Covalent Linkage of a Protein to a Defined Nucleotide Sequence at the 5'-Terminus of Virion and Replicative Intermediate RNAs of Poliovirus

James B. Flanegan, Ralf F. Pettersson, Victor Ambros, Martinez J. Hewlett, and David Baltimore

PNAS 1977;74;961-965
doi:10.1073/pnas.74.3.961

Proc. Natl. Acad. Sci. USA
Vol. 74, No. 3, pp. 961–965, March 1977
Biochemistry

Covalent linkage of a protein to a defined nucleotide sequence at the 5′-terminus of virion and replicative intermediate RNAs of poliovirus

(picornaviruses/RNA synthesis/paper ionophoresis/mRNA)

JAMES B. FLANEGAN, RALF F. PETTERSSON, VICTOR AMBROS, MARTINEZ J. HEWLETT*, AND DAVID BALTIMORE

Department of Biology and Center for Cancer Research, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge, Mass. 02139

Contributed by David Baltimore, December 27, 1976

ABSTRACT **The 5′-terminus of poliovirus polyribosomal RNA is pUp. A candidate for the 5′-terminus of poliovirion RNA was recovered as a compound migrating toward the cathode when ^{32}P-labeled virion RNA was completely digested with ribonucleases T_1, T_2, and A and analyzed by paper ionophoresis at pH 3.5. Treatment with proteinase K reversed its direction of migration, indicating the presence of protein. Treatment with venom phosphodiesterase liberated all of the radioactivity as pUp, suggesting that poliovirion RNA has a protein-pUp 5′-terminus. Treatment of virion RNA with T_1 ribonuclease alone generated a proteinase K-sensitive oligoribonucleotide. Analysis of the oligoribonucleotide using ribonucleases A and U_2 showed its structure to be protein-pU-U-A-A-A-A-C-A-G. Digests of replicative intermediate RNA contained sufficient protein-pUp to suggest that this structure is at the 5′-end of most nascent poliovirus RNA molecules. We suggest that a protein-nucleotide structure acts as a primer for initiating synthesis of poliovirus RNA.**

Although the $m^7G^{5'}ppp^{5'}N(m)pNp$ "capping" group has been found on virtually all known mammalian mRNAs (1), poliovirus polyribosomal RNA has pUp at its 5′-end (2–4). Because poliovirion RNA will direct viral protein synthesis in a cell-free system (5), it was thought to be identical to polyribosomal poliovirus RNA. Instead, we found that less than 10% of the virion molecules contained a pUp 5′-end (2).

Lee *et al.* (6) recently presented evidence that a protein might be linked to the 5′-terminus of poliovirion RNA. We also have obtained evidence for a 5′-terminal protein in virion RNA. When total RNase digests of ^{32}P-labeled virion RNA were examined by paper ionophoresis at pH 3.5, some labeled material was found moving toward the cathode, the direction opposite that of pure nucleotides. This material had the properties of a protein-pUp 5′-terminus of the RNA, a structure also suggested by Lee *et al.* (6). We have further found that cellulose acetate electrophoresis of a RNase T_1 digest of virion RNA separates a protein-linked oligonucleotide. We show here that it consists of the structure protein-pU-U-A-A-A-A-C-A-G, which appears to be the 5′-terminus of poliovirion RNA. The protein is also found on replicative intermediate RNA, suggesting that it represents an initiating structure for viral RNA synthesis.

MATERIALS AND METHODS

Preparation of Poliovirus RNA. Poliovirus-specific RNA labeled with carrier free [^{32}P]orthophosphate (New England Nuclear Corp.) was prepared by infecting actinomycin D-treated HeLa cells with poliovirus type 1 as described (2). To prepare ^{32}P-labeled virions, cells were harvested 6-hr after infection, washed once with Earle's saline, and broken by suspension in cold 1% Nonidet P-40/10 mM Tris·HCl (pH 7.5)/10 mM NaCl/1.5 mM $MgCl_2$. The nuclei were immediately removed by centrifugation (5,000 × *g*, 5 min) and the virions were collected by centrifugation at 45,000 rpm for 2 hr in a type 65 Spinco rotor. The virions were purified by sucrose gradient centrifugation (7), and the RNA was extracted with either 0.5% sodium dodecyl sulfate ($NaDodSO_4$)/0.1 M acetic acid (pH 3.5) (7) or with 0.5% $NaDodSO_4$/phenol/chloroform/isoamyl alcohol (25:24:1) (7). The RNA was purified by sucrose gradient centrifugation and precipitated by ethanol (7). ^{32}P-Labeled replicative intermediate RNA isolated from a cytoplasmic extract of infected cells harvested at 3.5 hr was purified by precipitation with 2 M LiCl, phenol extraction, and chromatography on Sepharose 2B (7). The purified replicative intermediate RNA did not contain any labeled single-stranded 35S RNA when analyzed by electrophoresis on a 1% agarose gel (8).

Enzymatic Digestions. Polypropylene tubes and pipettes were used for all enzymatic digestions. Limit digestion of RNA with RNases T_1, T_2, and A was for 1 hr at 37° in 15 μl of 0.05 M ammonium acetate (pH 5.0) containing 10 μg of RNA, 40 μg of bovine serum albumin carrier protein (electrophoretically pure, Sigma), 5 units/ml of RNase T_2 (Calbiochem), 200 units/ml of RNase T_1 (Calbiochem), and 150 units/ml of pancreatic RNase A (Worthington Biochemicals). Limit digestion of RNA was also performed at 37° for 30 min in 5 μl of 6 mM sodium acetate (pH 4.5)/0.6 mM EDTA containing 30 μg/ml of heat-treated takadiastase extract (from Calbiochem; Sanzyme-R) and 0.42 mg/ml of RNase A. The takadiastase extract was prepared as described (9) except 1 mM EDTA was added during the final dialysis step and the extract was used without lyophilization. Reaction conditions for RNase U_2 (Calbiochem) (0.1 unit/ml) (10), RNase T_1 (11), RNase A (11), bacterial alkaline phosphatase (Boehringer Mannheim) (12), and venom phosphodiesterase (Boehringer Mannheim) (12) were as described. Bovine serum albumin (30 μg) was added when samples were digested with RNase A. Bovine serum albumin was also present in the RNase U_2 digests (10).

Digestion with proteinase K was for 1 hr at 37° in a 100-μl solution of 0.5% $NaDodSO_4$/0.1 M NaCl/0.01 M Tris·HCl (pH 7.5)/0.001 M EDTA/proteinase K (200 μg/ml) (EM Laboratories). The RNA was then extracted with phenol/chloroform/isoamylalcohol (25:24:1) and precipitated with ethanol as described (7).

Analytical Separations. Ionophoretic separations on cellulose acetate, DEAE-paper, and Whatman 3MM paper at pH 3.5 have been described (10, 12). Compounds labeled with ^{32}P were located by autoradiography with Kodak No-Screen Film. La-

Abbreviations: $NaDodSO_4$, sodium dodecyl sulfate; P_1, *Penicillium* nuclease.

* Present address: Department of Cellular and Developmental Biology, University of Arizona, Tucson, Ariz. 85721.

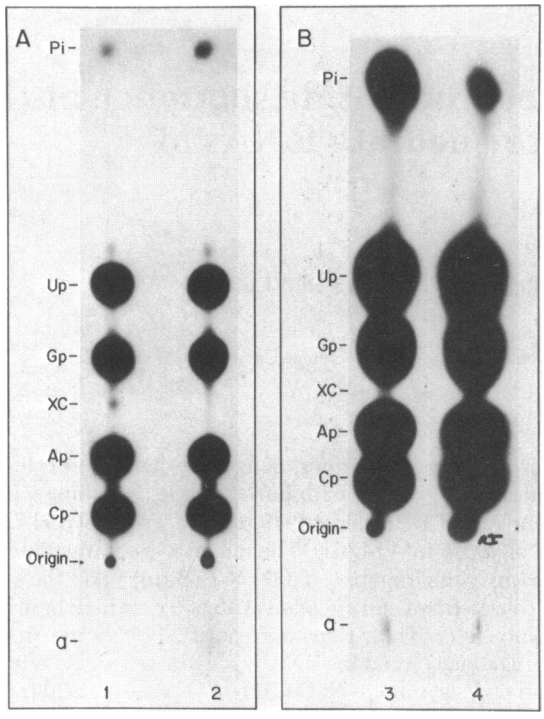

FIG. 1. Isolation of a proteinase K-sensitive structure from ^{32}P-labeled poliovirion and replicative intermediate RNA. *A*. Equivalent amounts of labeled virion RNA with (lane 1) and without (lane 2) prior treatment with proteinase K were digested to completion with RNases T_1, T_2, and A. Digested samples were spotted on Whatman 3 MM paper and subjected to pH 3.5 ionophoresis at 30 V/cm until the blue dye, xylene cyanol (XC), had moved about 8 cm. The labeled products were located by subsequent autoradiography. *B*. An equivalent amount of labeled virion and replicative intermediate RNA was dissolved in 100 μl of water, heated to 100° for 2 min, and then rapidly frozen and lyophilized. The RNA was digested with a takadiastase-RNase A mixture and subjected to ionophoresis as in part *A* until the XC marker was 4.5 cm from the origin. Labeled products from virion RNA (lane 3) and replicative intermediate RNA (lane 4) were located by autoradiography.

beled compounds were quantitated by cutting out spots from the paper and measuring radioactivity either by Cherenkov counting or by counting in a toluene-based scintillation fluid (Econofluor, New England Nuclear Corp.) using a Beckman LS-330 scintillation spectrometer.

RESULTS

Identification of a Candidate 5′-End. In our previous analysis of the digestion products of poliovirion RNA using glass capillaries and analyzing products by electrophoresis on DEAE-paper, the 5′-terminus was not identified (2). To reinvestigate the problem, ^{32}P-labeled poliovirion RNA was digested with RNases T_1, T_2, and A and the products were separated by ionophoresis at pH 3.5 on Whatman 3 MM paper (Fig. 1*A*). All manipulations used plasticware. Along with the expected four nucleoside monophosphates migrating toward the anode, there was a broad smear of radioactivity that moved toward the cathode (marked *a* in Fig. 1*A*, lane 2). This was the only candidate 5′-end because all of the other radioactivity in the digest could be accounted for by inorganic phosphate (P_i), some cyclic mononucleotides (e.g., just over the Up spot), and radioactivity that remained fixed to the origin and that gave rise to mononucleotides after elution and redigestion.

When spot *a* was quantitated in three separate experiments, values of 0.026, 0.027, and 0.028% of the total radioactivity in the digest were obtained. This agrees well with the theoretical molar yield of 0.027% for two phosphates out of 7500 (assuming uniform labeling and a molecular weight of 2.5×10^6; ref. 13). As will be discussed later, the specific radioactivity of the phosphates associated with the four ribonucleosides was not the same and the almost exact agreement with the theoretical value for two phosphates was fortuitous. Nevertheless the yield of spot *a* suggests that it contains approximately two phosphates out of the 7500 in the RNA. This yield of spot *a* also agrees with the average yield of 0.027% for pUp from poliovirus polyribosomal RNA (2).

When virion RNA was treated with proteinase K prior to RNase digestion, spot *a* was no longer found (Fig. 1*A*, lane 1). Instead, a new spot with a varying mobility close to that of the xylene cyanol dye marker was observed. The protease sensitivity of spot *a* suggests that it consists of a protein attached to a labeled nucleotide. The protein is apparently covalently linked to virion RNA because spot *a* was recovered after the RNA was subjected to treatments that normally disrupt noncovalent protein-nucleic acid interactions. Treatment of virion RNA with 0.5% $NaDodSO_4$/0.1 M acetic acid/7 M urea at pH 3.5, repeated extraction with phenol/chloroform/isoamylalcohol (25:24:1), or boiling at 100° for 2 min did not significantly reduce the yield of the protein-linked nucleotide from 35S RNA separated in sucrose gradients. Care was needed, however, to insure that the material in spot *a* was not lost during handling. Significant losses were observed when any glass apparatus was used or when carrier protein was not added to the solution of RNA digestion products.

When equal amounts of radioactivity in virion and replicative intermediate RNA were completely digested to 3′-monophosphates, an equivalent yield of spot *a* was obtained from both samples (Fig. 1*B*). A discussion of the expected yield of a 5′-terminal end for replicative intermediate RNA will be given later. Both RNAs were heated to 100° for 2 min and quickly cooled prior to digestion to help insure the complete digestion of the replicative intermediate RNA.

Analysis of the 5′-End. Spot *a* (Fig. 1*A*) was analyzed to determine its nucleotide composition. The labeled material was eluted from the paper and analyzed by paper ionophoresis at pH 3.5 after no additional treatment, digestion with proteinase K, or digestion with venom phosphodiesterase. As expected, the undigested material again migrated toward the cathode (Fig. 2*A*, lane 2). Proteinase K removed the cathodal material and generated a new compound designated as spot *b* (Fig. 2*A*, lane 3), which migrated with approximately the same mobility as the xylene cyanol dye marker. A compound with the same mobility was seen after treatment of virion RNA with proteinase K (Fig. 1*A*, lane 1). After treatment with venom phosphodiesterase, an exonuclease that will digest from a nonphosphorylated or phosphorylated 3′-end (10), a labeled compound with the mobility of pUp was the only evident product (Fig. 2*A*, lane 4). Elution of spot *b* and digestion with either P_1 nuclease or alkaline phosphatase (a nonspecific phosphomonoesterase; ref. 15) released exactly half of the radioactivity as free phosphate and half as a new compound designated as spot *c* (Fig. 2*A*, lanes 5 and 6). P_1 nuclease cleaves 3′,5′-phosphodiester bonds in polynucleotides generating 5′-monophosphates without regard to base or ribose modifications and cleaves the phosphate away from 3′-phosphomonoesters of nucleosides (16, 17). Elution of spot *c* and treatment with venom phosphodiesterase generated pU as the only labeled product (Fig. 2*B*, lane 8).

The above results are consistent with the material in spot *a* being [protein-pUp] and the material in spot *b* and spot *c* being [(amino acid)$_n$-pUp] and [(amino acid)$_n$-pU], respectively. No

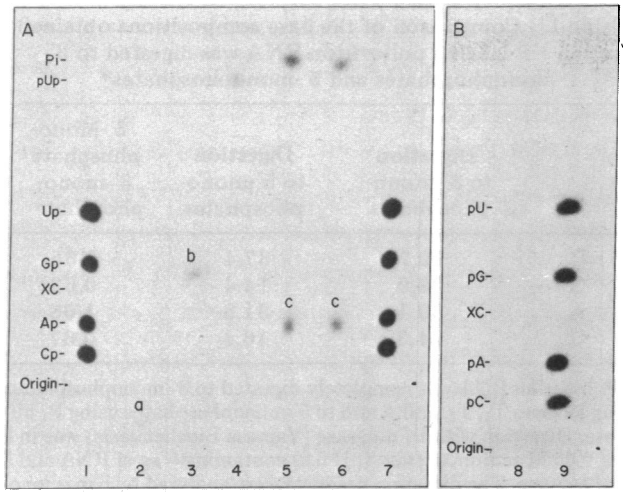

FIG. 2. Components of the 5′-terminal protein-nucleotide complex. *A*. Analysis of spot *a* (Fig. 1*A*) by paper ionophoresis at pH 3.5 for nucleotide components. Spot *a* in Fig. 1*A* was eluted from the paper with water and rerun without additional treatment (lane 2), after digestion with proteinase K (lane 3), or after digestion with venom phosphodiesterase (lane 4). Ribonucleoside 3′-monophosphate markers in lane 1 were run simultaneously with lanes 2, 3, and 4. Spot *b* in lane 3 was eluted and rerun after digestion with *Penicillium* (P_1) nuclease (lane 5) or bacterial alkaline phosphatase (lane 6). Lane 7 shows 3′-monophosphate markers for lanes 5 and 6. Conditions for P_1 nuclease digestion were as described in ref. 14. *B*. Identification of ribonucleoside 5′-monophosphate component of spot *c*. Spot *c* from lane 5 was eluted and rerun after digestion with venom phosphodiesterase (lane 8). Ribonucleoside 5′-monophosphate markers are shown in lane 9.

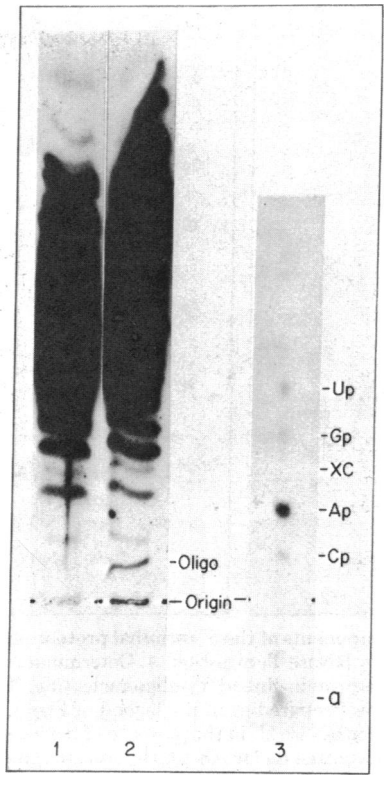

FIG. 3. Isolation and base composition of a proteinase K-sensitive T_1-oligonucleotide from ^{32}P-labeled poliovirion RNA. Poliovirion RNA with (lane 1) and without (lane 2) prior treatment with proteinase K was digested to completion with RNase T_1. Digested samples were spotted on cellulose acetate strips (3 × 55 cm) and subjected to pH 3.5 ionophoresis at 5500 V for 30 min. Lane 3 shows an autoradiogram of pH 3.5 paper ionophoresis of a limit takadiastase-RNase A digest of the T_1-oligoribonucleotide marked in lane 2. The region on the cellulose acetate strip containing the oligoribonucleotide was cut out and the labeled material was transferred to DEAE-paper by methods similar to those described by Barrell (10). The DEAE-paper was washed in ethanol, and the labeled oligoribonucleotide was eluted using 30% triethylamine bicarbonate (TEAB) as described (10). The TEAB was removed by lyophilization and the oligoribonucleotide was digested with a takadiastase-RNase A mixture and subjected to paper ionophoresis as in Fig. 1*A*.

other labeled phosphates appear to be attached to the protein. The size of the protein and the exact nature of the linkage between the protein and pUp have not been investigated.

Isolation of a Protein-Linked T_1-Oligoribonucleotide. Poliovirion RNA was digested to completion with RNase T_1 and analyzed by ionophoresis at pH 3.5 on cellulose acetate strips to detect an oligoribonucleotide that might be protease sensitive. An oligoribonucleotide was found proximal to the origin that was absent if the RNA was treated with proteinase K prior to RNase T_1 digestion (Fig. 3). The disappearance of this band in the autoradiogram was the only observed change caused by proteinase K, and was reproducible.

The protein-linked oligoribonucleotide was eluted from the cellulose acetate strip, digested to completion with a takadiastase-RNase A mixture (or RNase T_1, T_2, and A in other experiments), and analyzed by pH 3.5 paper ionophoresis. Heat-treated takadiastase extracts contain RNases T_1 and T_2 and will specifically digest RNA to 3′-mononucleotides (9). All four ribonucleoside 3′-monophosphates were obtained along with labeled material (designated spot *a*) that migrated toward the cathode (Fig. 3, lane 3). Elution of the labeled material in spot *a* and digestion with venom phosphodiesterase again yielded pUp (as determined by its mobility when analyzed by paper ionophoresis). We therefore conclude that the proteinase K-sensitive oligonucleotide is derived from the 5′-end of poliovirion RNA.

Sequence of Protein-Linked Oligoribonucleotide. To determine its structure, we digested the protein-linked oligoribonucleotide with RNase A and analyzed the products by pH 3.5 ionophoresis on DEAE-paper (Fig. 4*A*). Products labeled A_4C, AG, and U were identified by their mobility relative to marker oligoribonucleotides as well as by base composition analysis (Fig. 4*B*). To clearly identify A_4C, it was necessary to run a longer ionophoresis (30 V/cm, 5 hr) of the digestion products (data not shown). The labeled material marked as spot *d* was shown to contain the 5′-end protein because it migrated toward the cathode when eluted and analyzed by paper ionophoresis (Fig. 4*B*, lane 5). There was no material migrating toward the cathode when the other spots (A_4C and AG) were analyzed. The fact that the material in spot *d* does not migrate as a distinct spot and that it ran to the side of the other oligonucleotides in lane 1, Fig. 4*A*, appears to be a result of uneven wetting of the DEAE-paper before ionophoresis. Apparently the protein-linked oligonucleotide was not as firmly bound as the pure oligonucleotides to the DEAE residues on the paper and so moved slightly out of the lane. Similar behavior of a protein-linked oligonucleotide is evident in lane 2 (see below).

To determine the order of the RNase A digestion products, we digested the T_1-oligoribonucleotide with the purine-specific RNase U_2 (Fig. 4*A*, lane 2). The products found included G and A (not resolved but evident by further analysis), CA, AA [due to some internal cuts by RNase U_2 in the oligo(A) stretch], and spot *e*. The composition of the RNase U_2 products was shown to correspond to the indicated structures by analysis of complete digestion products (Fig. 2*B*, lanes 6–8). Spot *e* did not move

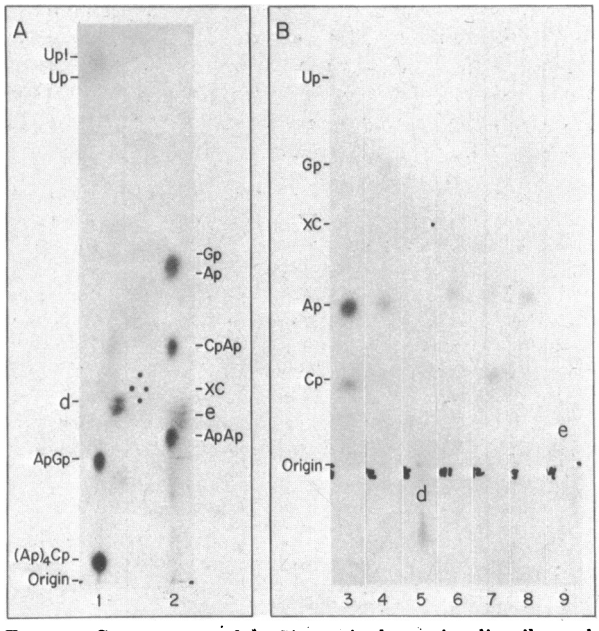

FIG. 4. Components of the 5′-terminal protein-oligoribonucleotide generated by RNase T_1 digestion. *A*. Determination of nucleotide sequence in the protein-linked T_1-oligonucleotide. The T_1-oligoribonucleotide was prepared as in the legend of Fig. 3, digested with either RNase A or RNase U_2 in the presence of bovine serum albumin carrier protein, spotted on DEAE-paper, and analyzed by pH 3.5 ionophoresis at 20 V/cm for 3 hr. The identity of marker oligoribonucleotides comigrating with the RNase A digestion products (lane 1) were identified by their known mobilities (10). Markers for the RNase U_2 products (lane 2) were identified by direct determination of their base composition and by comparison with a previous analysis of RNase U_2 digestion products at pH 3.5 (C. Squires and J. Rose, unpublished data). Up! is uridine 2′:3′-cyclic phosphate. *B*. Analysis of spots *d* and *e* by paper ionophoresis at pH 3.5 and the base composition of oligoribonucleotides in panel *A*. Spots *d* and *e* were eluted and dissolved in 5 μl of a bovine serum albumin solution (10 mg/ml) and spotted on Whatman 3MM paper. The oligoribonucleotides were eluted and digested with RNases T_1, T_2, and A. Paper ionophoresis (as described in legend of Fig. 1*A*) of A_4C, AG, spot *d*, AA, CA, A plus G, and spot *e* are shown in lanes 3, 4, 5, 6, 7, 8, and 9, respectively.

from the region of the origin during ionophoresis on Whatman 3 MM paper (Fig. 4*B*, lane 9) and therefore appeared to represent the protein-linked oligonucleotide(s) left by RNase U_2.

These data show that the sequence of the protein-linked T_1-oligonucleotide must be protein-pU-U-A-A-A-A-C-A-G. The four RNase A digest products found are consistent with this structure, and the recovery of CA after RNase U_2 digestion shows that the A_4C must be next to the AG. The protein could only be at the 5′-terminus linked by pUp to the rest of the oligonucleotide.

Varying Specific Radioactivity in ^{32}P-labeled Poliovirus RNA. To quantitatively analyze the digestion products it was necessary to determine if the phosphates associated with each of the four ribonucleosides in poliovirus [^{32}P]RNA had the same specific radioactivity. To this end the base compositions obtained by digestion to 3′-monophosphates were compared to the results obtained by digestion to 5′-monophosphates with P_1 nuclease. When RNA is digested to 3′-monophosphates, each nucleotide is labeled with the phosphate of its nearest neighbor and thus differences in specific radioactivity are averaged out. Digestion of RNA to 5′-monophosphates, however, yields nucleotides labeled with their original α-phosphates and any differences in specific radioactivity will result in a base composition different from that obtained by digestion to 3′-monophosphates. Differences between the two digestion products were evident for the poliovirus RNA used in this study, which was labeled throughout infection with ^{32}P (Table 1). The ratio of the values obtained from 5′-phosphates to the values obtained from 3′-phosphates reflects the ratio of specific radioactivity of a given nucleoside 5′-phosphate relative to the average specific radioactivity for all phosphates in the RNA. Thus, the specific radioactivity of pU is 1.65 times higher than the average and 2.6 times greater than the specific radioactivity of pG.

Table 1. Comparison of the base compositions obtained when ^{32}P-labeled poliovirion RNA was digested to 3′-monophosphates and 5′-monophosphates*

	Digestion to 3′-monophosphates	Digestion to 5′-monophosphates	5′-Monophosphate/3′-monophosphate
U	22.7	37.4	1.65
G	22.9	14.4	0.63
A	30.1	31.8	1.06
C	24.3	16.4	0.67

Poliovirion RNA was completely digested to 3′-monophosphates using RNases T_1, T_2, and A and to 5′-monophosphates using P_1 nuclease. Digestion with P_1 nuclease (Yamasa Biochemicals) was in 5 μl of 0.05 M sodium acetate (pH 5.0) containing 5 μg of RNA and 5 μg of enzyme. The digestion products were analyzed by paper ionophoresis as in Fig. 1.
* Values given are percent of total cpm.

To be certain that two residues of uridylic acid were part of the sequence and that the Up recovered after RNase A digestion (Fig. 4*A*, lane 1) and takadiastase-RNase A digestion (Fig. 3, lane 3) was not derived by partial digestion of protein-pUp, the yields of the limit digestion products in Fig. 3, lane 3 (i.e., 3′-monophosphates) were corrected for the relative specific radioactivities given in Table 1. From all of the nearest neighbors in the sequence described above (except for Gp), the theoretical yields for Up and protein-pUp were calculated. This calculation predicted that 19% of the radioactivity in Ap and Cp residues would be found in Up (if pA was its nearest neighbor). An experimental value of 21 ±3% (three experiments) was obtained. A similar determination for the yield of protein-pUp (with pU as its nearest neighbor) gave a theoretical value of 60% and an experimental value of 27 ±6%. These calculations are consistent with two residues of uridylate being part of the sequence, one of which is recovered in molar yield, the other of which, being protein-linked and very susceptible to losses during handling, is recovered in about 50% yield. This conclusion is also strengthened by the results in Fig. 2, which show that treatment of the labeled material in spot *b* [(amino acid)$_n$-pUp] with P_1 nuclease or alkaline phosphatase released exactly half of the counts, suggesting that its nearest neighbor was pU.

DISCUSSION

The results in this paper indicate that the 5′-terminal end of poliovirion RNA has the following structure: protein-pU-U-A-A-A-A-C-A-G. The nature of the linkage between the protein and the RNA has not yet been determined, but the protein was not removed by conditions that normally disrupt noncovalent protein-nucleic acid interactions. The ability of venom phosphodiesterase to release pUp from protein association is the strongest argument for a covalent link of protein to the 5′-end of viral RNA.

The results of Lee *et al.* (6) also suggest that the 5′-terminal protein is covalently linked to pUp. A difference from the

present report is their finding that a very large amount of P_1 nuclease (100 μg) could remove pU and P_i from trace amounts of protein-pUp, while we found that sufficient P_1 nuclease (0.05 μg of nuclease per 5 μg of total RNA) to digest all other 3',5'-phosphodiester bonds in RNA (14) was unable to remove the pU although it could remove P_i. Lee *et al.* (6) suggest from their results and the known specificity of P_1 nuclease that there might be a nucleoside between the protein and pUp. Our results make this possibility seem less likely unless the nucleoside is modified in such a way as to make it very resistant to P_1 digestion.

The identification of protein-pUp in replicative intermediate RNA at the same relative concentration as in virion RNA implies that protein-pUp is on many of the nascent chains. The replicative intermediate RNA has a mass about four times that of virion RNA (a minus strand plus six nascent chains, each nascent chain averaging one-half of a finished molecule; ref. 18). There must therefore be about four protein-pUp moieties per replicative intermediate. At most, one protein-pUp could be on the minus strand so that the others must be on nascent chains. We cannot at the present exclude the possibility that some of the nascent chains lack the protein.

Finding protein on the nascent RNA suggests that protein-pU (or an elongated form) might serve as a primer for initiating synthesis of poliovirus RNA. The requirement for such a unique priming mechanism by the poliovirus RNA polymerase is supported by the recent isolation of a poliovirus-specific RNA polymerizing enzyme from infected HeLa cells that will begin synthesis on poly(A) only if provided with a preformed primer [oligo(U) in this circumstance] (J. B. Flanegan and D. Baltimore, unpublished data). The model of a protein-oligonucleotide primer for initiating RNA synthesis may also be relevant to certain types of cellular RNA synthesis. The occurrence of A-A-C-A-G at the 5'-end of vesicular stomatitis virus mRNAs (J. Rose, unpublished observations; and ref. 19), the same sequence we find in the 5'-terminus of poliovirus RNA, raises the possibility of a common initiation mechanism for various types of viral RNA synthesis.

Assuming that all poliovirus RNA synthesis is initiated by a protein-nucleotide, the occurrence of a molar yield of pUp in polyribosomal poliovirus mRNA requires that the protein be removed after synthesis of the RNA. To determine whether the protein is removed neat or is removed as protein-pU (or even protein-oligonucleotide) will require sequence analysis of the 5'-terminal oligonucleotide of mRNA.

The presence of a protein at the 5'-end of the RNA may be a prerequisite to its packaging. This could explain why polyribosomal RNA does not function as a precursor for virion formation (18). The protein, however, does not appear to be required to initiate an infection. When the infectivity of purified RNA was tested in four experiments, average values of 1.55 $\times 10^4$ plaque-forming units/μg and 1.65 $\times 10^4$ plaque-forming units/μg were obtained for virion and polyribosomal RNA, respectively (M. J. Hewlett, V. Ambros, and D. Baltimore, unpublished data). This contrasts with the decreased infectivity that was observed when the poly(A) stretch was removed from the 3'-end of poliovirus RNA (20).

We are grateful to Dr. Eckard Wimmer for communicating to us results from his laboratory prior to their publication. We thank Dr. John Rose for many invaluable suggestions that greatly assisted in the completion of this work. Helpful suggestions by Dr. Naomi Guttman and the technical assistance of Kahan Smith are gratefully acknowledged. This work was supported by Grants AI08388, CA 12174, and CA 14051 from the National Institutes of Health. J.B.F. is the recipient of a National Institutes of Health Postdoctoral Fellowship F32 AI05179. R.F.P. is the recipient of a Public Health Service International Fellowship F05 TW-2245. M.J.H. was a postdoctoral fellow of the American Cancer Society. D.B. is a Research Professor of the American Cancer Society.

1. Griffin, B. (1976) *Nature* **263**, 188–190.
2. Hewlett, M. J., Rose, J. K. & Baltimore, D. (1976) *Proc. Natl. Acad. Sci. USA* **73**, 327–330.
3. Nomoto, A., Lee, Y. F. & Wimmer, E. (1976) *Proc. Natl. Acad. Sci. USA* **73**, 375–380.
4. Fernandez-Munoz, R. & Darnell, J. E. (1976) *J. Virol.* **18**, 719–726.
5. Villa-Komaroff, L., Guttman, N., Baltimore, D. & Lodish, H. F. (1975) *Proc. Natl. Acad. Sci. USA* **72**, 4157–4161.
6. Lee, Y. F., Nomoto, A., Detjin, B. M. & Wimmer, E. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 59–63.
7. Spector, D. H. & Baltimore, D. (1975) *J. Virol.* **15**, 1418–1431.
8. Hewlett, M. J., Rozenblatt, S., Ambros, V. & Baltimore, D. (1977) *Biochemistry*, in press.
9. Niramaru, M., Uchida, T. & Egami, F. (1966) *Anal. Biochem.* **17**, 135–142.
10. Barrell, B. G. (1971) in *Procedures in Nucleic Acid Research*, eds. Cantoni, G. L. & Davies, D. R. (Harper and Row, New York), Vol. II, pp. 751–828.
11. Rose, J. K. & Knipe, D. (1975) *J. Virol.* **15**, 994–1003.
12. Rose, J. K. (1975) *J. Biol. Chem.* **250**, 8098–8104.
13. Granboulan, M. & Girard, M. (1969) *J. Virol.* **4**, 475–479.
14. Rose, J. K. & Lodish, H. F. (1976) *Nature* **262**, 32–37.
15. Stadtman, T. C. (1961) in *The Enzymes*, eds. Boyer, P. A., Lundy, H. & Myback, K. (Academic Press, Inc., New York), Vol. 5, 2nd ed., p 55.
16. Fujimoto, M., Kuninaka, A. & Yoshino, H. (1974) *Agr. Biol. Chem.* **38**, 1555–1561.
17. Fujimoto, M., Fujyama, K., Kuninaka, A. & Yoshino, H. (1974) *Agr. Biol. Chem.* **38**, 2141–2147.
18. Baltimore, D. (1969) in *Biochemistry of Viruses*, ed. Levy, H. B. (Marcel Dekker, New York and London), pp. 101–176.
19. Rhodes, D. P. & Banerjee, A. K. (1976) *J. Virol.* **17**, 33–42.
20. Spector, D. H. & Baltimore, D. (1974) *Proc. Natl. Acad. Sci. USA* **71**, 2983–2987.