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The structure of poliovirus replicative form

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ABSTRACT

The structure of polio replicative form (RF) has been investigated by 3' end labeling and the use of polynucleotide phosphorylase to now allow a complete composite of the RF structure. The evidence presented indicates that the 3' terminal sequence of the minus strand is an exact complement to the 5' end of polio RNA. This suggests that the 5' terminal U of polio RNA is genetically coded. Other data is presented to show that in addition to the genetically coded poly(A) tract of the plus strand in RF, a single-stranded poly(A) tail protrudes beyond the double-stranded RNA.

INTRODUCTION

Poliovirus double-stranded RNA (the "replicative form", RF) is an interesting molecule for several reasons: (i) Based upon kinetic (1) and biochemical (2) data, RF is synthesized during poliovirus replication and is not an artifact of isolation procedures. (ii) RF is infectious (3), but has been shown to be a potent inhibitor of protein synthesis (4). In contrast to single-stranded viral RNA, infectivity of RF requires host cell nuclear function(s), presumably for the conversion of the double-stranded RNA to mRNA (5). (iii) Both strands of polio RF are polypeptide-linked at the 5' ends (6,7) to a small, basic, viral protein called VPg (8). Degradation of the terminal proteins by protease does not diminish the infectivity of the molecule (Reich and Wimmer, unpublished results). (iv) The plus strand of RF (which is of the same polarity as virion RNA) is 3' polyadenylated (2). The 5' end of the minus strand, on the other hand, is polyuridylated (9), an observation suggesting that the poly(A) of poliovirus RNA is genetically coded (10,11,12). (v) As determined by polyacrylamide gel electrophoresis, poly(A) of RF plus strands appears to be longer than the complementary poly(U) of minus strand (2,12).

VPg is linked to all intracellular virus-specific RNAs except for viral mRNA. Particularly interesting is the observation that nascent strands of

replicative intermediate contain VPg. This has led us to suggest that the polypeptide is involved in the initiation of RNA synthesis (8,6,14) a view shared by others (15). VPg or a polypeptide precursor thereof may directly function as a primer (for a discussion, see reference 14). On the other hand, RNA synthesis could also be initiated with VPg-pU, the uridine residue subsequently becoming the 5' terminal base of the newly synthesized polynucleotide chain (6). In such case, the 5' terminal uridine may in fact not be genetically coded. This latter consideration has prompted us to analyze the 3'-terminal nucleotide sequence of poliovirus minus strands.

We also report an enzymatic assay providing evidence that single-stranded poly(A) in RF protrudes beyond the double-stranded molecule. These data allow us to describe the structure of RF isolated from poliovirus infected HeLa cells.

MATERIALS AND METHODS

Synthesis of carrier free [γ-^{32}P]ATP and [5'-^{32}P]pCp: Carrier free [γ-^{32}P]ATP was synthesized by the procedure of Johnson and Walseth (16) with the following modification (S.I.T. Kennedy, personal communication): After synthesis of ATP, 400 µl of H_2O is added to the reaction mixture that is subsequently extracted once with an equal volume of water saturated phenol: chloroform (1:1 v/v), and twice with ether. The aqueous phase containing the [γ-^{32}P]ATP is then mixed with an equal volume of ethanol and stored at -20°C.

Cytidine 3',5'-bis(phosphate) was synthesized by the procedure of England and Uhlenbeck (17) with minor modifications. 1.5 mCi of [γ-^{32}P]ATP was lyophilized in a 1.5 ml Eppendorf tube immediately before use. The reaction mixture (50 µl) contained 25 mM Tris-HCl, pH 9.0, 5 mM MgCl, 3 mM dithiothreitol, 50 µg/ml bovine serum albumin, 1 mM cytidine 3'-monophosphate, and 150 units/ml polynucleotide kinase (P.L. Biochemicals), and was incubated for 1 hour at 37°C.

The extent of the reaction was monitored by spotting 0.5 µl, before and after incubation, onto a PEI thin-layer plate that was developed by ascending chromatography in 0.75 M potassium phosphate (pH 3.5). The R_f of pCp is approximately 0.77. The mixture was diluted to 1 ml with water and applied to a column (1.0 x 10.0 cm) containing DEAE-cellulose equilibrated in 0.1 M triethylammonium acetate (TEAA), pH 5.0. A linear elution gradient of 60 ml was then applied ranging from 0.1 M TEAA to 0.7 M TEAA. Sixty 1 ml fractions were collected and small aliquots were monitored for Cerenkov radiation.

The material eluting between [^{32}P]P_i and [^{32}P]ATP is [^{32}P]pCp and was collected and lyophilized. It was redissolved in water and lyophilized again to remove any residual volatile salt, and finally dissolved in ethanol/water (1:1) for storage at -20^{o}C.

3' End-labeling of RF: Polio replicative form (RF) was purified as described by Detjen et al. (5). Before 3' end labeling, the RF was treated with proteinase K to degrade VPg (18,19), which is juxtaposed to the 3' hydroxyl of the minus strand. The reaction mixture (20 µl) contained: proteinase K (2.0 mg/ml), RF (25 µg), NaCl (0.1 M), Tris HCl (10 mM) pH 7.5, EDTA (1 mM), pH 7.5, and sodium dodecylsulfate (0.5%). After incubation for 1 hour at 37^{o}C, the mixture was extracted with phenol/chloroform and the RNA precipitated with ethanol.

The 3' hydroxyls were labeled with [5'-^{32}P]pCp and RNA ligase as described (17) with minor modifications. The reaction mixture (30 µl) contained: HEPES, pH 8.3 (50 mM), $MgCl_2$ (15 mM), dithiothreitol (3.3 mM), dimethyl sulphoxide (10%, v/v), glycerol (15%, v/v), ATP (7.5 µM), [5'-^{32}P]pCp (0.5 µM) polio RF (125 nM) and RNA ligase (580 units ml^{-1}). Incubation was for 1 hour at 37^{o}C and was terminated by ethanol precipitation. End-labeled [^{32}P]RF was purified by sedimentation through a 15-30% sucrose gradient (2). One peak was observed that sedimented at 18S, characteristic for poliovirus double-stranded RNA. It was recovered by ethanol precipitation.

Isolation of the 3'-terminal T_1 oligonucleotide from minus strand RNA: 3' end-labeled RF was dissolved in 30 µl of NaCl (10 mM), Tris-HCl (1 mM), pH 7.5, and EDTA (0.1 mM). RF in 10 µl of this solution was denatured by heating to 100^{o} for 2 minutes, followed by a quick cooling. 3.5 units of RNase T_1 were added and the mixture incubated for 15 minutes at 37^{o}C. It was then adjusted to a final concentration of 7M urea, 0.04% bromophenol blue, 1 mM EDTA and applied directly to a polyacrylamide slab gel consisting of 20% (w/v) acrylamide, 0.67% (w/v) N,N'-methylene-bis-acrylamide, 7M urea, 50 mM Tris-borate (pH 8.3) and 1 mM EDTA (20). Electrophoresis was terminated when the bromophenol blue dye migrated 32 centimeters.

Recovery of the 3' labeled T_1 fragment of minus strand RNA: Material that we assumed to correspond to the 3'-terminal oligonucleotide was cut from the 20% acrylamide gel, and placed directly into a column (disposable 5 ml plastic pipette cut to appropriate length) containing 2 ml of DEAE-cellulose equilibrated in 10 mM TEAA, pH 5.0. The RNA was transferred from the gel fragment to the DEAE-cellulose by pumping 100 ml of 10 mM TEAA

through the column. The RNA was subsequently eluted from the DEAE-cellulose with 0.5 ml of 2.0 M TEAA. The eluant was diluted with water and lyophilized. The radioactive material was finally dissolved in water.

Determination of the 3'-terminal nucleotides of the oligonucleotide: The 3'-terminal nucleotides of the 3'-labeled oligonucleotide were determined by complete digestions using RNase T_2, RNase U_2 and nuclease P1, followed by paper electrophoresis. Marker nucleotides were generated by digesting poliovirus [^{32}P]RNA with the same enzymes. Enzyme to substrate ratios were 0.05 units of RNase T_2/µg RNA; 0.1 units of RNase U_2/µg RNA; and 0.1 µg of nuclease P1/µg RNA. Incubations were at 37°C for 30 minutes (RNase T_2) or for 1 hour (RNase U_2 and nuclease P1). T_2 and U_2 digests were carried out in 50 mM NH_4OAc, pH 4.5, 1 mM EDTA; P1 digests in 50 mM NH_4OAc, pH 6.0.

The products were applied to Whatman 3MM paper and electrophoresed in pyridine/acetic acid, pH 3.5, for 1.5 hours at 4,000 volts. The paper was then dried and autoradiographed.

To determine the penultimate base of the minus strand the oligonucleotide was digested with RNase A, at a ratio of 6.7×10^{-2} µg enzyme/µg RNA, in 50 mM Tris-HCl, pH 7.5, 2 mM EDTA, for 1 hour at 37°C. The product was applied to DEAE-paper, along with marker [^{32}P]ApApCp, a gift from Akiko Kitamura. Electrophoresis in pyridine/acetic acid, pH 3.5, was carried out for 5 hours at 3,000 volts. The paper was then dried and autoradiographed.

Mobility shift analysis: The nucleotide sequence of the T_1 fragment was determined by mobility shift analysis as described by Silberklang et al. (21) except that the partial hydrolysis of the labeled fragment was accomplished with sodium carbonate instead of nuclease P1 as follows: The oligonucleotide was lyophilized together with 5 µg carrier tRNA. The mixture was then dissolved in 15 µl of 50 mM $NaHCO_3/Na_2CO_3$, pH 9.0, 1 mM EDTA and the solution was transferred to a capillary tube, sealed at both ends and incubated for 20 minutes at 90°C. The sample was subsequently lyophilized and redissolved in 1-2 µl of water and subjected to electrophoresis on cellulose acetate at pH 3.5 (1st dimension), followed by homochromatography using a 75 mM KOH-strength "homomix", as previously described (22).

Treatment of RF with polynucleotide phosphorylase: [^{32}P]RF was isolated from poliovirus infected cells as described (2). 0.12 µg [^{32}P]RF (100,000 cpm) in 4 µl of 0.1 M NaCl, 0.01 M Tris-HCl, pH 7.5 were mixed with 1 µl 0.1 M sodium phosphate, pH 8.5, 1 µl 0.5 M Tris-HCl, pH 8.5, 1 µl 0.5 M $MgCl_2$, and 2 µl H_2O. 0.1 Unit polynucleotide phosphorylase (Micrococus lysodeiktikus, Type 15; P.L. Biochemicals, Inc.) in 1 µl of 0.2 M NaCl, 0.1 M Tris-HCl,

pH 8.5, 0.001 M $MgCl_2$ was added to the RF mixture and 1 µl of the mixture was withdrawn immediately and applied to a polyethyleneimine (PEI) plate (Macherey - Nagel & Co.). The other 9 µl were rapidly transferred to a capillary and heated to 60°C on the surface of a heater block. The ends of the capillary remained open. At various times approximately 1 µl was withdrawn with a 1 µl glass pipette (Pederson, Denmark) and immediately applied to the PEI-plate to which marker nucleoside diphosphates had been applied before. After withdrawal of the aliquot the reaction mixture was quickly moved back into the center of the capillary. At the end of the incubation, the PEI plate was developed with 4 M sodium formate, pH 2.5 (9) and autoradiographed. Marker nucleotides were identified under UV light. Radioactive spots were cut out and counted. The yield of released nucleotides was calculated as percent of total radioactivity applied to the lane (approximately 99% of the radioactivity remained at the origin as undergraded RF).

RESULTS

3' labeling of poliovirus RF: We assumed that VPg linked to the 5' end of plus-strand RNA would interfere, possibly by steric hindrance, with the ligase-catalyzed condensation of pCp to the end of minus-strand RNA in RF. The 3'-terminal poly(A) of plus strand RNA, on the other hand, would be expected to label well because its 3' end extends beyond complementary minus strand RNA (see below). To avoid the problem of steric hindrance with end-labeling, polio RF was incubated with proteinase K, an enzyme known to degrade RNA-linked VPg (18,19). Proteinase K-treated RF was subsequently incubated with RNA ligase and [5'-^{32}P]pCp under the conditions described in "Materials and Methods". Recovered, labeled RF sedimented through a sucrose gradient as a single peak at 18S (data not shown). As calculated from the recovery of a 3'-terminal oligonucleotide, labeling of the minus strand proceeded to at least 5% of the theoretical yield. A value similar to that was found in labeling studies of reovirus dsRNA (34).

Analysis of the 3'-end labeled RNA: Poliovirus RF labeled with [^{32}P]pCp at the 3' ends was denatured and hydrolysed with RNase T1. The products were separated by gel electrophoresis as shown in Figure 1. Some undigested material remained at the origin. The single strong band that migrated to the lower portion of the gel (as indicated by the arrow) was a candidate for the 3'-terminal hexa- or heptanucleotide [UUUUA(A)pCp] of the minus strand which we expected on the basis of the 5'-terminal sequence of plus strand RNA, VPg-pUUAAAACAG (18,6). The oligonucleotide marked by the arrow was extracted

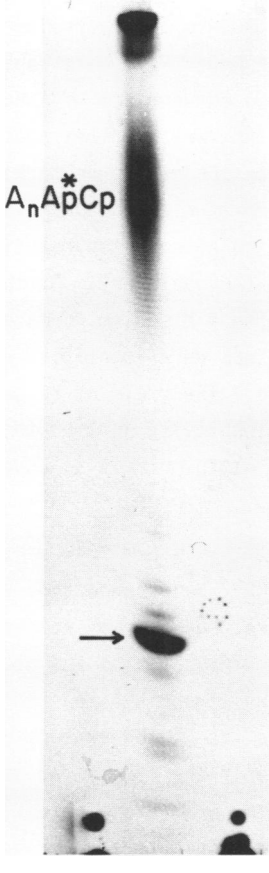

Fig. 1 Autoradiogram of a 20% acrylamide, 7 M urea slab gel. The arrow indicates the position of the putative 3' terminal RNase T_1 fragment of minus strand and the dotted semicircle corresponds to the position of the bromophenol blue dye. Gel dimensions were 50 cm x 8 cm x 1.5 mm.

from the gel and purified by column chromatography. Its sequence was determined as follows:

(i) Digestion with RNase T_2 or with RNase U_2 followed by paper electrophoresis yielded [^{32}P]Ap as the only labeled nucleotide (Fig. 2A, lanes 2 and 3). Digestion with nuclease P1 yielded only [^{32}P]pC (Fig. 2A, lane 4). These results show that the oligonucleotide was indeed labeled with [5'-^{32}P]pCp and that the nearest neighbor of pCp is A. Digestion of the oligonucleotide with RNase A yielded AACp as identified by electrophoresis on DEAE-paper (Fig. 2B, lane 1), indicating that the sequence of the oligo-

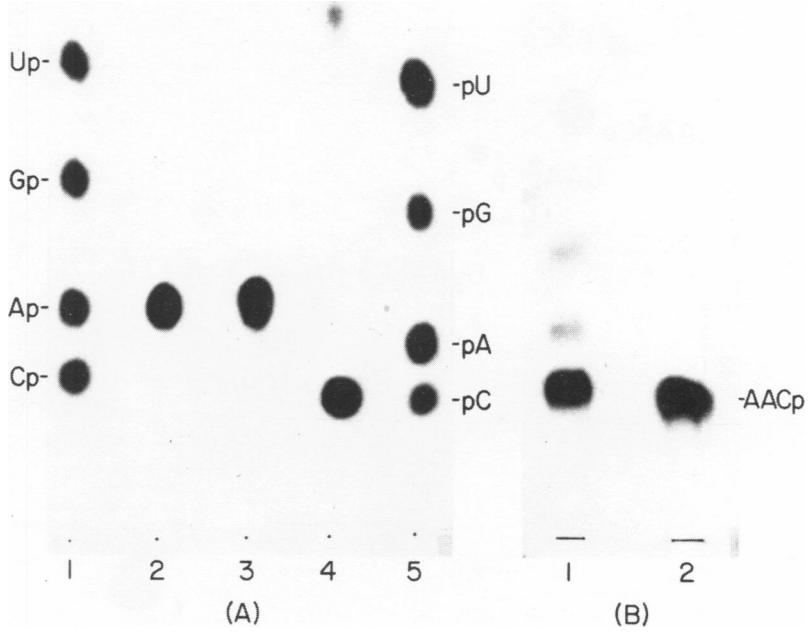

Fig. 2 Terminal and penultimate nucleotide determination of minus strand RNA. The T_1 oligonucleotide obtained in Fig. 1 was subject to the following digestions and electrophoresed either on Whatman 3MM (A) or DEAE-paper (B). RNase T_2 (A-2), RNase U_2 (A-3), nuclease P1 (A-4) and RNase A (B-1). Polio [^{32}P]RNA was digested with RNase T_2 (A-1) and nuclease P1 (A-5). Marker [^{32}P]AACp is seen in (B-2).

nucleotide is $(N)_nYAACp$.

(ii) The nature of the unknown bases was determined by the "wandering-spot" method (21) as shown in Fig. 3. The oligonucleotide was partially digested such that at most one phosphodiester per molecule was cleaved, and the products were separated by electrophoresis followed by homochromatography. The autoradiogram of the homochromatogram shown in Fig. 3 revealed 4 consecutive U shifts and 1 A shift which is compatible with the sequence UUUUAACp. The majority of the material remained undigested (Fig. 3) whereas a portion of the incomplete digest yielded a product that migrated to the upper right of the heptanucleotide (C-shift) and therefore is U_4AAp. Two independent experiments of labeling RF and sequence analysis have given

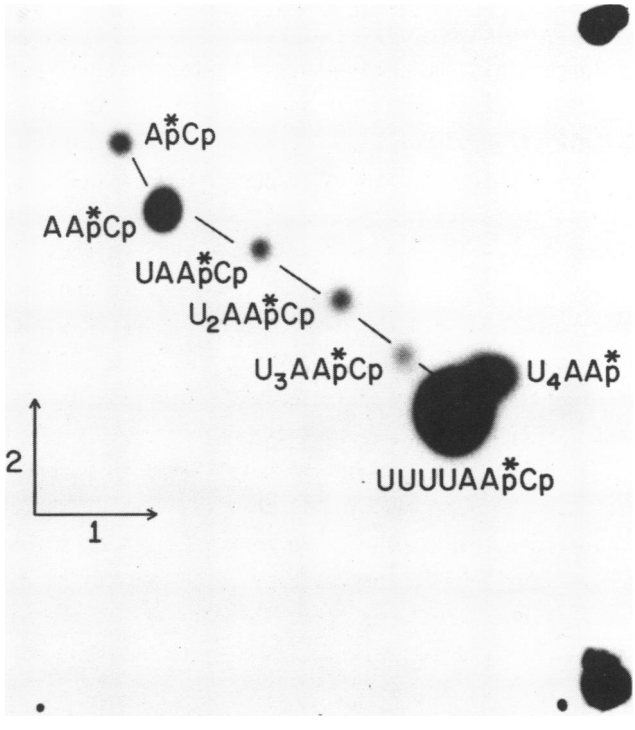

Fig. 3 Autoradiogram analysis of a two dimensional "mobility shift" of the T_1 oligonucleotide obtained in Fig. 1. Partial hydrolysis of the fragment resulted from sodium carbonate treatment. The first dimension was on cellulose acetate, pH 3.5 and the second was on a DEAE-cellulose plate using "homomix". For conditions, see "Materials and Methods".

identical results. The sequence of U_4AApCp was also confirmed (data not shown) by enzymatic sequence analysis according to the method of Donis-Keller et al. (20).

The material that appeared broadly distributed in the upper half of the gel shown in Fig. 1 is presumably 3'-end labeled poly(A) of plus strand RNA. Poly(A) of poliovirus RF is known to be very heterogeneous in size (2) and the sharp bands on either side of the broad band are likely to be minor species in length of poly(A). Longer electrophoresis on sequencing gels should be sufficient to separate the poly(A) into discrete bands allowing one to determine accurately the average chain length of poly(A). This, in fact, has recently been accomplished using poliovirion RNA (23).

Treatment of [^{32}P]RF with polynucleotide phosphorylase: Gel electrophoresis of RNase T_1-released poly(A) of poliovirus RF has suggested that the homopolymer is longer than the complementary 5'-terminal poly(U) of minus strand RNA (2,12,13). It follows that one end of poliovirus RF must contain a segment of single-stranded poly(A). We have tested this hypothesis by treating [^{32}P]RF with polynucleotide phosphorylase in Tris-HCl/phosphate buffer under conditions enabling the enzyme to act as a 3' exonuclease with consequent release of [α-^{32}P]-labeled nucleoside 5'-diphosphates (24). Polynucleotide phosphorylase is known to be sensitive to inhibition by secondary structure of RNA. In the case of weak secondary structure this steric inhibition can be overcome by digesting the RNA at 60°C (25). To assure that single stranded poly(A) was accessible to the action of the enzyme, we incubated RF with polynucleotide phosphorylase at 60°C. As can be seen in Fig. 4 only ppA to 0.6% of the total label in [^{32}P]RF was released. This was also observed even if the dsRNA was incubated with the enzyme at 60°C for more than 3 hours. In contrast, 25% of the label in single-stranded virion [^{32}P]RNA was released in 26 minutes to yield all 4 nucleoside 5'-diphosphates (data not shown). Analysis of the phosphorylase treated RF for poly(A) by denaturation, RNase T_1 release and gel electrophoresis (2) revealed that a segment of poly(A), approximately 60-80 nucleo-

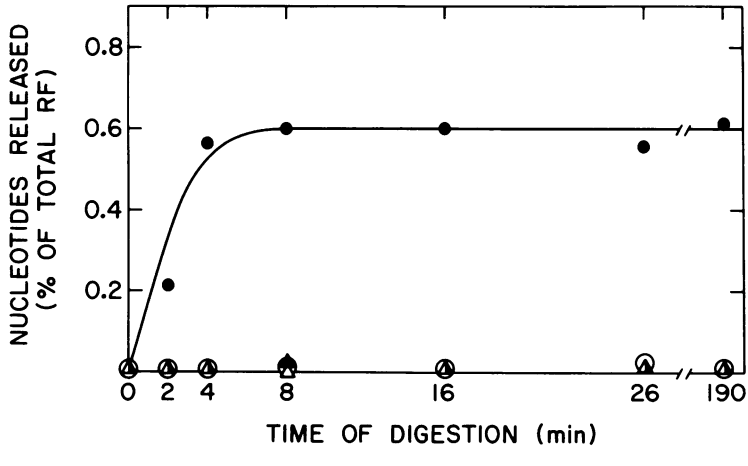

Fig. 4. Release of nucleoside 5'-diphosphate from [^{32}P]RF catalysed by polynucleotide phosphorylase at 60°C. Only [α-^{32}P]ppA was liberated and this amount is given in percentage of total radioactivity in each aliquot. For conditions, see "Materials and Methods".

tides long, remained undigested. We conclude that the ppA released from polio RF originates from a protruding single-stranded tail of poly(A). The yield of 0.6% corresponds to approximately 90 adenosine residues, a value that would be expected from the estimated length of poly(A) (2). It should be noted that ^{32}P-labeled poliovirus RNA isolated from infected HeLa cells is not uniformly labeled (18,6,26). Deviation from equal distribution of label, however, has been found only for pG, pC and pU nucleotides whereas pA is labeled to the expected extent (26). Therefore, the yield of [α-^{32}P] ppA need not be corrected. It is of interest to mention that the polynucleotide phosphorylase released ppA also at room temperature to the same extent but at a slower rate. However, the enzyme used in this study contains a phosphodiesterase activity, cleaving ppA to pA, that is present at room temperature but was found to be inactive at 60°C.

DISCUSSION

The results presented here together with our data published previously (9,6,7), allow us to draw a structure of poliovirus RF that is shown in Fig. 5. Clearly this structure is much more complex than previously anticipated (27).

The sequence study of the 3' end of minus strand RNA of RF shows that the molecule forms a perfect duplex at the left hand side of the molecule (Fig. 5), an observation suggesting that the 5'-terminal U of poliovirion RNA is specified by the 3'-terminal A of minus strand RNA. This does not exclude a mechanism by which a VPg-pU complex functions as primer although this nucleotidyl-polypeptide has not been detected in poliovirus infected cells (14).

The release of only [α-^{32}P]ppA from [^{32}P]RF by polynucleotide phosphorylase is direct evidence that a single-stranded poly(A) tail protrudes beyond the double-stranded RNA at the right hand side of the molecule. Although the T_m of poly(U)·poly(A) is 56.5°C in 0.1 M NaCl, the presence of an additional 5 mM $MgCl_2$ stabilizes the homopolymeric duplex in poliovirus RF to inhibit

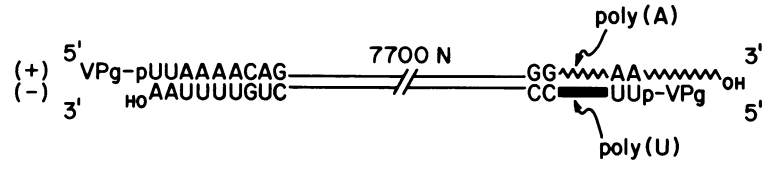

Fig. 5 Structure of poliovirus RF. (+) and (-) indicate plus strand and minus strand RNAs. VPg is the genome-linked viral protein (8).

degradation of that segment of the 3'-terminal poly(A) complexed to poly(U) (28). Since we have obtained evidence that the poly(A) in polio RNA is synthesized by transcription from poly(U) (11) the presence of the poly(A)-tail in polio RF is puzzling. We consider it likely, however, that, following transcription, the poly(A) is elongated by cellular 3' adenylate transferase. Alternatively, the longer poly(A) could have arisen by a slippage mechanism (2).

There is no apparent sequence homology between the 3' ends of plus strand RNA (29,30; Kitamura and Wimmer, in preparation) and the minus strand RNA with the exception of two terminal A residues. These two A residues, however, may be crucial in that the replicase which synthesizes plus and minus strands may only initiate RNA synthesis with two U residues. In minus strand RNA synthesis the first 2 U residues are followed by more U residues to form the 5'-terminal poly(U). In plus strand RNA synthesis the first 2 U residues are followed by a heteropolymeric region. Interestingly, VPg-linked aphthovirus RNA also contains two uridine residues at the 5' end (31). The fact that poly(A) is absent from the 3' end of minus strand RNA suggests that the viral replicase can initiate RNA synthesis without a homopolymeric region. This must be kept in mind when the role of virion RNA-associated poly(A) in poliovirus replication is being debated (see, for example, reference 32).

Finally, it should be noted that Richards et al. (33) have been able to complex VPg of plus strand RNA in RF with biotin/avidin spheres. Labeling of the VPg attached to the poly(U) of minus strand RNA in RF by the same method, however, failed. We suggest that this failure may be correlated with the presence of a single-stranded poly(A)-tail. For example, the poly(A) may complex to the positively charged VPg thereby preventing biotin/avidin labeling. This consideration may explain the failure to label VPg of single-stranded virion RNA by the same method (O.C. Richards, personal communication).

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