

# Two autolytic processing reactions of a satellite RNA proceed with inversion of configuration

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## ABSTRACT

Both polarities of the satellite RNA of tobacco ringspot virus occur in infected cells in multimeric forms which are capable of autolytic processing, using different sequences and structures [Feldstein, P.A., et al., Proc. Nat. Acad. Sci. USA (1990) 87 (in press)]. These transesterification reactions generate a 2',3'-cyclophosphate and a 5'-hydroxyl as the two new end groups. Cleavage is at a CpA for the (+) polarity RNA and at an ApG for the (-) polarity RNA. We enzymically synthesized oligoribonucleotides with processing capability and with specific <sup>35</sup>S-labeled phosphorothioate diesters in the *R<sub>p</sub>* configuration. After processing had occurred, the terminal nucleoside-2',3'-cyclophosphorothioate diester residues were recovered from the appropriate product by digestion with nuclease and phosphatase. Comparisons with specially prepared endo- and exo-isomer reference compounds by thin layer chromatography and autoradiography revealed that the [<sup>35</sup>S]cytidine- and [<sup>35</sup>S]adenosine-2',3'-cyclophosphorothioate both were endo-isomers. The results are consistent with transesterification occurring by an in-line *S<sub>N</sub>2(P)* attack of the 2'-hydroxyl group in the autolytic processing reactions of both polarities of the satellite RNA.

## INTRODUCTION

The satellite RNA of tobacco ringspot virus (sTobRV RNA) replicates in association with tobacco ringspot virus and becomes encapsidated in the virus coat protein. Extracts of infected tissue contain repetitive sequence multimers of the 359 nucleotide residue (nt) 'monomeric' sTobRV RNA (1) as well as circular forms (2,3). The existence of circular and multimeric RNA molecules suggests that the replication of sTobRV RNA occurs by rolling circle transcription, with circular RNA as template and multimeric RNA as transcript (4,5). Endonucleolytic processing of at least one of the multimeric forms would be essential to such a scheme. Multimeric RNA both of the most abundant polarity, arbitrarily designated as (+), and its

complement, sTobRV (-)RNA, exhibit autolytic processing *in vitro* (6,7). Each RNA cleaves at a specific phosphodiester to create a 5'-hydroxyl group and a 2',3'-cyclophosphate at the new polyribonucleotide chain ends. Thus these examples of autolytic processing are transesterification reactions.

The self-cleaving sTobRV (+)RNA sequence corresponds to a short, contiguous segment of polyribonucleotide chain which encompasses the cleaved CpA phosphodiester that is the junction (J(+), Fig. 1A) between the monomeric units of the multimeric RNA. The pattern of base-paired segments and connecting sequences displayed in Fig. 1A is termed a 'hammerhead' structure (8). The hammerhead structure is based on comparisons of the self-cleaving sequences of several replicating RNAs (9) and is consistent with several studies of mutant sequences (10-17).

Sequences required for the self-cleavage (7) of sTobRV (-)RNA were mapped to two regions of the polyribonucleotide chain by insertional mutagenesis (15). These two regions were prepared as two oligoribonucleotides: an endoribonucleolytic oligoribonucleotide E (Fig. 1B) and an oligoribonucleotide S whose cleavage is facilitated by E (18). Cleavage is at the ApG phosphodiester of S that is cleaved in multimeric sTobRV (-)RNA. The sequences in these two oligoribonucleotides do not conform to the hammerhead structure. The base pairing between E and S shown in Fig. 1B is based on observed compensating effects of mutations in E and S and other results (19). The stem in E is suggested by a phylogenetic comparison (15,20). Although critical aspects of the secondary structures of actively cleaving sequences from the two sTobRV RNAs have been identified, the mechanisms of the two reactions remain obscure.

Bacteriophage T7 RNA polymerase (21), like other RNA polymerases (22), accepts the *S<sub>p</sub>* diastereomer of a nucleoside-5'-O-(1-thiotriphosphate), abbreviated rNTP $\alpha$ S, as a substrate and synthesizes phosphorothioate diesters of the *R<sub>p</sub>* configuration. The *R<sub>p</sub>* configuration of a phosphorothioate diester is shown in Fig. 2A. Both polarities of sTobRV RNA exhibit autolytic processing even when the junction phosphodiester (Fig. 1A,B) is replaced by a phosphorothioate diester (23,24). Here we take advantage of the chiral center at

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the phosphorus atom in the phosphorothioate diester and in the 2',3'-cyclophosphorothioate to determine the stereochemical relationship between reactant and product in the self-cleavage reactions of both polarities of sTobRV RNA.

## MATERIALS AND METHODS

### Reference nucleoside-2',3'-cyclophosphorothioates

Syntheses of the mixed endo- and exo-diastereomers of the 2',3'-cyclophosphorothioates of adenosine (cAMPS) and cytidine (cCMPS) were initiated from salicyl phosphorochloridite and, respectively, 5'-acetyladenosine and 5'-*O*-,*N*<sup>4</sup>-diacetylcytidine, according to Ludwig and Eckstein (25). Separation of the endo- and exo-forms was by reversed-phase HPLC (25). The orientation of the exocyclic, phosphorus-bound S and O atoms in, and hence the absolute configuration of, the two uridine-2',3'-cyclophosphorothioate (cUMPS) isomers is known from x-ray structural analysis of endo-cUMPS (26). The assignment of the absolute configuration of the two diastereomers of cAMPS and cCMPS rests on their <sup>31</sup>P NMR chemical shifts, which are nearly identical to those of endo- and exo-cUMPS (25).

### Thin layer chromatography of cAMPS and cCMPS

Thin layer sheets were 20 cm long. Cellulose thin layers with fluorescent indicator (Eastman No. 13254) were developed with saturated ammonium sulfate:1 M sodium acetate:isopropanol, 40:9:1. Polyethyleneimine-cellulose thin layers with fluorescent indicator (PEI-cellulose, Macherey-Nagel Polygram 300/UV 254) were pre-chromatographed with 0.5 M KH<sub>2</sub>PO<sub>4</sub> and rinsed with water and methanol. PEI-cellulose plates were developed with 0.3 M KH<sub>2</sub>PO<sub>4</sub> (pH not adjusted) for adenosine compounds and with 0.1 M KH<sub>2</sub>PO<sub>4</sub> for cytidine compounds. For cellulose thin layers, and for PEI-cellulose thin layers chromatographing adenylates, Whatman 3MM filter paper was stapled to the top of the thin layer, and the solvent migrated approximately 10 cm into the paper. Reversed phase thin layers (Analtech Uniplate type RPSF) were developed with 50 mM Tris-HCl, pH 7.5, 9% by volume acetonitrile. The mobility of each nucleoside-2',3'-cyclophosphorothioate isomer was calculated relative to that of the corresponding nucleoside-2',3'-cyclophosphate.

### Plasmid templates

The template for the synthesis of a self-cleaving oligoribonucleotide with a sTobRV (+)RNA sequence (Fig. 1A) was derived from four oligodeoxyribonucleotides (Fig. 1C). These were electrophoretically purified in 20% polyacrylamide gels and were enzymically 5'-phosphorylated as described (27). pd(+)<sub>355</sub>\*46 and pd(-)<sub>46</sub>\*1, each at 5.6 μM, were mixed with 1.4 μM each of pdT7(+) and pdT7(-)<sub>359</sub>\*355 in 100 μl of 20 mM Tris-HCl, pH 7.5, 50 mM NaCl, 10 mM MgCl<sub>2</sub>, 1 mM DTT and incubated at 70°C for 5 min and 37°C for 1 hr. Products were incubated at 20°C with 8 U/μl bacteriophage T4 DNA ligase, and the ends were filled in by the action of Klenow fragment DNA polymerase with 0.1 mM dNTPs as substrate (28). Ligation into EcoRI- and HincII-digested product into similarly-cut plasmid pIC19H (29) and transformation (30) into *E. coli* strain 71-18 gave a plasmid with a pentameric version of the sequence derived from d(+)<sub>355</sub>\*46 and d(-)<sub>46</sub>\*1, as detected by screening for insert size after digestion with HindIII. The plasmid was digested with SalI, ligated, and used to transform *E. coli*. Selected plasmid pT7(+)<sub>355</sub>\*46 has a unit insert.

Plasmids which were the templates for the synthesis of the two oligoribonucleotides E and S, shown in Fig. 1B, are respectively pT7(-)<sub>54</sub>\*40(CU) and pT7(-)<sub>222</sub>\*163(AG), described by Feldstein et al. (19).

### Transcription of oligoribonucleotides from plasmid templates

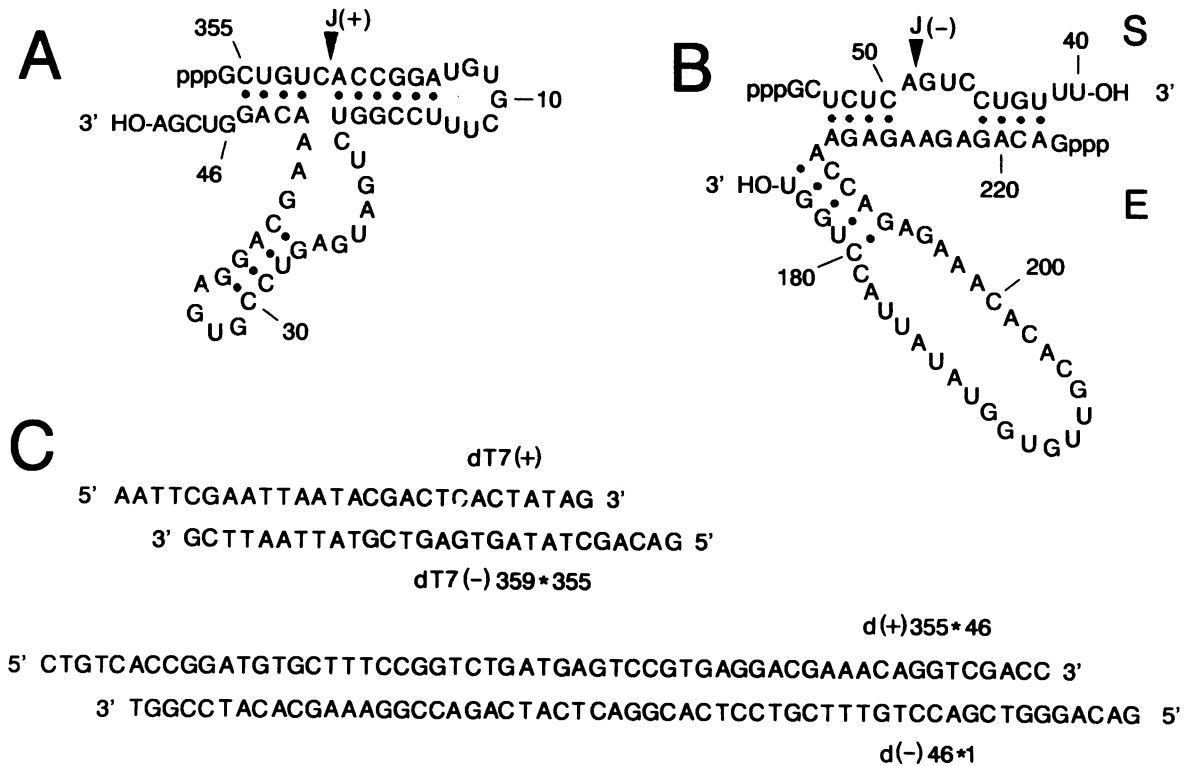
Two μg of plasmid pT7(+)<sub>355</sub>\*46 were linearized by digestion with SalI and transcribed by 2 U/μl bacteriophage T7 RNA polymerase in 100 μl of 1× transcription buffer (40 mM Tris-HCl, pH 7.5, 20 mM NaCl, 6 mM MgCl<sub>2</sub>, 2 mM spermidine-HCl) containing 10 mM DTT, 0.8 U/μl ribonuclease inhibitor RNasin (Promega Biotech), 0.5 mM of each of rCTP, rGTP, and rUTP, 90 μM rATP, and 85 μM S<sub>p</sub>-[<sup>35</sup>S]rATPαS (23 Ci/mmol, Amersham; unlabeled rATPαS from Boehringer Mannheim). Transcription product [<sup>35</sup>S]P<sub>5</sub>-D<sub>46</sub> (Fig. 1A) has nt 355 through 46 of circularly permuted sTobRV (+)RNA with 5' and 3' flanking sequences pppG and UCGA.

pT7(-)<sub>54</sub>\*40(CU) was linearized with DraI and was transcribed (23) in 100 μl reaction mixtures which contained 0.5 mM each of rATP, rCTP and rUTP, 0.1 mM rGTP, and 50 μCi of S<sub>p</sub>-[<sup>35</sup>S]rGTPαS (New England Nuclear, 1320 Ci/mmol) to generate the S oligoribonucleotide [<sup>35</sup>S]P<sub>6</sub>-D<sub>9</sub>(CU) (S in Fig. 1B). E222\*176(AG) was transcribed from RsaI-cut plasmid pT7(-)<sub>222</sub>\*163(AG) as described (19). The two transcripts are composed entirely of sTobRV (-)RNA sequences except for the 5'-terminal pppG and two pairs of compensating mutations. C52 and U51 replace G52 and A51 of the wild type S sequence; A213 and G212 replace U213 and C212 of the wild type E sequence (Fig. 1B; 19). [<sup>35</sup>S]P<sub>5</sub>-D<sub>46</sub>, pT7(-)<sub>222</sub>\*163(AG), and [<sup>35</sup>S]P<sub>6</sub>-D<sub>9</sub>(CU) were electrophoretically purified (28) through 8%, 8%, and 12% polyacrylamide gels, respectively, all in 7 M urea.

### Oligoribonucleotide cleavage reactions and recovery of [<sup>35</sup>S]cAMPS and [<sup>35</sup>S]cCMPS

Approximately 3 μg of [<sup>35</sup>S]P<sub>5</sub>-D<sub>46</sub> was incubated at 37°C for 16 hr in 10 μl of 20 mM Tris-HCl, pH 7.5, 10 mM NaCl, 3 mM MnCl<sub>2</sub> (Dahm, S., and Uhlenbeck, O.C., personal communication). Less than 0.5 μg of [<sup>35</sup>S]P<sub>6</sub>-D<sub>9</sub>(CU) and 2 μg of pT7(-)<sub>222</sub>\*163(AG) were incubated in 10 μl of 20 mM Tris-HCl, pH 7.5, 12 mM MgCl<sub>2</sub>, 4 mM spermidine-HCl for 7 hr at 37°C. Reactions were terminated by addition of 0.6 to 1 volume of formamide and heating to 80°C for 30 sec. Electrophoresis was through 20% polyacrylamide gel in 7 M urea. Oligoribonucleotides [<sup>35</sup>S]pppGCUGUC>pS and [<sup>35</sup>S]ppp(S)GCUCUCA>pS were located by autoradiography and were recovered from gel slices by soaking (31). Additions of 10 μg of glycogen (Boehringer Mannheim) and three volumes of ethanol precipitated the oligoribonucleotides.

Digestion of oligoribonucleotides [<sup>35</sup>S]pppGCUGUC>pS and [<sup>35</sup>S]ppp(S)GCUCUCA>pS was with nuclease P<sub>1</sub> (7). Incubation with 0.5 mU/μl calf intestinal alkaline phosphatase was in 10 μl of 0.1 M NH<sub>4</sub>HCO<sub>3</sub>, pH 9, for 30 min at 37°C and then for 2 min at 100°C. Initial purification of adenosine- and cytidine-2',3'-cyclophosphorothioate was by chromatography on 0.4 to 0.6 ml bed volume columns of DEAE-Sephadex A50 (Pharmacia) and DE-52 DEAE-cellulose (Whatman), respectively. Elutions were in a centrifugal field for 1 min at a few hundred rpm. Prior to use, water-washed columns were exposed to one bed volume each of 0.1 M NaOH and 1 M NH<sub>4</sub>HCO<sub>3</sub>, then two bed volumes of water. Digests were diluted to 0.2 ml with water and mixed with



**Fig. 1.** Autolytically processing sequences derived from the satellite RNA of tobacco ringspot virus (sTobRV RNA). A. Transcript P<sub>5</sub>-D<sub>46</sub> containing nt 355 through 46 of a circularly permuted sTobRV (+)RNA sequence. J(+) locates the phosphodiester, between C359 and A1, which is the junction that is cleaved in multimeric sTobRV (+)RNA. B. Mutated sTobRV (-)RNA sequences of the endonucleolytic transcript E222\*176(AG), indicated by E, and the corresponding junction-containing sequence P<sub>6</sub>-D<sub>9</sub>(CU) (19), indicated by S. J(-) locates the phosphodiester between A49 and G48 that is cleaved in multimeric sTobRV (-)RNA. All numbering is based on the sTobRV (+)RNA sequence (38). C. Four oligodeoxyribonucleotides, dT7(+), dT7(-)359\*355, d(+)\*355\*46, and d(-)\*46\*1, arranged to indicate complementary sequences. These contributed the insert of plasmid pT7(+)\*355\*46, from which P<sub>5</sub>+D<sub>46</sub> was transcribed.

10  $\mu$ g of one or both isomers of the reference nucleoside-2',3'-cyclophosphorothioate. The sample was applied to the column, which subsequently was washed with less than one column volume, 0.2 ml, of 0.25 M NH<sub>4</sub>HCO<sub>3</sub>. DE-52 was washed with 5  $\times$  0.5 ml of 0.07 M NH<sub>4</sub>HCO<sub>3</sub> and then with 0.2 ml of 0.25 M NH<sub>4</sub>HCO<sub>3</sub>. Elution of cAMPS or cCMPS was with 4  $\times$  0.5 ml of 0.25 M NH<sub>4</sub>HCO<sub>3</sub>. The eluate was combined with 2 drops of triethylamine and evaporated under vacuum. The sample was twice dissolved in 25  $\mu$ l 25% ethanol and dried.

The adenosine compound was further purified by thin layer chromatography on PEI-cellulose. The major radioactive spot was located by autoradiography, and recovered thin layer powder was twice extracted with 20  $\mu$ l of 1 M NH<sub>4</sub>HCO<sub>3</sub>. Extracts, combined with 5  $\mu$ l of triethylamine, were dried, dissolved in 0.5 ml water and chromatographed on DE-52, as described in the previous paragraph. The adenosine- and cytidine-2',3'-cyclophosphorothioates were compared with the endo- and exo-isomer reference standards by thin layer chromatography and autoradiography.

## RESULTS

The oligoribonucleotides designed to undergo the processing reactions of sTobRV (+)RNA and sTobRV (-)RNA are here designated in the form P<sub>n</sub>-D<sub>m</sub>. P<sub>n</sub>-D<sub>m</sub> is cleaved to form the

promoter-proximal oligoribonucleotide P<sub>n</sub>, with a new 2',3'-cyclophosphate end, and the promoter-distal portion of the polyribonucleotide chain, D<sub>m</sub>, with a new 5'-hydroxyl end group. Subscripts designate numbers of sTobRV RNA-derived nt.

The 5' extent of sTobRV (+)RNA sequence in P<sub>5</sub>-D<sub>46</sub> (Fig. 1A) was selected to have no adenylate residues in the P<sub>5</sub> product, [<sup>35</sup>S]pppGCUGUC > pS, and hence <sup>35</sup>S only in the 2',3'-cyclophosphorothioate. We were able to control the extent of self-cleavage of P<sub>5</sub>-D<sub>46</sub> during the transcription reaction, based on the observation (23) that an sTobRV (+)RNA-derived oligoribonucleotide, when synthesized from rCTP, rGTP, rUTP and rATP $\alpha$ S, exhibited only limited self-cleavage, even on long incubation. Replacing only a few NpA phosphodiesters with phosphorothioate diesters would have allowed most of the cleavage to occur during the transcription reaction period. Our approach here was to synthesize P<sub>5</sub>-D<sub>46</sub> from a reaction mixture with rATP $\alpha$ S and rATP in approximately equimolar amounts, causing retention of 80% of the P<sub>5</sub>-D<sub>46</sub> in the uncleaved form at the end of the transcription reaction. This allowed us to electrophoretically purify intact P<sub>5</sub>-D<sub>46</sub> from premature termination products. Recovery of [<sup>35</sup>S]pppGCUGUC > pS directly from the transcription reaction mixture would have been difficult because of prematurely-terminated transcription products which migrate similarly to [<sup>35</sup>S]pppGCUGUC > pS. Processing of the phosphorothioate diester CpA of electrophoretically-purified [<sup>35</sup>S]P<sub>5</sub>-D<sub>46</sub> was enhanced by manganese ions (Dahm, S., and Uhlenbeck, O.C., personal communication).

**Table 1.** Mobilities of cAMPS and cCMPS relative to the corresponding cyclophosphates.

Thin layer	endo-cAMPS	exo-cAMPS	endo-cCMPS	exo-cCMPS
PEI-Cellulose	0.81	0.83	0.83	0.85
Cellulose	not resolved		0.52	0.59
Reversed phase silica gel	0.90	1.05	0.93	1.02

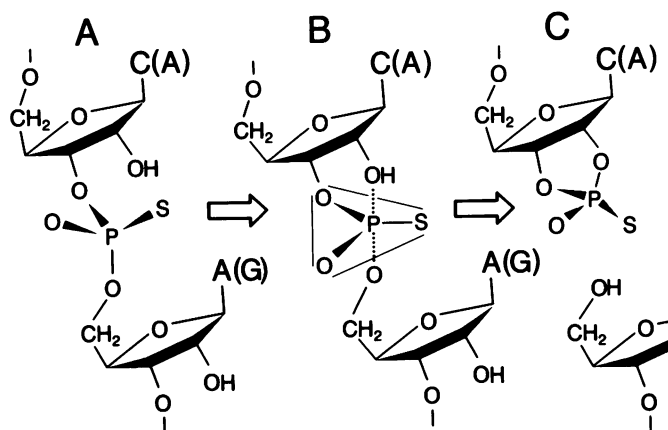
Incorporating rGTP $\alpha$ S into sTobRV (-)RNA-derived oligoribonucleotides was found to have little effect on their self-cleavage (24). The action of E222\*176(AG) on [ $^{35}$ S]P $_6$ -D $_9$ (CU) generated two labeled products, and one of these, [ $^{35}$ S]ppp(S)GCUCUCA>pS, was recovered. The mutant sequence of P $_6$ -D $_9$ (CU) (Fig. 1B) resulted in [ $^{35}$ S]ppp(S)GCUCUCA>pS having only one guanylate residue and hence  $^{35}$ S label only in the 3'-terminal adenosine-2',3'-cyclophosphorothioate and at the  $\alpha$  position of the 5'-terminal rGTP $\alpha$ S residue. The conditions for phosphatase treatment were sufficient to remove most of the relatively resistant [ $^{35}$ S]phosphorothioate monoester (22) during degradation of the terminal rGTP $\alpha$ S residue. Thus, presumably virtually all phosphate monoesters should have been hydrolyzed. The 2',3'-cyclophosphate and cyclophosphorothioate are not opened under these conditions, allowing cCMPS and cAMPS to be recovered from the sTobRV (+)RNA sequence and the sTobRV (-)RNA sequence, respectively.

Table 1 reports the mobilities of the endo- and exo-isomers of cCMPS and cAMPS relative to the corresponding phosphate compounds, as analyzed by chromatography in three thin layer systems. Using these systems, only the endo-isomers of [ $^{35}$ S]cCMPS and of [ $^{35}$ S]cAMPS (Fig. 3) were detected in digests of the respective P $_n$  oligoribonucleotides.

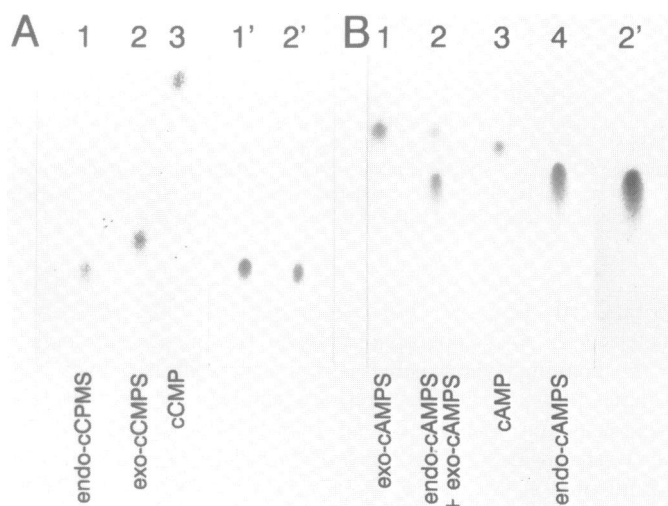
## DISCUSSION

In each of the thin layer chromatography systems in which separation of the endo- and exo-isomers of cAMPS and cCMPS was achieved, the endo-isomer had a lesser mobility than that of the exo-isomer, as also was observed during reversed-phase column chromatography (Table 1; 25). Because only the endo-isomer of cAMPS and of cCMPS (Fig. 3) was identified after digestion of the  $^{35}$ S-labeled P $_n$  oligoribonucleotides, the configuration at the phosphorus atom was shown directly to be inverted in the product, relative to the R $_p$  reactant (Fig. 2).

This result is consistent with an in-line attack of the 2'-hydroxyl group in an S $_N$ 2(P) transesterification reaction of a phosphorothioate diester in the R $_p$  configuration, as diagrammed in Fig. 2. Although more complex mechanisms can account for the stereochemical relationship of reactants and products (22,32,33), the simplicity of the oligoribonucleotide reactants investigated here, with only a few tens of nt, strongly suggests that the S $_N$ 2(P) displacement mechanism describes the autolytic processing of the multimeric forms of both sTobRV (+)RNA and sTobRV (-)RNA, as well as of the ribozymes (14) derived from sTobRV (+)RNA, and that no covalent intermediates are involved. Recently, Mei et al. (34), using a molecular dynamics simulation, found a conformation of a hammerhead structure which is consistent with a direct, in-line attack in the self-cleavage reaction. Differences in structural requirements (Fig. 1AB) for the participating sequences of sTobRV (+)RNA and sTobRV



**Fig. 2.** Postulated stereochemical course of phosphorothioate diester cleavage in sTobRV RNA. A. R $_p$  configuration of a CpA phosphorothioate diester of sTobRV (+)RNA or an ApG phosphorothioate diester of sTobRV (-)RNA. B. Postulated trigonal bipyramidal transition state for an S $_N$ 2(P) transesterification reaction initiated as an attack by the 2'-OH. C. New ends of the products of an S $_N$ 2(P) transesterification reaction: a 5'-OH group and the endo-isomer of a cyclophosphorothioate.



**Fig. 3.** RNA-mediated cleavage of a specific phosphorothioate diester in each polarity of sTobRV RNA generated the endo-isomer 2',3'-cyclophosphorothioate at new chain end. In each part, A and B, detection was by photography under ultraviolet light (left panel) and by autoradiography (right panel, indicated by primes). A. The cleavage of the sTobRV (+)RNA-derived fragment P $_5$ -D $_{46}$  (Fig. 1A) released [ $^{35}$ S]pppGCUCUC>pS, which was electrophoretically purified and enzymically digested. Two aliquots of the radioactive product were mixed, each with one of the two diastereomers of unlabeled cCMPS: the endo-isomer (lane 1,1') and the exo-isomer (lane 2,2'). cCMPS was analyzed in lane 3. Chromatography was on a cellulose thin layer. B. sTobRV (-)RNA-derived, oligoribonucleotide-E-cleaved oligoribonucleotide [ $^{35}$ S]P $_6$ -D $_9$ (CU) (Fig. 1B). Digestion of the electrophoretically-purified reaction product [ $^{35}$ S]ppp(S)GCUCUCA>pS released [ $^{35}$ S]thiophosphate and [ $^{35}$ S]cAMPS. cAMP (lane 3), the exo-isomer (lane 1), the endo-isomer (lane 4), and the two isomers combined with [ $^{35}$ S]cAMPS (lane 2,2') were analyzed on a reversed-phase thin layer.

(-)RNA, however, require that the overall mechanisms differ in detail for the two polarities of RNA. sTobRV (-)RNA exhibits an efficient, spontaneous ligation reaction (7), whereas self-

cleavage of sTobRV (+)RNA was reversible to only a very limited extent (6). The spontaneous ligation of sTobRV (-)RNA molecules, which regenerates a 3'-to-5' phosphodiester bond (35), presumably proceeds as the reverse of the cleavage reaction shown in Fig. 2.

In another system which exhibits RNA self-cleavage, the intervening-sequence-containing ribosomal RNA precursor of *Tetrahymena* retains the 5'-O-P bond, rather than the 3'-O-P that is retained in the case of the sTobRV RNA reactions. The *Tetrahymena* RNA transesterification reaction, with a phosphorothioate at the cleaved bond, proceeds by inversion (36,37). Thus phosphorothioate transesterification reactions within two sTobRV RNA sequences and the *Tetrahymena* RNA sequence, as well as in reactions catalyzed by ribonucleases (22) have a common stereochemical course. McSwiggen and Cech (36) summarize the evidence in favor of the same mechanisms applying in the phosphate and the phosphorothioate reactions.

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