5'-Terminal Structure of Poliovirus Polyribosomal RNA is pUp

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5′-Terminal structure of poliovirus polyribosomal RNA is pUp

(DEAE-paper ionophoresis/mRNA/7-methylguanosine in 5′-5′-pyrophosphate linkage)

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ABSTRACT **Poliovirus RNA purified from virus-specific polyribosomes does not contain m^7G in a 5′-5′-pyrophosphate linkage at its 5′-end. The only potential 5′-end found in ribonuclease digests of this RNA is pUp, which is present in a yield of 1 mole/mole of poliovirus RNA. We conclude that a 5′-terminal m^7G is not required for translation of at least one RNA species in animal cells.**

Recent evidence indicates that messenger RNA molecules of eukaryotic cells and their viruses have an unusual 5′-terminal structure of the general form $m^7G(5')ppp(5')Nm\text{-}N(m)\text{-}\ldots$ This structure has been found on viral mRNAs synthesized by virion RNA polymerases of viruses containing double- and single-stranded RNA genomes (1, 2) as well as double-stranded DNA genomes (3). Viral mRNAs synthesized in virus-infected cells also contain this structure (4), and in the case of vesicular stomatitis virus mRNAs as many as 5 base and ribose methylations may occur near the 5′-end (5, 6). Animal cell mRNAs also contain a variety of "m^7G-blocked" 5′-terminal structures (7, 8). It has been proposed that the 5′-terminal m^7G is required for translation of viral RNAs, since reovirus and vesicular stomatitis virus mRNAs lacking this nucleotide are translated poorly in a cell-free system derived from wheat embryo (9). It has also been suggested that this nucleotide may be required for translation of all mRNAs in eukaryotic cells, since its chemical removal from globin mRNA prevents translation (10).

Virions of poliovirus contain a single-stranded RNA, the major 5′-end of which has been reported to be pAp (11); this RNA can be completely translated in a cell-free system (12). To examine whether the 5′-end of the viral mRNA active in infected cells might have the m^7G blocking group, we have analyzed the structure of the 5′-terminus of poliovirus polyribosomal RNA prepared from infected HeLa cells.

MATERIALS AND METHODS

Preparation of Poliovirus RNA. The growth of HeLa cells in suspension using Joklik's modified minimal essential medium plus 7% horse serum and their infection by type 1 poliovirus have been described (13). For the preparation of ^{32}P-labeled virus-specific RNA, 4×10^8 HeLa cells were harvested and washed three times with phosphate-free minimum essential medium buffered at pH 7.2 with 25 mM Hepes (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) and 10 mM Tes [*N*-tris(hydroxymethyl)methylaminoethanesulfonic acid]. The cells were resuspended at approximately 4×10^7 cells per ml in phosphate-free medium containing sufficient poliovirus to give a multiplicity of infection of 20. After room temperature adsorption for 30 min, the cells were diluted to 4×10^6/ml in phosphate-free medium plus 5% dialyzed horse serum (100 ml final volume). At 15 min post-infection, the culture was treated with 5 μg/ml of actinomycin D and at 30 min post-infection, 600 μCi/ml of carrier-free ^{32}P were added. The course of the infection was monitored by [^{14}C]uridine uptake into RNA in a portion of the culture that did not receive ^{32}P.

At 210 min post-infection, the cells were harvested, washed free of medium, swelled for 10 min at 4° in 10 mM Tris, 10 mM NaCl, 1.5 mM $MgCl_2$, pH 7.5, and broken with a Dounce homogenizer. Polyvinyl sulfate (4 μg/ml) was added to the cytoplasmic extract and virus-specific polyribosomes were prepared by method 2 described in detail by Spector and Baltimore (14). The polyribosomes were extracted with phenol/$NaDodSO_4$ (sodium dodecyl sulfate), and ethanol precipitated, and the resulting material was bound to a column of oligo(dT)-cellulose (0.1 g) in the presence of 0.4 M NaCl, 0.01 M Tris (pH 7.4), and 0.05% $NaDodSO_4$. Material bound to the column was eluted with 0.01 M Tris (pH 7.4), 0.05% $NaDodSO_4$ and recovered by ethanol precipitation. Intact 35S poliovirus RNA was recovered after sedimentation through a 15–30% (weight/weight) sucrose gradient in 0.5% $NaDodSO_4$, 0.10 M NaCl, 0.01 M Tris (pH 7.5), and 0.001 M EDTA. The gradients were centrifuged in an SW27 rotor at 64,000 × *g* for 14 hr at 21°, and RNA was recovered from the peak fractions by two successive ethanol precipitations. This procedure yields about 10^7 cpm of ^{32}P-labeled poliovirus polyribosomal RNA.

Enzymatic Digestions. Limit digests of RNA with a mixture of RNase A, RNase T_1, and RNase T_2 were performed as previously described (5). Procedures for alkaline phosphatase and *Penicillium* (P1) nuclease digestions were as described (5).

Analytical Separations. Ionophoresis of limit RNase digests on DEAE-paper at pH 3.5 and of isolated nucleotides on Whatman 3 MM paper at pH 3.5 have been described (5, 15). Two-dimensional thin-layer cellulose analysis of nucleotides was as described previously (5). The positions of ^{32}P-labeled material were determined by autoradiography using Kodak Royal RP X-Omat film.

Materials. Pancreatic RNase A was obtained from Worthington Biochemicals and RNase T_1 and RNase T_2 were from Calbiochem. P1 nuclease was purchased from Yamasa Shoyu Biochemicals, Tokyo, Japan. Alkaline phosphatase (*Escherichia coli*) was obtained from Boehringer-Mannheim. [^{14}C]Uridine and carrier-free ^{32}P (as phosphoric acid) were purchased from New England Nuclear. Actinomycin D was a kind gift of Merck, Sharp and Dohme. Oligo(dT)-cellulose (T-3) was from Collaborative Research.

Abbreviations: $NaDodSO_4$, sodium dodecyl sulfate; P1, *Penicillium* nuclease.

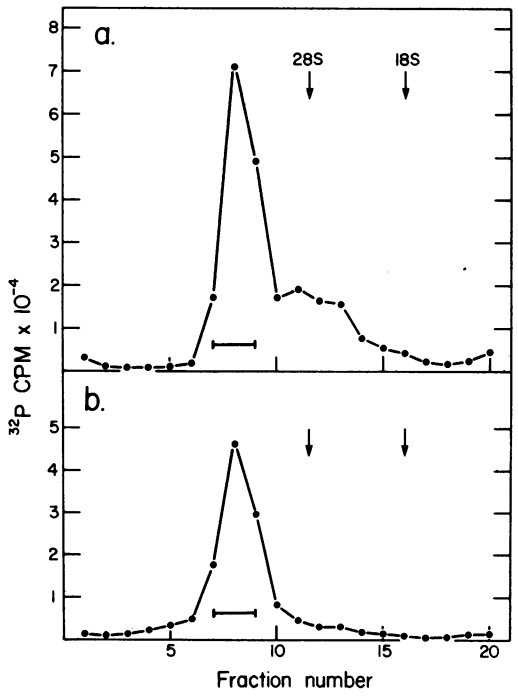

FIG. 1. Sedimentation analysis of poliovirus RNA. (a) RNA was extracted from ^{32}P-labeled poliovirus virions (12) and layered onto a 15–30% sucrose/NaDodSO$_4$ gradient as described in the *text*. The direction of sedimentation is from right to left. The positions of HeLa cells ribosomal RNA marker were determined in a parallel gradient. (b) ^{32}P-Labeled poliovirus polyribosomal RNA was eluted from oligo(dT)-cellulose as described and layered onto a 15–30% sucrose/NaDodSO$_4$ gradient. Sedimentation direction and marker positions were as described above. Radioactivity was determined by Cherenkov counting. Fractions were pooled as indicated by the bars.

RESULTS

Isolation of poliovirus polyribosomal RNA

^{32}P-Labeled poliovirus RNA was prepared from the virus-specific polyribosomes of infected cells (14) and the poly(A)-containing viral RNA was selected by binding to oligo(dT)-cellulose. Approximately 70% of this RNA bound to the oligo(dT)-cellulose and the sedimentation profile of this material is shown in Fig. 1. The sedimentation of ^{32}P-labeled poliovirus virion RNA is also shown in Fig. 1. Spector and Baltimore (14) have shown that virus-specific polyribosomal RNA prepared by the methods we have used contains a small amount (<10%) of replicative intermediate RNA. The distinct peaks of intact 35S poliovirus RNA were pooled as indicated, and ethanol precipitated and the 5′-ends were isolated as described below.

5′-End isolation

To isolate potential 5′-end structures from poliovirus RNA, the RNA was digested with RNases A, T_1, and T_2. This combination of enzymes will digest RNA to 3′-mononucleotides with the exception of the following: 5′-terminal nucleotides containing 5′-phosphates, 5′-terminal structures of the form m^{7}G(5′)ppp(5′)Nm-Np, and internal 2′-*O*-methylated oligonucleotides. The ribonuclease digest was then subjected to pH 3.5 ionophoresis on DEAE-paper to separate mononucleotides from potential 5′-end structures (5). Autoradiograms of these separations are shown in Fig. 2. Lanes 1 and 2 show the separations of poliovirus polyribosomal RNA and virion

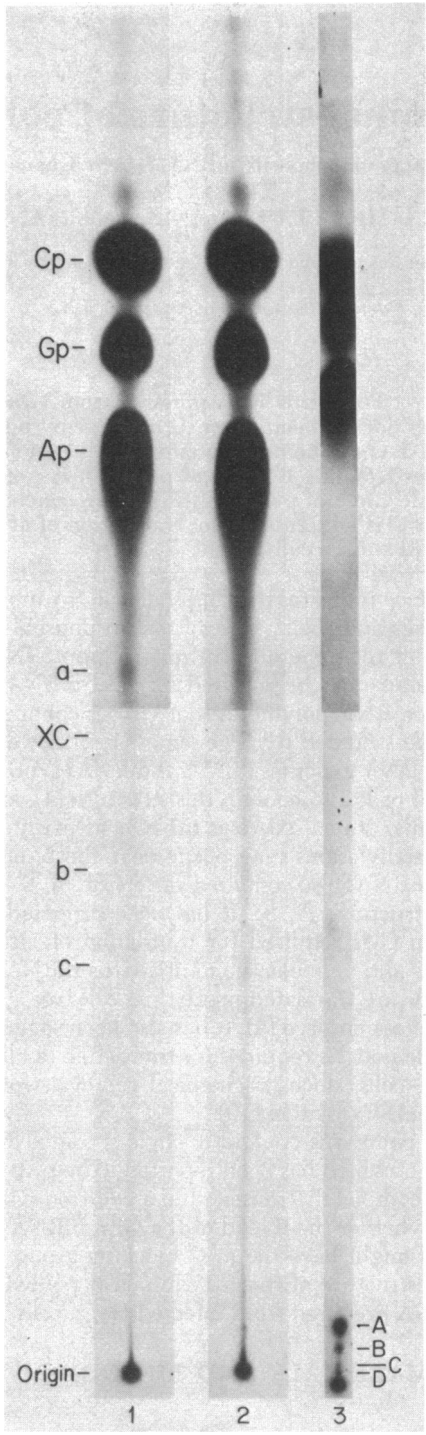

FIG. 2. Autoradiograms of ionophoretic separations of 5′-ends from limit RNase T_1, T_2, and A digests of poliovirus and vesicular stomatitis virus mRNAs. Digested samples were spotted on DEAE-paper and subjected to pH 3.5 ionophoresis for 8 hr at 30 V/cm. Lanes 1 and 2 show the separation of limit digests of poliovirus polyribosomal RNA and poliovirus virion RNA, respectively. Lane 3 shows the separation of the products of a limit digest of total vesicular stomatitis virus mRNA which has been described previously (5). XC indicates the position of the blue dye xylene cyanol. The positions of the 3′ mononucleotides are as shown (Up has migrated off the paper). Material remaining at the origin was found to contain mononucleotides after elution and redigestion. The discontinuity between XC and *a* reflects the fact that the 100 cm long ionopherogram was cut at this point and autoradiograms of each section were prepared.

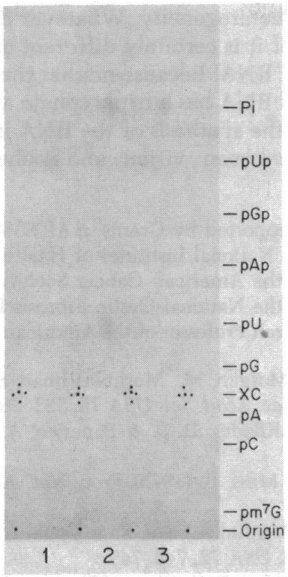

FIG. 3. Autoradiograms of pH 3.5 paper ionophoretic separations of spot *a* and its digestion products. Material migrating in the position of spot *a* (lane 1, Fig. 2) was eluted and repurified by electrophoresis on DEAE-paper, pH 3.5, to free it of contaminating mononucleotides. The material which again ran with the mobility of spot *a* was eluted and subjected to pH 3.5 paper ionophoresis on Whatman 3 MM paper at 40 V/cm for 35 min after no digestion, lane 1; P1 digestion, lane 2; alkaline phosphatase digestion, lane 3.

RNAs, respectively, and lane 3 shows total vesicular stomatitis virus mRNA for comparison. In each case, as expected, the major products are the mononucleotides. The spots labeled A and B (lane 3) contain the "m^7G-blocked" ends of vesicular stomatitis virus mRNAs which have the structure $m^7G(5')ppp(5')(m)Am\text{-}Ap$ and $m^7G(5')ppp(5')(m)Am\text{-}(m)A(m)\text{-}Cp$ (5) with m in parentheses indicating partial base or ribose methylations. The two spots below A and B are the unblocked tetraphosphate ends from vesicular stomatitis virus mRNA (5). The m^7G-blocked ends of HeLa cells mRNA are also found in the region near spots A and B from vesicular stomatitis virus mRNA (M. Hewlett, J. Bergmann, and J. Rose, unpublished results). There is a clear absence of radioactivity in this region of the separation of RNase digests of poliovirus RNA, lanes 1 and 2. If poliovirus RNA contained a 5′-terminus of the type $m^7G(5')ppp(5')Nm\text{-}Np$, a spot of intensity equivalent to spot A from vesicular stomatitis virus mRNA would be found in this region. However, the only material migrating slower than the mononucleotides in poliovirus RNA from polyribosomes (lane 1) is indicated as spot *a*, and this material migrates much faster than any "m^7G-blocked" structures. The poliovirus virion RNA (lane 2) also shows no candidate for the "m^7G-blocked" structures.

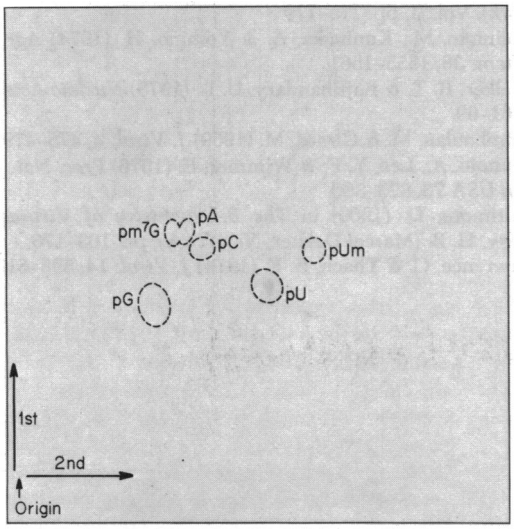

FIG. 4. Autoradiogram of a two-dimensional cellulose thin-layer separation of the pU derived from material in spot *a*. The material migrating with pU marker Fig. 3, lane 2 was eluted, lyophilized, and then spotted on a cellulose thin-layer plate. The solvent system of Saneyoshi described by Walter and RajBhandary (17) was used. First dimension: isobutyrate–NH_3–water, 152:10:100; 2nd dimension: *t*-butanol–HCl–water, 70:15:15. The positions of marker nucleotides which were located by UV illumination are indicated by the broken circles.

Structure of material in spot *a* from poliovirus polyribosomal RNA

Polyribosomal RNA does yield material other than the mononucleotides, as indicated by the spot labeled *a* in Fig. 2, lane 1. To determine its structure, this material was eluted from the paper and treated with alkaline phosphatase of P1 nuclease, and the resulting products were electrophoresed at pH 3.5 on Whatman 3 MM paper, along with marker nucleotides. The autoradiograms of these analyses are shown in Fig. 3. The untreated material migrates very rapidly, and has the mobility of pUp (lane 1). Treatment with alkaline phosphatase converts the material to free phosphate (lane 3), indicating that the phosphate(s) in spot *a* are external and not internucleotide phosphates. Treatment with P1 nuclease, which has a 3′ phosphatase activity (16), yields equal amounts of radioactivity migrating with marker pU and P_i. This analysis indicates that material in spot *a* has the structure pUp. To confirm that the 5′ mononucleotide derived from spot *a* was in fact pU and not some other modified nucleotide we eluted this material from the paper and analyzed it in a two-dimensional thin-layer cellulose chromatography system designed for identification of modified nucleotides from tRNA (17). As shown in Fig. 4 this material migrates exactly with a pU marker and is clearly separated from pUm as well as the other indicated markers. Thus we conclude that the structure of spot *a* is pUp.

Molar yield of the 5′-end material

If the 5′-terminus of poliovirus polyribosomal RNA is the 3′-5′-diphosphate pUp, then the expected molar yield for two phosphates out of 7500 phosphates (assuming a molecule of 2.5×10^6 daltons; ref. 18) is 0.027%. Table 1 lists the molar yields found for pUp in two separate preparations of

Table 1. Molar yield of material found in spot *a* of RNase digest of polyribosomal RNA

	% of total cpm	
	Actual	Theoretical*
Experiment I	0.030	0.027
Experiment II	0.023	0.027

* Assuming material contains two phosphates of a molecule of 7500 nucleotides.

polyribosomal RNA. Since in both cases the actual yield is approximately equal to the theoretical yield, we conclude that the pUp recovered from this RNA is the structure at the 5′-end of virtually all of the poliovirus mRNA.

5′-Terminus of virion RNA

While poliovirus virion RNA also does not have a m^7G-blocked 5′-end, the 5′-end of this RNA species appears to differ from that of the polyribosomal RNA. We have determined that for virion RNA (Fig. 2, lane 2) there is less than 10% of the yield of pUp (spot *a*), found for polyribosomal RNA. Instead, material is found in two slower migrating positions, indicated by spots *b* and *c*. We have not yet determined the structures of the material migrating at these positions nor are we certain that they derive from the 5′-end.

DISCUSSION

All eukaryotic mRNA molecules so far examined contain m^7G in an inverted 5′-5′ linkage at their 5′-termini, except for poliovirus virion RNA (ref. 11 and this report) and poliovirus polyribosomal RNA, as shown above. The identification of pUp as the 5′-end of poliovirus mRNA has also been accomplished by Nomoto *et al.* (19) and R. Fernandez-Muñoz and J. Darnell (personal communication).

Since poliovirus mRNA purified from polyribosomes where it is actively translated lacks an "m^7G-blocked" 5′-terminus, we conclude that this structure is not required for translation of all mRNAs in eukaryotic cells as has been suggested (9, 10). However, it is possible that poliovirus infection alters the specificity of ribosome recognition of mRNA. In fact, within 2- to 3-hr post-infection, poliovirus has inhibited the cell's translation activity, such that less than 5% of the HeLa mRNA can be found in polyribosomes (20). The disappearance of cellular polyribosomes is followed by the rapid appearance of virus-specific polyribosomes, and the onset of viral protein synthesis (20). However, poliovirus does not appear to alter either the size or blocked 5′-end of HeLa cell mRNA molecules after infection (M. Hewlett, J. Rose, and J. Bergmann, unpublished results; R. Fernandez-Muñoz and J. Darnell, personal communication). Therefore, it appears that host mRNA 5′-ends are not the direct target for inhibition of host mRNA translation. In addition, experiments with encephalomyocarditis virus have shown that the translational capacity of cellular mRNA is not altered by infection (21).

The exact end of poliovirus virion RNA remains to be determined. We cannot find any single structure in molar yield, suggesting heterogeneity. Whatever the end of the virion RNA, most of it is certainly different at its 5′-end from the polyribosomal RNA. Because neither the virion RNA nor the polyribosomal RNA has a triphosphate at its 5′-terminus, it is unclear how the synthesis of the RNA initiates and how the difference between virion and polyribosomal RNA comes about.

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