

The 5'-terminal structures of poliovirion RNA and poliovirus mRNA differ only in the genome-linked protein VPg*

(two-dimensional gel electrophoresis/5'-terminal oligonucleotide/nucleotide sequence/proteinase K/transfection)

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ABSTRACT The 5'-terminal, RNase T1-resistant oligonucleotide of poliovirus mRNA has been isolated. Its sequence is pU-U-A-A-A-C-A-Gp, which is identical to that of virion RNA except that the genome-linked protein VPg is absent [Nomoto, A., Detjen, B., Pozzatti, R. & Wimmer, E. (1977) *Nature* 268, 208-213]. Because all newly synthesized viral RNAs are VPg-linked, we propose that VPg is cleaved from progeny RNA at the linkage between protein and nucleic acid prior to polyribosome formation. This may represent a new mode of processing of viral macromolecules. Virion RNA from which VPg has been cleaved proteolytically retains its specific infectivity, an observation suggesting that VPg is not involved in early steps (penetration and translation) of the infectious cycle initiated by RNA.

The genome of poliovirus, a plus-strand RNA virus (1), is covalently linked to a small basic protein, which we have called VPg (2-4). VPg is attached to the 5'-terminal uridine of the single-stranded RNA via an unknown linkage group (5).

After penetration into the host cell, the RNA of the infecting poliovirion is thought to serve a dual function: first as mRNA in virus-specific protein synthesis and second as template in RNA replication (6). Poliovirus mRNA, that is, viral RNA isolated from polyribosomes of infected cells, has previously been considered to be identical to virion RNA because mRNA and virion RNA have the same sedimentation coefficient of 35 S, have the same base composition (7, 8), and yield indistinguishable fingerprints (9). In contrast to virion RNA, however, the 5' end of poliovirus mRNA is not linked to a protein. Poliovirus mRNA begins with pUp (9-11) and is the only mammalian mRNA known to date that is "uncapped" (12).

Available evidence suggests that nascent strands of the replicative intermediate, minus strands of replicative intermediate and of double-stranded RNA, and progeny virion RNA are VPg-linked (5). Thus, all species of newly synthesized viral RNAs appear to be attached to a protein with the notable exception of mRNA. This observation has led us to propose that the protein or an (oligo)nucleotidyl-protein is cleaved from plus-strand RNA prior to polyribosome formation (3, 5). In order to identify the mode of cleavage we have isolated and sequenced the 5'-terminal, RNase T1-resistant oligonucleotide of polio mRNA and compared it to the corresponding oligonucleotide of virion RNA (4, 5). Here we report that the 5'-terminal nucleotide sequence of poliovirus mRNA is pU-U-A-A-A-C-A-Gp and is identical to the 5'-terminal VPg-pU-U-A-A-A-C-A-Gp of virion RNA (4, 5). This result indicates that the site of cleavage is the linkage between protein and RNA and not an internucleotide bond.

We also report that proteolytic removal of VPg does not alter the specific infectivity of virion RNA, an observation suggesting that VPg is not involved in an early step of viral replication.

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MATERIALS AND METHODS

³²P-Labeled poliovirus type 1 (Mahoney) was grown in spinner cultures of S3 HeLa cells, and poliovirus [³²P]mRNA was isolated from polyribosomes as previously described (9, 13). Enzymatic digestions and separation of products by one- or two-dimensional gel electrophoresis, paper electrophoresis, or column chromatography were performed as published (3, 5, 9, 13). For further details see the legends to the figures. The transfection assays of viral RNA were carried out according to a modified procedure of Bishop and Koch (ref. 14; B. Detjen, J. Lucas, and E. Wimmer, unpublished). Briefly, RNA was added to HeLa cells that were suspended in a cold solution of 0.02 M sodium phosphate, pH 7.0/0.15 M NaCl/DEAE-dextran at 300 µg/ml/10% (vol/vol) dimethyl sulfoxide. After rapid shaking of the suspension for 10 min at 37°, more HeLa cells and a solution of 1.4% agar were added, and the mixture was poured onto a base layer. Plaques were scored after incubation at 37° for 40-48 hr. Specific infectivities of the RNA were typically 10⁵-10⁶ plaque-forming units (PFU)/µg.

RESULTS

Isolation and sequence of the 5'-terminal oligonucleotide of mRNA

Fingerprints of RNase T1-resistant oligonucleotides of poliovirion RNA and of polio mRNA analyzed by two-dimensional gel electrophoresis are identical (9). The same result was obtained with protease-treated virion RNA (data not shown). These results precluded the possibility of a straightforward identification and isolation of the 5'-terminal fragment of mRNA by two-dimensional gel electrophoresis. Because of the high resolution of large oligonucleotides obtained by two-dimensional gel electrophoresis (13) these results also revealed that the fragment is shorter than 15 nucleotides.

We then attempted to isolate the 5'-terminal oligonucleotide by the following procedures: (i) the "diagonal procedure" of Dahlberg (ref. 15; two-dimensional paper electrophoresis of RNase T1-resistant oligonucleotides in combination with phosphatase treatment); and (ii) derivatization of the 5' end according to Rosenberg *et al.* (ref. 16; condensation of sorbitol to the 5'-terminal phosphate of the RNA followed by chromatography of the RNase T1 digest on a DBAE-cellulose N-[N'-(m-dihydroxyboryl) phenyl]succinamoyl]aminoethyl-cellulose column). Neither of the procedures yielded satisfactory results. This may be due to large size of the mRNA (7700 nucleotides)

Abbreviation: PFU, plaque-forming unit.

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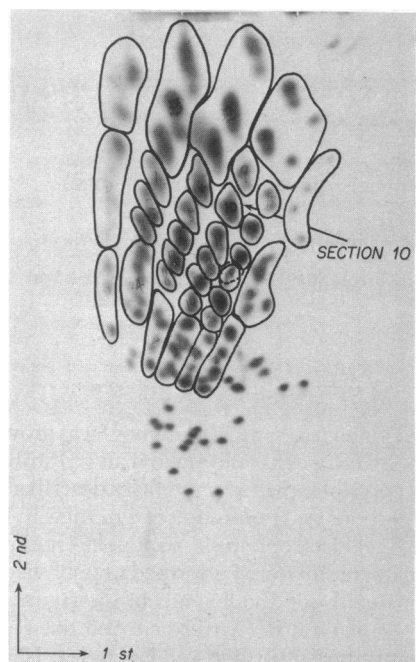


FIG. 1. Two-dimensional gel electrophoresis of polio mRNA. A culture of 10^9 HeLa S3 cells was infected with poliovirus at a multiplicity of infection of 50 PFU per cell and labeled with 120 mCi of carrier-free ^{32}P at a concentration of 10^7 cells per ml in the presence of actinomycin D at $5 \mu\text{g}/\text{ml}$. At 3.25 hr after infection the cells were harvested and polyribosomal RNA was isolated as previously described (9) except that the precipitation with Mg^{2+} was omitted. A sample containing 10^8 cpm of polio mRNA ($15 A_{260}$ units of RNA) was digested with 50 units of RNase T1 at 37° for 1 hr and the hydrolysate was separated by two-dimensional gel electrophoresis (9, 13). After autoradiography the slab gel was divided into sections as indicated and the radioactive materials were eluted with water. Note that at the top of the gel the oligonucleotides Gp, U-Gp, U-U-Gp, U-U-U-Gp, and U-U-U-U-Gp (13) have been run off.

and the corresponding small molar content of the 5'-terminal nucleotide. Method *ii* yielded a 5'-terminal fragment with a base composition similar to that of the 5' end of the virion RNA (data not shown). The yield, however, was too small for proper sequence analysis.

We therefore decided to analyze different sections of a slab gel in which the RNase T1 digest of poliovirus [^{32}P]mRNA had been separated (Fig. 1) to look for a pUp-containing oligonucleotide. Material in each section of the slab gel was eluted, and an aliquot of each was digested with RNase T2 to yield the four nucleotide 3'-monophosphates (13). Only section 10 (as indicated by an arrow in Fig. 1) was found to contain material that produced pUp with RNase T2 (compare Fig. 3). Ion exchange chromatography in 7 M urea of material in section 10 yielded two peaks (Fig. 2). Oligonucleotides in both peaks were recovered and analyzed separately for pUp. Only peak II of Fig. 2 yielded the nucleoside 3',5'-diphosphate upon digestion with RNase T2 (Fig. 3), an observation suggesting that this peak contained the 5'-terminal oligonucleotide. Sequence analysis (see below) showed that other oligonucleotides were absent from this peak. On the other hand, peak I of Fig. 2 was found to be a mixture of isopleths with the estimated chain length of eight nucleotides (data not shown). The separation of the 5'-terminal oligonucleotide (peak II) from the isopleths (peak I) in Fig. 2 was successful because ion exchange chromatography in 7 M urea separates predominantly according to charge (17) (Fig. 2), whereas the second dimension of the slab gel electrophoresis separates according to size and charge (13).

Secondary enzymatic digestion of the RNase T1-resistant

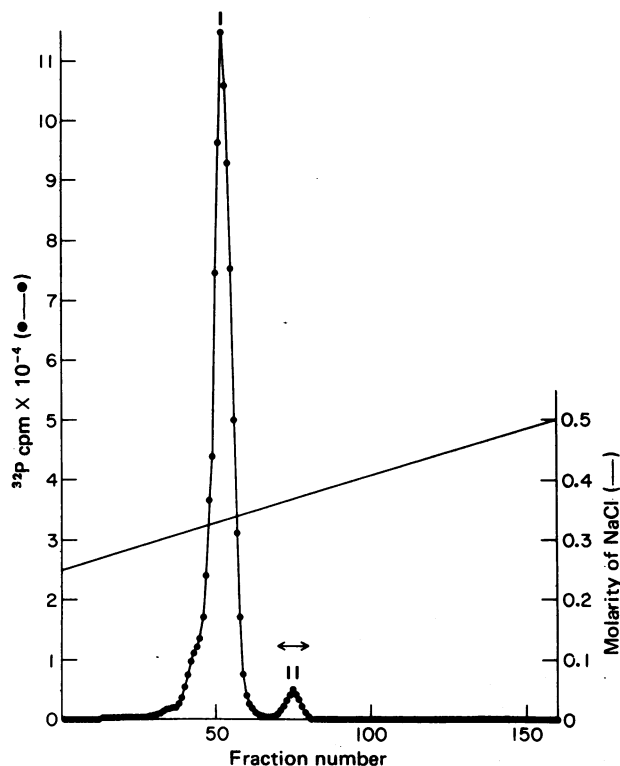


FIG. 2. Purification of the 5'-terminal oligonucleotide of polio mRNA. Materials eluted from each section of the slab gel in Fig. 1 were divided, an aliquot (5%) was digested with RNase T2, and the products were separated by electrophoresis on DEAE-paper (see Fig. 3). In some cases the separation was poor (tail formation was presumably due to salt contamination); in that case the oligonucleotides were eluted and electrophoresed again. Only material of section 10 (as indicated by an arrow in Fig. 1) yielded pUp. The aqueous solution of oligonucleotides in section 10 was adjusted to 0.02 M Tris-HCl, pH 7.5/7 M urea and applied to a 5-ml column of DEAE-Sephadex A25. The materials were eluted with a linear gradient (total volume 200 ml) of NaCl (0.25 M to 0.5 M) in 0.02 M Tris-HCl, pH 7.5/7 M urea. Oligonucleotides in peaks I and II were desalted for further analysis.

oligonucleotide in peak II (Fig. 2) was carried out as previously described (5). Separation of the products by paper electrophoresis (Fig. 4) revealed that this fragment yields a pattern of mono- and oligonucleotides similar to that of the 5'-terminal RNase T1-resistant fragment of poliovirion RNA (5), with the exception of pUp-containing oligonucleotides. The secondary digestion products and their molar ratios are listed in Table 1. As previously reported (4, 5) the bases of the viral RNA are not uniformly labeled with ^{32}P . We have compensated for this observation by multiplying the counts in each nucleoside phosphate with a correction factor (ref. 5; and Table 1). The characterization of the RNase U2 digestion products is shown in Fig. 5. The largest RNase U2 oligonucleotide, pU-U-A>p, is identified as being the 5'-terminal trinucleotide of polio mRNA due to its content of pUp.

The results (Table 1; Figs. 4 and 5) show that the material in peak II (Fig. 2) contains only one oligonucleotide. The nature and quantitation of the secondary digestion products determine unambiguously the sequence of that oligonucleotide as pU-U-A-A-A-C-A-Gp. This nucleotide sequence is identical to that of the 5' end of virion RNA.

Infectivity of protease-treated virion RNA

Absence of VPg from virus-specific mRNA suggests that the presence of the protein on the messenger may interfere with

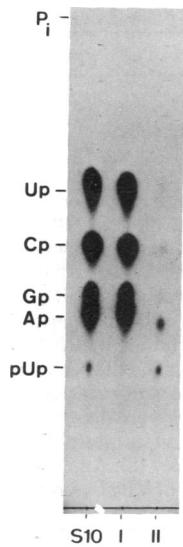


FIG. 3. Paper electrophoresis of digestion products of oligonucleotides. Aliquots (5%) of the oligonucleotides in section 10 (Fig. 1) and of peaks I and II in Fig. 2 were digested with 0.1 unit of RNase T2 at 37° for 30 min. Samples were electrophoresed in 5% acetic acid, adjusted to pH 3.5 with pyridine, on DEAE-paper at 3000 V for 2.5 hr. The identity of pUp was confirmed by two-dimensional thin-layer chromatography (3).

some steps in translation. If so, VPg would have to be cleaved from the RNA of the infecting virus soon after uncoating because the viral genome serves as mRNA. On the basis of this hypothesis one can predict that removal of the protein from virion RNA *in vitro* may not interfere with the biological ac-

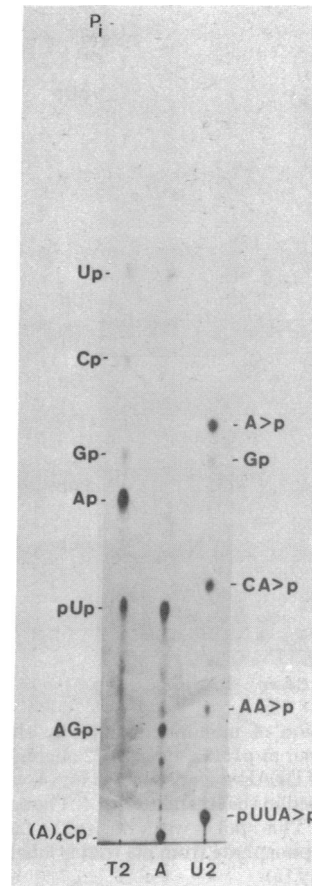


FIG. 4. Separation of digestion products of the 5'-terminal oligonucleotide (peak II of Fig. 2) by electrophoresis on DEAE-paper in pyridinium acetate, pH 3.5 (see Fig. 3) at 3000 V for 4 hr. Aliquots of the 5'-terminal oligonucleotide were digested in 10- μ l reaction mixtures for 1 hr at 37° with 0.1 unit of RNase T2, 1 μ g of RNase A, or 0.05 units of RNase U2. For buffers see ref. 5. Digestion with RNase U2 under these conditions is incomplete and yields cyclic phosphates. The identities of the oligonucleotides were subsequently verified by digestion with RNase T2 and electrophoresis. The characterization of the RNase U2 oligonucleotides is shown in Fig. 5.

Table 1. Products of nuclease digestions of the 5'-terminal oligonucleotide of polio mRNA

Products	Yield, cpm		Molar ratio	
	Observed	Corrected*	Observed	Theoretical
RNase A				
A-A-A-A-Cp	481	430	1.0	1.0
A-Gp	97	212†	1.23	1.0
pUp	240	132	0.77	1.0
Up	72	77	0.90	1.0
Nuclease P1				
pG	22	253	1.0	1.0
pA	1490	1252	4.95	5.0
pC	231	280	1.11	1.0
pU	820	451	1.78	2.0
Pi	177	214†	0.85	1.0
RNase U2‡				
pU-U-A>p, A-A>p, C-A>p, Gp, A>p				

RNase T1-resistant oligonucleotide of Fig. 2 (peak II). Digestion with RNase A and RNase U2 was as described in the legend to Fig. 4. Another aliquot of the oligonucleotide was digested with 1 μ g of nuclease P1 in 10 μ l of 0.01 M sodium acetate, pH 6, for 1 hr at 37°.

* The observed molar ratios were corrected for nonuniform label in phosphates as recently described (5). The correction factor for each 5'-phosphate of this RNA preparation was pG = 11.47, pA = 0.84, pC = 1.21, and pU = 0.55.

† Assuming that the nearest 3' neighbor is C. This assumption is supported by the yield of Pi that is found after digestion with nuclease P1, because the Pi originates from the 3'-terminal G of the oligonucleotide.

‡ Digestion with RNase U2 is incomplete. The cleavage products have 2',3'-cyclic phosphates.

tivity of the RNA. We have tested this by assaying proteinase K-treated virion RNA for infectivity. As shown in Table 2, no decrease of specific infectivity of virion RNA is observed after proteolysis of the genome-linked protein.

It should be stressed that virion RNA purified by our standard procedures (9) consistently shows specific infectivities of 10⁵-10⁶ PFU/ μ g. This infectivity, albeit lower than that reported by Bishop and Koch (14) is fully sensitive to treatment with RNase (B. Detjen, J. Lucas, and E. Wimmer, unpublished results). Moreover, our RNA preparations are infectious with L-cells at the same specific infectivity as with HeLa cells (B. Detjen, J. Lucas, and E. Wimmer, unpublished results). Thus, the possibility that the titers in Table 2 are due to residual virions or subviral particles can be excluded.

DISCUSSION

We have recently reported that the nascent strands of the poliovirus replicative intermediate are protein linked (5). Furthermore, we have failed to identify any pppN termini in replicative intermediate preparations in experiments that would have allowed us to find one pppN-terminus per six to eight termini of nascent strands (5). These observations strongly

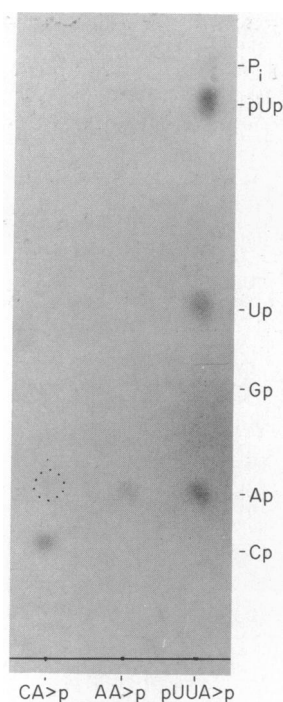


FIG. 5. Separation of mononucleotides by electrophoresis on Whatman 3MM paper at pH 3.5. RNase U2 oligonucleotides, which were eluted from the DEAE-paper shown in Fig. 4, were digested with RNase T2 as described in the legend to Fig. 4. The spot encircled with dots represents Ap. This spot is weak due to the fact that the Ap of C-A>p received its phosphate from pG that is labeled with very low specific radioactivity (5).

suggest that initiation of RNA synthesis does not occur *de novo* with a nucleotide 5'-triphosphate but instead occurs with a primer. We have proposed that the primer is VPg (3, 5). On the other hand, newly synthesized viral RNA found in polyribosomes has a free pUp terminus. It is therefore likely that VPg is removed from that fraction of newly synthesized RNA that is destined to become mRNA. This immediately raises the question of whether cleavage occurs at the linkage between protein and nucleic acid. Alternatively, cleavage could occur at an internucleotide bond by endonucleases such as RNase III, resulting in the removal of a nucleotidyl-protein or an oligonucleotidyl-protein. The data presented here show that virion RNA and mRNA have the identical 5'-terminal nucleotide sequence. This, together with the fingerprints recently published (9), suggests that the only difference between the two RNAs is the presence or absence of VPg. Cleavage of VPg, therefore, occurs at the linkage between protein and RNA, which may represent a new type of processing of viral macromolecules.

Why is VPg absent from mRNA? The most plausible answer is that VPg interferes with a certain step in protein synthesis *in vivo*. This answer does not necessarily contradict the fact that polio virion RNA can be translated into proper viral proteins *in vitro* (18) because it is unknown whether or not VPg is cleaved from virion RNA during incubation of the RNA with the cell-free system. In addition, the conditions that are selected for translation of poliovirus RNA *in vitro* may overcome the block normally caused by VPg *in vivo*. The nature of the activity that we suggest is responsible for cleavage of VPg from newly synthesized RNA *in vivo* is certainly of interest. If VPg is removed from the infecting RNA, the cleavage enzyme might be of cellular origin. The fact that VPg can be proteolytically

Table 2. Infectivity of poliovirion RNA after removal of VPg

RNA treatment	Titer, (PFU/ μ g RNA) $\times 10^{-5}$
1 hr at 37° with proteinase K	2.6
1 hr at 37° without proteinase K	2.1
Untreated	2.6

Samples containing 25 μ g of poliovirion [32 P]RNA (at 2×10^5 cpm/ μ g) were incubated in 100 μ l of 0.01 M Tris-HCl, pH 7.5/0.1 M NaCl/0.001 M EDTA/0.5% sodium dodecyl sulfate with or without 20 μ g of proteinase K. After 1 hr at 37° samples were deproteinized by phenol/chloroform extraction and RNA was precipitated by ethanol. The infectious titers of both experimental samples and an untreated control were determined by RNA plaque assay as described in *Materials and Methods*. The titer given is the average of duplicate determinations. In order to verify susceptibility of VPg to proteinase K treatment, both samples were digested with ribonuclease T2 and subjected to sodium dodecyl sulfate/polyacrylamide gel electrophoresis as described previously (3). Analysis of the autoradiogram indicated complete removal of VPg by proteinase K (data not shown).

cleaved from virion RNA without loss of the infectivity of the RNA (Table 2) is in agreement with the notion that the protein is not essential for the infecting genome to function either as messenger or as template. It also suggests that VPg plays no role in early events (those up to and including translation) of transfection with purified virion RNA. In agreement with this is the observation that poliovirus mRNA is infectious (4).

What, then, is the function of VPg? As mentioned before, we have suggested that VPg is involved in initiation of virus-specific RNA synthesis. A similar conclusion has been reached by Flanagan *et al.* (4). It should be emphasized that our model of RNA replication (5) predicts that template RNA need not be linked to the protein; thus, protease-treated RNA can function as template at the onset of RNA replication.

If mRNA has the same nucleotide sequence as virion RNA, why are mRNA strands not encapsidated into virions? A trivial explanation would be that viral RNA, once it has combined with ribosomes, may subsequently not exist free of ribosomes. However, free viral RNA (not bound to ribosomes) that is 5'-terminated with pUp has been found in cytoplasmic extracts of infected cells (19). Thus there exists the intriguing possibility that, in addition to the role in replication, VPg linked to the 5' end of plus-strand RNA is also a prerequisite for virion formation.

After this manuscript was completed, a paper by Pettersson *et al.* (20) appeared, also reporting that the 5'-terminal nucleotide sequences of polio virus polyribosomal RNA and virion RNA are identical.

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