Specific association between an endoribonucleolytic sequence from a satellite RNA and a substrate analogue containing a 2'–5' phosphodiester

(catalytic RNA/synthetic oligoribonucleotide/competitive inhibitor/plant virus/phosphotransfer reaction)

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ABSTRACT Both polarities of the satellite RNA of tobacco ringspot virus are sources of self-cleaving sequences. RNA of the less abundant, negative polarity, designated sTobRV(–)RNA, has cleaving activity that was mapped previously to two noncontiguous regions of the polyribonucleotide chain. Endoribonucleolytic oligoribonucleotides (E) corresponding to the larger of the two regions cleaved smaller substrate oligoribonucleotides, at the ApG phosphodiester that is cleaved in sTobRV(–)RNA. An analogue of the substrate, which has a 2'–5' ApG phosphodiester, was not cleaved by E but acted as a competitive inhibitor of the cleavage of substrate. The analogue served as a primer, and E served as template, for reverse transcriptase-catalyzed copying of specific E sequences. The sequences transcribed suggest base pairing between the 5' region of E and a portion of the substrate that is located 3' to, but does not include, the ApG phosphodiester. Results from other experiments indicate this base pairing is a part of the functional cleavage complex. The association of the ends of E and substrate anticipates a second, 4-base-pair association between E and a portion of substrate that is 5' to, but does not include, the ApG phosphodiester. The effects of compensating mutations in E and substrate oligoribonucleotides support the existence of this second association in the active cleavage complex.

Several RNA molecules undergo autolytic cleavage to generate a 2',3'-cyclic phosphodiester and a 5'-hydroxyl as the new chain end groups. Most of these self-cleaving RNA sequences are derived from small satellite RNAs associated with plant viruses of the nepovirus and sobemovirus groups (1, 2), and most conform to a consensus secondary structure, termed a "hammerhead structure," which also applies to a self-cleaving viroid RNA (2, 3). The form of the satellite RNA of tobacco ringspot that is most abundantly encapsidated has a self-cleaving sequence that conforms to the hammerhead structure, whereas the self-cleaving complement of this RNA, designated sTobRV(–)RNA, does not have the conserved sequences of the consensus secondary structure. The products of the cleavage of sTobRV(–)RNA, unlike those of the RNAs from the consensus group, join efficiently in a spontaneous ligation reaction (4, 5). The cleavage reaction generates unit length RNA from repetitive sequence multimeric forms of the cited RNAs.

Mutagenesis experiments of Haseloff and Gerlach (6) identified two regions of the sTobRV(–)RNA sequence within which insertions inactivated the self-cleaving activity. One of the two mutationally sensitive regions includes the ApG phosphodiester that is the junction at which multimeric

sTobRV(–)RNA sequences cleave (4). Feldstein *et al.* (7) produced the two mutationally sensitive regions in the form of separate oligoribonucleotides: a substrate, containing the cleavable ApG phosphodiester, and the endoribonucleolytic oligoribonucleotide (E). By truncating these molecules they identified the minimum sequences that are sufficient for mutual recognition by the two oligoribonucleotides in the cleavage reaction. The minimum substrate is P₄-D₆, which has 4 nucleotide (nt) residues to the 5' promoter-proximal side of the junction and 6 nt to the 3' promoter-distal side. P₄-D₆ is composed of nt 52–43, numbered according to the positive polarity sTobRV RNA sequence (8). The minimum form of active E corresponds to nt 222–177 and is designated E222*177. The system exhibited turnover; one E molecule cleaved many molecules of substrate (7). Here we report the synthesis of an analogue of a substrate, which has a 2'–5' form of the ApG junction, map the site of association, and determine the cleaving activity of other altered oligoribonucleotides.

MATERIALS AND METHODS

Materials. Plasmid pIBI20 was from International Biotechnologies. Plasmid pTZ18R(Apa9) was modified from pTZ18R (United States Biochemical) to give restriction sites downstream of the bacteriophage T7 promoter in the order *Apa* I, *Pst* I, *Sph* I, and *Hind* III. The sequence in the region at which transcription by bacteriophage T7 RNA polymerase is initiated on pTZ18R(Apa9) is ATAGGGCCCC. Enzymes were from commercial suppliers and ³²P-labeled compounds were from Amersham. Oligodeoxyribonucleotides were synthesized by Operon Technologies (Alameda, CA), and were electrophoretically purified in 12% polyacrylamide gels in 7 M urea and 90 mM Tris borate/1 mM EDTA buffer (9).

Starting materials for the chemical synthesis of oligoribonucleotides were the barium salts of 5'-O-dimethoxytrityl-2'-O-(o-nitrobenzyl)-3'-(p-chlorophenyl phosphate) derivatives of 6-N-benzoyladenine, 4-N-benzoylcytidine, 3-anisoyluridine, and 2-N-phenoxyacetyl-6-O-(o-nitrobenzyl)guanosine. Details of the syntheses of these monomers and preparation of the nucleoside terminator 3-anisoyl-2',3'-O-dibenzoyluridine will be reported elsewhere.

A specially prepared monomer unit, 5'-O-dimethoxytrityl-3'-O-(o-nitrobenzyl)-6-N-benzoyladenine-2'-(p-chloro-

Abbreviations: nt, nucleotide(s); sTobRV(–)RNA, RNA complementary to the most abundantly encapsidated form of satellite RNA associated with tobacco ringspot virus; E, an endoribonucleolytic sequence from sTobRV(–)RNA; P_n and D_m, respectively, the 5'-most and 3'-most products from an oligoribonucleotide cleaved by E, with subscripts indicating the numbers of nt derived from the sTobRV RNA sequence.

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phenyl phosphate), was the source of the adenosine-2'-phosphoryl group. Synthesis of this derivative began with the *O*-nitrobenzoylation of adenosine (10) and *N*-benzoylation of the resulting mixed 2' and 3' products according to the transient protection method of Ti *et al.* (11). The mixed products were chromatographically isolated from a silica gel column and converted to their 5'-*O*-dimethoxytrityl derivatives. The 2'- and 3'-*O*-(*o*-nitrobenzyl) isomers then were separated by preparative centrifugal thin-layer chromatography on silica gel (Chromatotron, Harrison Research, Palo Alto, CA) to obtain the pure 3' isomer. This was phosphorylated with excess *p*-chlorophenyl phosphorodichloridate and triazole in pyridine (12), and the product was recovered as its barium salt (13). To establish the validity of the compound as a reactant in forming 2'-5' phosphodiester, it was condensed with 6,6-*N*-2',3'-*O*-tetrabenzoyladenine (14) by the action of 1-(*p*-toluenesulfonyl)-3-nitro-1,2,4-triazole (15), which also was the condensing reagent in oligoribonucleotide synthesis (see below). Products were analyzed by paper chromatography and by anion-exchange chromatography (Dowex AG 1-X2; 400 mesh; linear gradient elution in 10% ethanol, from 0.04 M NaCl/0.02 M NH₄OH to 0.4 M NaCl/0.2 M NH₄OH).

Chemically Synthesized Oligoribonucleotides. HO-CUGA-CAGUCCUGUUU-OH, corresponding to nt 54-40 of sTobRV(-)RNA, was synthesized in the all 3'-5' form (P₆-D₉) and also with a 2'-5' phosphodiester at the junction position [P₆(2'-5')D₉]. Synthesis was in solution by a phosphotriester method (13, 16, 17) adapted for oligoribonucleotide synthesis (18, 19). The dimers AC, GU, and UU and the trimer CUG were constructed from the protected ribonucleotide barium salts. 3-Anisoyl-2',3'-*O*-dibenzoyluridine was added to the 3' end of UU to give UUU. A portion of the trimer CUG derivative was coupled to the cytidine mononucleotide derivative to give CCUG. The dimer GU was condensed in separate reactions with both the 2' and 3' Ap derivatives to give A(2'-5')GU and A(3'-5')GU, respectively. The scheme for the assembly of the two oligoribonucleotides from these precursors was (CUG + {AC + [AGU + (CCUG + UUU)]}), with purifications of the intermediates at each stage. Protecting groups were removed (18), and the oligoribonucleotides were purified by preparative high-performance liquid chromatography (20). The base compositions were determined by alkaline hydrolysis followed by anion-exchange chromatography and had the expected value for both oligoribonucleotides.

[5'-³²P]P₆-D₉ of known concentration was prepared by incubating ≈5 μg of P₆-D₉ and 10 units of bacteriophage T4 polynucleotide kinase in 50 μl of 70 mM Tris-HCl, pH 7.6/10 mM MgCl₂/5 mM dithiothreitol/60 μM (spectrophotometrically determined) [γ-³²P]ATP (33 Ci/mmol; 1 Ci = 37 GBq) at 37°C for 1 hr. The mass of the electrophoretically purified [5'-³²P]P₆-D₉ was calculated from its Cerenkov radioactivity and that of a weighed aliquot of the original reaction mixture. [5'-³²P]P₆(2'-5')D₉ was prepared similarly from [γ-³²P]ATP (0.3 Ci/mmol).

Other Junction-Containing Oligonucleotides. An excess of 5'-phosphorylated and electrophoretically purified oligodeoxyribonucleotide dGTTCCGATCCCCGGGTACCG (d225) was coupled by the action of 20 units of bacteriophage T4 RNA ligase to 0.5 μg, of P₆-D₉ in a 25-μl solution of ligase buffer and dimethyl sulfoxide (21). Products were 5'-labeled by phosphorylation as described above, and [³²P]P₆-D₉-d225 was recovered after preparative electrophoresis through a 12% polyacrylamide gel.

The transcribed sequence of *Dra* I-cut pT7(-)54*40, pppgP₆-D₉, has a pppG residue preceding sTobRV(-)RNA nt 54-40. pppgP₆-D₉(CU), transcribed from pT7(-)54*40-(CU), has G-52 and A-51 of the sTobRV(-)RNA sequence replaced by C-52 and U-51. Oligodeoxyribonucleotides

d(+)*40*54 (dAGCTTTAAACAGGACTGTCAGGGCC) and d(+)*40*54(CU) (dAGCTTTAAACAGGACTGAGAGGCC) have terminal sequences complementary to the single strand ends of *Apa* I- and *Hind*III-cut DNA. In a modification of the procedure of Haseloff and Gerlach (22), aliquots of plasmid pTZ18R(Apa9) cut with *Apa* I and *Hind*III were incubated with a 100-fold excess of phosphorylated d(+)*40*54 or d(+)*40*54(CU), DNA ligase, and subsequently with Klenow fragment DNA polymerase. Transformations of 71-18 *Escherichia coli* cells and selection for resistance to ampicillin were according to Seidman (23). Colonies were selected (9) using as probes [5'-³²P]d(+)*40*54 or [5'-³²P]d(+)*40*54(CU). Treatment with *Apa* I and bacteriophage T4 DNA polymerase and then with DNA ligase gave, after transformation, *Apa* I-insensitive plasmids pT7(-)54*40 and pT7(-)54*40(CU). Transcription by bacteriophage T7 RNA polymerase (24) was in a solution 0.5 mM in each NTP as described (25).

Synthesis of Endonucleolytic RNA. Oligoribonucleotide E227*176 was transcribed from plasmid pT7(-)227*176, which had been linearized with *Sal* I (7). E227*176 has the 52 nt of the sTobRV(-)RNA sequence from nt 227-176 flanked on the 5' and 3' sides by 30 and 3 nt, respectively, of vector-derived sequence.

E222*163 and E222*163(AG) have the same sequence except that U-213 and C-212 of the former sTobRV(-)RNA sequence are replaced by A-213 and G-212 in the latter mutated sequence. The inserts for plasmids pT7(-)222*163 and pT7(-)222*163(AG) were each prepared from two oligodeoxyribonucleotides: dT7(-)222*195 and d(+)*163*209 for pT7(-)222*163 and dT7(-)222*195(GA) and d(+)*163*209 for pT7(-)222*163(AG). The sequences of the oligodeoxyribonucleotides are as follows:

dT7(-)222*195: dATCGAATTAATACGACTCACTATAG-ACAGAGAAGTCAACCAGAGAAACACACG

dT7(-)222*195(AG): dATCGAATTAATACGACTCACTATAGACAGAGAAGAGAACCAGAGAAACACACG

d(+)*163*209: dCTAGTCAAGGCGTACCAGGTAATATACCACAACGTGTGTTTCTCTGG.

Each pair of the 5'-phosphorylated oligodeoxyribonucleotides was hybridized and end-filled and then ligated into pBI20 that had been cut with *Eco*RV and *Sal* I and end-filled (9). Ligation and transformation generated β-galactosidase-negative clones bearing a plasmid with both *Eco*RV and *Spl* I sites. The transcript of *Spe* I-cut plasmid pT7(-)222*163 is E222*163, which is expected to have a pppG residue followed by the sTobRV(-)RNA sequence from nt 222-163. E222*176 is the transcript of pT7(-)222*163 digested with *Rsa* I. The concentrations of E222*176 and E227*176 were determined by synthesizing them in solutions containing a spectrophotometrically determined concentration of [α-³²P]CTP (≈0.08 Ci/mmol) and obtaining the Cerenkov radioactivity of weighed aliquots. Nucleotide sequence analysis confirmed the structures of the template portions of the substrate- and E-encoding plasmids.

Endoribonucleolytic Reaction and Association of E with Substrate Analogues. Mixtures of E and P₆-D₉ or P₆(2'-5')D₉ were incubated in 1× processing buffer (10 mM Tris-HCl, pH 7.5/6 mM MgCl₂/2 mM spermidine hydrochloride). Products of the reaction were mixed with 4 vol of a solution of dyes in formamide and were heated to 80°C for 30 sec before analysis by electrophoresis through 20% polyacrylamide gel in 7 M urea. Detection was by autoradiography or two-dimensional scanning with a β-particle detector (Ambis Systems, San Diego). The extent of processing at the end of the reaction was calculated from the radioactivities of P and P-D as the

ratio $P/(P + P-D)$. For the reaction kinetics reported here, the ratio did not exceed 0.28. The substrate concentration for initial velocity kinetics was calculated as the average of the initial and final concentrations of $P-D$, which is expected to cause a deviation of no more than 1% from a true initial velocity at an extent of reaction of 0.28 (26).

Transcription of 1.2 μM E was catalyzed by 1.7 units of avian myeloblastosis virus reverse transcriptase per μl (Life Sciences, Saint Petersburg, FL) and primed by 30 nM $[5'\text{-}^{32}\text{P}]\text{P}_6(2'\text{-}5')\text{D}_9$ or $[5'\text{-}^{32}\text{P}]\text{D}_9$ in 66 mM Tris-HCl, pH 8.3/50 mM KCl/8 mM MgCl_2 /11 mM dithiothreitol/4 mM sodium pyrophosphate/0.8 mM each added dNTP/1 unit of RNasin ribonuclease inhibitor per μl . The solution was incubated, without prior hybridization, at room temperature for 5 min and then at 37°C for 45 min.

RESULTS

The blocked adenosine precursor of 2'-5' phosphodiester was tested in the synthesis of ApA. After deblocking (18), the product was identified chromatographically as A(2'-5')A with no more than 1% contamination by the 3'-5' isomer. Periodate oxidation of the ApA followed by β elimination released adenosine 2'-phosphate as the only phosphorylated product detected. The two synthetic oligoribonucleotides $[5'\text{-}^{32}\text{P}]\text{P}_6\text{-D}_9$ and $[5'\text{-}^{32}\text{P}]\text{P}_6(2'\text{-}5')\text{D}_9$ were partially degraded with base and ribonucleases T1 and U2, and the products were analyzed by electrophoresis and autoradiography (data not shown). The pattern of degradation products was consistent with the expected nucleotide sequence and structures: a zone was observed for cleavage by ribonuclease U2 at the ApG of $[5'\text{-}^{32}\text{P}]\text{P}_6\text{-D}_9$ but not at the corresponding phosphodiester of $[5'\text{-}^{32}\text{P}]\text{P}_6(2'\text{-}5')\text{D}_9$. Both ApG phosphodiester were susceptible to base. Thus, the analogue has an ApG phosphodiester with the characteristics expected for a 2'-5' internucleoside linkage with a free 3'-hydroxyl group.

E227*176 cleaved $[5'\text{-}^{32}\text{P}]\text{P}_6\text{-D}_9$ (Fig. 1A, lane 1). In contrast, $[5'\text{-}^{32}\text{P}]\text{P}_6(2'\text{-}5')\text{D}_9$ was not a substrate for E227*176 (lane 3) or other forms of E tested. Although it was not cleaved, $[5'\text{-}^{32}\text{P}]\text{P}_6(2'\text{-}5')\text{D}_9$ associated with the endonucleolytic oligoribonucleotide: it acted as an inhibitor during the cleavage of $[5'\text{-}^{32}\text{P}]\text{P}_6\text{-D}_9$ by E227*176. The intercept of each of the two lines in the Lineweaver-Burk plot (Fig. 1B) is within the range established by the standard deviation of the other.

From a previously reported (7) time course of E227*176 acting on a 20-fold excess of $[5'\text{-}^{32}\text{P}]\text{P}_6\text{-D}_9$, we estimate the turnover number to be 9.5 per hr, based on the initial rate. Using the same oligoribonucleotides and the methods reported here for determining the concentrations, we obtained a turnover number of 8.3 per hr and a Michaelis constant, K_m , of 0.29 μM from the initial rates at several concentrations of $[5'\text{-}^{32}\text{P}]\text{P}_6\text{-D}_9$. E227*176 proved to be slightly less active, cleaving $[5'\text{-}^{32}\text{P}]\text{P}_6\text{-D}_9$ with a turnover number of 3.7 per hr and a K_m of 0.60 μM (Fig. 1B). The velocity of the cleavage reaction was not increased by prior heating and snap cooling of E227*176 alone or mixed with $[5'\text{-}^{32}\text{P}]\text{P}_6\text{-D}_9$. At a $[5'\text{-}^{32}\text{P}]\text{P}_6\text{-D}_9$ concentration of 5 times K_m , the reaction velocity was proportional to the concentration of E227*176 over the range tested, 9–130 nM. Similar velocities were observed with either $[5'\text{-}^{32}\text{P}]\text{pppg}(\text{P}_6\text{-D}_9)$ or $[5'\text{-}^{32}\text{P}]\text{P}_6\text{-D}_9$ as substrates. With $[5'\text{-}^{32}\text{P}]\text{P}_6\text{-D}_9$ as substrate, an apparent inhibitor constant (26), K_i , for the 2'-5' analogue was estimated to be 0.22 μM for E227*176 and 0.49 μM for E227*176 (Fig. 1B).

To map the position on E with which the 3' end of $\text{P}_6\text{-D}_9$ associates, we used $[5'\text{-}^{32}\text{P}]\text{P}_6(2'\text{-}5')\text{D}_9$ as a primer and E as a template in reverse transcriptase-catalyzed reactions. One dNTP was omitted from each of four reaction mixtures for each of the two primer-template pairs defined by Fig. 2A and B. For both pairs, no extension was obtained when dGTP was

FIG. 1. Association of an analogue of a substrate with an endonucleolytic oligoribonucleotide. (A) Substrate $[5'\text{-}^{32}\text{P}]\text{P}_6\text{-D}_9$ (lanes 1 and 2) and its analogue $[5'\text{-}^{32}\text{P}]\text{P}_6(2'\text{-}5')\text{D}_9$ (lanes 3 and 4) were incubated at 0.2 μM in 1 $\times$ transcription buffer for 13 hr at 37°C either alone (lanes 2 and 4) or with 7 μM E227*176 (lanes 1 and 3, indicated by E). Analysis of products was by electrophoresis and autoradiography. The symbol $\text{P}_6\text{-D}_9$ on the left locates the substrate and its analogue; P_6 designates the radioactive cleavage product $[5'\text{-}^{32}\text{P}]\text{P}_6$. (B) Initial rates at 37°C of cleavage of $\text{P}_6\text{-D}_9$ by 34 nM E227*176, alone ($\blacksquare$) and in the presence of 1 μM $[5'\text{-}^{32}\text{P}]\text{P}_6(2'\text{-}5')\text{D}_9$ (+). Data are presented by double-reciprocal plots.

omitted, and an extension of 1 nt was obtained when dTTP was omitted. With E247*176 (Fig. 2A) as template, omitting dCTP and dATP allowed extensions of 7 and 8 nt, respectively. The corresponding extensions with E227*176 (Fig. 2B) as template were 3 and 6 nt (data not shown). These templates gave the same results when $[5'\text{-}^{32}\text{P}]\text{D}_9$ was substituted for $[5'\text{-}^{32}\text{P}]\text{P}_6(2'\text{-}5')\text{D}_9$ as the primer. The pattern of extensions observed for each of the four primer-template pairs is consistent with only one alignment of $[5'\text{-}^{32}\text{P}]\text{P}_6(2'\text{-}5')\text{D}_9$ or of $[5'\text{-}^{32}\text{P}]\text{D}_9$ on E, as indicated in Fig. 2A and B.

To test whether the base pairing identified above is part of the catalytically productive complex, $[5'\text{-}^{32}\text{P}]\text{P}_6\text{-D}_9$ -d225 and E227*176 (Fig. 2C) were treated under conditions that favor molecular hybridization but not endonucleolytic cleavage, including heating and cooling in a solution that was 0.5 M in NaCl and 1 mM in EDTA. Subsequent dilution of NaCl and additions of magnesium ions and spermidine induced efficient cleavage of $[5'\text{-}^{32}\text{P}]\text{P}_6\text{-D}_9$ -d225 (Fig. 2D, lane 8). The pre-formed complexes of E227*176 and $[5'\text{-}^{32}\text{P}]\text{P}_6\text{-D}_9$ -d225 also cleaved when incubated in EDTA solution (lane 7), under conditions not conducive to cleavage of $[5'\text{-}^{32}\text{P}]\text{P}_6\text{-D}_9$ (compare lanes 4 and 3). When E227*176 and $[5'\text{-}^{32}\text{P}]\text{P}_6\text{-D}_9$ -d225 were submitted to the hybridization regimen but without NaCl (lane 5), only very limited cleavage was observed.

If a helical segment incorporating the sequence at the 3' end of $\text{P}_6\text{-D}_9$ (Fig. 2A and B) is an essential part of the endonucleolytic complex, then the 4-nt UGAC sequence to the 5' side of the cleaved ApG sequence in $\text{P}_6\text{-D}_9$ presumably will have an opportunity to base pair with the GUCA sequence at nt 214–211 within the E oligoribonucleotide. This potential base pairing was tested with mutants of $\text{pppg}(\text{P}_6\text{-D}_9)$ and E227*163 as reported in Fig. 3. The mutant and wild-type E and substrate oligoribonucleotides were mixed in four combinations and incubated under conditions suitable for autolytic cleavage. The two heterologous combinations of wild-type and mutant oligoribonucleotides were inactive (Fig. 3D, lanes 1 and 4). Both the wild-type combination and the mutant combination resulted in cleavage (lanes 2 and 3), showing that the mutations are compensating.

FIG. 2. Mapping associations between two forms of E and analogues of a substrate. (A and B) A set of 6 contiguous base pairs between the analogue [5'-³²P]P₆(2'-5')D₉, represented by the upper lines, and two versions of E, E247*176 (A) and E227*176 (B). (C) Extended base pairing between E227*176 and the 3' region of [5'-³²P]P₆-D₉-d225 (italics, deoxyribonucleotide portion). Lowercase letters, vector sequences. (D) [5'-³²P]P₆-D₉ (lanes 1-4) and [5'-³²P]P₆-D₉-d225 (lanes 5-8) were incubated at 15 μ M alone (lanes 1 and 5) or with excess (35 μ M) unlabeled E227*176 (E, above lanes 2-4 and 6-8). Incubation was for 1 min at 90°C; cooling was from 60°C to 30°C over 90 min in 10 mM Tris-HCl (pH 7.8; 25°C)/1 mM EDTA (lanes 2 and 6) or in the same buffer containing 500 mM NaCl. Subsequent incubation for 4.5 hr at room temperature was in 10 mM Tris-HCl, pH 7.5/100 mM NaCl/1 mM NaEDTA (lanes 2, 3, 6, and 7) or in the same buffer containing 7 mM MgCl₂/2 mM spermidine hydrochloride [lanes 1, 4, 5, and 8 (+)].

DISCUSSION

Results presented here and previously (7) show that any of several E oligoribonucleotides containing nt 222-177 of the sTobRV(-)RNA sequence behaves as an enzyme, as a specific ribonuclease. The system exhibited turnover (7), and when substrate oligoribonucleotide was present in great excess over E, the initial reaction velocity was proportional to the concentration of E. Initial rate kinetics exhibits saturation with regard to the substrate, and the uncleaved analogue P₆(2'-5')D₉ inhibited the reaction competitively (Fig. 1B). Because K_i for the analogue is not significantly smaller than K_m , P₆(2'-5')D₉ appears to be a substrate analogue rather than a more interesting transition state analogue.

Results from reverse transcriptase-catalyzed extensions show that P₆(2'-5')D₉ and D₉ and, presumably, P₆-D₉ bind to the same site on E, all forming a 6-base-pair helix with the 5' end of E. That this binding is productive is indicated by the cleavage P₆-D₉-d225 by E227*176 when the two oligoribonucleotides are hybridized to form a long DNA-RNA helix, which is expected to lock in place the 6 base pairs of the RNA-RNA helix (Fig. 2 C and D). The stability of the long helix is indicated by the retardation of the zone for [5'-³²P]P₆-D₉-d225 in Fig. 2D (lanes 6-8). Apparently, the complex with E227*176 is dissociated slowly during electrophoresis in the hot, urea-containing gel. The cleavage of the preformed complex of E227*176 and P₆-D₉-d225 even in EDTA solution (cf. lanes 7 and 6) indicates the stability of the complex and further supports the contention (4) that magnesium and spermidine ions serve as stabilizers of structure rather than cofactors in the cleavage of sTobRV(-)RNA.

When a mock hybridization reaction between E227*176 and P₆-D₉-d225 was carried out, at a low salt concentration designed to discourage hybrid formation, cleavage of P₆-D₉-d225 was greatly reduced (Fig. 2D, compare lane 6 with lanes 8, 7, and 5). Under these circumstances, apparently productive complexes did not form in sufficient concentration before or during the incubation to allow extensive cleavage of [5'-³²P]P₆-D₉-d225. It is possible that the complexes implied by results in lane 6 of Fig. 2D were produced only at the end of the reaction period when NaOAc and ethanol were added.

Combinations of mutant and wild-type E and substrate that were designed to prevent the formation of a 4-base-pair helix also prevented the cleavage reaction (Fig. 3 A and B and Fig.

3D, lanes 1 and 4). These results strongly support the participation of the postulated 4-base helix in active E-substrate complexes. Observations of products at an earlier time (data not shown) indicate that compensating mutations (Fig. 3C) gave cleavage with an efficiency comparable to that observed with the wild-type sequence. Thus, the results from experiments with three kinds of substrate analogues, and the corresponding E oligoribonucleotides, suggest that an extended, imperfect helix that encompasses the ApG junction is critical to the cleavage reaction. This imperfect helix includes the 6-

FIG. 3. Compensating nucleotide sequence substitutions in substrate and E. (A-C) The upper sequences define a substrate oligoribonucleotide that is initiated with a guanosine triphosphate residue, pppg. Lowercase letters represent deviations from the sTobRV(-)RNA sequence in pppg(P₆-D₉)(CU) and E227*163(AG). In C, compensating mutations in the two strands restore the 2 base pairs that were prevented by mutations in A and B. J(-) indicates the junction phosphodiester that is cleaved in sTobRV(-)RNA. (D) Unmutated and mutated 0.82 μ M E and 3.5 μ M substrate, synthesized in reaction mixtures containing [α -³²P]CTP, were incubated in four combinations in 10 μ l of 1 $\times$ processing buffer at 37°C for 2 hr. Samples were E227*163 and mutant pppg(P₆-D₉)(CU) [lane 1 (sequences in B)], E227*163 and pppg(P₆-D₉) (lane 2), the two mutant oligoribonucleotides [lane 3 (sequences in C)], and mutant E227*163(AG) and P₆-D₉ [lane 4 (sequences in A)]. The locations of E227*163, pppg(P₆-D₉), and the products D₉ and pppg(P₆) are indicated in the margin.

and 4-base-pair helices described above, all of the sequences of substrate previously shown to be essential for E-substrate recognition (7), and the 5' portion of the essential E sequences (7). The results presented here do not suggest what contributions to the cleavage reaction are derived from the 3' two-thirds of the essential nucleotides of E.

sTobRV RNA and another nepovirus-associated RNA, satellite RNA of Arabis mosaic virus (sArMV RNA), for which self-cleavage has not been demonstrated, have sequence similarities of 65–70% (27) in two regions. These two regions in sTobRV RNA include the sequences required for self-cleavage. Based on the similarities, Haseloff and Gerlach (6) proposed a partial secondary structure model for the sTobRV(–)RNA and sArMV(–)RNA, which has, in addition to the ApG-encompassing imperfect helix, a stem and large loop in the 3' portion of E. Not all aspects of the loop are essential for the cleavage reaction, because two insertional mutations within the loop were not inactivating (6). Fig. 4 has an extension of the earlier (6) phylogenetic comparison, which generates a second imperfect helix. This structure clearly is unlike that of a hammerhead (2, 3) and is, unfortunately, less predictive because of its small proportion of the paired bases. Self-cleaving sequences from hepatitis δ virus RNA (28–31) also exhibit a ligation reaction (32) and have proposed secondary structures (30, 31, 33) unlike either a hammerhead or the structure in Fig. 4. Two other, precursor, RNA molecules also self-cleave to generate 2',3'-cyclophosphate and 5'-hydroxyl ends (34, 35). The two do not seem to conform to the documented secondary structures. However, comparisons are difficult because the nucleotides essential for the cleavage reaction have not been defined completely.

While this communication was in preparation, a publication of Hampel and Tritz (36) appeared that describes cleavage kinetics using constructions that, in the nomenclature of this paper, may be designated E224*176 and pppgCG(P₆-D₉). In 40 mM Tris-HCl, pH 7.5/12 mM MgCl₂/2 mM spermidine at 37°C, the reported turnover number and K_m are 2.1 per min and 0.03 μ M, respectively. From the dependence of the reaction on the magnesium ion concentration, the turnover number would be expected to be 1.5 per min in a buffered solution of 6 mM MgCl₂. We have no explanation for the

FIG. 4. Secondary structure model for self-cleaving sequence of sTobRV(–)RNA, based on a comparison with an analogous region (27) of the satellite RNA of Arabis mosaic virus [sArMV(–)RNA]. Numbering is according to the corresponding positive polarity sequences (8). Isolates of sTobRV RNA may have either a C or an A at position 54 of the sTobRV(–)RNA sequence (8). Lowercase letters indicate portions of the sequence that are not essential for self-cleavage (7). J(–) marks the junction phosphodiester at which self-cleavage of the sTobRV(–)RNA sequence occurs in both oligoribonucleotides and the multimeric RNA. Self-cleavage of sArMV(–)RNA is assumed but has not been reported.

dissimilar catalytic efficiencies reported for the apparently similar oligoribonucleotides studied in this work and by Hampel and Tritz (36).

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