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The 5′ end of poliovirus mRNA is not capped with m^{7}G(5′)ppp(5′)Np

(polyribosomes/fingerprints)

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ABSTRACT **Poliovirus was grown in HeLa cells in the presence of phosphorus-32 and actinomycin D. Three to four hours after infection, viral mRNA was recovered from polyribosomes and its identity verified by two-dimensional gel electrophoresis of RNase T1 digests. Digestion of the viral [^{32}P]mRNA with RNase T2 and separation of the products by ion exchange chromatography at pH 5 yielded pUp as possible 5′ terminus but no "capping group" of the structure m^{7}G(5′)ppp(5′)Np. Total cytoplasmic [^{32}P]RNA of HeLa cells, on the other hand, was found to contain capping groups. Neither the capping group nor ppNp or pppNp was found in an RNase T2 digest of poliovirion [^{32}P]RNA, in agreement with previous results [Wimmer, E. (1972) *J. Mol. Biol.* 68, 537–540]. The data indicate that 5′-terminal m^{7}G(5′)ppp(5′)Np is absent from poliovirus RNAs and, therefore, is not involved in poliovirus protein synthesis.**

mRNAs of a number of animal viruses contain an unusual group of methylated nucleotides with the general structure m^{7}G(5′)ppp(5′)N^{m}p (2) at the 5′ terminus. This group, which we shall refer to as a "capping group" (3, 4), has been found in mRNAs of cytoplasmic polyhedrosis virus (2), human reovirus (5), vaccinia virus (6, 7), and vesicular stomatitis virus (8). More recent reports indicate that the capping group is present in mRNA of every mammalian cell that has been analyzed so far, for example, in mRNA of HeLa cells (4, 9) mouse myeloma cells (10), rabbit reticulocytes (11), and monkey kidney cells (12). Finally, viral RNAs that are synthesized in the nucleus are also capped at the 5′ end, as has been shown for mRNA of simian virus 40 (12) and for Rous sarcoma virus RNA (13).

Available evidence suggests that the capping group plays an important role in host cell and viral protein synthesis (11, 14), presumably during the formation of the initiation complex (15). While the capping group may be universally found in mRNA of eukaryotic cells, a crucial question remains unanswered: is the presence of 5′-terminal m^{7}G(5′)ppp(5′)N^{m}p obligatory for a mRNA to function in protein synthesis? If so, *all* species of mRNA translated in the cytoplasm should be terminated with the capping group.

Poliovirus contains a single-stranded RNA genome which, immediately after penetration and uncoating of the virus in the host cell, functions as mRNA in viral protein synthesis (for reviews see refs. 16 and 17). Earlier studies have shown that the poliovirion RNA contains a 5′ end with the general structure pNp (1). The terminal base was found to be adenosine (1), although recently, alkaline digests of virion RNA yielded more pUp than pAp as possible 5′-termini (Jacobi and Wimmer, unpublished). Lack of the capping group in poliovirion RNA does not exclude the possibility that m^{7}G(5′)ppp(5′)N^{m}p is involved in poliovirus protein synthesis, since cell-free extracts have been found to block and methylate unmethylated viral mRNAs (11, 14). Thus, poliovirus RNA might be modified in the cytoplasm of HeLa cells prior to or during complex formation with ribosomes. We have, therefore, analyzed poliovirus-specific mRNA isolated from polyribosomes of infected HeLa cells. We were unable to identify a capping group in the viral mRNA, but obtained evidence that suggests that pUp is the 5′ end. To our knowledge this is the only mRNA so far isolated from a mammalian cell that is uncapped.

MATERIALS AND METHODS

Poliovirus type 1 (Mahoney) was grown and labeled in spinner cultures of HeLa S3 cells, and its RNA was extracted, as published previously (1, 18). Poliovirus [^{32}P]mRNA was isolated as follows: 100 ml of HeLa cells (4×10^6 cells per ml) were infected with 30–50 plaque-forming units per cell of poliovirus (19) and incubated at 37°. Ninety minutes after infection the cells were washed and suspended in ice-cold phosphate-free medium containing 5% dialyzed calf serum at 4×10^6 cells per ml. The cells were kept at 0° for 90 min (1), then mixed with actinomycin D (5 μg/ml) and carrier-free phosphorus-32 (0.6 mCi/ml), and incubated at 37°. After a total incubation time at 37° of 3.2–4 hr the cells were washed twice with ice-cold 0.14-buffer (0.14 M NaCl, 0.01 M KCl, 0.01 M Tris·HCl, pH 7.5, 1.5 mM $MgCl_2$), suspended in 6 ml of 0.14-buffer, and layered in portions of 2 ml onto stepwise sucrose gradients. The sucrose gradients contained (from bottom to top): 2 ml of 60% sucrose in RSB + Mg buffer (0.01 M KCl, 0.01 M Tris·HCl, pH 7.5, 1.5 mM $MgCl_2$), 30 ml of a gradient (15–30%) of sucrose in RSB + Mg buffer, and 3 ml of 10% sucrose, 1% deoxycholate, 1% Brij 58 in RSB + Mg buffer. The gradients were spun in a Spinco SW 27 for 90 min at 26,000 rpm and 4° and were fractionated at 4°. The contents of each fraction were monitored for absorbance at 260 nm and radioactivity. Fractions containing polyribosomes were combined, mixed with 1/9 volume of 0.3 M $MgCl_2$ (20), and kept for 30 min at 0°. Precipitates of polyribosomes were collected by centrifugation, dissolved in 0.02 M EDTA, 1% sodium dodecyl sulfate, and treated three times with phenol/chloroform (18, 21). The deproteinized RNA was sedimented through a sucrose gradient (15–30%) in 0.1 M NaCl, 0.01 M Tris·HCl, pH 7.5, 1 mM EDTA, 0.5% sodium dodecyl sulfate. 35S RNA was collected and used for further analyses.

Total cytoplasmic HeLa cell [^{32}P]RNA was prepared from cells that had been labeled with ^{32}P (0.3 mCi/ml) in the presence of 0.05 μg/ml of actinomycin D (4, 21). RNA sedimenting between 10 and 28 S was isolated by zonal centrifugation.

Other procedures are outlined in the legends to the figures.

RESULTS

Isolation of Poliovirus mRNA. During infection with poliovirus, cellular polyribosomes are replaced by the much larger virus-specific polyribosomes, resulting in an inhibition

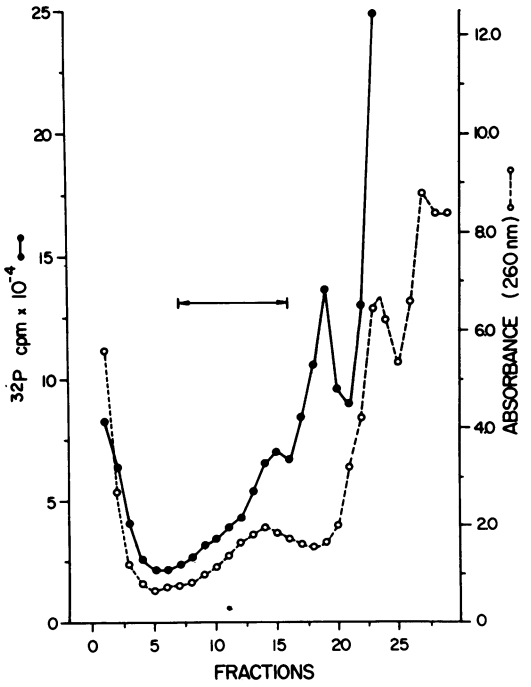

FIG. 1. Centrifugation of poliovirus-infected, ^{32}P-labeled HeLa cells through a stepwise sucrose density gradient. The top layer of the gradient contained 1% deoxycholate, 1% Brij-58. The bottom of the tube is at the left. Material indicated by the double-headed arrow was collected and analyzed.

of host-cell protein synthesis. This process is accelerated in the presence of actinomycin D (16, 17). Fig. 1 shows the sedimentation profile of a detergent lysate of infected HeLa cells. The cells were labeled from 90 to 210 min after infection with phosphorus-32 in the presence of 5 μg/ml of actinomycin D. As found previously (16, 17), the peak at 150 S (fraction 18) represents newly synthesized poliovirions. Virus-specific polyribosomes sediment faster than virions, with S-values between 150 and 400 (16, 17). From the sucrose gradient of Fig. 1 we isolated fractions as indicated by the double-headed arrow. Viral polyribosomes in these fractions precipitated in 30 mM $MgCl_2$ (20) and yielded 35S single-stranded RNA when treated with EDTA and phenol. As can be seen in Fig. 2, most of the ^{32}P-labeled RNA recovered from deproteinized polyribosomes sediments faster than 28S ribosomal RNA, although some RNA, sedimenting slower than 28 S, can also be observed. We consider the slowly sedimenting RNA to be breakdown products of the viral mRNA and not contaminations of other RNA (see below). It is unlikely that an appreciable amount of the labeled RNA in Fig. 2 originates from a virus-specific RNA replication complex (16) because lysis of the cells with deoxycholate/Brij 58 releases the replication complex from membranous material and thereby lowers its S-value to approximately 150 (22, 23). Further support that the ^{32}P-labeled RNA, which was isolated from polyribosomal fractions, is indeed pure poliovirus RNA comes from fingerprint analyses. Fig. 3A shows the separation of a complete RNase T1 digest of poliovirion [^{32}P]RNA by two-dimensional gel electrophoresis*. The pattern of the larger oligonucleotides in the lower portion of the autoradiograms is highly diagnostic of a high-molecular-weight RNA (25). When a fingerprint was prepared of [^{32}P]mRNA isolated from the polyribosomal fractions, an identical pattern was obtained (Fig. 3B). All spots of the large oligonucleotides of Fig. 3A and B are superimposable. These fingerprints not only identify the labeled RNA found in polyribosomal fractions as poliovirus plus-strand RNA but also show that the viral mRNA is free of contamination by labeled cellular RNA or polio minus-strands.

* Y. F. Lee and E. Wimmer (1976), submitted for publication.

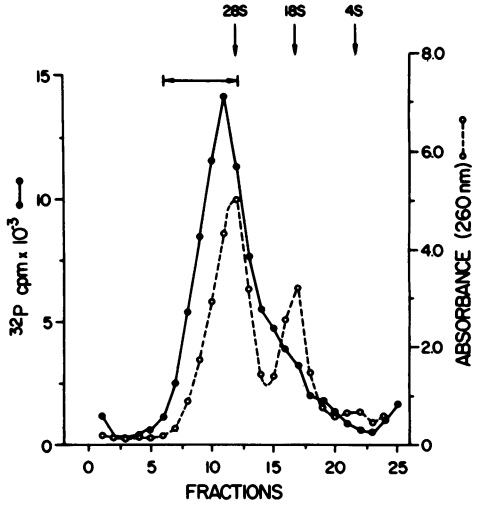

FIG. 2. Zonal centrifugation of phenol/chloroform-treated polyribosomes. Deproteinized polyribosomal RNA was sedimented through 15–30% sucrose as described in *Materials and Methods.*

End Group Analysis of the mRNAs. Analyses for the capping group were performed according to established procedures (1, 2) in which RNA is digested exhaustively with alkali or RNase T2. Nucleotides are separated by column chromatography on DEAE-cellulose. Triethylammonium acetate (Et_3N·HOAc), pH 5, was used as eluent because (*i*) excellent separation of nucleotides was achieved under these conditions (Fig. 4A), (*ii*) the capping group might be degraded at alkaline pH (26), and (*iii*) Et_3N·HOAc is volatile

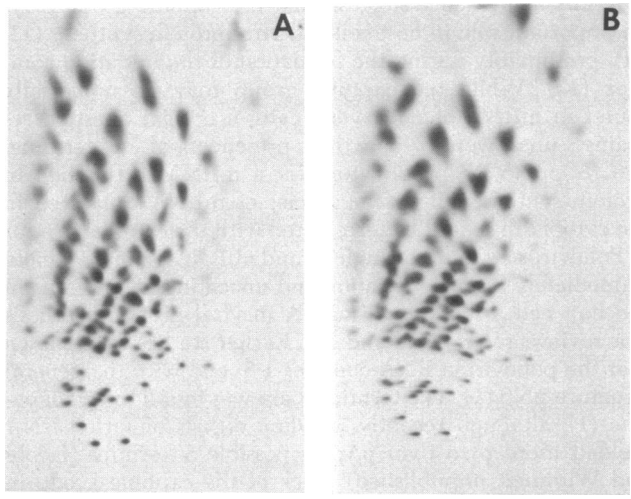

FIG. 3. Fingerprints of complete RNase T1 digests of ^{32}P-labeled poliovirion RNA (A) and polio mRNA (B). Digests were separated first (left to right) in a 8% polyacrylamide slab gel (4 × 28 cm) in 6 M urea at pH 3.3. Separation in the second dimension (bottom to top) was in a 22% polyacrylamide, 1.5% bisacrylamide slab gel (24 × 44 cm) at pH 8. Details of the procedure, which is a modification of that by DeWachter and Fiers (24) will be published elsewhere*.

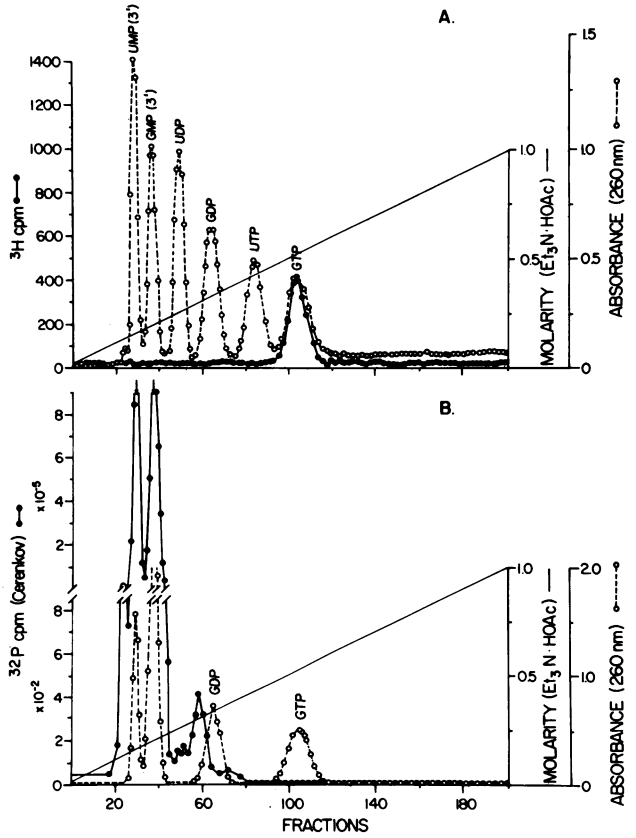

FIG. 4. Column chromatography of nucleotides on DEAE-cellulose at pH 5. (A) An RNase T2 digest of reovirus [*methyl*-^{3}H]mRNA was applied to the column (0.25 × 40 cm) together with marker nucleotides. The column was developed with a linear gradient from 0.02 to 1 M (total volume: 200 ml) of Et_3N·HOAc. Aliquots of the fractions were monitored for tritium (solid circles) and absorbance (open circles). Radioactive material was recovered by lyophilization. (B) Separation as in (A) of an RNase T2 digest (20 units of enzyme per 2 A_{260} units of RNA, 2 hr, 37° at pH 4.5) of polio [^{32}P]mRNA in the presence of marker GDP and GTP. The nucleoside monophosphates, which are detected by the absorbance of 260 nm, originate mostly from unlabeled rRNA of the polyribosomal RNA.

and permits desalting of fractions by lyophilization. Fig. 4 (solid circles) shows the elution of the capping group from a RNase T2 digest of reovirus [*methyl*-^{3}H]mRNA, which has the structure $m^7G(5')ppp(5')G^m$-Cp (5). Unexpectedly, this capping group elutes together with GTP. Its identity, however, was verified by phosphatase and/or snake venom exonuclease treatment followed by paper electrophoresis (2). When poliovirus mRNA is digested and the products are separated under identical conditions, no material with a net charge greater than that of GDP elutes in appreciable amounts from the column (Fig. 4B). This indicates that no capping group of the general structure $m^7G(5')ppp(5')N^mp$ is present in poliovirus mRNA. Absence of the capping group was observed with digests from three different preparations of poliovirus mRNA.

Analyzed in the experiment shown in Fig. 4B was a digest of 2.5 × 10^7 cpm of poliovirus [^{32}P]mRNA. This amount should yield 1.6 × 10^4 counts for a possible $m^7G(5')ppp(5')N^m$-Np, 1.3 × 10^4 for a possible $m^7G(5')ppp(5')$Np (if the nucleoside N proximal to the triphosphate were not *O*-methylated), and 6.6 × 10^3 cpm for a possible pNp group. The cpm per nucleotide were calculated by assuming a chain length of 7500 nucleotides of the RNA, in which case 3.3 × 10^3 cpm are associated with each phosphate (1). Either terminus could, therefore, be easily detected by our procedure.

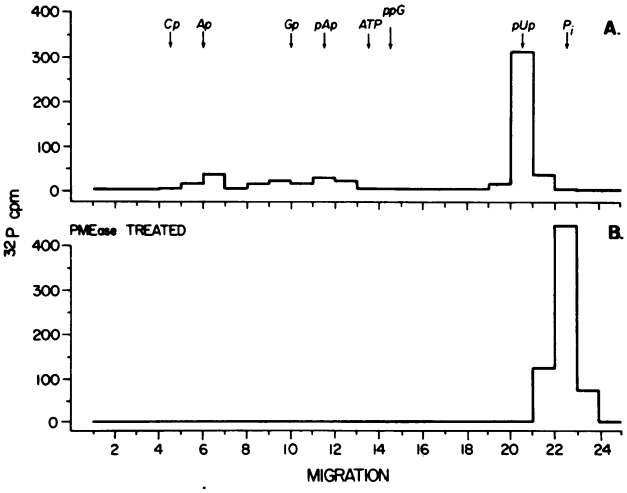

FIG. 5. Paper electrophoresis at pH 3.5 of (A) untreated and (B) phosphomonoesterase-treated material of fractions 54–64 in Fig. 4B.

Radioactive material eluting ahead of GDP (fractions 54–64 of Fig. 4B) was desalted by lyophilization and analyzed by paper electrophoresis (Fig. 5) and by thin-layer chromatography on polyethyleneimine plates (Fig. 6). As can be seen in Figs. 5A and 6A, the majority of counts of fractions 54–64 migrate with marker pUp. Upon treatment with alkaline phosphomonoesterase, all the radioactivity is converted to inorganic phosphate (Figs. 5B and 6B). Taking into consideration that UDP migrates in both systems (chromatography and electrophoresis) slightly more slowly than pUp (not shown), these data suggest that the material eluting ahead of GDP from DEAE-cellulose at pH 5 is pUp. Total

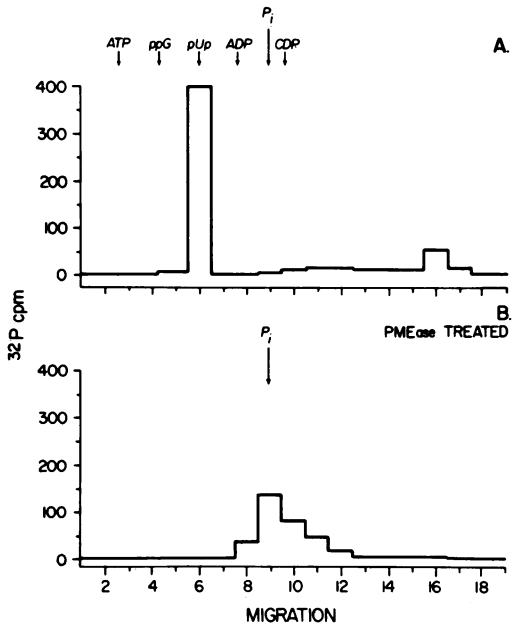

FIG. 6. Thin-layer chromatography on polyethyleneimine thin-layer plates of (A) untreated and (B) phosphomonoesterase-treated material of fractions 54–64 in Fig. 4B. Plates were developed with 4 M sodium formate, pH 2.5 (27).

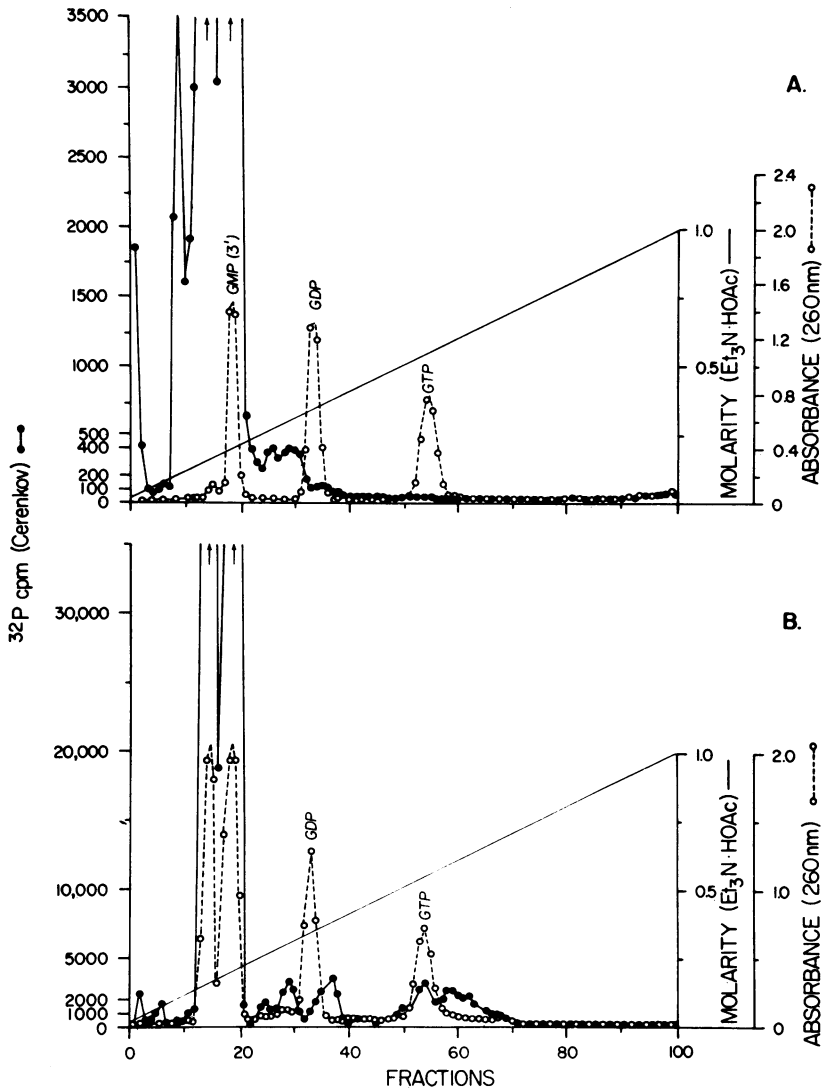

FIG. 7. Column chromatography of nucleotides on DEAE-cellulose at pH 5. Conditions are as in Fig. 4 except that fractions were collected at twice the volume of those shown in Fig. 4. (A) Separation of an RNase T2 digest of 3×10^7 cpm of poliovirion [^{32}P]RNA in the presence of Gp, GDP, and GTP. (B) Separation of an RNase T2 digest of total cytoplasmic [^{32}P]RNA (2×10^7 cpm) of HeLa cells.

recovery of counts in this peak was 6740 cpm, from which must be subtracted a small amount of counts that migrated differently from pUp in both systems. These compounds are presumably mononucleotides, since they are also sensitive to phosphomonoesterase. In any event, the recovery of pUp can account for most of the 5′ termini of poliovirus mRNA.

The identity of the small peak eluting after GDP in Fig. 4B (fractions 68–80) is unknown. This peak is found in fractions in which m^{7}G(5′)ppp(5′)Np might be expected to elute, since authentic marker m^{7}G(5′)ppp(5′)G elutes two fractions after UDP (not shown). The material in fractions 68–80 of Fig. 4B, however, is present in too small an amount (<1000 cpm) to represent the 5′ end of the viral mRNA.

Analysis of Poliovirion [^{32}P]RNA. Previous studies have shown that genome RNA of poliovirus was terminated in pNp (ref. 1; Jacobi and Wimmer, unpublished). These data were based on analyses of alkaline digests of [^{32}P]RNA and on labeling experiments with polynucleotide kinase. To confirm the absence of the capping group or of a ppNp or pppNp terminus, we digested poliovirion [^{32}P]RNA with RNase T2 and separated its products on DEAE-cellulose at pH 5. As shown in Fig. 7A, an elution profile similar to that in Fig. 4B of poliovirus mRNA was obtained. No counts eluted after GDP, an observation indicating the absence of ppNp, pppNp, or the capping group in the digest. We have, however, reproducibly (three experiments) observed distinct differences between elution profiles of Figs. 4B and 7A. First, materials in front of GDP elute as a double peak (fractions 24–32) in Fig. 7A, rather than as a single peak as in Fig. 4B. When analyzed by paper electrophoresis, these peaks contain, besides pUp and pAp, a number of other nucleotides (see below). Second, ^{32}P-labeled material which is of neutral charge at pH 5 elutes with the void volume of the column in Fig. 7A. This compound is also neutral at pH 3.5, since it remains at the origin during paper electrophoresis. All the compounds in fractions 1–2 and 24–32 must be phosphomonoesters, since their counts are converted to inorganic phosphate with alkaline phosphatase. The nature of these compounds and their relationship to virion RNA are currently under investigation.

Analysis of HeLa Cytoplasmic RNA. To show that our method used here would lead to the detection of capping groups, we have analyzed HeLa cytoplasmic [^{32}P]RNA that was labeled in the presence of low levels of actinomycin.

Fig. 7B shows the separation of an RNase T2 digest of 10–28S RNA. In contrast to analyses of poliovirus RNAs, radioactive material elutes at the position of GTP and after. Oligonucleotides in fractions 48–51, 52–56, and 57–69 were analyzed (2) and found to contain 7mpG (data not shown). The result confirms the presence of capping groups in HeLa cytoplasmic RNA (4, 9).

DISCUSSION

During the infectious cycle, newly synthesized poliovirus RNA might (*i*) associate with an as yet uncharacterized viral replicase to form a replication complex, or (*ii*) associate with ribosomes, or (*iii*) associate with viral proteins during assembly of progeny virus (16, 17). Since viral mRNA in polyribosomes does not appear to become encapsidated (16), a modification of mRNA that occurred during viral protein synthesis would not be evident in progeny virion RNA. Our previous results (1) and the data presented here show that the capping group is absent not only in genome RNA (Fig. 7A), but also in poliovirus mRNA (Fig. 4B), whereas, it can be found in HeLa cell cytoplasmic RNA (Fig. 7B). Poliovirus RNA can, therefore, be translated in the host cell cytoplasm without the capping group.

This conclusion is supported by our observation that in a wheat germ cell-free extract (28) poliovirion RNA was not labeled with [^{3}H]methyl in the presence of *S*-[*methyl*-^{3}H]adenosylmethionine (compare refs. 11 and 14). We found that protein synthesis (incorporation of [^{3}H]leucine) is stimulated 80-fold by poliovirion RNA in the absence of *S*-adenosylmethionine, while addition of *S*-adenosylmethionine or *S*-adenosylhomocysteine (5 or 10 μmol/liter) did not affect the stimulation. Incorporation of one [^{3}H]methyl group into one viral RNA molecule would have yielded theoretically 20,000 cpm. No incorporation above background, however, was observed (Marcu and Wimmer, unpublished). Furthermore, we have failed so far to label poliovirion RNA in HeLa cells *in vivo* with [*methyl*-^{3}H]methionine (Nomoto and Wimmer, unpublished) under the conditions of Furuichi *et al.* (4), which readily yield methyl-labeled HeLa mRNA (4). Absence of methylation of poliovirus RNA *in vivo* has been reported previously (29).

Picornaviruses, such as the poliovirus, effectively inhibit host-cell protein synthesis (16, 17). The mechanism of inhibition is probably an active shut-off of cellular protein synthesis caused by a virus-specific protein (16, 17). Alternatively, inhibition could be simply the result of competition between cellular and viral mRNA for ribosomes in which the viral mRNA has an advantage (30). In any event, translation of poliovirus RNA in the cytoplasm of HeLa cells *in vivo* or in a HeLa cell-free extract *in vitro* is efficient (31). Although the nature of the ribosome-binding site of picornavirus mRNA is unknown to date, one might speculate that it is distant from the 5′ end, that is, is preceded by a sequence of 100 or more untranslated nucleotides. As has been speculated for coliphage RNA (32), these untranslated nucleotides may be involved in RNA replication. The 5′ end might then be so distant from the ribosome binding site that it is not involved in the formation of the initiation complex; hence the capping group is unnecessary for protein synthesis. Picornaviruses are unique among cytocidal viruses in that during the replicative cycle they do not synthesize viral mRNAs that are shorter than the viral genome. In contrast, other single-stranded RNA viruses synthesize mRNAs that are subgenome size and are not involved in replication (33). In respect to the position of their binding site relative to the 5′ end, these smaller viral mRNAs may resemble cellular mRNAs and are therefore capped.

The nature of the 5′ end of the poliovirus genome remains puzzling. pAp was detected after polynucleotide kinase labeling (1), and pAp and pUp have been found in alkaline digests of virion RNA (ref. 1; Jacobi and Wimmer, unpublished). These data may indicate heterogeneity of the 5′ end of the polio genome. On the other hand, pUp is the only 5′-phosphorylated nucleotide found in digests of poliovirus mRNA (see Figs. 5 and 6). In comparison with other RNA-synthesizing systems, however, one would expect a 5′-terminal purine triphosphate at the 5′ end of polio RNA, if the nascent polio RNA strands in the replicative intermediate were initiated *de novo* (34). It is possible that initiation of polio RNA synthesis involves a primer that is cleaved at some point after chain termination to yield terminal pNp. In this respect it is of interest that we have failed to find pppNp in alkaline digests of the poliovirus replicative intermediate (Wimmer, unpublished).

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