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Autolytic processing of a phosphorothioate diester bond

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ABSTRACT

A small satellite RNA of tobacco ringspot virus replicates in tissues infected with tobacco ringspot virus and accumulates in virus capsids, forming virus-like particles. Previous research showed that multimeric forms of this satellite RNA have tandem repeats of the "monomeric" satellite RNA sequence of 359 or 360 nucleotide residues. The multimeric RNAs undergo autolytic processing at a specific CpA phosphodiester bond, the junction, to generate the monomeric RNA. We substituted phosphorothioate diester bonds for various sets of phosphodiester bonds, in dimeric and truncated forms of the satellite RNA. The degree of reduction in autolytic cleavage varied both with the sites of substitution and the size of the RNA molecules. Analyses of a product of the autolysis reaction suggest that one phosphorothioate diester bond most strongly interferes with processing, the one introduced at the CpA junction during its synthesis from adenosine-5'-O-(1-thiotriphosphate). However, extensive introduction of phosphorothioate diester bonds elsewhere in the molecule also decreased processing, possibly by altering conformation.

INTRODUCTION

A satellite RNA replicates extensively only in plants that also are infected with a specific virus or one of a small group of closely related viruses. The smallest such satellite RNAs are of fewer than 400 nucleotide residues (nt). They encode no coat protein gene and instead become encapsidated in the coat protein of the virus on which they depend, to form virus-like particles with capsids indistinguishable from virus capsids. Although a small satellite RNA has an intimate association with its supporting virus, it lacks any extensive nucleotide sequence homology with the virus genomic RNA(s). Some small satellite RNAs reduce the accumulation of the associated virus and the severity of symptoms that the virus induces when inoculated alone; they act as parasites of the respective virus and are potentially useful as antiviral agents. These and other characteristics of small satellite RNAs have been reviewed extensively (1-5).

Most, but not all, strains of tobacco ringspot virus (TobRV) will support the replication and encapsidation of any of several isolates of the small sat-

ellite RNA of tobacco ringspot virus (STobRV RNA;6,7). The 359 or 360 nucleotide residue (nt) "monomeric form" of the isolates of STobRV RNA thus far analyzed differ from the TobRV genomic RNAs not only in size and nucleotide sequence but also in terminal groups (7-9). The monomeric STobRV RNA sequence is tandemly repeated in multimeric forms of STobRV RNA, which are found in infected tissue and in limited amounts in virus-like particles. The multimeric RNAs may be intermediates in the replication of the satellite RNA. Nonenzymic, autolytic processing of the multimeric RNAs at a specific CpA phosphodiester bond, the junction, gives a 5'-adenosine and a cytidylate-2':3'-cyclic phosphodiester as the new terminal residues. This reaction is a step in proposed schemes for the replication of STobRV RNA (5,10,11).

We are interested in the possible effects of small changes in RNA structure on the autolytic processing reactions of STobRV RNA. Phosphorothioate diester bonds, with stereospecific replacement of a non-bridging oxygen atom of the ordinary phosphodiester bonds, were introduced at specific sets of sites in the polyribonucleotide chain by in vitro transcription from plasmids bearing STobRV RNA sequences. We observed the extents of autolytic processing of various transcripts, with various substitutions of phosphorothioate diester bonds for phosphodiester bonds. These substitutions offer the possibility of making alterations in the RNA conformation and chemical reactivity that are of modest proportions, as is indicated by the abilities of enzymes to recognize the phosphorothioate diester bond-containing analogues of natural RNAs (12).

MATERIALS AND METHODS

Plasmids

The inserts for plasmids pT7+117/M/D and pT7+39/58 were constructed from derivatives of plasmid pBB1, which has a permuted, dimeric copy of the STobRV RNA sequence. The authenticity of the insert in pBB1 is indicated by the ability of the plasmid to engender STobRV RNA when it is co-inoculated with TobRV (13). Plasmid pT7+117/M/D has the circularly permuted dimeric STobRV RNA sequence of the pBB1 derivative plasmid pSP651 (13) inserted as the EcoRI-BamHI fragment into the bacteriophage T7 promoter-bearing plasmid pIBI20 (International Biotechnologies, Inc.) that had been cut with these same enzymes.

The insert of plasmid pT7+39/58 has the junction-containing small HaeIII fragment of STobRV RNA cDNA from plasmid pSP65H13 (14). It was constructed by ligating the products from a digest of 1 µg of pIBI20 and 2 µg of pSP65H13 with BamHI and EcoRI. The sequences derived from the vector portion of pSP65H13 were cut with NarI before transformation and selection on 100 µg/ml

ampicillin. Plasmids from individual colonies were screened for the size of vector and insert after digestion with BamHI and EcoRI, and for the ability to serve as template for bacteriophage T7 RNA polymerase, to identify pT7+39/58.

The insert for plasmid pT7+15/52 was derived from two oligodeoxyribonucleotides, which encode a T7 promoter, STobRV RNA sequences, and sites for restriction endonucleases ApaI and SalI: the 51 nt dCCGTCGACAGTCCTGTTTCGTCCTCACGGACTCATCAGACCGGAAAGCACA and the 58 nt dGAATTAATACGACTCACTATAGGGAGGGCCCGATACCCTGTCACCGGATGTGCTTTCC. These were synthesized by the phosphoramidite triester method in the Protein Structure Laboratory of this Campus. Nine µg of each electrophoretically-purified oligodeoxyribonucleotide were dissolved in a total of 30 µl of 23 mM Tris-HCl pH 9.0, 2.3 mM DTT, 2.3 mM $MgCl_2$, 93 µl/ml glycerol. The reaction mixture contained 20 µCi [γ-^{32}P]rATP and 12 units of bacteriophage T4 polynucleotide kinase (Pharmacia). The incubation was for 100 min at 37°, with additions of 12 more units of enzyme and 200 nmoles of rATP at 30 min. Proteins were removed by extraction with phenol and chloroform. The oligodeoxyribonucleotides, concentrated by precipitation with ethanol, were dissolved in 150 µl of hybridization buffer (100 mM Tris-HCl, pH 7.5, 0.33 M sodium acetate), heated to 90° for 2 minutes, then transferred to a 60°, 300 ml water bath for cooling to 32° over a period of 150 minutes. The hybrid was precipitated and dried under vacuum.

The extension reaction, at 20° for 2 hr, was in 50 µl of 50 mM Tris-HCl, pH 7.2, 10 mM Mg_2SO_4, 0.1 mM DTT, 50 µg/ml bovine serum albumin, 0.8 mM of each dNTP, and 10 units of Klenow fragment of DNA polymerase I (New England Biolabs). After removal of proteins and precipitation, the DNA was dissolved in 100 µl of water. To 5 µl of this solution was added 0.5 µg of pIBI20 that had been cut with EcoRI and EcoRV and blunted at the EcoRI-derived ends by incubation with dATP, dTTP and Klenow fragment of DNA polymerase I. After ligation and transformation, plasmids from single colonies were tested for susceptibility to cleavage by EcoRI, to reveal those plasmids with insert oriented so that transcription proceeds towards the multiple cloning site. Analyses of the transcripts synthesized by bacteriophage T7 polymerase re-vealed plasmids with a single insert, one of which was designated pT7+15/52.

Purification of bacteriophage T7 RNA polymerase

Bacteriophage T7 RNA polymerase was from commercial sources and laboratory preparations. The latter was recovered from 100 ml cultures of Escherichia coli HMS174 bearing plasmid pAR1219 by a procedure of Davanloo et al. (15), only the deviations from which are reported here. Cells were incubated for 2-3 hr, rather than 4 hr, after induction with isopropyl-beta-D-thiogalac-

toside. The amount of lysozyme was tripled to 27 mg. As suggested in a protocol received from J.J. Dunn of Brookhaven National Laboratory, phenylmethylsulfonyl fluoride (0.04 ml of 23 mg/ml solution in acetone) was added to the suspension at the time of digestion. After 30 min at 4^{o} sodium deoxycholate was added to 0.4 mg/ml, and the incubation was continued for an additional 30 min. The precipitate that formed after addition of streptomycin sulfate was extracted three times with 5 ml of 0.2 M NH_4Cl. The subsequent $(NH_4)_2SO_4$-induced precipitate was dissolved in only 3 ml of buffer and was dialyzed overnight against 500 ml of buffer.

The dialyzed sample was applied to a 3.5 ml bed volume Affi-Gel Blue (BioRad Laboratories) column, which was held for 2 hr before beginning the elution schedule. Each elution step was continued until the A_{280} had decreased to baseline. The fraction that eluted with 1.5 M NH_4Cl was dialyzed and applied to a 3 ml bed volume diethylaminoethyl-cellulose column (Whatman DE52). This column was eluted with five 6 ml steps with 100 mM increments, 100 mM to 500 mM NH_4Cl. Eluted fractions were assayed by electrophoresis through 12.5% polyacrylamide gel (16), and fractions (usually those eluted with 200 mM NH_4Cl) that had a strong zone of protein of apparent molecular weight 108,000 were pooled. Pooled fractions were concentrated 5 fold by pressure dialysis, combined with one-half volume of glycerol and stored at -20^{o} in aliquots. The preparations had a concentration of about 35 U/µl. One unit catalyzes the incorporation of 1 nmole of nt from one labeled rNTP in 1 hr under conditions described in the next paragraph.

Transcription of plasmids

Plasmid pT7+117/M/D was linearized by treatment with restriction endonuclease BamHI. The DNA recovered after phenol/chloroform extraction and precipitation with ethanol was incubated (6 µg/reaction) at 37^{o} for 1 hour in 100 µl 1X transcription buffer (40 mM Tris-HCl, pH 7.5, 20 mM NaCl, 6 mM $MgCl_2$, 2 mM spermidine-HCl), 10 mM dithiothreitol (DTT), 0.5 mM each rNTP, 20 µCi [α-^{32}P]rCTP, 125 units ribonuclease inhibitor RNAsin (Promega Biotec) and 125 units of bacteriophage T7 RNA polymerase (United States Biochemicals).

The expected transcript of BamHI-linearized plasmid pT7+117/M/D is P_{117}-M-D_{247}, where P_{117} and D_{247} are the promoter-proximal and promoter-distal bordering sequences, respectively, and M is the nucleotide sequence of monomeric STobRV RNA. Subscripts of P and D indicate the number of nucleotide residues that correspond to STobRV RNA sequences. P_{117}-M-D_{247} has 723 nt of STobRV RNA sequences, preceded by 21 nt derived from the vector.

Plasmid pT7+39/58, linearized with HindIII, and plasmid pT7+15/52, linearized with SalI, were transcribed as for pT7+117/M/D except that 2.5 µg of

DNA template was supplied per reaction, the reaction was catalyzed with 250 units of T7 RNA polymerase purified in this laboratory and 20 μCi of [α-^{32}P]rATP replaced the radioactive rCTP. The expected nucleotide sequence of the transcript P_{39}-D_{58} has 24 non-STobRV RNA nt to the 5' side, and 55 nt to the 3' side, of its STobRV RNA sequences. Similar results were obtained when pT7+39/58 was cut with BamHI to leave only 7 plasmid-derived nt at the 3' end of the transcript. For P_{15}-D_{52} derived from pT7+15/52 fragmented with SalI, the corresponding 5' and 3' values are 6 nt and 2 nt, respectively.

Phosphorothioate diester bonds were introduced into transcripts by replacing individual, or sets of, rNTPs with derivatives that have one of the diastereotopic oxygens at Pα replaced by sulfur. This nucleoside-5'-O-(1-thiotriphosphate) is here abbreviated [αS]rNTP. It is the Sp configuration epimer that is incorporated by the actions of RNA polymerases (12,17,18) to produce phosphorothioate diester bonds of the Rp configuration. Sp-[αS]rATP was from Boehringer Mannheim, stated analysis 87% with 10% Sp-[αS]rADP and 3% rATP. [αS]rUTP, 55% Sp and 45% Rp, was from New England Nuclear. F. Eckstein generously supplied Sp-[αS]rGTP and the mixed Sp- and Rp-diastereomers of [αS]rCTP. Except as noted in the legend for Figure 3, [αS]rATP, [αS]rGTP and [αS]rUTP replaced the corresponding rNTPs at the same concentration, 0.5 mM, where indicated in the table and figures. In order to achieve the same extent and approximately the same rate of incorporation that was achieved from rNTPs and other [αS]rNTPs, [αS]rCTP was supplied at 1.0 mM in place of 0.5 mM rCTP, and, in experiments with [αS]rCTP and the pT7+117/M/D template, the concentration of T7 RNA polymerase was quadrupled to 5 U/μl. With pT7+39/58 or pT7+15/52 as templates, 2.5 U/μl of T7 RNA polymerase was sufficient to compensate for the inhibitory effects of the [αS]rCTP on RNA synthesis.

Analysis of products of autolytic processing reactions

Autolytic processing of transcripts occurred during the transcription reaction and, in some experiments, during separate incubations. Electrophoretically resolved zones that had been located by autoradiography or toluidine blue O staining were recovered by soaking (19). The ^{32}P content of zones was determined by Cerenkov counting (lower ^{3}H channel) of gel fragments and 0.3 ml of eluting solution.

P_{15}-D_{52} was synthesized from [αS]rATP and [α-^{32}P]rATP, and the P_{15} derived from it was analyzed for the [^{32}P]cytidine-2':3'-cyclic phosphodiester terminus. Transcription reaction mixtures of 100 μl contained 0.5 mM each of rCTP, rGTP and rUTP, 200 μCi [α-^{32}P]rATP and 0.2 mM [αS]rATP. Taking into

account the rATP content of the [αS]rATP preparation, the molar ratio of [αS]rATP to rATP in the reaction mixture was expected to be 29:1. Since P_{15}-D_{52} has only 15 adenylate residues, on the average a P_{15}-D_{52} molecule has less than one NpA phosphodiester bond, the others being NpA phosphorothioate diester bonds. Electrophoretically purified P_{15} was combined with 0.6 µg of monomeric STobRV RNA as carrier and was digested with 1.2 units of nuclease P_1 in 9 µl of 1:2:250 acetic acid:pyridine:water for 70 min at 37° to release [^{32}P]pA and the cytidine-2':3'-cyclic phosphodiester-5'-phosphate, [2':3'-^{32}P]pC>p. The dried products were hydrolyzed in 40 µl 20% piperidine for 80 min at 95° to open the 2':3'-cyclic phosphodiester, combined with 10 µg pCp and 40 µg pA, dried, analyzed by two-dimensional thin layer chromatography (9), and located by autoradiography. Cellulose powder scraped from zones was analyzed by scintillation counting.

RESULTS

Incubation of plasmid pT7+117/M/D with bacteriophage T7 RNA polymerase and four rNTPs gave the expected transcript, P_{117}-M-D_{247} (14), and its autolytic processing products (Figure 1, lane 1). The latter result from the cleavage of the transcript at two junction sites of nucleotide sequence CpA (9,10), promoted by the Mg^{2+} ions and spermidine (10) that are components of the polymerase reaction mixture.

Phosphorothioate diester bonds in P_{117}-M-D_{247}

The substitution of [αS]rATP for rATP in the transcription reaction mixture, introducing a phosphorothioate diester bond in place of NpA phosphodiester bonds, did not decrease the extent of transcription but apparently prevented autolytic processing (Figure 1, lanes 2,5). Not even the incorporation of 3% of rATP from the [αS]rATP preparation was sufficient to allow a detected level of autolytic processing (Table 1). The P_{117}-M-D_{247} derived from reactions incorporating [αS]rATP was electrophoretically purified and was incubated for 165 min at room temperature in 1X transcription buffer. Autolytic processing was not detected (data not shown). A minor transcript, that is prematurely terminated near nt 230 of the M sequence (14), is designated as "1" in Figure 1. This transcript was more evident when [αS]rATP was substituted for rATP (Figure 1, lane 2) because it failed to process to P_{117} and RNA fragment "2" and because it was not obscured by processing product M from P_{117}-M-D_{247} (Figure 1, compare lanes 2 and 1).

Replacing single rNTPs other than rATP with the corresponding [αS]rNTP did not prevent autolytic processing of P_{117}-M-D_{247} (Figure 1, lanes 6 and 7; Table 1; similar results for [αS]rCTP not shown). Even substituting the com-

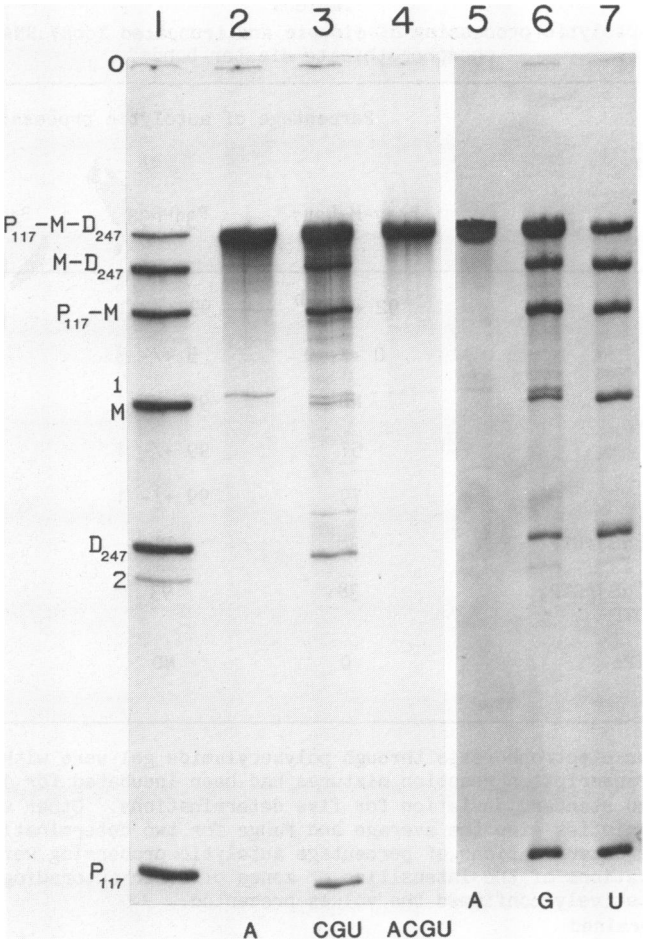

Figure 1. Effects of phosphorothioate diester bonds derived from various [αS]rNTPs on the autolysis of circularly-permuted, dimeric STobRV RNA. Linearized plasmid pT7+117/M/D was incubated with bacteriophage T7 RNA polymerase, rNTPs, including [α-^{32}P]rCTP, and the specified [αS]rNTPs for 1 hr at 37°. Electrophoresis through 6.5% polyacryamide gel in 7.0 M urea and autoradiography revealed the products of transcription and autolytic processing. The reaction mixture for lane 1 had rNTPs only. For the other reaction mixtures, as indicated by letters below the lanes, substitutes for the corresponding rNTP(s) were: lanes 2 and 5, [αS]rATP; lane 3, [αS]rCTP, [αS]rGTP, and [αS]rUTP; lane 4, all four [αS]rNTPs; lane 6, [αS]rGTP; lane 7, [αS]rUTP. M is monomeric STobRV RNA and P_{117} and D_{247} are the flanking RNA fragments that are released by autolytic processing of the transcript, P_{117}-M-D_{247}, and its partial autolysis products, P_{117}-M and M-D_{247}. The zone labeled 1 contains a prematurely terminated transcript which autolytically processed at the junction to release P_{117} and the ca. 230 nt RNA fragment of zone 2 (14). The origin of electrophoresis is indicated by O. Lanes 1 through 4 and lanes 5 through 7, respectively, are from separate experiments.

Table 1
Extent of autolytic processing of dimeric and truncated TobRV RNAs that have phosphorothioate diester bonds

Incorporated [αS]rNTP	Percentage of autolytic processing[a] P_{117}-M-D_{247}	P_{39}-D_{58}	P_{15}-D_{52}
None	92 +/- 2[b]	99 +/- 1	73
[αS]rATP	0 +/- 0	5 +/- 3	13
[αS]rCTP	ND	99 +/- 1	63
[αS]rGTP	57	99 +/- 1	0
[αS]rUTP	79	99 +/- 1	29
[αS]rGTP, [αS]rUTP	38	ND	ND
[αS]rCTP, [αS]rGTP, [αS]rUTP	38	93	0
all [αS]rNTPs	0	ND	ND

[a] Samples for electrophoresis through polyacrylamide gel were withdrawn after the transcription reaction mixtures had been incubated for 1 hr at 37°.
[b] Average and standard deviation for five determinations. Other values with uncertainties give the average and range for two determinations. Where single determinations of percentage autolytic processing were made, observations of the intensities of zones of other autoradiograms qualitatively confirmed the values presented.
ND: not determined

bination of [αS]rCTP, [αS]rGTP and [αS]rUTP for the combination rCTP, rGTP and rUTP (Figure 1, lane 3) allowed a reduced extent of processing (Table 1), which was greater than that found when most NpA sequences in P_{117}-M-D_{247}, including the junction CpA, had phosphorothioate diester bonds.

Phosphorothioate diester bonds in P_{39}-D_{58} and P_{15}-D_{52}

P_{39}-D_{58} synthesized from [αS]rCTP, [αS]rGTP or [αS]rUTP readily cleaved to the corresponding P_{39} and D_{58} (Figure 2, lanes 3-5; Table 1). Although the incorporation of [αS]rATP drastically reduced the autolytic processing of P_{39}-D_{58} (Figure 2, lane 2; Table 1) during the period of the transcription reaction, on further incubation P_{39}-D_{58} with NpA phosphorothioate diester bonds slowly converted to P_{39} and D_{58} (Figure 2, lane 8). P_{39}-D_{58} synthesized

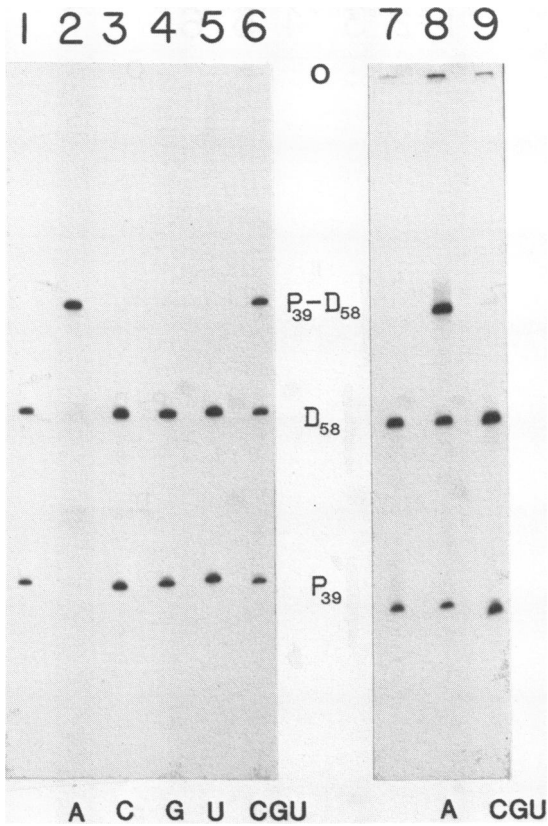

Figure 2. Effects of phosphorothioate diester bonds on the rate of autolysis of a junction-containing, truncated STobRV RNA. Linearized plasmid pT7+39/58 was incubated under conditions for transcription to generate P_{39}-D_{58} and its autolytic processing products. The [α-^{32}P]rATP-labeled reaction products were analyzed by electrophoresis, through 8.0% polyacrylamide gel in 8.0 M urea, and autoradiography. Substitutions of [αS]rNTPs for the corresponding rNTPs were for lane 1, none; lane 2, [αS]rATP; lane 3, [αS]rCTP; lane 4, [αS]rGTP; lane 5, [αS]rUTP; lane 6, [αS]rCTP, [αS]rGTP and [αS]rUTP. For lanes 7, 8 and 9, unfractionated nucleic acids, recovered from a reaction mixtures like those for lanes 1, 2 and 6, respectively, were incubated for 150 min in 2X transcription buffer at 37° before analysis. Further incubation of material for lane 8 for an additional 150 min resulted in complete conversion to P_{39} and D_{58} (not shown).

from rATP, [αS]rCTP, [αS]rGTP and [αS]rUTP was intermediate with regard to its extent of autolytic processing (Figure 2, lanes 6,9; Table 1).

P_{15}-D_{52} autolytically cleaved to P_{15} and D_{52} (Figure 3, lane 1). In these experiments bacteriophage T7 polymerase purified in this laboratory was more useful than commercial preparations of the enzyme then available, which

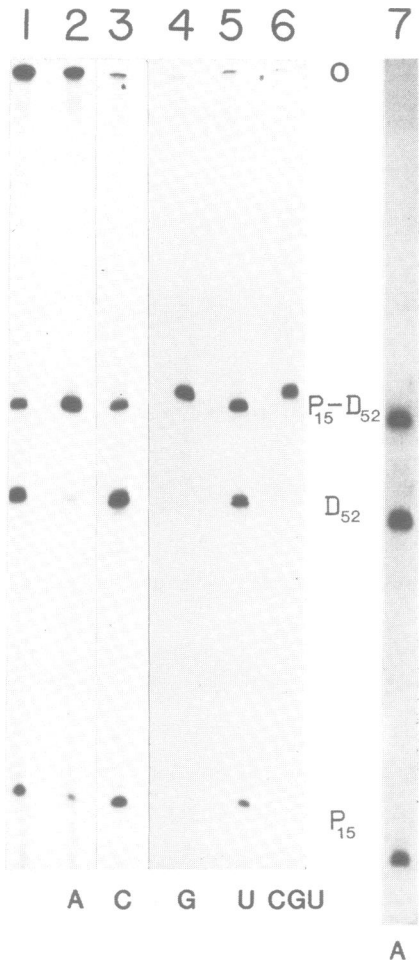

Figure 3. Effects of phosphorothioate diester bonds on the rate of autolysis of a near-minimum-size, autolytically processing RNA. SalI-linearized plasmid pT7+15/52 was transcribed to generate P_{15}-D_{52} and its autolytic processing products. [α-^{32}P]rATP-labeled reaction products were analyzed by electrophoresis through 12% polyacrylamide gel in 7.7 M urea and autoradiography. Substitutions of [αS]rNTPs for the corresponding rNTPs were for lane 1, none; lane 2, [αS]rATP; lane 3, [αS]rCTP; lane 4, [αS]rGTP; lane 5, [αS]rUTP; lane 6, [αS]rCTP, [αS]rGTP and [αS]rUTP. For lane 7, electrophoretically-purified P_{15}-D_{52} from a transcription reaction mixture that contained 0.2 mM [αS]rATP and 2 μCi/μl [α-^{32}P]rATP in place of rATP was incubated in transcription buffer for 2 hr at room temperature and 1 hr at 37°. Excised zones of P_{15} and D_{52} contained 34% of the radioactivity of the three zones combined. Note that P_{15}-D_{52} derived from [αS]rGTP showed reduced electrophoretic mobility compared to the corresponding sulfur-free RNA.

for reasons unknown generated an RNA fragment that migrated between P_{15}-D_{52} and D_{52} (data not shown; our enzyme preparations also generated cleaner patterns for transcripts from pT7+39/58). P_{15}-D_{52} is nearly the smallest truncated STobRV RNA sequence that exhibits autolytic processing (D.J. Siler, personal communication). P_{15}-D_{52} always processed less efficiently than P_{39}-D_{58} (Table 1), when the two RNAs were incubated under similar conditions. The incorporation of [αS]rGTP, but not [αS]rCTP or [αS]rUTP, was significantly more effective than incorporation of [αS]rATP in slowing the processing of P_{15}-D_{52} (Table 1; Figure 3, lanes 2-5). On further incubation (Figure 3, lane 7) P_{15}-D_{52} derived from [αS]rATP processed to a limited extent, whereas the RNA derived from [αS]rGTP did not generate a detected amount of processing products even after 2.5 hr at 37° in 2X transcription buffer (data not shown).

Phosphodiester and phosphorothioate diester CpA junctions

The reduced extent of autolysis of P_{15}-D_{52}, that had been synthesized from [αS]rATP, allowed P_{15} to be recovered at two stages: just after the transcription reaction and after a subsequent incubation of P_{15}-D_{52}, electrophoretically purified from the same transcription reaction mixture, for 2 hr at room temperature and 1 hr 37° in 1X transcription buffer. P_{15}-D_{52} was synthesized in a reaction mixture that contained [αS]rATP and [α-^{32}P]rATP. The nucleotide sequence of P_{15} predicts that its digestion with nuclease P_1 should yield pA and pC>p in a 3:1 ratio. The observed radioactivity ratio, [^{32}P]pA to [2':3'-^{32}P]pC>p, was 1.17:1 after the first stage and 4.14:1 after the second.

DISCUSSION

Unlike many other enzyme-catalyzed reactions of phosphorothioate nucleotides, RNA synthesis, catalyzed by RNA polymerases, has been found to be slowed only slightly by replacement of rNTPs by Sp-[αS]rNTPs (reviewed in 12). Transcripts were efficiently synthesized from [αS]rNTPs by bacteriophage T7 RNA polymerase in research reported here, with little modification of the transcription conditions. In contrast to transcription, autolytic processing of the phosphorothioate diester bonds derived from the [αS]rNTPs was in some instances dramatically reduced. Replacement of NpA phosphodiester bonds by phosphorothioate diester bonds in P_{117}-M-D_{247} or P_{39}-D_{58} inhibited processing more effectively than the substitution of any NpN' or group of NpN's, where N' is C, G or U (Table 1 and results not presented). The triple substitution also did not cause any large deviation in the electrophoretic mobilities of these transcripts or processing products, giving no evidence of a gross change in conformation of the molecules under the partially denaturing conditions of

gel electrophoresis. Previous analyses (14) of the autolytic processing of truncated STobRV RNAs revealed that P_{39}-D_{58} processed more efficiently than any of the longer, junction-containing RNAs tested. P_{39}-D_{58} also processed more efficiently than P_{15}-D_{52} and was the transcript least affected in its processing by the incorporation of [αS]rNTPs other than [αS]rATP.

Of the transcripts tested, only P_{15}-D_{52} failed to process when it was synthesized from [αS]rGTP, rATP, rCTP and rUTP or from [αS]rGTP, [αS]rCTP, [αS]rUTP and rATP. The incorporated phosphorothioate diester NpG bonds decreased the mobility of P_{15}-D_{52} (Fig. 3, lane 4, compared to lanes 1-3 and 5), and this was the most extreme mobility shift observed by incorporation from a single [αS]rNTP into any of the transcripts or processing products. We believe that because P_{15}-D_{52} is small, the incorporation of 25 NpG phosphorothioate diester bonds, among its total 74 phosphodiester bonds, is sufficiently disruptive to change its conformation and thereby reduce autolytic processing. That the autolytic reaction was prevented by an altered conformation of P_{15}-D_{52}, rather than a chemical reaction of the phosphorothioate group or a localized steric effect, is suggested by the efficient autolytic processing of P_{39}-D_{58} containing NpG phosphorothioate diester bonds. This P_{39}-D_{58} has all of the NpG phosphorothioate diester bonds of the P_{15}-D_{522} derivative, yet its electrophoretic mobility is unaltered compared to that of the P_{39}-D_{58} with all phosphodiester bonds.

The nucleotide sequence of P_{15} predicts that if P_{15}-D_{52} is synthesized from [α-^{32}P]rATP as the only source of adenylate nucleotide, P_{15} digested with nuclease P_1 will release [^{32}P]pA and [2':3'-^{32}P]pC>p in a 3:1 ratio. A similar synthesis, but from a mixture of [α-^{32}P]rATP and [αS]rATP, should generate the same ratio of products if two conditions hold: (1) [α-^{32}P]rATP and [αS]rATP are incorporated with the same relative efficiency at any site in the polyribonucleotide chain and (2) replacing any NpA phosphodiester bond in P_{15}-D_{52} with a phosphorothioate diester bond had an equally inhibitory effect on autolytic processing. Two observations indicate that condition (1) is valid. First, [αS]rATP and rATP were incorporated into transcripts of STobRV RNA sequences with apparently equal or nearly equal efficiency by bacteriophage T7 RNA polymerase at the high substrate concentrations used here. Second, the ratios of pA to pC>p, 1.17 and 4.14, for P_{15} derived from initial and later processing, bracketed the theoretical 3:1, indicating that the weighted average ratio may have been close to 3:1.

We believe that condition (2) does not hold. P_{15}-D_{52} molecules from the initial processing reaction are enriched in 2':3'-cyclic phosphodiester ter-

mini, relative to cyclic phosphorothioate diester termini, as indicated by the 1.17:1 pA to pC>p ratio. We propose that the enrichment occurs because the CpA phosphodiester junctions of P_{15}-D_{52} autolytically processed more rapidly than the CpA phosphorothioate diester junctions, regardless of the distribution of other NpA phosphorothioate diester bonds. The observed ratio of 1.17:1 provides indirect evidence that, of all of the possible single substitutions of phosphorothioate diester bonds for phosphodiester bonds in P_{15}-D_{52}, the substitution at the CpA junction most strongly inhibits processing. Presumably the same specificity would be observed for single substitution in the larger transcripts.

In experiments not reported, we attempted to confirm the apparently greater reactivity of the junction CpA phosphodiester bond, relative to its phosphorothioate-substituted analogue. The transcription reaction mixtures contained rATP, with a small addition of [α-^{35}S]rATP designed to give on the average less than one phosphorothioate NpA bond per P_{15}-D_{52}. The experiments proved to be technically difficult because the [^{35}S-phosphorothioate]P_{15} from the initial and later processing reactions sufficiently resisted digestion by nuclease P_1 so as to produce a complex mixture of radioactive products that did not allow an unequivocal determination of the pA to pC>p ratio. Probably the observed resistance to digestion by nuclease P_1 was obvious in this experiment because of the types of bonds that the nuclease P_1 must cleave to release the radioactive pA: phosphorothioate diester bonds in this experiment and phosphodiester bonds in the previous experiment.

Except for the systems in which autolytic processing was undetected, i.e., P_{117}-M-D_{247} synthesized from [αS]rATP and P_{15}-D_{52} synthesized from [αS]rGTP, the equilibrium for each reaction presumably corresponded to nearly complete conversion to products because continued incubation converted more reactant to products. Prody et al. (10) showed that the back reaction, as represented by the ligation of two monomeric STobRV RNA molecules, proceeded to a fraction of a percent. We have been unable (unpublished results) to detect any ligation of P and D RNA fragments derived from truncated STobRV RNA transcripts such as P_{15}-D_{52} or P_{39}-D_{58}. That is, the transcripts of most sizes and locations of phosphorothioate substitutions may have similarly large equilibrium constants for autolysis. Thus the extent of the reaction after a 1 hr incubation may be taken as an indicator of the relative rates of autolysis for those reactions that had proceeded but not reached completion. If the extents of reaction we observe represent relative rates, then substitution of phosphorothioate diester bonds for phosphodiester bonds must influence the

rate limiting step for autolytic processing. Our other observations suggest that this can be accomplished by local chemical or steric changes (20) at the CpA junction, or by changes in conformation of the RNA molecule, or both. Chemical effects from phosphorothioate diester bonds at a distance from the junction also must be considered, but we do not have evidence for such effects.

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