, lane 2). Substitution of all four

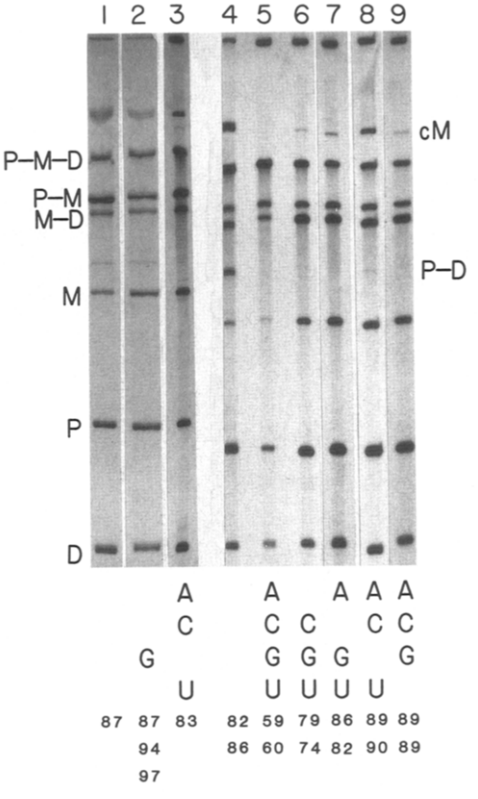

FIGURE 1. Autolytic processing of STobRV (-)RNAs that contain phosphorothioate diester bonds. The transcripts of plasmids pSP641 (lanes 1-3) and plasmid p20SD(-)3 (lanes 4-9) partially autolytically processed and spontaneously ligated during the time of incubation of the transcription reaction mixtures. Products were resolved by 6.5% PAGE and were detected by autoradiography. The zones for the transcript P-M-D and its autolytic processing products are marked to the left of the lanes; zones for the spontaneous ligation products P-D and circularized monomer, cM, are indicated to the right. The letters below each lane designate the rNTPs that were replaced by the respective [αS]rNTPs in the transcription reaction mixtures: no substitution for the samples applied to lanes 1 and 4, [αS]rGTP for rGTP in lane 2, etc. Each of the 17 numbers below the lanes is the result of a separate determination of the percentage of the transcript that was converted to products of autolytic processing and spontaneous ligation. The first line of percentages corresponds to the gel of the autoradiogram shown.

STobRV (-)RNA also proceeds to a significant extent, generating P-D from P and D as well as cM from M (e.g., Fig. 1, lane 1). The autolytic processing and spontaneous ligation reactions are possible steps in proposed schemes for the replication of STobRV RNA (9,10,12,13).

That phosphorothioate diester bonds may only slightly alter the conformation and reactivities of some RNA molecules is indicated by their recognition by enzymes and activity in cells (14-16). Each phosphorothioate diester bond has a non-bridging oxygen atom of the corresponding phosphodiester replaced stereospecifically by a sulfur atom. RNA polymerases (EC2.7.7.6) accept as substrates the Sp diastereomers of [αS]rNTPs. The resulting phosphorothioate diester bonds have the Rp configuration (14). We report properties of STobRV (-)RNA sequences synthesized in reaction mixtures with up to four of the rNTPs replaced by the corresponding [αS]rNTPs.

Materials and Methods

Plasmids and transcripts: Plasmids pSP641 (10) and p20SD(-)3 (17) have circularly permuted STobRV RNA dimeric cDNA inserts oriented for transcription of STobRV (-)RNA from bacteriophage SP6 and T7 promoters, respectively. Bacteriophage SP6 RNA polymerase is expected to transcribe from restriction endonuclease (EC3.1.21.4) SmaI-linearized pSP641 a 759 nt P-M-D with 199 and 165 nt of STobRV RNA sequences in the P and D portions. An additional vector-derived 34 and 2 nt are at the 5' and 3' ends, respectively. The bacteriophage T7 RNA polymerase-catalyzed, 807 nt transcript of HindIII-linearized p20SD(-)3 is similar, but with 48 and 36, respectively, 5' and 3' vector derived nt.

Reactions of 1 hr duration were at 40° with SP6 RNA polymerase (1 U/μl) and 0.09 μCi/μl [α^{32}P]rCTP or at 37° with T7 RNA polymerase (3.6 U/μl) and 0.25 μCi/μl [α^{32}P]rGTP in 1X transcription buffer (40 mM Tris-HCl, pH 7.5, 20 mM NaCl, 6 mM $MgCl_2$, 2 mM spermidine-HCl) containing 10 mM dithiothreitol and 0.5 mM of each rNTP. The mixed diastereomers of [αS]rUTP and [αS]rCTP, at 0.5 mM and 1.0 mM, respectively, were substituted for the corresponding rNTPs. The substitutions for rATP and rGTP were by the corresponding Sp-[αS]rNTPs at 0.5 mM. The buffer for PAGE in 7 M urea was 90 mM Tris, 90 mM boric acid, 2.5 mM disodium EDTA. Gel zones for each P-M-D and its corresponding autolytic processing and spontaneous ligation products were soaked for 24 hr, and the suspension was subjected to Cerenkov counting as described (20). The percentage of processing was calculated from T, the combined radioactivity of all zones, and S, the radioactivity of the P-M-D zone, from the formula 100 x (T-S)/T.

Spontaneously-ligated RNA and its junction-containing fragment: RNA cM, spontaneously formed from M (10), was hybridized to the oligodeoxyribonucleotide d35-68(+), which has the nucleotide sequence dGGACGAAACAGGACTGTCATGTGGCCGAAAGCCA. d35-68(+) is complementary to the sequence variant of cM used here except at position 54 (11), as indicated by the break in the vertical bar in Fig. 3. The molecular hybrid of d35-68(+) and cM was partially digested (21) by incubation with ribonuclease T_1 (EC3.1.27.3). The DNA-RNA hybrids were melted, and the oligoribonucleotide products were separated by 20% PAGE.

Oligoribonucleotides, located by autoradiography, were eluted from gel slices, 5'-^{32}P-labeled in a bacteriophage T4 polynucleotide kinase-catalyzed (EC.2.7.1.78) reaction, and electrophoretically purified before being partially degraded by ribonucleases and base (22,23). Oligoribonucleotides also were cleaved partially at phosphorothioate diester bonds by incubation with iodoethanol at 95° for 3 min under conditions similar to those described by Gish and Eckstein (24) except that iodoethanol was dissolved in the 95% formamide solution of dyes to a concentration of 6% rather than 7%. This solution was

rNTPs did signficantly reduce processing (lane 5), but even the most inhibitory tri-substitution, for rCTP, rGTP and rUTP (lane 6), still allowed more than 70% of the autolytic processing observed for the control with no substitutions of rNTPs by [αS]rNTPs.

Effects of phosphorothioate diester bonds on spontaneous ligation: The ligation of M to cM is more robust than the joining of P and D, occurring even in buffered solution of the metal ion chelator EDTA (10). The conversion of M to cM during the 1 hr transcription in a solution containing either all rNTPs or rATP, rCTP, rUTP and [αS]rGTP gave, respectively, cM values, as a percentage of total monomer, of 64 +/- 5% and 15 +/- 12% (average and standard deviation from three experiments). M and cM from the [αS]rGTP-derived transcript were electrophoretically purified and incubated in 1X transcription buffer for 150 min at room temperature, giving percentages of cM of 45% and 60%, respectively. Apparently phosphorothioate substitution had less effect on the M to cM equilibrium than on the rate of cM formation. Synthesis from all four [αS]rNTPs greatly reduced but did not prevent cM formation (Fig. 2, lanes 1 and 2). Of the four tri-substitutions of [αS]rNTPs tested, substitution of

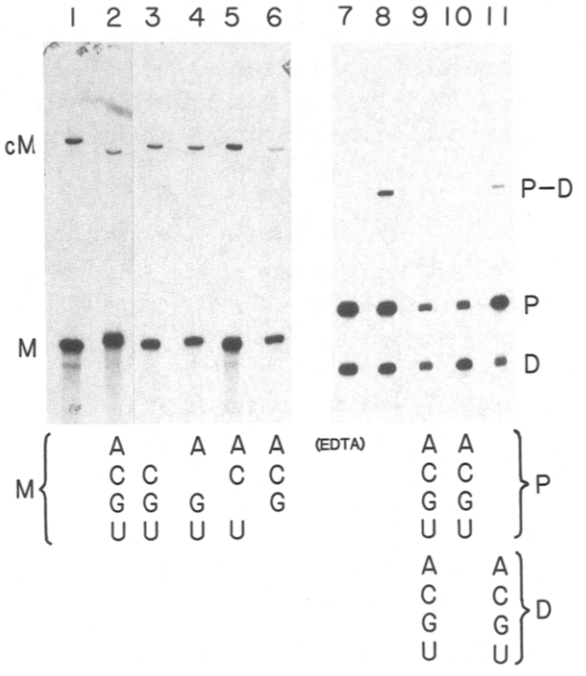

FIGURE 2. Spontaneous ligation of STobRV (-)RNAs that contain phosphorothioate diester bonds. RNAs P, M and D were purified by 6.5% PAGE, from reaction mixtures in which HindIII-linearized p20SD(-)3 was transcribed by bacteriophage T7 RNA polymerase. From 0.5 to 1 μg of monomeric RNA M was incubated in 7 μl of 1X transcription buffer for 150 min at room temperature (lanes 1-6). From 0.4 to 1.2 μg each of P and D were incubated in 10 μl of 1X transcription buffer (lanes 8-11) or 1X transcription buffer with 10 mM EDTA in place of $MgCl_2$ and spermidine (lane 7) for 90 min at room temperature. Reactions were stopped by an addition of 1 μl of 200 mM EDTA and 5 μl of tracking dye solution in formamide. Detection of reactants and products was by 6.5% PAGE and autoradiography. The letters below each lane designate the rNTPs that were replaced by the respective [αS]rNTPs in the source transcription reaction mixtures.

all but rGTP allowed the greatest accumulation of cM (Fig. 2, lanes 3-6). Fig. 2, lanes 7-11 shows the effects of synthesis from four [αS]rNTPs on the ligation of P to D.

Formation of a phosphorothioate diester bond during RNA-directed spontaneous ligation. Monomeric STobRV (-)RNA, derived from transcript synthesized from [αS]rGTP, was allowed to circularize. An RNA fragment recovered from the junction region of cM was too small to be capable of autolytic processing (21). Two such oligoribonucleotide preparations were purified, end-labeled, and partially degraded to determine their structure (Fig. 3, panels A and B). Within the range of digestion products resolved in Fig. 3., ribonucleases T_1 and U_2 (EC3.1.27.4) cleaved to the 3' side of the guanylate and purine (adenylate > guanylate) residues, except at those positions that have phosphorothioate diester bonds derived from [αS]rGTP, corresponding to A49 and G60. The phosphodiester bond to the 3' side of G59, which is next to two phosphorothioate diester bonds, cleaved inefficiently (lanes 6 and 2). The phosphodiester forms of these three bonds are known to be cleaved efficiently by ribonucleases U_2, T_1 and T_1, respectively (21; Fig. 4 of 13).

Iodoethanol is expected to cleave bonds to the 5'-side of guanylate residues in RNA synthesized from [αS]rGTP, rATP, rCTP and rUTP (24). The family of oligoribonucleotides generated by iodoethanol thus should migrate more rapidly than the family generated by ribonuclease T_1. The locations of guanylate residues in the sequence and a relationship between the mobilities of ribonuclease T_1- and iodoethanol-generated zones are indicated in lane 3, Fig. 3.

Discussion

Several small RNAs that replicate in plants, including plant virus-associated satellite RNAs (25) and one viroid (26), exhibit autolytic processing to generate cleavage products which have as their new ends a 5'-hydroxyl group and a 2':3'-cyclic phosphodiester bond. Of these RNAs, including STobRV (+)RNA, only STobRV (-)RNA has exhibited an extensive back reaction, i.e., spontaneous, not enyzme-catalyzed ligation. We found that even STobRV (-)RNA synthesized from all four [αS]rNTPs processed efficiently (Fig. 1, lane 5). In contrast, autolytic processing of circularly-permuted, dimeric STobRV (+)RNA was not detected when the transcript was synthesized in the presence of [αS]rATP as the only [αS]rNTP, which introduces phosphorothioate diester bonds at the CpA junction and other NpA bonds (20).

Ribonucleases A (EC3.1.27.5), T_1 and T_2 (EC3.1.27.1) cleave Rp phosphorothioate diester bonds more slowly than the corresponding phosphodiester bonds (14,27). The two curved arrows in Fig. 3 define the ends of the oligoribonucleotide as expected from the combined effects of resistant phosphorothioate diester bonds and the hybridized d35-68(+). The phosphorothioate diester junction, at nt 49-48, resisted cleavage by ribonuclease U_2 under conditions of partial digestion and was specifically cleaved by iodoethanol (Fig. 3). We conclude that the STobRV (-)RNA sequence directed the spontaneous formation of an ApG phosphorothioate diester bond, which presumably is 3'-to-5' (21). Since the spontaneous ligation reaction is expected to be the reverse of autolysis, ligation should stereospecifically

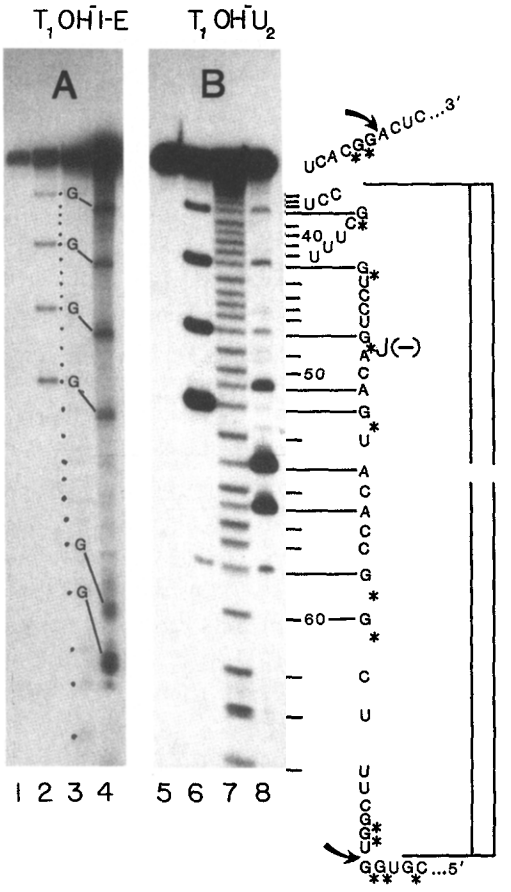

FIGURE 3. A phosphorothioate diester bond in the junction of spontaneously formed STobRV (-)RNA circles. STobRV (-)RNA was transcribed from p20SD(-)3 in the presence of [αS]rGTP, in place of rGTP, and 80 μCi [α^{32}P]rCTP per 200 μl reaction mixture. Electrophoretically purified RNA M circularized spontaneously when incubated as described in the Fig. 2 legend. A ribonuclease T_1-resistant oligoribonucleotide, protected by hybridization of cM to oligodeoxyribonucleotide d35-68(+), was electrophoretically purified, 5'-terminally labeled, and subjected to sequence analysis (21). Partial degradations were with the guanylate specific ribonuclease T_1 (lanes 2 and 6), the phosphorothioate diester bond-specific reagent iodoethanol (lane 4), base (lanes 3 and 7), and the purine-specific (adenylate > guanylate) ribonuclease U_2 (lane 8). The controls (lanes 1 and 5) were incubated as for the ribonuclease T_1 reaction but with no enzyme. Dots in lane 3 locate zones generated by partial base hydrolysis, which were insufficiently exposed to photographically reproduce. The bar on the right corresponds to the region of the RNA sequence that is complementary to oligodeoxyribonucleotide d35-68(+) and J(-) locates the ApG junction phosphodiester bond. Asterisks indicate phosphorothioate diester bonds expected to be derived from [αS]rGTP. The long horizontal lines to the right of the autoradiograms designate the nt identified by combining information from the partial degradations of the oligoribonucleotide by ribonucleases and by iodoethanol. A secondary indication of the locations of guanylate residues in the sequence is the exceptionally wide spacing between the zones for oligoribonucleotides that differ by removal of a 3'-terminal guanylate residue (compare Fig. 1 of ref. 30).

generate the Rp configuration of a phosphorothioate diester bond as the newly formed junction (14,28).

Results in lanes 10 and 11 of Fig. 2 show that, for RNA synthesized from all four [αS]rNTPs, ligation was reduced less by phosphorothioate diester

bonds to the 3' side of the junction than to those of RNA P, which are to the 5' side of, and actually form, the junction. RNA M synthesized from [αS]rATP, [αS]rCTP, rGTP and [αS]rUTP apparently circularized as readily as M with all phosphodiester bonds (Fig. 2, lanes 5 and 1) and more readily than any of the RNA M molecules sythesized from [αS]rGTP and two other [αS]rNTPs (Fig. 2, lanes 3-6). These results imply that the [αS]rGTP substitution was the most influential in retarding cM formation. RNA M molecules with phosphorothioate diester bonds derived from [αS]rGTP and any other two or the other three [αS]rNTPs had a reduced electrophoretic mobility (Fig. 1, lanes 4-9; Fig. 2, lanes 1-6). Thus, although incorporation from [αS]rGTP alone did not cause a detected decrease in the mobility of M (Fig. 1, lanes 1 and 2), phosphorothioate diester bonds derived from [αS]rGTP may influence the conformation of RNA M as well as the chemical structure of the junction.

The phosphorothioate P-S bond is expected to be longer than the corresponding non-bridging phosphodiester P-O bond, and the bond angles of the two 2':3'-cyclic ester compounds are different (29). Our results show that the substitution of phosphorothioate diester bonds for phosphodiester bonds resulted in no detected slowing of base-catalyzed chain cleavage (Fig. 3), little alteration of the equilibrium between the unligated and ligated forms of the monomeric STobRV (-)RNA, and a slight slowing of the rate-limiting step in the ligation (and presumably the autolysis) reactions.

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