

# Protein Is Linked to the 5' End of Poliovirus RNA by a Phosphodiester Linkage to Tyrosine\*

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## SUMMARY

Purification and partial characterization of the poliovirus RNA-linked protein (VPg) are described. VPg has been freed from the RNA by ribonuclease digestion and phenol extraction. Gel filtration chromatography of VPg-pUp (labeled with  $^{32}\text{P}$ ) in 0.5% sodium dodecyl sulfate or 6 M guanidine HCl indicates that it has a molecular weight of about 12,000. VPg is bound to the 5' end of poliovirus RNA by a phosphodiester bond between a tyrosine residue in the VPg molecule and the 5'-terminal uridine. After acid hydrolysis of [ $^3\text{H}$ ]tyrosine-labeled VPg-pU, free tyrosine can be released by venom phosphodiesterase. Acid hydrolysis of VPg-p labeled with either  $^{32}\text{P}$  or [ $^3\text{H}$ ]tyrosine yields tyrosine-phosphate. There appears to be only 1 tyrosine residue per VPg molecule.

The single-stranded RNA genome of poliovirus is covalently bound through its 5'-terminal phosphate to a virus-specified protein (VPg<sup>1</sup>) (1, 2). VPg is also found both on negative strands of viral RNA and on nascent poliovirus RNA molecules (2, 3). It therefore must be attached to the RNA at a very early stage in the synthesis of each strand, perhaps functioning as a primer. Poliovirus messenger RNA—in contrast to virion RNA, negative strand RNA, and nascent chain RNA—contains no covalently bound protein, and terminates at its 5' end with a phosphate followed by exactly the nucleotide sequence found in virion RNA (UUAAAACAG) (4, 5). It therefore appears that the protein-RNA linkage is broken in the case of RNA destined to act as messenger RNA whereas it remains intact in RNA used for the formation of virions.

To better understand the function of VPg in poliovirus replication, and the mechanism of formation and breakage of the protein-nucleic acid linkage, it is necessary to know the chemical nature of that linkage. In this paper we demonstrate that VPg is linked to the 5' terminus of poliovirus RNA through a phosphodiester bond to a tyrosine residue. We also describe a method for rapid purification of VPg from virion RNA and show that VPg has a molecular weight of about 12,000.

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<sup>1</sup> The abbreviation used is: VPg, poliovirus RNA-linked protein.

## EXPERIMENTAL PROCEDURES

**Cells and Virus**—HeLa cells were infected with type I poliovirus and virions were purified as previously described (6). Virion RNA was labeled with  $^{32}\text{P}$  and purified as described elsewhere (7). Poliovirus was labeled with [ $^3\text{H}$ ]tyrosine by infecting  $4 \times 10^8$  HeLa cells in 100 ml of Earle's saline, supplemented with all amino acids except tyrosine and including 5% dialyzed fetal calf serum. Actinomycin D (5  $\mu\text{g}/\text{ml}$ ) was added 15 min postinfection and 5 mCi of [ $^3\text{H}$ ]tyrosine (40 to 50 Ci/mmol, New England Nuclear Corp.) was added 1 h postinfection. Virus was harvested at 6 h after infection and purified by high speed centrifugation and sodium dodecyl sulfate-sucrose gradient sedimentation (7). Virions were lysed by extraction with phenol:chloroform:isoamyl alcohol (50:48:2) and 35 S RNA was isolated by sedimentation through a 15 to 30% sucrose gradient in 0.5% sodium dodecyl sulfate, 0.1 M NaCl, 50 mM Tris, pH 7.5, 1 mM EDTA. Fractions were collected and a portion of each fraction was counted in Bray's solution. RNA was recovered from the peak of [ $^3\text{H}$ ]tyrosine radioactivity by ethanol precipitation.

**Enzymatic Digestion**—RNA was digested with P1 nuclease (8), a mixture of nucleases T1, T2, and pancreatic ribonuclease A (1), or micrococcal nuclease. The conditions for micrococcal nuclease digestion were 20  $\mu\text{g}$  of carrier yeast tRNA, 150 units of nuclease (P-L Biochemicals) in 10  $\mu\text{l}$  of 20 mM glycine, pH 9.0, 5 mM  $\text{CaCl}_2$ , 37°C, 2 h. Proteinase K digestion was in 10  $\mu\text{l}$  of 20 mM Tris, pH 7.5, 2 mM EDTA, 200  $\mu\text{g}/\text{ml}$  of protease K, at 37°C for 2 h. The conditions for venom phosphodiesterase and bacterial alkaline phosphatase digestion are described elsewhere (9).

**Purification of VPg-pUp, VPg-pU, and VPg-p**—Poliovirus RNA labeled with either [ $^3\text{H}$ ]tyrosine or  $^{32}\text{P}$  was digested with the appropriate nuclease. After digestion, the sample volume was adjusted to 50  $\mu\text{l}$  with 20 mM Tris, pH 7.5, 2 mM EDTA and the digest was extracted twice with 50  $\mu\text{l}$  of phenol:chloroform:isoamyl alcohol (50:48:2). The pooled organic phases plus interfaces were then washed four times with 100  $\mu\text{l}$  of 20 mM Tris, 2 mM EDTA to remove contaminating ribonucleotides. The material contained in the organic phase and interface was precipitated with 10 volumes of acetone at  $-20^\circ\text{C}$ . The precipitate was recovered by centrifugation, and the pellet was dried and dissolved in 50 mM Tris, 0.1 M NaCl, 1 mM EDTA buffer plus 0.5% sodium dodecyl sulfate. The material recovered from the phenol was chromatographed through a column of Sephadex G-25 (0.5  $\times$  10 cm) and the void volume fractions were precipitated with acetone. This excluded material consisted of radioactive VPg attached to a phosphate or uridylate residue. Digestion of RNA with nucleases T1, T2, and A yields VPg-pUp (1); P1 digestion yields VPg-pU (1). Micrococcal nuclease digestion of VPg-pUp yields VPg-p.

**Molecular Weight Estimation of VPg-pUp**—Gel filtration of  $^{32}\text{P}$ -labeled VPg-pUp was carried out on a column (1.0  $\times$  30 cm) of Bio-Gel A-1.5m equilibrated with 0.5% sodium dodecyl sulfate, 50 mM Tris, 0.1 M NaCl, 1 mM EDTA. Blue dextran 2000, bromphenol blue, and 50  $\mu\text{g}$  of cytochrome *c* were included as visible markers. For molecular weight determination, the column was calibrated with a sample containing  $^{125}\text{I}$ -labeled Moloney leukemia virus p12 and p30, 300  $\mu\text{g}$  of insulin, 10  $\mu\text{g}$  of cytochrome *c*, plus bromphenol blue and blue dextran 2000.  $^{32}\text{P}$  was detected by Cerenkov radiation,  $^{125}\text{I}$  by  $\gamma$  emission, cytochrome *c* by  $A_{410\text{nm}}$ , bromphenol blue and blue dextran by  $A_{600}$ , and insulin by  $A_{280}$ . VPg was recovered from the peak fractions by acetone precipitation. Gel filtration chromatography of VPg-pUp in 6 M guanidine HCl, 0.1 M  $\beta$ -mercaptoethanol on a Bio-Gel A-5m was exactly according to Fish *et al.* (9) except that the column dimensions were 1.0  $\times$  30 cm.

**Acid Hydrolysis of VPg**—VPg was hydrolyzed in sealed glass ampules under nitrogen in the presence of 20  $\mu\text{g}$  of bovine serum albumin carrier protein in 200  $\mu\text{l}$  of 2 M HCl at 110°C for 24 h (10). The hydrolysate was freeze-dried and dissolved in water, and a portion was treated with venom phosphodiesterase or alkaline phosphatase.

**3MM Paper Ionophoresis**—Acid hydrolysates were spotted on a 34-cm length of Whatman No. 3MM paper and subjected to electrophoresis in pH 3.5 pyridine/acetic acid buffer (11) at 2400 V for 1 h. Each lane was then cut into 0.5-cm slices, and each slice was soaked in 0.5 ml of water for 30 min and then counted in 10 ml of Bray's solution.

Electrophoretic markers of phosphoserine, phosphothreonine, and tyrosine (Sigma) were detected by spraying the dried Whatman No. 3MM paper with a 0.1% solution of ninhydrin in ethanol followed by heating at 65°C for 5 min.

**Thin Layer Chromatography**— $^3\text{H}$  Tyrosine-labeled VPg-pU was prepared as described above. After the pU was removed by digestion with venom phosphodiesterase, the protein was hydrolyzed as described above, except the bovine serum albumin carrier was omitted. The hydrolysate was freeze-dried and redissolved in 20  $\mu\text{l}$  of  $\text{H}_2\text{O}$  plus 5  $\mu\text{g}$  of tyrosine carrier. The sample was spotted onto a cellulose-coated glass thin layer chromatography plate (10  $\times$  10 cm) and two-dimensional development was carried out. First dimension, butanol:acetic acid: $\text{H}_2\text{O}$ , 18:2:5. Second dimension, butanol:pyridine:acetic acid: $\text{H}_2\text{O}$ , 32.5:29:5:20 (v/v) (12). 2,5-Diphenylxazole (PPO) was included in the second dimension solvent at a concentration of 6.6 g/100 ml.  $^3\text{H}$  radioactivity was detected by 2,5-diphenylxazole fluorography at  $-70^\circ\text{C}$  for 10 days using Kodak XR-5 film. The tyrosine marker was stained with ninhydrin as described above.

## RESULTS

To purify labeled VPg, infected cells were labeled with  $^{32}\text{P}$ , virions were purified, and virion RNA was phenol-extracted. The 35 S RNA was recovered from the aqueous phase and digested with ribonucleases T1, T2, and A. Such a digest should contain mainly  $^{32}\text{P}$ -mononucleotides and about 0.042% of the  $^{32}\text{P}$  in VPg-pUp (5). The digest was then extracted again with phenol and proteins in the phenol phase were recovered by acetone precipitation. When this material was fractionated by chromatography in 0.5% sodium dodecyl sulfate on Sephadex G-25, an excluded peak of  $^{32}\text{P}$  label was found (Fig. 1A). As previously demonstrated for VPg-pUp (5), most of the excluded  $^{32}\text{P}$  migrated toward the anode during pH 3.5 electrophoresis on Whatman No. 3MM paper. The included peaks from Sephadex G-25 contained a small amount of ribonucleotides contaminating the phenol layer. Thus, phenol extraction of a ribonuclease digest followed by gel filtration can rapidly purify VPg-pUp.

To size VPg-pUp, it was chromatographed in 0.5% sodium dodecyl sulfate on Bio-Gel A-1.5m (Fig. 1B). The majority of the  $^{32}\text{P}$  migrated as a peak close to the positions of cytochrome *c* ( $M_r = 12,500$ ). To further analyze the molecular weight of VPg-pUp, it was chromatographed on Bio-Gel A-5m in 6 M guanidine HCl, 0.1 M  $\beta$ -mercaptoethanol (Fig. 1C). Again it appeared to be 12,000 in molecular weight. The electrophoretic mobility of VPg in the presence of sodium dodecyl sulfate would suggest a molecular weight less than 10,000 (Ref. 5 and Footnote 2). The estimate of 12,000 from gel exclusion chromatography is probably a better approximation of the true molecular weight of the protein (9).

The combination of sequential Sephadex and Bio-Gel A-1.5 chromatography of the phenol phase gave a yield of  $^{32}\text{P}$  in VPg-pUp of about 0.032% of the starting material. When this was corrected by the unequal labeling of the 4 nucleotides (1), it represented a recovery of about 75% of the theoretical yield.

Digestion of VPg-pUp with venom phosphodiesterase released pUp and digestion of VPg-pU with venom phosphodiesterase released pU (1, 2). These results suggest that the linkage between VPg and the 5'-terminal uridine is a phosphodiester bond. Preliminary studies (data not shown) showed that the linkage was stable under acidic conditions that would hydrolyze phosphoramidate or carboxyl-phosphate anhydride linkages. Under such conditions, however, phosphoester bonds to threonine, serine, or tyrosine would be stable (13).

To begin identification of the phosphate-linked amino acid,  $^{32}\text{P}$ -labeled VPg-p was hydrolyzed in 2 M HCl to break all peptide bonds. The products were analyzed by pH 3.5 ionophoresis (Fig. 2A). The  $^{32}\text{P}$  radioactivity migrated as a peak of material (compound I) faster than either the phosphoserine

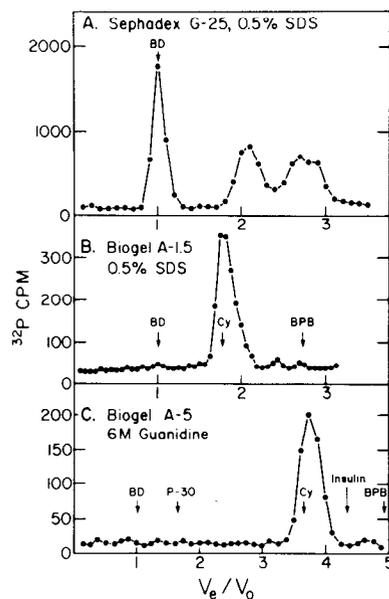


FIG. 1. Chromatography of VPg-pUp.  $^{32}\text{P}$ -labeled poliovirus RNA was digested with ribonuclease and VPg-pUp was recovered from the digest by phenol extraction and acetone precipitation as described under "Experimental Procedures." A, the material recovered from the phenol was chromatographed through a column of Sephadex G-25, equilibrated with 50 mM Tris, 0.1 M NaCl, 1 mM EDTA buffer plus 0.5% sodium dodecyl sulfate (SDS) and the void volume fractions were precipitated with acetone, collected by centrifugation, dried, and redissolved in the same buffer. B, gel filtration of VPg-pUp was then carried out on a column (1.0  $\times$  30 cm) of Bio-Gel A-1.5m equilibrated with 0.5% sodium dodecyl sulfate, 50 mM Tris, 0.1 M NaCl, 1 mM EDTA. C, VPg-pUp was chromatographed on a column of Bio-Gel A-5m in 6 M guanidine HCl, 0.1 M  $\beta$ -mercaptoethanol. Blue dextran 2000 (BD), bromphenol blue (BPB), and cytochrome *c* (Cy) were included as visible markers. Inulin and Moloney leukemia virus p30 protein markers were detected as described under "Experimental Procedures."

or phosphothreonine markers. Treatment of this material with bacterial alkaline phosphatase released all of the radioactivity as free phosphate (Fig. 2B). Because compound I did not appear to be either phosphoserine or phosphothreonine, we investigated whether it might represent phosphate linked to tyrosine.

Poliovirus was labeled with  $^3\text{H}$  tyrosine as described under "Experimental Procedures." Purified virions were lysed by phenol extraction, and the RNA was sedimented through a sodium dodecyl sulfate-sucrose gradient. A peak of  $^3\text{H}$  radioactivity was recovered at 35 S, co-sedimenting with  $^{32}\text{P}$ -labeled 35 S poliovirus RNA that was analyzed on a parallel gradient. The RNA was recovered from the gradient fractions, digested with ribonucleases T1, T2, and pancreatic ribonuclease A, and chromatographed on a column of Sephadex G-25. All of the  $^3\text{H}$  radioactivity appeared in the excluded volume where VPg-pUp would be expected and no radioactivity was detected in the included volume coincident with the nucleoside monophosphates. The  $^3\text{H}$  tyrosine-labeled VPg-pUp was recovered from the excluded fractions and digested with proteinase K, and the products were separated by pH 3.5 paper ionophoresis (Fig. 3A). The  $^3\text{H}$  radioactivity appeared as a single peak (compound II), with a mobility similar to that of the product produced by digesting  $^{32}\text{P}$ -labeled VPg-pUp with proteinase K (1). The mobility of compound II was altered greatly by removal of pUp with venom phosphodiesterase prior to proteinase K digestion (Fig. 3B) indicating that in it, pUp is covalently bound to  $^3\text{H}$  tyrosine-containing material. The phosphate-free  $^3\text{H}$  tyrosine-labeled peak in Fig. 3B, however, did not co-migrate with tyrosine marker, and probably is a

<sup>2</sup> V. Ambros and D. Baltimore, unpublished results.

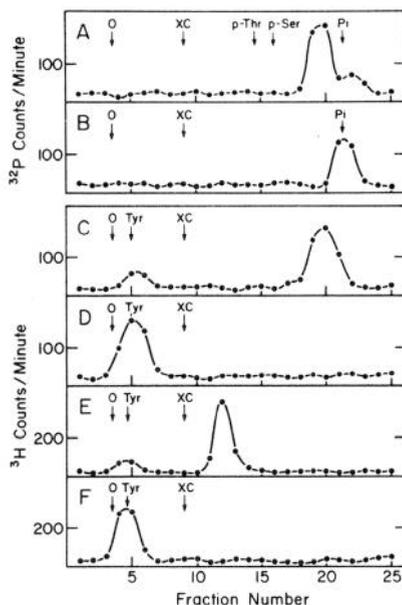


FIG. 2. Ionophoretic separation of acid hydrolysates at pH 3.5. Radioactively labeled VPg-p and VPg-pU were prepared as described under "Experimental Procedures."  $^{32}\text{P}$ -labeled VPg-p was hydrolyzed in 2 M HCl at  $110^\circ\text{C}$  for 24 h and the products were analyzed before (A) and after (B) bacterial alkaline phosphatase treatment.  $^3\text{H}$  Tyrosine-labeled VPg-p was acid-hydrolyzed in an identical manner and the products were analyzed before (C) and after (D) bacterial alkaline phosphatase treatment.  $^3\text{H}$  Tyrosine-labeled VPg-pU was acid-hydrolyzed as above and the products were analyzed before (E) and after (F) treatment with venom phosphodiesterase. Cathode is on the left. O, indicates position of sample application. XC marks xylene cyanol blue dye marker. Amino acid markers tyrosine (Tyr), phosphothreonine (p-Thr), and phosphoserine (p-Ser) were run in a parallel lane and detected by ninhydrin staining.

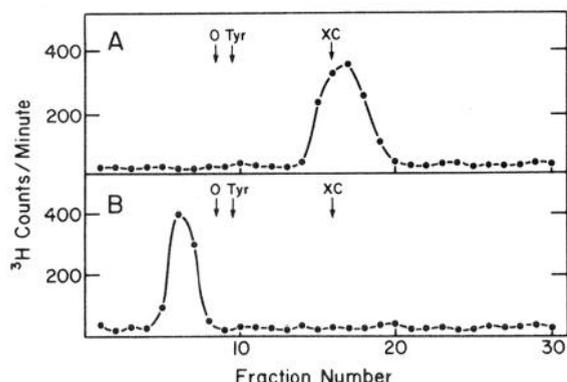


FIG. 3. Ionophoretic separation at pH 3.5 of  $^3\text{H}$  tyrosine-labeled products of proteinase K digestion of VPg-pUp and VPg.  $^3\text{H}$  Tyrosine-labeled VPg-pUp was purified as described under "Experimental Procedures" and digested with proteinase K directly (A) and after prior treatment with venom phosphodiesterase (B). Procedures and conditions of ionophoresis and amino acid markers used were identical to those in Fig. 1.

proteinase K-resistant oligopeptide containing one or more amino acids in addition to tyrosine (2). The linkage to pUp must be through one of those amino acids.

To determine whether tyrosine was involved in the linkage,  $^3\text{H}$  tyrosine-labeled VPg-p was hydrolyzed with 2 M HCl and the products were analyzed by pH 3.5 paper ionophoresis both before (Fig. 2C) and after (Fig. 2D) bacterial alkaline phosphatase treatment. Over 90% of  $^3\text{H}$  tyrosine label was recovered in material migrating at the position of the  $^{32}\text{P}$ -labeled compound I shown in Fig. 2A. After phosphatase treatment, all of the  $^3\text{H}$  tyrosine label migrated like free tyrosine (Fig.

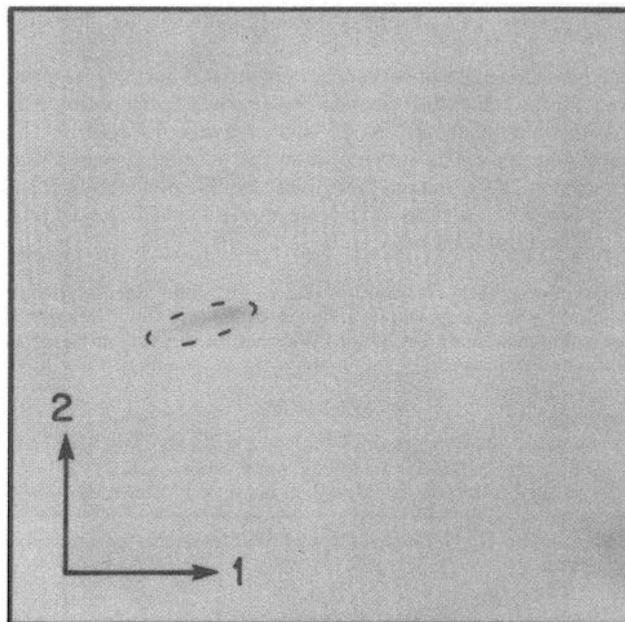


FIG. 4. Two-dimensional thin layer chromatography.  $^3\text{H}$  tyrosine-labeled VPg-pU was digested with venom phosphodiesterase to remove pU and hydrolyzed in 2 M HCl, and the hydrolysate was chromatographed in two dimensions on a cellulose-coated thin layer plate. Dimension 1, *n*-butanol:acetic acid: $\text{H}_2\text{O}$ , 12:2:5. Dimension 2, *n*-butanol:pyridine:acetic acid: $\text{H}_2\text{O}$ , 32.5:29.5:20.  $^3\text{H}$  radioactivity was detected by 2,5-diphenyloxazole fluorography. The position of unlabeled tyrosine marker was determined by ninhydrin staining and is indicated by the dashed outline.

2D). The phosphate that links VPg to viral RNA therefore appears to be covalently bound to tyrosine.

To further characterize the linkage,  $^3\text{H}$  tyrosine-labeled VPg-pU was analyzed by acid hydrolysis and pH 3.5 electrophoresis exactly as above (Fig. 2E). The mobility of the major peak in Fig. 2E was unaffected by phosphatase (data not shown) yet free  $^3\text{H}$  tyrosine was generated by venom phosphodiesterase digestion (Fig. 2F). To confirm the identity of this released material, the pU was removed from  $^3\text{H}$  tyrosine-labeled VPg-pU by venom phosphodiesterase. The protein was then hydrolyzed in 2 M HCl as above, and the hydrolysate was analyzed by two-dimensional thin layer chromatography (Fig. 4). The  $^3\text{H}$  radioactivity migrated as a spot coincident with the ninhydrin-stained tyrosine marker included in the sample. This confirms that the 5'-terminal uridine of poliovirus RNA is linked to VPg by a phosphodiesterase bond to tyrosine. It also appears that the RNA-linked tyrosine is the only tyrosine residue in the VPg protein molecule, because over 90% of the  $^3\text{H}$  tyrosine radioactivity was phosphate-linked (Fig. 2, C and E).

#### DISCUSSION

We have shown that the molecular weight of poliovirus RNA-linked protein is 12,000 and the protein is bound to the 5' end of poliovirus RNA through a tyrosine residue. The acid stability of this linkage, and its sensitivity to cleavage by venom phosphodiesterase, suggests that the linkage is a phosphodiester bond to the tyrosine side chain, presumably to the hydroxyl group at position 4 of the ring.

If the proposed role of VPg as a primer (1) for RNA synthesis is true, the initial covalent bond formed during chain elongation would be a phosphodiester bond between the tyrosine side chain of VPg and the  $\alpha$ -phosphate of an activated UMP moiety probably in UTP. This could be analogous to the reaction of the *E. coli* glutamine synthetase regulatory protein PII with UTP to form 5' UMP covalently bound to

the PII protein by a phosphodiester linkage through tyrosine (14).

We have recently observed<sup>3</sup> an enzymatic activity in uninfected HeLa cells that cleaves the protein from poliovirus RNA leaving intact RNA. If this cleavage represents the normal activity of the enzyme, then the possibility arises that the tyrosine-RNA linkage described here for poliovirus RNA is an example of a class of protein-nucleic acid linkages normally found in HeLa cells.

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