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Stable Hairpin Structure Within the 5'-Terminal 85 Nucleotides of Poliovirus RNA

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The primary sequence of a 5'-terminal fragment of poliovirus type 1 RNA, generated by digestion with RNase III, has been determined. This sequence reveals the presence of a stable hairpin structure beginning nine nucleotides from the terminally linked protein VPg. The sequence does not contain (i) the initiation codons AUG or GUG or (ii) the putative ribosome-binding sequence complementary to the 3' end of eucaryotic ribosomal 18S RNA. The stem-and-loop structure identified can be drawn in either plus or minus RNA strands. It is unclear to which strand functional significance (if any) can be assigned. It is possible that the hairpin structure is involved in ribosomal recognition and translation or in RNA synthesis by interacting with replicase molecules.

Plus-strand RNA viruses, such as picornaviruses, are unique biological entities in that their genome functions as mRNA after the entry of the virus into the host cell (3). Although the invading virion RNA will engage in protein synthesis, virion RNA and viral mRNA need not be identical molecules. Indeed, poliovirion RNA has a small protein called VPg (26, 27) linked to the 5' terminus of the RNA via an O^4-(5'-uridylyl)-tyrosine bond (2, 32, 41), whereas the viral mRNA isolated from polyribosomes is 5' terminated in pUp (11, 18, 37). The presence or absence of VPg appears to be the only difference between virion RNA and mRNA, as both RNAs have the same sedimentation coefficient of 35S (46, 47), yield indistinguishable fingerprints of RNase T_1-resistant oligonucleotides by two-dimensional gel electrophoresis (37), and have identical 5'-terminal nonanucleotides (13, 32, 35, 39). Moreover, poliovirus mRNA is infectious (13). Sequence analysis of poliovirion RNA can therefore be considered not only a study of the viral genome but also a study of viral mRNA. The 5'-terminal sequence of poliovirus RNA is particularly interesting because it may contain the initiation site of viral protein synthesis as well as unusual structural features of the noncoding region which may be involved in RNA replication.

We have previously developed a method to determine the primary structure of poliovirus RNA without recourse to molecular cloning. This method involves sequencing of complementary DNA (cDNA) by DNA synthesis in the presence of chain terminators (43) and primed by virus-specific RNase T_1- or RNase A-resistant oligonucleotides (21). Our procedure cannot be applied to a study of the 5' end of poliovirus RNA since we were unable to generate full-length cDNA to virion RNA. The analysis of the 5'-terminal sequence of poliovirus RNA is further complicated by the protein block at the 5' end that prevents labeling with [γ-^{32}P]ATP and polynucleotide kinase. Nomoto and Imura (33) and Hewlett and Florkiewicz (17) have overcome this problem by radioiodination of VPg at the 5' end followed by rapid sequencing techniques. So far, this method has yielded an unambiguous sequence only up to 22 nucleotides (17; Nomoto, personal communication). Harris (15), on the other hand, has isolated a 5'-terminal fragment of aphthovirus (foot-and-mouth disease virus) mRNA and sequenced 70 nucleotides.

We have found previously that poliovirus RNA can be cleaved with RNase III at a low salt concentration into numerous fragments (34, 36). A 5'-terminal fragment, estimated to be 100 nucleotides long, was identified within the RNase III digestion products and purified (16). Here, we report the nucleotide sequence (n = 85) of this fragment. The sequencing data reveal the presence of a very stable hairpin structure beginning at residue 9 from the 5' terminus of the RNA.

MATERIALS AND METHODS

Virion RNA. Poliovirus type I (Mahoney) was isolated from infected suspension cultures of S3 HeLa cells as previously described (25). Poliovirus RNA was extracted from purified virus with phenol-chloroform-isoamyl alcohol (50:48:2) and further purified by zonal sedimentation through sucrose gradients (25).

5'-terminal fragment. Poliovirus RNA (1.0 mg) was digested with RNase III at 20 mM NH_4Cl as previously described (16). The digest was analyzed on 3 to 20% polyacrylamide gradient gels and stained for 1 h in 0.2 M sodium acetate (pH 5.0) containing

ethidium bromide (1 μg/ml). Band 14 was cut from the gel and extracted as described previously (8).

Proteinase K treatment of fragment 14. Half of the purified fragment 14 was digested with proteinase K (2 mg/ml) in 15 μl of 0.1 M NaCl–0.01 M Tris-hydrochloride–0.001 M EDTA (pH 7.5)–0.1% sodium dodecyl sulfate for 1 h at 37°C. An equal volume of 10 M urea–0.4% xylene cyanol was then added to each sample of treated or untreated fragment 14 (control). The mixtures were heated for 5 min at 50°C and loaded directly on a 15% acrylamide slab gel.

3' Labeling of fragment 14 with RNA ligase. Fragment 14 was condensed to [5'-^{32}P]pCp (~6,000 Ci/mmol) by using RNA ligase (P.L. Biochemicals) as described previously (10), except 6 pmol of fragment 14 and 30 pmol of [5'-^{32}P]pCp were incubated in the presence of 15 mM ATP (24). The products were then precipitated with ethanol and purified by gel electrophoresis.

5'-Labeled, RNase T_1-resistant oligonucleotides of fragment 14. RNase T_1-resistant oligonucleotides were generated from fragment 14, 5' labeled, and purified as follows. A 6-μl mixture of 0.1 M Tris-hydrochloride (pH 8.0) containing 0.4 μg of fragment 14, 2.5 U of RNase T_1 (Sankyo), and 0.1 U of calf intestine alkaline phosphatase (Boehringer Mannheim) was incubated for 1 h at 37°C. Next, 1.8 μl of 0.5 N HNO_3 was added, and the mixture was incubated for 15 min at room temperature. The solution was then mixed with 7.2 μl of 10 mM spermidine–1 mM EDTA–0.15 M Tris-hydrochloride (pH 9.0), heated to 50°C for 5 min, and subsequently chilled. The mixture was adjusted to 50 mM Tris-hydrochloride (pH 9.0), 10 mM $MgCl_2$, and 5 mM dithiothreitol, and 10 U of polynucleotide kinase were added. The solution was then transferred to a tube containing 3 mCi of lyophilized [γ-^{32}P]ATP, incubated for 30 min at 37°C, and mixed with an equal volume of 10 M urea containing 0.04% dye (Y. F. Lee, personal communication). The 5'-labeled oligonucleotides were purified by two-dimensional gel electrophoresis (25) and eluted as previously described (21).

Mobility shift analysis. The nucleotide sequences of the T_1 oligonucleotides were determined by mobility shift analysis as previously described (44). Briefly, a limited alkaline digest of the oligonucleotides was prepared as previously described (8). After lyophilization the fragments were suspended in 2 μl of water and subjected to electrophoresis on cellulose acetate at pH 3.5 (first dimension) followed by homochromatography with a 50 mM KOH "homomix."

Isolation of poliovirus mRNA. Intracellular poliovirus RNAs were labeled in separate experiments with $^{32}P_i$ and [^{3}H]guanosine, and the viral mRNA was isolated from infected HeLa S3 cells as previously described (37), except that Triton X-100 was used in place of Brij 58. Fractions corresponding to polyribosomes were pooled, adjusted to 0.02 M EDTA and 1% sodium dodecyl sulfate, treated twice with phenol-chloroform, and precipitated with ethanol (49). The precipitate was sedimented at 24°C through a 15 to 30% sucrose gradient in 0.1 M NaCl–0.01 M Tris-hydrochloride (pH 7.5)–1 mM EDTA–0.5% sodium dodecyl sulfate with a Beckman SW41 at 40,000 rpm for 4.75 h. Only peak fractions corresponding to 35S were selected. All further gradient selections were conducted similarly.

The 35S peak was again ethanol precipitated, and polyadenylic acid-containing RNA was selected by using oligodeoxythymidylic acid-cellulose (Collaborative Research type III) as previously described (42). Polyadenylic acid-containing RNA was then purified by zonal centrifugation, and 35S RNA was selected and precipitated. This 35S mRNA was then treated with calf intestine alkaline phosphate to remove the 5'-terminal phosphate. The enzyme was removed by phenol-chloroform extraction, and the mRNA was purified again by sucrose gradient centrifugation. Only peak fractions were selected, yielding approximately 25% of the original polyribosomal mRNA.

5' labeling of mRNA. Nuclease-free polynucleotide kinase was purified by the procedure of Richardson (40). [γ-^{32}P]ATP (~6,000 Ci/mmol) was prepared as previously described (19, 24); 2 to 3 mCi of [γ-^{32}P]ATP was used to label 10 to 15 μg of mRNA by the procedure of Richardson (40). Unreacted ATP was removed from labeled RNA by using a G-100 Sephadex column. RNA eluting in the void volume was precipitated with ethanol and purified by zonal centrifugation. Peak fractions were pooled and used for rapid-sequence analyses.

Enzymatic sequence determination. Enzymatic reactions were carried out using 5 μg of carrier tRNA in 8-μl reaction volumes. Reactions using pancreatic RNase and RNases T_1, U_2, and Phy M, were carried out in 7 M urea–20 mM sodium citrate (pH 5.0)–1 mM EDTA–0.06% bromophenol blue-xylene cyanol as previously described (8). Partial cleavages using *Bacillus cereus* RNase (U+C specific) were carried out in the same buffer, but urea was omitted (29). Additional RNase U_2 and *Staphylococcus aureus* nuclease digests (U+C specific) were conducted under a modification of conditions previously described (23). The modification is inclusion of 20 mM citric acid in the buffer to digest the RNA with RNase U_2 at pH 3.5, at a lower enzyme/substrate ratio. Reactions were terminated after incubation for 15 min at 50°C by adding 4 μl of deionized formamide followed by freezing. Formamide "ladders" were generated by adding deionized formamide to lyophilized RNA and heating the mixture to 100°C in sealed capillaries for 1.5 and 3 h.

RESULTS

It has been shown that under low-monovalent salt conditions a small 5'-terminal fragment of poliovirus RNA can be generated by using RNase III. This RNA fragment, estimated to be 100 nucleotides in length, has been called band 14, based upon its migration in acrylamide gels (16).

A typical digest of poliovirus RNA with RNase III is shown in Fig. 1A. The lowest band, designated as band or fragment 14, has been shown to contain VPg (16), an observation demonstrating that it is a 5'-terminal fragment of the virion genome.

Before conducting further studies, it was necessary to prove that all RNA in band 14 was

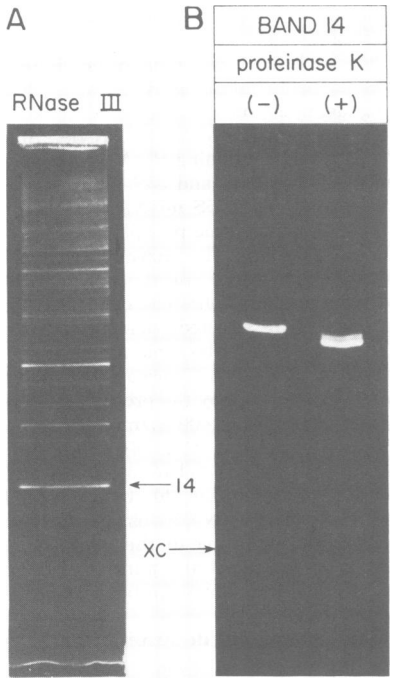

FIG. 1. *Analysis of a 5'-terminal fragment. (A) Virion RNA was digested with RNase III and applied to a 3 to 20% polyacrylamide gradient gel. Electrophoresis was terminated when the bromophenol dye reached the gel bottom and the RNA was stained with ethidium bromide. Band 14 (5'-terminal fragment) is denoted by the arrow. (B) Purified RNA (band 14 in A) was treated (+) with proteinase K and electrophoresed with untreated RNA (-), in a 15% acrylamide-7 M urea-50 mM Tris-borate (pH 8.3)-1 mM EDTA gel. The RNA was visualized by ethidium bromide staining. The xylene cyanol (XC) dye migrated 9.5 cm.*

VPg linked. This was accomplished by treating purified fragment 14 with proteinase K, an enzyme previously shown to degrade RNA-linked VPg (13, 35), which results in a shift in electrophoretic mobility of fragment 14 (Fig. 1B). The double band in Fig. 1B appears to be due to incomplete proteolysis, since higher proteinase K concentrations in the incubation mixture yielded only the lower band (data not shown). Note that all of band 14 is protein linked, since no RNA in the proteinase K-treated lane comigrates with the untreated control, and because under these conditions proteinase K does not alter RNA (35).

Untreated fragment 14 was extracted from gels and labeled with [5'-^{32}P]pCp by using T4 RNA ligase. It was not necessary to dephosphorylate fragment 14 at the 3' end, since RNase III yields only 3' hydroxyls (9). Half of the ligation mixture was again treated with proteinase K, and a similar shift of gel mobility was observed (data not shown).

Purified 3'-labeled fragment 14 was then subjected to rapid enzymatic sequence analysis. An autoradiogram of a sequencing gel is shown in Fig. 4B. Two problems in determining the sequence were encountered. First, it was sometimes difficult to read the gel due to the presence of "doublets." These could be observed at nucleotides 54 to 55 for U, 57 to 58 for G, and 60 to 61 for A. These doublets are likely to be analogous to the "ghost" spots reported by Bruenn and Brennan (6). Second, there were two areas in the autoradiogram where no specific enzymatic cuts were observed. These problems led us to an alternative approach of isolating poliovirus mRNA from polyribosomes of virus-infected HeLa cells.

mRNA: enzymatic sequencing. It has been shown (37) that poliovirus mRNA is not covalently linked to VPg at the 5' end. Instead, the 5' terminus is pUp (37). Therefore, it seemed possible to isolate viral mRNA, dephosphorylate it at the 5' end, and label the newly generated 5' hydroxyl group by using [γ-^{32}P]ATP and polynucleotide kinase. Viral mRNA labeled with $^{32}P_i$ or [^{3}H]guanosine to facilitate detection by scintillation counting was purified and labeled at the 5' end as described above. The RNA was then subjected to partial base-specific digestion, and the products were analyzed by gel electrophoresis.

Each of the labeled mRNA's was first analyzed on 20% polyacrylamide gel to determine whether the known sequence of the 5' end of virion RNA and mRNA (17, 32, 35) could be deduced. The nucleotide ladders concurred with the published sequences (data not shown), an observation demonstrating that only full-length mRNA had been 5' end labeled.

Analyses of the partial digests on 15 and 8% gels are shown in Fig. 2A and B. The interpretation of the autoradiograms was simplified by the discovery that any bands in the sequencing gel not corresponding exactly with the partial formamide ladder were nonspecific, anomalous cuts. This is because both the enzymes and formamide produce oligonucleotides with 2',3'-cyclic phosphate end groups that comigrate (45). Note in Fig. 2A that the Phy M (U+A) cleavages at residues 11, 13, and 18 are usually and reproducibly weak. Nucleotides 11 and 13 correspond correctly to uridylate residues (Table 1). Since residue 18 was not analyzed in a similar manner, we consider it possible but unlikely that nucleotide 18 is a C. In addition, faint bands corresponding to T_1 (G specific) cleavages at nucleo-

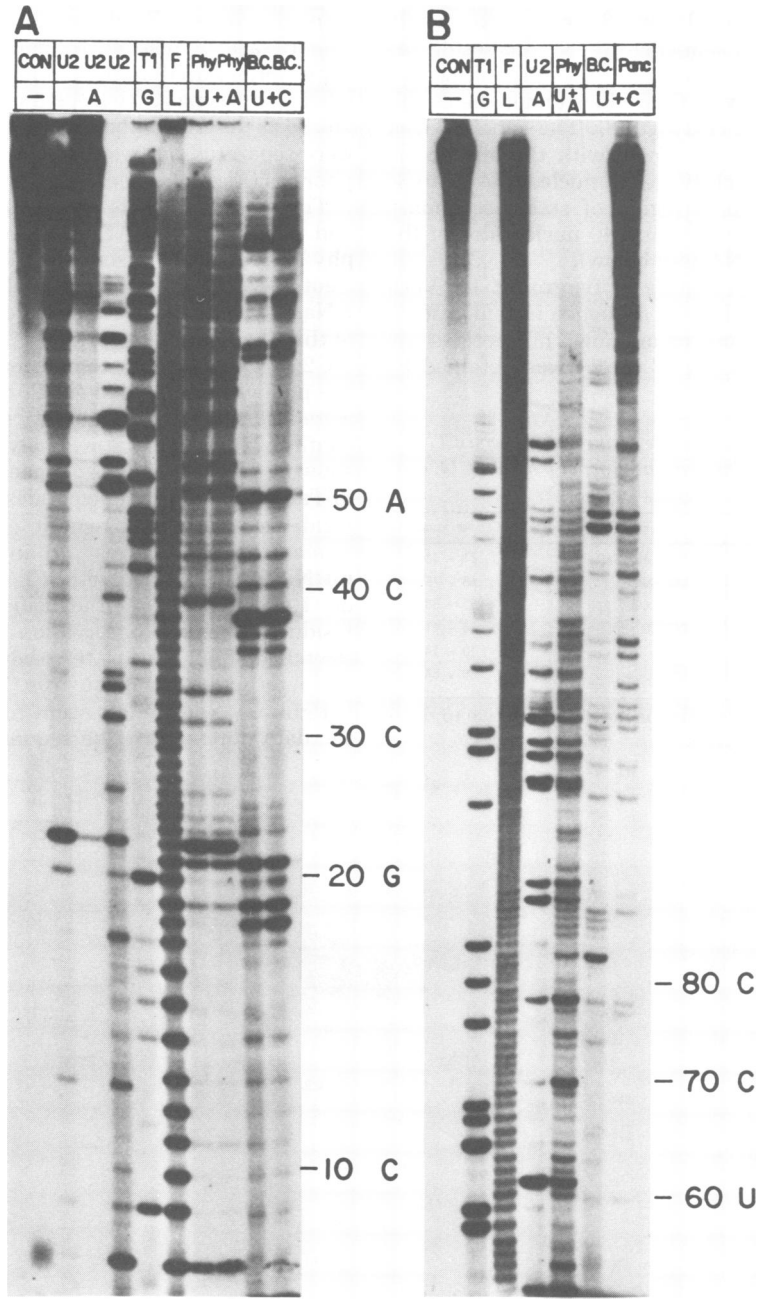

FIG. 2. *Sequencing of [5′-^{32}P]mRNA by partial enzymatic degradation. Shown are autoradiographs of thin (0.4-mm-thick) sequencing gels. The components of the gels were as described in the legend to Fig. 1B, except that the acrylamide/bis ratio was 18:1. Partial base-specific enzymatic cleavage products of end-labeled mRNA were electrophoresed on a 15% acrylamide gel (A), or a similar digest was applied to an 8% acrylamide gel (B). Nucleotide identification and location in relation to the 5′ terminus are indicated to the right of both figures. Indicated above each figure in the bracketed region are the nucleases used, with corresponding specificity. These are, respectively: CON, untreated; U2, A; T_1, G; F, formamide ladder; Phy, Physarum M (U+A); B.C., B. cereus (U+C); Panc (U+C). The fourth lane in A is a U_2 digest at pH 3.5.*

tides 14 to 17, 32, 34, and 35 were reproducibly observed in our sequencing gels. This result was peculiar since RNase T_1 has been shown to cleave faithfully at G residues under the conditions of partial cleavage (8). The generation of very weak bands coupled with the absence of any enzymatic cleavages at nucleotides 27 to 30 suggested to us a region of stable secondary structure within the first 40 nucleotides of the poliovirus mRNA (see below).

RNase T_1 products of fragment 14. Verification of the known sequence and identification of ambiguous or unknown nucleotides (including nucleotides 27 to 30) was obtained by complete RNase T_1 digestion of fragment 14. The RNase T_1-resistant oligonucleotides were dephosphorylated at the 3′ end, followed by 5′ labeling by using [γ-^{32}P]ATP and polynucleotide kinase as described above. The oligonucleotides were separated by two-dimensional gel electrophoresis (Fig. 3A) as described by Lee et al. (25).

The six major spots were eluted with water and purified by DEAE-cellulose chromatography as previously described (21). The oligonucleotides were then further analyzed by partial RNase digestion as described above. An example of this is shown in Fig. 3B for T_1 oligonucleotide no. 2 that corresponds to nucleotides 68 to 75 of the poliovirus RNA. All six T_1 oligonucleotides were also subjected to mobility shift analysis to confirm the enzymatic analyses (data not shown). The resulting sequences are presented in Table 1. Note that nucleotides 27 to 30, which yielded no specific enzymatic cleavages in the sequencing procedures of intact 5′-labeled mRNA, are contained within T_1 oligonucleotide no. 1 and have all been identified as cytidylate residues. A similar observation was made for a stretch of four cytidylate residues found near the 5′ terminus of aphthovirus (15).

TABLE 1. *Nucleotide sequence of the six T_1 oligonucleotides generated from fragment 14, as shown in Fig. 3*[a]

T1 Oligonucleotide	Sequence (5'→3')	Genome Location
#1	UACCCACCCCAG	21 - 32
#2	UACCCUUG	68 - 75
#3	UACUCCG	52 - 58
#4	CCCACG	36 - 41
#5	CUCUG	10 - 14
#6	UAUUG	60 - 64

[a] The genome location is indicated relative to the 5′ terminus of the RNA.

Primary sequence: stem and loop. From the data obtained by the sequencing strategies

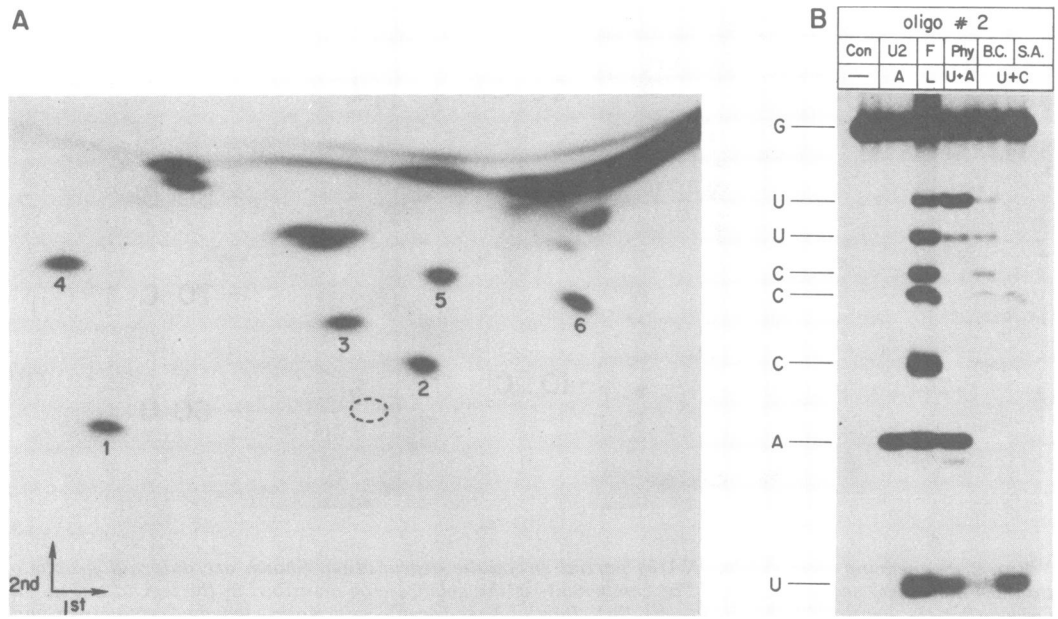

FIG. 3. *Analysis of fragment 14 by complete digestion with RNase T_1. (A) RNase T_1-resistant 5′-^{32}P-labeled oligonucleotides were generated from fragment 14 as described in the text. Shown is an autoradiograph of the two-dimensional gel used to purify these T_1 oligonucleotides. Gel migration in each direction is indicated by the arrows. The dotted oval indicates the position of the bromophenol blue dye. The large oligonucleotides were numbered as indicated. (B) A typical sequencing autoradiogram is shown for T_1 oligonucleotide no. 2. Gel and sequencing conditions were the same as described in the legend to Fig. 2, except that the products were run on a 20% acrylamide gel and, in addition, the U+C-specific enzyme "S.A." (S. aureus) was used.*

described here, the primary sequence for the first 85 nucleotides of the 5' terminus of poliovirus type 1 RNA has been determined (Fig. 4A). The most striking feature of this 5'-terminal sequence is the presence of a stem-and-loop structure beginning nine nucleotides from the 5'-terminal protein, VPg. As drawn, the stem and loop contains 10 base pairs and represents the most energetically favorable structure. The presence of four consecutive G·C base pairs contributes strongly to the stability of the secondary structure, which has a calculated free energy of −21 kcal (ca. − 87.9 kJ)/mol (5, 48).

Evidence for the existence of the stem and loop comes from partial enzymatic cleavage of 3'-pCp-labeled fragment 14. Before addition of the enzymes the substrate was heated at 50°C for 10 min in 7 M urea, yet no enzymatic cleavage was observed in the regions corresponding to the stem structure (nucleotides 9 to 18 and 26 to 36) (Fig. 4B). This indicates that these regions are resistant to RNase and, hence, are base paired. Between these two regions, however, several base-specific cuts were observed. These cuts correspond to the UGUA stretch within the loop. Pyrimidine cuts that make up the remainder of the loop appear faintly in the *S. aureus* lane.

Additional support for the base pairing predicted in the stem structure is based on the unusually weak RNase T_1 cleavages (mentioned above) shown in Fig. 2A. Even after the [5'-^{32}P]mRNA substrate was boiled for 1 min before enzymatic digestion and gel loading, RNase resistance was still prevalent at the RNase T_1 cleavage sites in this region. We conclude that the stem-and-loop structure shown in Fig. 4A is

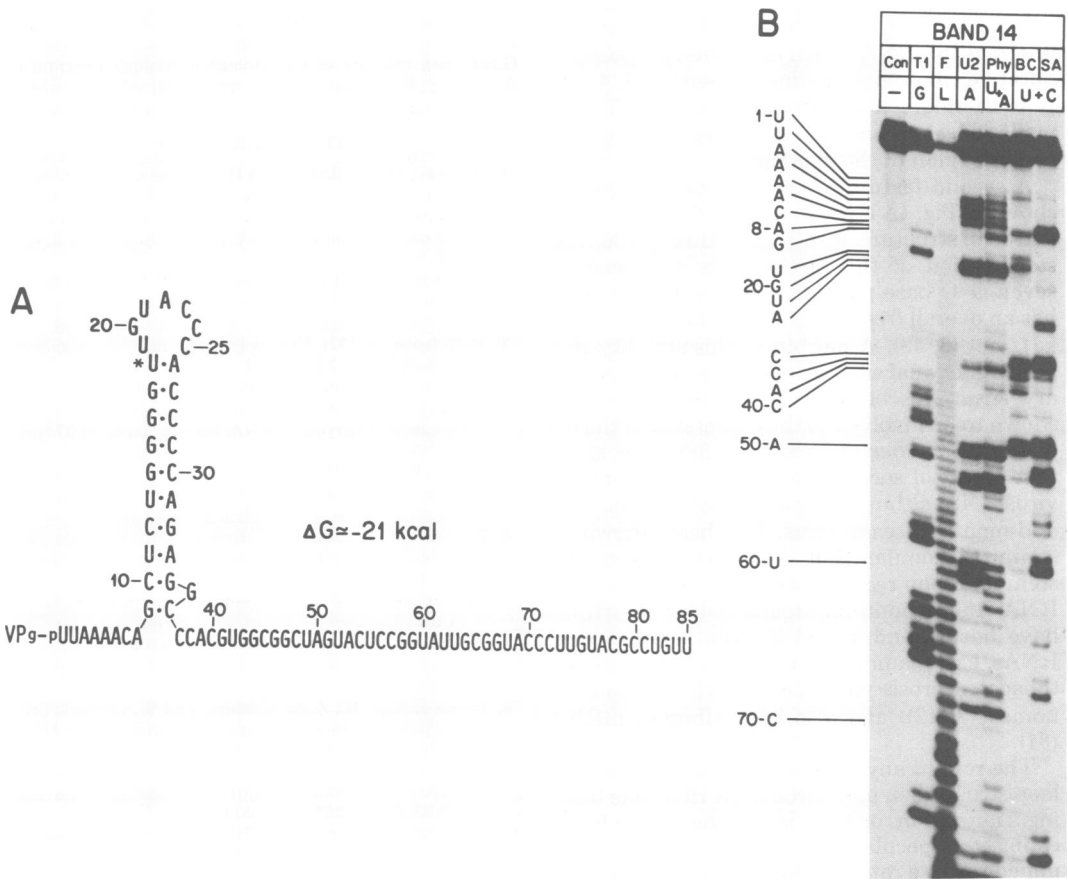

FIG. 4. *The primary and secondary structure of the 5'-terminal fragment of poliovirus RNA. (A) The primary sequence for the first 85 nucleotides of poliovirus RNA. VPg represents the viral protein which is covalently linked to the terminal uridylate residue of genome RNA. The free energy (ΔG) for the stem-and-loop structure drawn is indicated to the right of the hairpin. The asterisk represents a pyrimidine ambiguity. (B) Autoradiogram of a sequencing gel of 3'-pCp-labeled band 14. Sequencing conditions are as described in the text. The gel composition was 15% acrylamide–7 M urea. The nucleotide identification and location, distal to the 5' terminus, are indicated on the side.*

energetically stable and is likely to be an intrinsic property of poliovirus RNA.

DISCUSSION

We have determined the nucleotide sequence for the first 85 nucleotides of the 5′ terminus of poliovirus RNA. The sequencing methods described herein have enabled us to circumvent the problem of the 5′-terminus-linked protein, VPg, which is covalently bound to the 5′-terminal uridylate residue of the virion RNA and renders this resistant to polynucleotide kinase labeling. The sequence obtained for the 5′-terminal fragment generated by RNase III cleavage of poliovirion RNA is shown in Fig. 4A. One obvious feature of the sequence is the absence of an AUG or GUG initiator codon for protein synthesis within the first 85 nucleotides. In addition, there are no hexanucleotide sequences complementary to the 3′ end of eucaryotic ribosomal 18S RNA which have been proposed as putative ribosomal binding sequences (1, 4, 14). It is therefore likely that at least the first 85 nucleotides of poliovirus RNA represent a noncoding region of the genome.

A second feature of the nucleotide sequence shown in Fig. 4A is the presence of a stable stem-and-loop structure at residues 9 through 36. The stem region of this structure is stabilized by seven G·C base pairs, and the entire structure has an overall free energy of −21 kcal (ca. − 87.9 kJ)/mol (5, 48). A hairpin of this stability suggests functional significance since other secondary structures of this free energy have been shown to be involved in the expression of the *trp* operon in *Escherichia coli* (20, 38). This is the first report of such a 5′ structure for an enterovirus RNA, although another picornavirus, foot-and-mouth disease virus, has been shown to contain a similar (but somewhat less stable) stem-and-loop region near the 5′ terminus of its RNA (15). In addition, stem-and-loop structures have been found at the 5′ ends of other viral RNAs, e.g., mammalian retrovirus (30), satellite tobacco necrosis virus (28), the RNA phage genomes (7, 12), and also of ovalbumin mRNA (31).

The role (if any) of the 5′-terminal stem-and-loop structure in poliovirus RNA ribosome binding, translation, or both is unknown. It is interesting to speculate that this structure may impede or be a rate-limiting factor in the binding of ribosomes at the 5′ end of the RNA. This assumes that the ribosome recognizes the 5′ terminus of the poliovirus mRNA, as has been suggested for other mRNA's (22).

Lastly, it should be pointed out that because the very 3′ terminus of polio RNA minus strands is genetically coded by the 5′ end of plus strands (24), a stem-and-loop structure of similar stability will be present near the 3′ terminus of minus strands. Minus strands are found as templates for newly synthesized plus strands in replicative intermediate structures as well as the double-stranded replicative form. It will be important to determine whether the hairpin structure is involved in poliovirus RNA synthesis through an interaction with replicase molecules at the 3′ ends of RNA templates (minus strands) or in the displacement of newly synthesized minus strands from replicative intermediates.

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