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Structural Difference Between the 5′ Termini of Viral and Cellular mRNA in Poliovirus-Infected Cells: Possible Basis for the Inhibition of Host Protein Synthesis

RAFAEL FERNANDEZ-MUNOZ AND JAMES E. DARNELL*
The Rockefeller University, Department of Molecular Cell Biology, New York, New York 10021

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Host protein synthesis in poliovirus-infected HeLa cells is interrupted, but the host mRNA appears to remain completely intact and unmodified. The average size and poly(A) content of host mRNA was previously known to be unchanged (Koschel, 1974; Leibowitz and Penman, 1971), and this was confirmed. In addition, the 5′ terminal methylated "cap" structures remained intact, and no further base modifications at the level of 1 base in 1,000 could be detected. Poliovirus RNA from viruses was previously shown not to have "caps" (Wimmer, 1972), and in this work poliovirus RNA from polyribosomes was found to have pUp at its 5′ end. Since, initiation of protein synthesis is probably the basis for the inhibition of cellular protein synthesis in infected cells, the difference in the 5′ ends of the host cell and viral RNA could be the basis of selective translation of viral RNA during infection.

It was first observed in 1960 that the incorporation of amino acids into host proteins was decreased in HeLa cells infected by poliovirus (4). Later, host cell polyribosomes were shown to disappear during virus infection and to be replaced by poliovirus polyribosomes (15, 20). No molecular basis for this specific inhibition of host protein synthesis has been described. Penman and colleagues have presented evidence that infected cells do not properly initiate host protein synthesis, whereas initiation of polio-specific proteins proceeds well (7, 12, 16). However, the host cell mRNA appears to be largely intact (12, 21) and to have a normal segment of poly(A) at its 3′ terminus (11).

The 5′ end of most HeLa cell mRNA molecules (6, 18), like a wide variety of other cellular and viral mRNA molecules, consists of a blocked oligonucleotide structure m^7GpppNmpNp---- or m^7GpppNmpNmpNp----, termed a "cap." Reovirus, vesicular stomatitis virus, and hemoglobin mRNA molecules require this cap structure to participate in vitro in the initiation of protein synthesis (2, 18). Since during poliovirus infection the defect in host cell mRNA translation is probably in some step of initiation (12), we have examined host cell mRNA and poliovirus mRNA in infected cells for the presence of cap structures. The host cell mRNA still contains caps but the virus mRNA lacks them, perhaps providing a molecular basis for discrimination of the two mRNA classes, so that polio RNA can still be translated while host protein synthesis declines.

MATERIALS AND METHODS

Cell and virus growth. The growth of suspension cultures of HeLa cells and the growth, purification, and experimental infections (input multiplicity of infection about 2,000 particles/cell) with poliovirus were carried out as described earlier (1, 4, 10, 20). Actinomycin D was used during infection at 4 μg/ml and guanidine was used at 2 mM where indicated.

RNA digestion and product analysis. RNA samples were digested with T2 and pancreatic RNases as described (6) immediately after heating for 2 min at 100 C and quick chilling of the samples. DEAE-cellulose column chromatography procedure was as described (6). Selected fractions were desalted by a second DEAE-cellulose adsorption and elution with 1 M NH_4CO_3H or 1 M triethylaminebicarbonate, pH 9 (3). *Penicillium* nuclease and other enzymatic digestions as well as DEAE-paper electrophoresis (Whatman DE81) and cellulose thin-layer chromatography followed standard procedures (3, 6, 14). pUp, pGp, pAp, and pCp markers were obtained by transfer of [^{32}P]phosphate to the 5′ position of 3′-mononucleotides from [^{32}P]ATP, using T4-induced polynucleotide kinase (3). Poly(A) size was determined as described (19).

Materials. Carrier-free [^{32}P]phosphoric acid and methionine (specific activity, 10 Ci/mmol) were obtained from New England Nuclear Corp.; ^{3}H-labeled amino acids were from Schwarz/Mann. T2 RNase was purchased from Sankyo; *Penicillium* nuclease (P1) was from Yamasa Ltd., Tokyo; bacterial alkaline phosphatase and pancreatic A RNase were from Worthington; and T4-infected *Escherichia coli* B polynucleotide kinase was from PL Biochemicals. Puromycin was from Nutritional Biochemicals. Microcrystalline cellulose thin-layer plates (20 by 20 cm) were from Analtech.

Cellular mRNA labeling and purification. The poly(A)-containing RNA from the post-mitochondrial fraction of the cell cytoplasm was collected after ^{32}P and [methyl-^{3}H]methionine labeling (6). Poliovirus polysomal RNA was obtained from infected cells (3.5 h postinfection) by hypotonic swelling, Dounce homogenization, and zonal sedimentation (15) followed by EDTA treatment of polysomes and a second zonal sedimentation (9). The total labeled cytoplasmic non-encapsidated virus RNA, which is made up largely of poliovirus mRNA (9), was obtained by treating infected cell extracts with 1% sodium dodecyl sulfate, conditions where virion is stable, followed by zonal sedimentation (1, 9). 35S viral RNA was further purified by poly(U) Sepharose chromatography, using elution by 20 to 25% formamide (5). The 35S RNA eluted from such poly(U) Sepharose columns was considered to be purified viral mRNA and was analyzed as described in the text.

RESULTS

Poliovirus-induced inhibition of cell protein synthesis occurs even though poliovirus RNA replication is blocked by treatment of the infected cells with guanidine (16, 20). This situation allows the prior labeling of host cell mRNA, infection, and inhibition by actinomycin D treatment of further cell RNA labeling. Under these conditions, host protein synthesis declined to virtually zero within 2 h of infection (Fig. 1), and previously labeled host mRNA then could be examined. (It should be noted in Fig. 1 and reference 20 that this inhibition is virus induced and not due to actinomycin.)

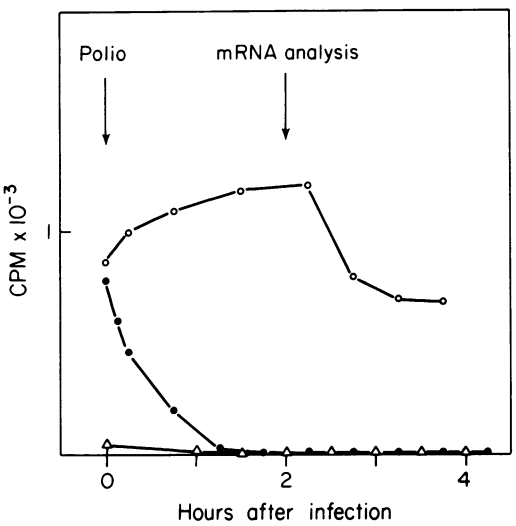

FIG. 1. *Protein synthesis in poliovirus-infected cells. HeLa cells were collected and labeled with ^{3}H-labeled amino acid mixture (Schwarz/Mann) after poliovirus infection (●), mock infection (○), and puromycin treatment at 100 μg/ml (△). Cells were treated with actinomycin (2.5 μg/ml) for 30 min and collected, and infection was carried out (2,000 particles/cell) at 10^{7} cells/ml in serum-free medium containing actinomycin for 30 min (20). Cells were then diluted to 2 × 10^{6} cell/ml in medium containing serum, actinomycin at the same concentration, and 2 mM guanidine. Trichloroacetic acid-precipitable, alkali-stable radioactivity was scored after 10-min pulses of ^{3}H-labeled amino acids. This experiment was a pilot to the experiment described in Fig. 2.*

In the experiment presented in Fig. 2, a culture was labeled with ^{32}P and divided into three portions: (i) one-third was infected with poliovirus and treated with guanidine and actinomycin; (ii) one-third was treated in the same way without infection; and (iii) one-third was the same as (ii) but was also treated with puromycin, a drug that, in common with poliovirus infection, inhibits protein synthesis and disaggregates host polyribosomes. The mRNA was selected from all three samples by poly(U) Sepharose chromatography. The sedimentation profile even after dimethyl sulfoxide treatment (data not shown), poly(A) content, and poly(A) size (data not shown) were found to be the same from the infected and uninfected cultures, as has been previously reported (11, 12). The three mRNA samples were digested with T2 RNase, which leaves the blocked methylated cap structures intact, and the digests were subjected to DEAE column chromatography. In all three samples about 0.3% of the ^{32}P label eluted from DEAE in the position of oligonucleotides with a charge of −5 to −6. Previous experiments have characterized oligonucleotides with this elution profile from DEAE to be cap structures of the type $m^{7}GpppN^{m}pNp$ or $m^{7}GpppN^{m}pNp$ (2, 6, 18). The number average length of HeLa cell mRNA is about 1,500 nucleotides, and 0.3% of PO_4^{3-} in caps equals about 1 cap per 1,500 to 1,800 nucleotides (5 to 6 PO_4^{3-} per 1,500 to 1,800 nucleotides). Thus most if not all the poly(A)-terminated, labeled mRNA in HeLa cells appeared capped both before infection and after virus inhibition of protein synthesis was complete. The radioactivity eluting from the column with approximate charge −3 and −4 was from rRNA because such material is not present when cells are labeled in the presence of 0.04 μg of actinomycin D per ml (6). The amount of radioactivity in these peaks corresponded to an approximate molar contamination of 2% in the poly(U) Sepharose eluate, in agreement with the estimated contamination by the pseudouridine content of the sample [see below]. Together with the previous studies showing the size of mRNA and poly(A) content to be unaffected by infection, we conclude that the cell mRNA from infected cells is not destroyed or chemically inactivated due to major changes at either end of the molecule.

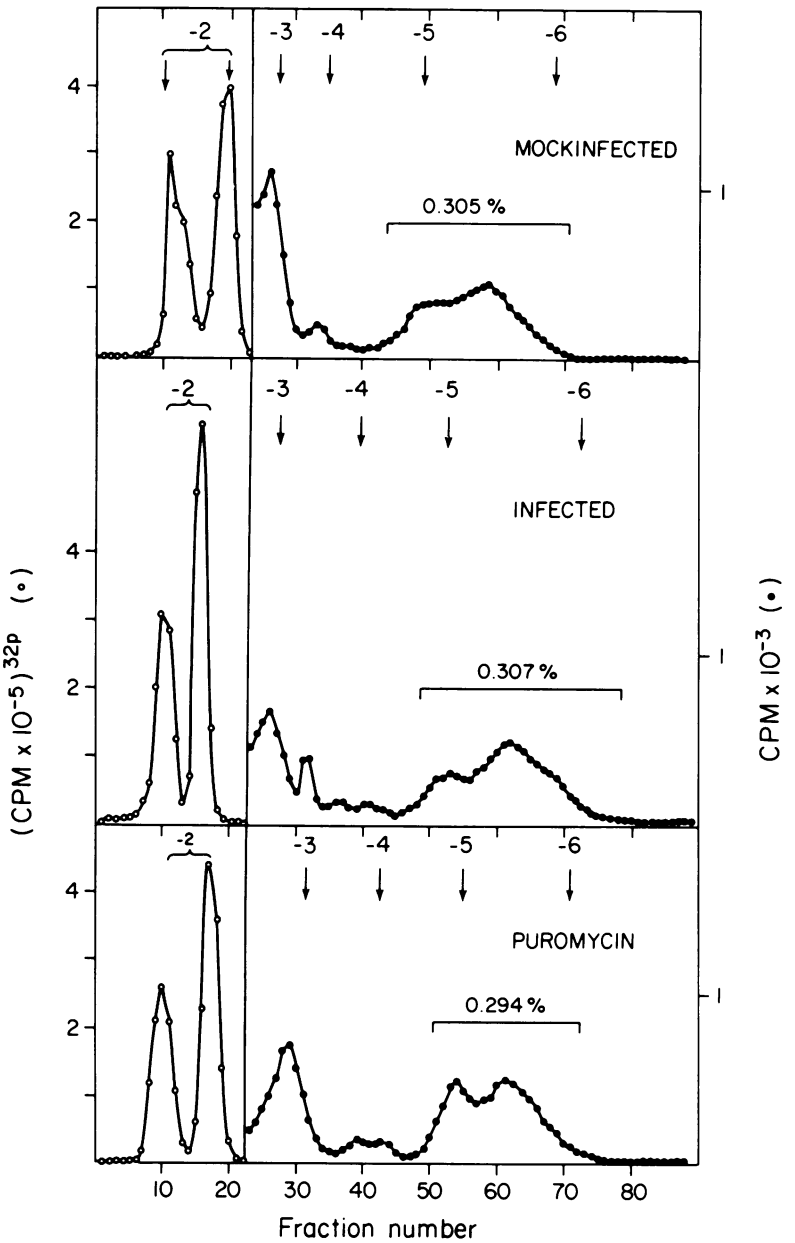

FIG. 2. *T2 RNase-resistant ^{32}P-labeled oligonucleotide in cell mRNA after poliovirus infection. HeLa cells were labeled with ^{32}P (6) for 3 h, collected, washed free of label, and divided into three parts: mock infected, infected, and puromycin treated. After 2 h of incubation the poly(A)-containing mRNA from each culture was purified, digested with T2 RNase (6), and analyzed by DEAE-urea chromatography. OD_{260} marker oligonucleotides were from yeast tRNA.*

To investigate the possibility of minor modification of bases in the host mRNA as a basis for translation inhibition, several additional experiments were carried out.

(i) Cells were labeled with *methyl*-^{3}H-labeled methionine, which labels both caps and *N*-6-methyl adenylic acid, and then infected in the presence of 0.04 μg of actinomycin per ml and guanidine as in Fig. 3. The methyl-labeled mRNA was then purified, digested, and analyzed by chromatography on DEAE-cellulose. The ratio of label in caps to label in mononucleotides (−2 charge) was 3.0 for infected cell mRNA and 2.5 for uninfected cells. This differ-

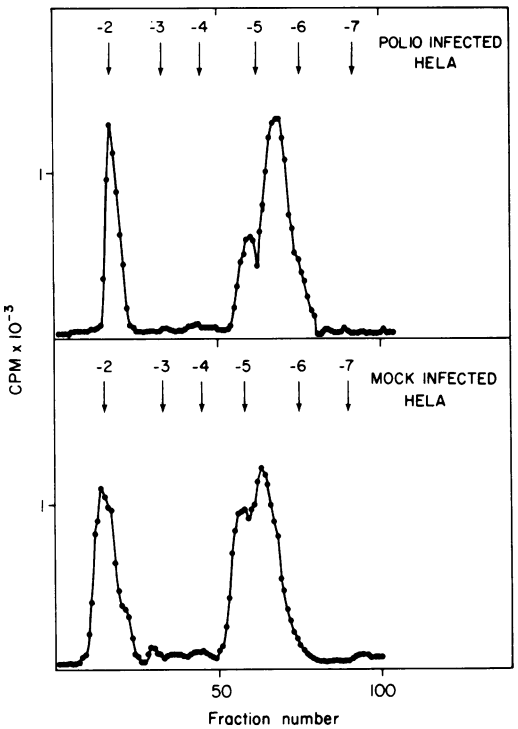

FIG. 3. *T2 RNase-resistant methyl-labeled oligonucleotides in cell mRNA after poliovirus infection. HeLa cells labeled for 3 h with [methyl-³H]methionine (6) were collected and either mock infected or infected with poliovirus as in Fig. 1 and 2. Two hours after infection the poly(A)-containing cytoplasmic mRNA was purified, digested, and analyzed by DEAE chromatography as in Fig. 2. A similar curve of cell protein synthesis inhibition to Fig. 1 was obtained in a pilot to this experiment.*

ence is probably not significant and shows very little or no change in methylation due to infection.

(ii) The ^{32}P-labeled caps isolated from infected and mock-infected cells were examined by electrophoresis on DEAE-cellulose paper at pH 1.7 in an attempt to detect any charge difference not observed by column chromatography that might have been the result of modifications of the basic cap structure (e.g., an additional 2′-*O*-methylation coupled with a "decapping," i.e., loss of terminal phosphate[s]). No differences in electrophoretic migration were detected (data not shown).

(iii) Minor bases, which constitute 0.1% of the total nucleotides, can be detected in ^{32}P labeled mRNA. If base modifications not involving *S*-adenosyl-methionine occurred (for example, conversion of uridylic acid to pseudouridylic acid or perhaps ribothymidylic acid), then the [*methyl*-³H]methionine experiment described in Fig. 3 above might not have demonstrated such changes. Therefore, ^{32}P-labeled mRNA from infected and uninfected cells was digested completely with T2 and subjected to two-dimensional chromatography on thin-layer cellulose plates (14). Figure 4 shows the expected four major spots (Ap, Cp, Gp, and Up) as well as two minor spots in both uninfected and infected samples. One of the minor spots corresponded to m^{6}Ap, which indicates the sensitivity of the method since about 1 base in 1,000 in HeLa cell mRNA should be m^{6}Ap (6; Salditt-Georgieff et al., in press), and that was the level observed here. The second small spot was in the position of pseudouridylic acid, but this spot proved to be an rRNA contaminant (approximately 2% contamination in molar terms calculated from the ~140 residues of pseudouridine per rRNA molecule [13]) because cells prelabeled with ^{32}P in the absence of rRNA synthesis did not contain the pseudouridine spot. Thus no change at the level of about 1 nucleotide per mRNA molecule could be detected as occurring after poliovirus infection.

Possible methylations and modifications of poliovirus mRNA. If in poliovirus infection the host mRNA remains intact yet is not translated, it seemed possible that the poliovirus RNA might be structurally different so that a distinction could be made between host and virus mRNA. The 3′ end of poliovirus RNA is a segment of poly(A) somewhat shorter than but similar to that of host mRNA (23). Thus, when the cap structures were found in host mRNA, it became of considerable interest to determine whether poliovirus mRNA differed at its 5′ end. It was already known that virion RNA, at least some of which must be translated upon entry into cells, did not contain a blocked 5′ terminal structure (22). Since modifications necessary for the translation of virion RNA after infection might be possible, particularly since some cytoplasmic extracts are able to add caps (2), it was necessary to isolate the functioning polio mRNA------, the polyribosomal polio RNA----, to compare with host mRNA.

Cells were infected, actinomycin treated, and labeled with either [³H]uridine to follow polio RNA synthesis or with [*methyl*-³H]methionine, and an uninfected control was also labeled with [*methyl*-³H]methionine. In the pilot study with the [³H]uridine-labeled cells, well over one-half of the total cytoplasmic 35*S* polio RNA was found in polyribosomes at 3.5 h after infection as described in an early study (9). Therefore, in the [*methyl*-³H]methionine-labeled culture the total cytoplasmic labeled RNA from the infected cells was released with sodium dodecyl sulfate; virions remained intact under these

circumstances, and the released 35S RNA (presumably mainly polysomal 35S RNA) was collected after zonal sedimentation. This RNA was further purified by adsorption to poly(U) Sepharose and a second sucrose gradient sedimentation, at which time the optical density tracing showed mainly 35S virus-specific RNA (Fig. 5). Approximately 12 μg of 35S polio RNA was recovered from the [*methyl*-^{3}H]methionine-labeled culture, but no *methyl*-^{3}H-labeled radioactivity was detectable. An equivalent amount of cellular mRNA purified from [*methyl*-^{3}H]methionine-labeled uninfected cells contained 40,000 counts/min. Each polio RNA molecule is about 7,500 nucleotides long (8), whereas cell mRNA averages 1,500 nucleotides; we would still have expected at least one-fifth as much radioactivity in the poliovirus 35S RNA if it were equally methylated compared with cell mRNA. In addition, poliovirus RNA was all newly synthesized in infected cells during the 3.5 h of this experiment, whereas cellular mRNA would have been at most one-half renewed during the 3.5-h labeling period, thus favoring the observation of methyl incorporation into polio RNA if methylation did indeed occur.

A further consideration indicates that ^{3}H from methyl-labeled methionine should have been detected if polio RNA were methylated. A parallel infected culture labeled with [^{3}H]uridine was also subjected to the same polysomal 35S RNA purification procedure and yielded 4×10^6 counts/min. Suppose the [^{3}H]uridine label in polio RNA to be derived from UTP and CTP pools that had reached maximum specific activity so that both C and U residues (4,000 total residues) were labeled at the maximum expected specific activity (approximately one-half the added [^{3}H]uridine specific activity of 20 mCi/μmol [17]). The [*methyl*-^{3}H]methionine and *S*-adenosylmethionine derived from it should exist in cells at the same specific activity as added to the medium (12 mCi/μmol). The [^{3}H]methionine should then contribute at least 1/4,000 as many counts per methyl group as [^{3}H]uridine contributes (1 methyl group per 4,000 C + U). This would have resulted in 1,000 counts/min for a single methyl group. Thus, it appears that poliovirus RNA lacks both the 5′ terminal cap structure as well as the m^{6}Ap and m^{5}C, which occur in cellular mRNA and in viral RNAs made in the cell nucleus (6, 18).

A detection of caps in polio RNA from infected cells was also attempted by labeling infected cells with ^{32}P during the first 3.5 h of infection. Structures sedimenting faster than virions were collected, and RNA was released and purified as for the *methyl*-^{3}H-labeled cells. The purified 35S virus-specific ^{32}P RNA was completely digested with nucleases, and the digest was subjected to DEAE-column chromatography. No nuclease-resistant material eluted in the position of caps, but about 1/4,000 of the radioactivity did elute at the position of −4 (Fig. 6). This material with a −4 charge was characterized as follows:

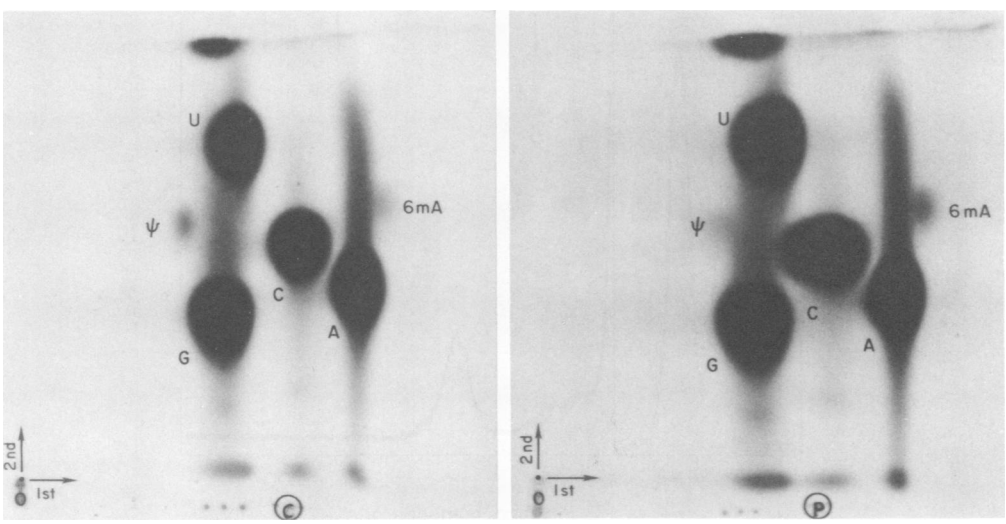

FIG. 4. *Two-dimensional chromatographic analysis of ^{32}P-labeled mono-nucleotides from cell mRNA (taken from Fig. 2) was carried out by techniques of Nishimura (14). First dimension was isobutyric acid-NH_4OH-water (66:1:33); second dimension was isopropanol-HCl-water (68:17.6:14.4). An autoradiogram from mock-infected (C) and infected (P) cells is shown.*

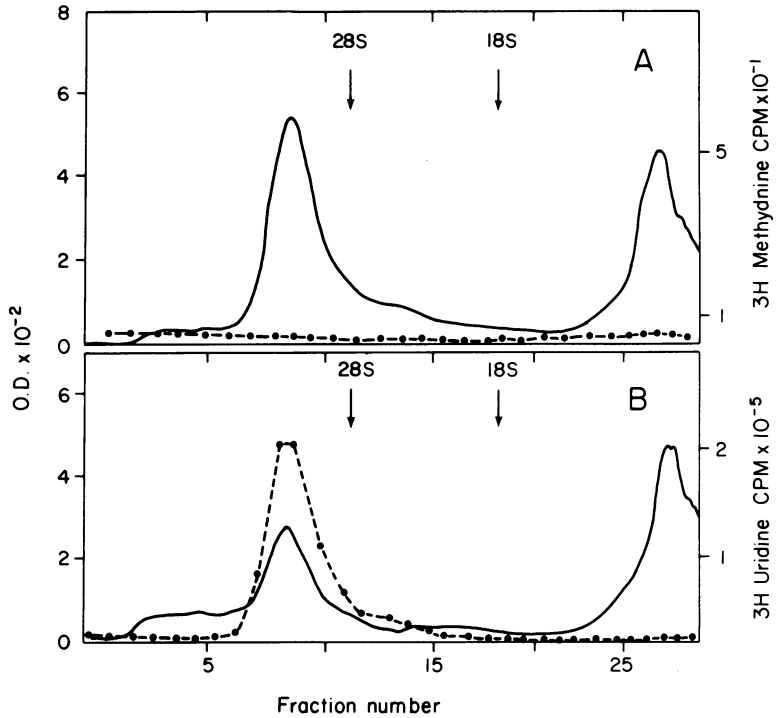

FIG. 5. *Failure of methyl labeling of poliovirus RNA. Infected cells labeled with [methyl-^{3}H]methionine (A) and [^{3}H]uridine (B). (A) 6 × 10^8 HeLa cells were infected with poliovirus and labeled for 3.5 h with 15 μCi of [methyl-^{3}H]methionine per ml at a final methionine concentration of 4 μM in the medium in the presence of 4 μg of actinomycin D per ml. 35S non-encapsidated viral RNA was purified as described in the text and then sedimented through a sucrose gradient. Adsorption at 260 nm was measured in a flow cell in a Gilford spectrophotometer, and total radioactivity in the aliquots was measured as indicated. (B) Parallel experiment where 10^8 HeLa-infected cells were labeled with 30 μCi of (^{3}H) uridine per ml.*

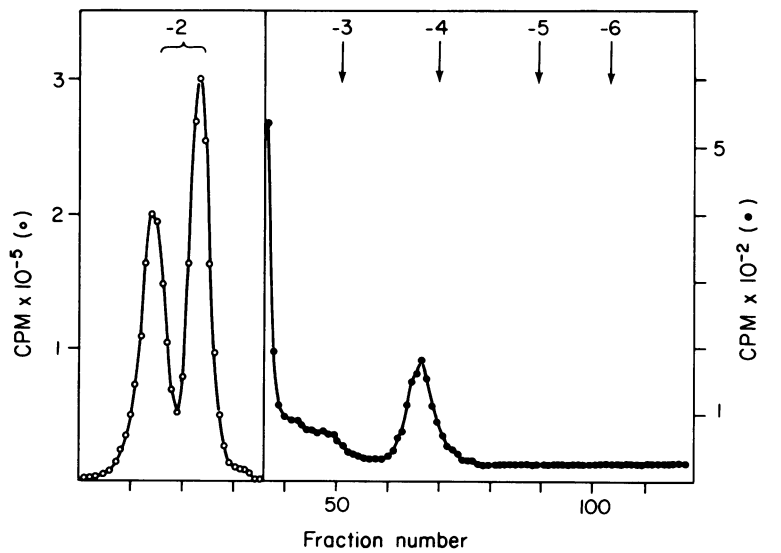

FIG. 6. *DEAE-cellulose column chromatogram of T2 RNase digestion products of ^{32}P-labeled poliovirus polysomal 35S RNA. 5 × 10^8 HeLa cells were infected with 60 PFU of poliovirus per cell for 3.5 h in the presence of 250 μCi of ^{32}P and 4 μg of actinomycin D per ml. 35S RNA was isolated from polysomes larger than 120S (the sedimentation coefficient of virions) as indicated in the text. The digestion and chromatography were done as for cellular mRNA (Fig. 2 and 3 and text).*

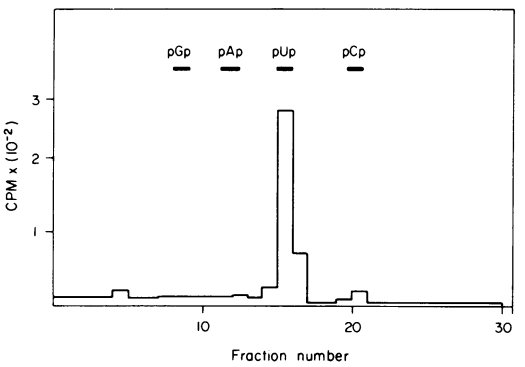

FIG. 7. *High-voltage DEAE-paper electrophoresis of ^{32}P-labeled oligonucleotide from poliovirus polysomal RNA. The −4 peak from chromatography (Fig. 6) was desalted and then electrophoresed on DEAE-paper (Whatman DE81) using pyridine-acetic acid-EDTA, pH 3.5, at 20 V/cm for 5 h with the four pXp markers. One-centimeter strips were cut, and the Cerenkov radioactivity was measured.*

(i) All the ^{32}P was released as PO_4^{3-} by alkaline phosphatase treatment (data not shown);

(ii) co-electrophoresis on DEAE-paper at pH 3.5 with markers of pUp, pCp, pAp, and pGp showed that 95% of the material migrated together with pUp and the remaining 5% as pCp (Fig. 7);

(iii) when treated with *Penicillium* nuclease, which contains a 3′-phosphatase (6), about 50% of the radioactivity was released as PO_4^{3-} and the remainder migrated during electrophoresis as 5′- pU, as did the ^{32}P-labeled pUp marker (data not shown);

(iv) finally, the presumptive pUp was chromatographed on cellulose thin-layer plates in isopropanol-water-HCl, which can distinguish phUp, prTp, pUmp, pdUp, and pUp; the −4 material derived from polio mRNA migrated as pUp (Fig. 8).

Thus there was one pUp derived from each 8,000 nucleotides, suggesting that pUpN----- is the 5′ end of the poliovirus mRNA.

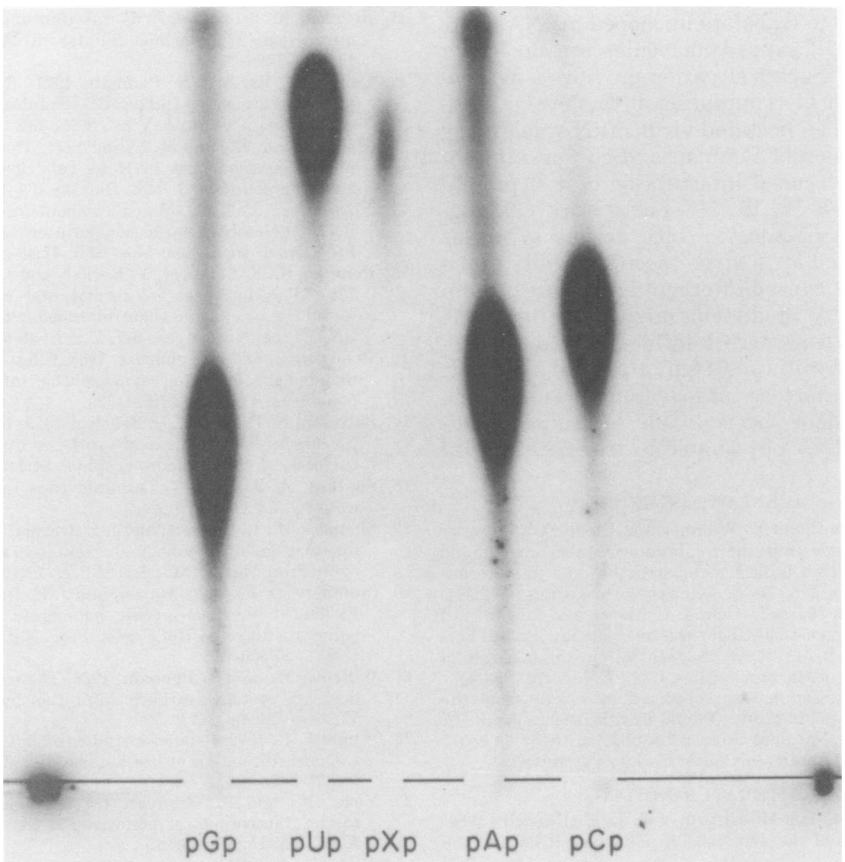

FIG. 8. *Thin-layer chromatography of "−4" oligonucleotode from poliovirus polysomal RNA. The −4 material, obtained after DEAE-urea column chromatography (Fig. 6) and DEAE-paper electrophoresis (Fig. 7), was eluted from the paper and chromatographed in cellulose thin layers using as solvent isopropanol-HCl-water, and an autoradiograph of the chromatogram was made.*

In contrast, virion RNA, isolated at two times after infection and analyzed in the same way, did show less than 1 pNp residue per 75,000 nucleotides (Fernandez-Munoz, unpublished observations).

DISCUSSION

The present experiments, like many other earlier ones, do not define the basis in infected cells for the complete inhibition of host protein synthesis while over one-half the ribosomes of the cell are engaged in synthesizing poliovirus protein (15, 20). These experiments do, however, complete the studies on host mRNA during infection, supporting the firm conclusion that host mRNA is not destroyed—the molecules are intact, both ends are normal, and the cell mRNA is not detectably modified at the level of less than 1 unusual base per 1,000 nucleotides. The fact that poliovirus mRNA itself does not have a 5′ methylated cap structure—a structure shown in at least three instances (2, 18) to be necessary for protein synthesis initiation—suggests that a mechanism exists in infected cells to translate uncapped mRNA molecules even if capped molecules are no longer translated. Such a situation provides a molecular basis for discrimination at the level of initiation between host and virus mRNA, following the prediction of Penman and co-workers that this is the locus of interruption of host protein synthesis (7, 12, 16, 21). Early work (16) suggested that in infected cells, protein synthesis was required for a virus-specific activity to develope that caused interruption in host protein synthesis. A prediction might now be made that this virus-specific influence occurs somehow in the initiation step of protein synthesis (initiation factors or ribosomal subunits?), which renders the cell able to translate uncapped mRNA but unable to translate capped mRNA.

ACKNOWLEDGMENTS

We wish to thank E. Wimmer and David Baltimore for communications that their laboratories also have found poliovirus mRNA to lack a cap structure and to be terminated by pUp. That work will appear elsewhere. We also wish to thank Maria L. Celma, Uri Lavi, and Edward Ziff for helpful suggestions about this manuscript. Rafael Fernandez-Munoz is on leave of absence from the Institute of Biochemistry of Macromolecules, C.S.I.C. Madrid, Spain.

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