Proc. Natl. Acad. Sci. USA
Vol. 75, No 10, pp. 4868–4872, October 1978
Biochemistry

O^4-(5′-Uridylyl)tyrosine is the bond between the genome-linked protein and the RNA of poliovirus*

(iodination/acid and alkali hydrolysis/enzymatic degradation/paper electrophoresis and chromatography)

PAUL G. ROTHBERG, TIMOTHY J. R. HARRIS†, AKIO NOMOTO‡, AND ECKARD WIMMER

Department of Microbiology, School of Basic Health Sciences, State University of New York at Stony Brook, Stony Brook, Long Island, New York 11794

Communicated by Seymour S. Cohen, August 3, 1978

ABSTRACT **Virion RNA of poliovirus type 1 has been analyzed for the linkage between genome-protein VPg and the polyribonucleotide chain. Hydrolysis of the linkage with acid or alkali and enzymatic degradation lead to the conclusion that the bond is neither a phosphodiester such as nucleotidyl-(*P-O*)-serine (or threonine) nor a phosphoramidate such as nucleotidyl-(*P-N*)-amino acid. VPg-RNA can be iodinated by the Bolton and Hunter reagent [iodinated 3-(4-hydroxyphenyl)propionic acid *N*-hydroxysuccinimide ester] but not by the chloramine-T or lactoperoxidase procedures, an observation suggesting that VPg does not contain accessible tyrosine. However, VPg can be labeled with [^{3}H]tyrosine *in vivo*. Hydrolysis of VPg-[^{32}P]pUp with 5.6 M HCl at 110° yielded ^{32}P-labeled O^4-(3′-phospho-5′-uridylyl)tyrosine that could be cleaved with micrococcal nuclease to O^4-[^{32}P]phosphotyrosine and uridine 3′-[^{32}P]phosphate. These data establish that VPg is linked to the poliovirus genome by a bond between the O^4 of tyrosine and the 5′-P atom of the terminal uridylic acid residue. The 5′ end of polio genome RNA can now be described as VPg(Tyr-*O*)-pU-U-A-A-A-A-C-A-G . . .**

Picornaviruses may be unique among animal RNA viruses because their single-stranded genome is covalently linked to a small protein, called VPg (1–7). The protein is virus-coded (2, 7) and linked to the 5′-terminal uridylate of the RNA (4, 7, 8). Polio VPg is hydrophobic (1, 2) and basic (net charge of +7 at pH 7.5, ref. 4) and has a molecular weight of 4000–6000 as determined by polyacrylamide gel electrophoresis (2, 5, 7).

Biochemical studies have shown that VPg is linked not only to virion RNA, but also to negative strand (genome complement) RNA (4) and to nascent strands of the replicative intermediate (3, 4). On the basis of these findings, we (2, 4) and others (3) have suggested that VPg may be involved in genome replication, possibly in the initiation of RNA synthesis (4).

Viral mRNA, on the other hand, may be the product of "processing" of newly made plus strand RNA because its 5′ terminus is pUp (refs. 9 and 10 and literature cited therein). We have speculated that cleavage of the bond between protein and RNA may have a regulatory function; it is possible that only protein-linked RNA is encapsidated, whereas RNA without the protein serves as mRNA or as a transcription template (2, 4, 9).

For an analysis of the function of VPg, the bond linking the protein to the RNA is of interest. Here we report that VPg of poliovirus type 1 (PV1) is bound to the RNA via a phosphodiester bond between the phenolic hydroxyl group of tyrosine and the 5′ phosphoric residue of uridylic acid. The 5′-terminal structure of the poliovirus genome can now be described as VPg(Tyr-*O*)-pU-U-A-A-A-A-C-A-G . . .

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

MATERIALS AND METHODS

Poliovirus was grown and labeled with phosphorus-32 in HeLa cell suspension cultures as previously described (9). VPg was labeled with [^{3}H]tyrosine as follows: 2.5×10^9 HeLa cells were washed twice with Earle's saline, suspended in 200 ml of Earle's saline containing 1 mg of actinomycin D, and infected with 50 plaque-forming units of PV1 per cell. After 25 min at room temperature, 200 ml of medium containing 168 ml of Earle's saline, 4 ml of an antibiotic solution (10,000 units of penicillin per ml, 10,000 μg of streptomycin per ml; GIBCO), 4 ml of vitamins (100×; GIBCO MEM vitamin solution), 20 ml of dialyzed calf serum, 4 ml of 10× amino acids (lacking cysteine and tyrosine) and 1 mg of cysteine were added to the infected culture, which was then incubated at 37°. One-half hour after infection, 10 mCi of [^{3}H]tyrosine (52.1 Ci/mmol, New England Nuclear) in 1 ml of 50% ethanol was added. Cells were harvested and virus and virion RNA were purified as previously described (9). Poliovirus was labeled with 50 μCi of [^{14}C]lysine (New England Nuclear, 250 Ci/mol) and purified essentially as described for [^{3}H]tyrosine except that labeling was in lysine-free F-14 medium (GIBCO) and started 2.5 hr after infection. Virus was recovered from sucrose gradient fractions by acetone precipitation (2 vol of acetone, −20°, overnight).

VPg-pUp labeled with ^{32}P or with [^{3}H]tyrosine was isolated by digesting purified virion RNA with RNase T2 and separating the nucleotidyl-protein from mononucleotides by ion-exchange chromatography on DEAE-cellulose at pH 5 (2, 4). [*tyrosyl*-^{3}H]VPg-pUp was always purified together with VPg-[^{32}P]pUp to facilitate detection of the protein by monitoring for Cerenkov radiation. VPg-pUp was digested with Pronase (Sigma protease type VI) to yield $(aa)_n$-pUp as described (2, 4), or with 1 ml of 5.6 M HCl (freshly distilled) in a vacuum-sealed hydrolysis tube (Pierce) for 2 hr at 110°. After hydrolysis the HCl was evaporated under reduced pressure and the residue was dissolved in water and lyophilized. Digestion products of VPg-pUp were further degraded with highly purified snake venom 3′-exonuclease (generous gift of M. Laskowski, Sr.), as previously described (2, 4); with 1.0 unit of bacterial alkaline phosphatase (Worthington Biochemical Co.) in 10 mM Tris·HCl, pH 7.5/1 mM EDTA, for 1 hr at 37°; or with 1.0 unit of micrococcal nuclease (*Staphylococcus aureus*, Foggi strain, grade VI; Sigma Chemical Co.) in 50 mM Tris·HCl, pH 8.5/10 mM $CaCl_2$ for 1 hr at 37°.

Abbreviations: VPg, the genome-linked viral protein of poliovirus; PV1, poliovirus type 1; $NaDodSO_4$, sodium dodecyl sulfate; Tyr-*P*,-O^4-phosphotyrosine; for further information on the nomenclature of phosphorus-containing compounds, see (1977) *Proc. Natl. Acad. Sci. USA* 74, 2222–2230.

* This is paper 5 in the series "The genome-linked protein of picornaviruses." Paper 4 is ref. 7.

† On leave from the Animal Virus Research Institute, Pirbright, Woking, Surrey, United Kingdom.

‡ Present address: Department of Public Health, School of Pharmaceutical Sciences, Kitasato University, 5-9-1 Shirokane, Minato-ku, Tokyo, Japan.

O^4-Phosphotyrosine was synthesized by heating 4.1 ml of 85% (wt/vol) phosphoric acid, 4.2 g of phosphoric acid anhydride, and 1.4 g of L-tyrosine to 100° for 72 hr (11). The mixture was then diluted to 65 ml with crushed ice and Tyr-*P* was isolated by a modified procedure of Mitchell and Lunan (12) as follows: The solution was percolated through a Dowex 50 column (3.1 × 28.5 cm, hydrogen form) and the column was washed with water. Fractions eluting from the column contained two peaks as monitored by absorbance at 280 nm: peak I (beginning at the void volume of the column) that contained Tyr and P_i, and peak II, eluting over a large number of fractions. Those fractions of peak II with an absorbance of >0.8 were combined, concentrated to 150 ml by flash evaporation, mixed with 160 ml of ethanol, and stored overnight at 4°. A white precipitate was recovered by filtration (800 mg) and shown to be Tyr-*P*, which was at least 97% pure as judged by thin-layer chromatography (see below) or by release of P_i after treatment with bacterial alkaline phosphatase.

Virion RNA was labeled with ^{125}I by one of three methods: (*i*) VPg-RNA (250 μg) in 150 μl of 0.1 M sodium phosphate, pH 7.4/0.1% sodium dodecyl sulfate ($NaDodSO_4$) was mixed with 2.5 μl of carrier-free $Na^{125}I$ (100 mCi/ml, 11–17 mCi/μg) and with 10 μl of chloramine-T (*N*-chloro-*p*-toluenesulfonamide; Sigma Chemical Co.) at 10 mg/ml (13, 14). The reaction was stopped after 1 min by the addition of 50 μl of sodium metabisulfite (2 mg/ml) and 100 μl of bovine serum albumin (1 mg/ml, in 100 mM NaI). The RNA was separated from unreacted ^{125}I by gel filtration with Sephadex G-25 in a disposable 25-cm plastic pipette in 0.1 M NaCl/0.1 M Tris·HCl, pH 7.5/5 mM EDTA/0.1 mM dithiothreitol/0.1% $NaDodSO_4$. The void volume (approximately 3.0 ml) was pooled and the RNA was precipitated with ethanol. (*ii*) VPg-RNA (250 μg) in 150 μl of 0.1 M sodium phosphate, pH 7.4, was mixed with 2.5 μl of $Na^{125}I$ and 5 μl of H_2O_2 (0.88 mM), and 2 μl of lactoperoxidase (200 μg/ml) was added (15). After 1 min, the reaction was stopped by the addition of 150 μl of 0.1 M sodium phosphate, pH 7.5/0.01 M NaI/0.25% $NaDodSO_4$/0.002 M EDTA and the RNA was purified as described for *i*. The lactoperoxidase (P-L Biochemicals, chromatographically purified) was rendered virtually RNase-free by gel filtration through Sephadex G-100, in 33 mM potassium phosphate, pH 6.5. (*iii*) VPg-RNA was incubated with 1 mCi of the Bolton and Hunter reagent [iodinated 3-(4-hydroxyphenyl)propionic acid *N*-hydroxysuccinimide ester; ref. 16] in 20–50 μl of 0.1 M sodium borate, pH 8.5/1 mM EDTA/0.1% $LiDodSO_4$ overnight at 4° (Harris, T. J. R., Dunn, J. J. & Wimmer, E., *Nucleic Acids Res.*, in press), and the RNA was purified as described for *i*. Carrier-free $Na^{125}I$ and the Bolton and Hunter reagent (1500 Ci/mmol) were purchased from Amersham Corp. and New England Nuclear, respectively. Poliovirus (25 μg in each reaction), purified by sucrose gradient centrifugation and subsequent isopycnic centrifugation in CsCl (17), was labeled with ^{125}I in 150 μl of sodium phosphate buffer by the chloramine-T or lactoperoxidase methods under conditions similar to those used to label VPg-RNA. Virus was separated from the unreacted ^{125}I by Sephadex G-25 gel filtration. The virus eluting in the void volume was purified further by sucrose gradient centrifugation and acetone precipitation.

Analysis of proteins was by 12.5% polyacrylamide slab gel electrophoresis according to Laemmli (18), or in 15% slab gels containing 4 M urea in the same buffer. Sample buffers (18) contained 1% (wt/vol) dithiothreitol instead of 2-mercaptoethanol, and sample disruption was at 60° for 10 min. Gels containing urea were processed for fluorography (19) in order to remove urea and to prevent loss of VPg by elution from the gels (1, 2).

Paper electrophoresis was carried out at pH 3.5 in pyridinium acetate as described (2, 4); thin-layer chromatography was on cellulose plates (Polygram CEL 300, Macherey, Nagel and Co.) or on EM silica gel F-254 plates (Brinkmann Instruments) in the following solvent systems: (A) saturated ammonium sulfate/1 M sodium acetate/isopropanol (40:9:1, vol/vol/vol; ref. 20); (B) first dimension: isobutyric acid/0.5 M ammonia (5:3, vol/vol), second dimension: isopropanol/concentrated HCl/water (70:15:15, vol/vol/vol; ref. 21); (C) isopropanol/concentrated ammonia/water (7:2:1, vol/vol/vol; ref. 22). Tyr-*P* was detected on cellulose plates by spraying them with 0.3% (wt/vol) ninhydrin in 1-butanol (Gelman Instrument) and heating to 100° for 5 min. The silica gel plates were precoated with a fluorescent indicator making it possible to visualize Tyr-*P* by absorbance under shortwave ultraviolet light.

Radioisotopes in dried gels or thin-layer plates were detected by autoradiography at −70° using Kodak SB5 film and a Du Pont Cronex intensifying screen.

RESULTS

Elimination of Phosphoramidates or Nucleotide-(*P-O*)-serine (or Threonine) as Linkage Groups. Initial studies on the linkage between VPg and RNA were carried out either with the Pronase-digestion product of VPg, a nucleotidyl-peptide called Y [$(aa)_m$-pUp; refs. 2 and 4] or with Y′ [$(aa)_n$-pUp; ref. 4]. Y′ is the product of aminopeptidase treatment of Y (4) and is resistant to 0.1 M NaOH for 2 hr at 37°, conditions that hydrolyze the phosphoester of Ser-*P* or Thr-*P*. Y′ can be cleaved with micrococcal nuclease to yield Y′-p and Up (data not shown). The purified Y′-p remained unchanged after incubation with 1 M NaOH at 100° for 1 hr as determined by paper electrophoresis and thin-layer chromatography. Again, both Ser-*P* and Thr-*P* are completely hydrolyzed under these conditions (23). The results strongly suggest that VPg is not linked to the RNA via a phosphodiester between the β-hydroxy group of serine or threonine and the 5′ phosphoric residue of uridylic acid.

A similar set of experiments excluded the possibility of a phosphoramidate—e.g., UMP-(5′-N^{ω})-Arg or UMP-(5′-N^{ϵ})-Lys—being the linking group between VPg and RNA. Nucleotidyl-(*P-N*)-peptide phosphoramidates are sensitive to treatment with acid (ref. 24 and literature cited therein). IMP-(5′-N^{ϵ})-Lys is completely cleaved to IMP and lysine in 0.1 M HCl at 37° for 1 hr (24), whereas Y′ was found to be resistant to 0.1 M HCl at 37° for 2 hr. Moreover, Bogdanov *et al.* (25) reported that phosphoramidates are not cleaved by snake venom exonuclease. We have synthesized *N*-(5′-uridylyl)alanine (26) and confirmed its stability to a large excess of snake venom exonuclease (data not shown). In contrast, both Y and Y′ are readily cleaved with snake venom exonuclease to yield pUp (2, 4).

Iodination of VPg-RNA. To facilitate a number of experiments designed to elucidate the function of VPg, it was desirable to prepare undegraded poliovirus RNA with VPg radioactively labeled to high specific activity, preferably in the absence of label in nucleotides. Labeling of VPg with ^3H- and ^{14}C-labeled amino acids however, failed to give sufficiently high specific activities, so the alternative procedure of *in vitro* labeling with carrier-free ^{125}I was adopted. The chemical method using chloramine-T as oxidizing agent in the presence of $NaDodSO_4$ was compared to the enzymatic method utilizing lactoperoxidase. The ^{125}I-treated virion RNA, which was not degraded by the iodination conditions, was purified by sucrose gradient centrifugation. RNA sedimenting at 35 S was precipitated by ethanol, dissolved in buffer, and digested with RNases A and T1. Finally, the products were analyzed by

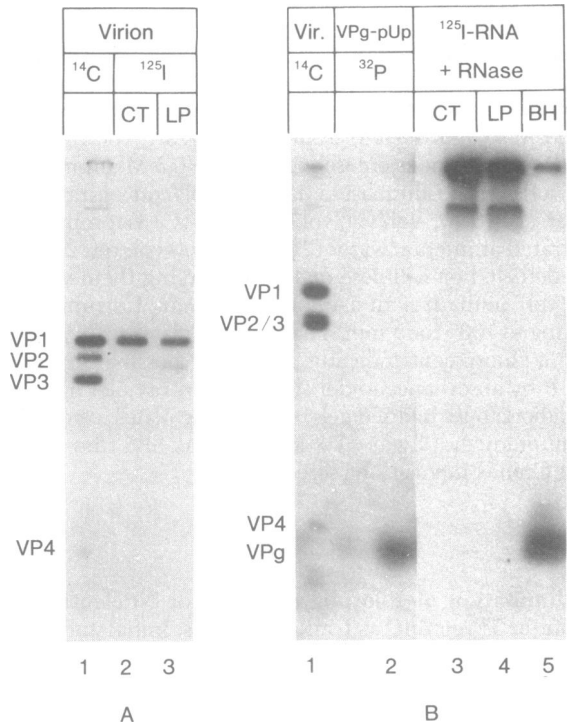

FIG. 1. Polyacrylamide gel electrophoresis of poliovirus proteins and poliovirus RNA labeled *in vitro* with ^{125}I. (*A*) Autoradiograph of a 12.5% gel of the polypeptides of poliovirus labeled with carrier-free ^{125}I by the chloramine-T (CT; track 2) or lactoperoxidase (LP; track 3) procedures, with ^{14}C-labeled poliovirus polypeptides (track 1) providing mobility markers. (*B*) Fluorograph of a 15% polyacrylamide gel of RNase-digested ^{125}I-labeled poliovirus RNA (^{125}I-RNA). Tracks 3–5: 10–20 μg of ^{125}I-RNA labeled by the chloramine-T or lactoperoxidase procedures or the Bolton and Hunter (BH) reagent was digested with 25 units of RNase T1/15 μg of RNase A in 20 μl of 50 mM Tris·HCl, pH 7.4/2 mM EDTA, for 2 hr at 37°, before disruption and electrophoresis. Track 1 is marker ^{14}C-labeled viral polypeptides and track 2 is VPg-[^{32}P]pUp prepared from [^{32}P]RNA by RNase T2 digestion and DEAE-cellulose chromatography (2, 4).

polyacrylamide gel electrophoresis. As can be seen in Fig. 1*B* (tracks 3 and 4) no radioactive material migrated to the position of marker VPg-[^{32}P]pUp (Fig. 1*B*, track 2). The nature of the radioactive material at the top of the stacking gel in tracks 3 and 4 (Fig. 1*B*) is unknown. It is probably not undigested RNA because it was also present in RNase T2-digested samples.

RNA isolated from virus particles by lysis with $NaDodSO_4$ at pH 3.5 (27) and sucrose gradient centrifugation in the presence of $NaDodSO_4$ was also iodinated by the lactoperoxidase or the chloramine-T procedure. This RNA was then digested with RNases A and T1, and the products were analyzed by electrophoresis on a 15% polyacrylamide gel. As before, no label could be detected in VPg, but ^{125}I was associated with polypeptides migrating to positions of viral capsid proteins (data not shown). This result indicates that trace amounts of capsid proteins are associated with RNA purified by acid lysis in $NaDodSO_4$, whereas in phenol/chloroform-extracted RNA the capsid proteins are absent. More importantly, the trace amounts of these capsid proteins can be labeled by oxidative iodination of tyrosine residues, whereas VPg cannot. Furthermore, poliovirus particles were also readily labeled *in vitro* with ^{125}I to high specific activities ($>5 \times 10^5$ cpm/μg) by the chloramine-T or lactoperoxidase procedures under conditions identical to those that failed to label VPg. The results of the iodination of virions can be seen in Fig. 1*A* (tracks 2 and 3). Predominantly VP1 is labeled with iodine, although some VP2 was also iodinated when lactoperoxidase was used. These results are in agreement with published studies concerning the iodination of enteroviruses (28–31) and other picornaviruses (32). Chloramine-T and lactoperoxidase facilitate iodination of the aromatic side chain of tyrosine. Thus, in poliovirions only capsid protein VP1 has easily accessible Tyr residues. In contrast VPg linked to RNA appears to contain no accessible Tyr.

To test whether VPg is "buried" within the RNA chain and so not accessible to chemical iodination, we attempted to label VPg by an alternative method using a modification of the procedure of Bolton and Hunter (16). As shown in Fig. 1*B*, track 5, this procedure readily labels VPg with ^{125}I, an observation demonstrating that VPg linked to RNA can be chemically modified under gentle conditions.

Labeling of VPg with [^{3}H]Tyrosine. *In vivo*, VPg could clearly be labeled with [^{3}H]tyrosine. Moreover, digestion of [*tyrosyl*-^{3}H]VPg-pUp with Pronase yielded [*tyrosyl*-^{3}H]Y (Fig. 2) with almost no loss of radioactivity. This result is at odds with our conclusion from the iodination studies. It can be explained, however, if one assumes that VPg contains only one Tyr residue, whose phenolic hydroxyl group is blocked. Derivatization of the hydroxyl group renders the aromatic side chain of Tyr incompetent for iodination with chloramine-T or lactoperoxidase (33). The most obvious blocking group on this hydroxyl group would be a phosphodiester linking the RNA to VPg.

Isolation of Tyr-O^4-[^{32}P]pUp from VPg-[^{32}P]pUp. On the basis of the above considerations, we synthesized O^4-phosphotyrosine (Tyr-*P*) and tested its stability to digestion with HCl. As reported previously, 87% of Tyr-*P* is hydrolyzed in 1 M HCl at 100° for 21 hr (11). We found, however, that in 5.6 M HCl at 110° for 2 hr, only 20% of Tyr-*P* was hydrolyzed to

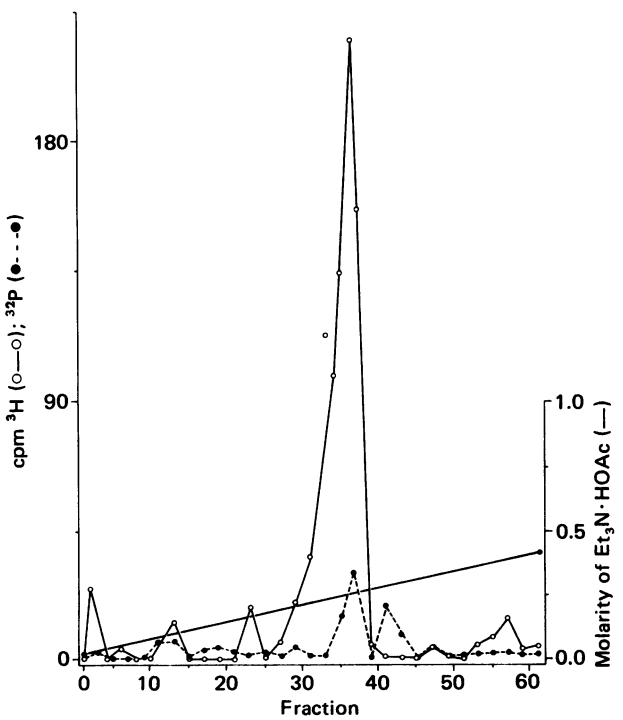

FIG. 2. Isolation of Y [$(aa)_m$-pUp]. [*Tyrosyl*-^{3}H]VPg-pUp (16,281 cpm) was digested with Pronase and the products were separated by ion-exchange chromatography on DEAE-cellulose at pH 5 (4). Aliquots of individual fractions were counted in Biofluor (New England Nuclear). The ^{3}H-labeled VPg-pUp contained a small amount of VPg-[^{32}P]pUp because ^{32}P-labeled virus was added to [^{3}H]tyrosine-labeled virus to aid detection of VPg-RNA and VPg-pUp by Cerenkov radiation. Recovery of [*tyrosyl*-^{3}H]Y was 10,648 cpm (65%). The yield of [^{32}P]Y from VPg-[^{32}P]pUp was 61%; if normalized to this value, the recovery of tyrosine label in Y from [*tyrosyl*-^{3}H]VPg-pUp was nearly 100%.

yield Tyr and P_i (data not shown). The latter conditions were therefore used to degrade VPg-[^{32}P]pUp. The products of hydrolysis were separated by paper electrophoresis at pH 3.5. Fig. 3*A* shows that the hydrolysis is incomplete, which was to be expected from similar studies on serine-phosphorylated proteins (see, for example, ref. 23). Spot III has been identified as P_i by electrophoresis on DEAE-paper at pH 3.5 (data not shown). Digestion for longer than 2 hr decreased the amount of spot I and resulted in a great increase in P_i. In one digestion for 2 hr, an increased amount of spot I and the appearance of slower migrating components were observed. We assume that spot I and slower moving materials are nucleotidyl-(*P-O*)-peptides (see below) of various amino acid contents. Their nature was not analyzed further.

The nature of spot II has been elucidated as follows: (*i*) Treatment of spot II with snake venom exonuclease yields pUp as the only labeled product (Fig. 3*B*). This suggests that the structure of spot II is $(aa)_n$-pUp (abbreviated as ZpUp). (*ii*) Treatment of spot II with bacterial alkaline phosphatase yields P_i and a slower migrating component (called ZpU) in a 1:1 ratio (Fig. 1*C*), an observation supporting the conclusion in *i*. (*iii*) Treatment of spot II with micrococcal nuclease yields what appears to be a single component as analyzed by electrophoresis on Whatman 3 MM paper (Fig. 3*D*). We had previously noted that authentic Tyr-*P* and Up do not separate by electrophoresis on Whatman 3 MM at any pH, or on DEAE-paper. The large spot shown in Fig. 3*D* was therefore eluted and further analyzed by thin-layer chromatography. As shown in Fig. 4, the ^{32}P-labeled material separated into two components (in a ratio of 1:1) that comigrated with authentic Tyr-*P* and Up in three different systems. This result is compatible with Tyr-O^4-pUp being the structure of spot II (Fig. 3*A*).

To support this conclusion, [*tyrosyl*-^{3}H]VPg-pUp was digested with HCl and radioactive material that migrated during paper electrophoresis to the position of spot II (Fig. 3) was isolated. Digestion of the ^{3}H-labeled material with snake venom exonuclease yielded only one radioactive product, which was identified by paper electrophoresis as [^{3}H]tyrosine (data not shown). The linking group between VPg and genomic RNA is thus identified as Tyr-O^4-pUp.

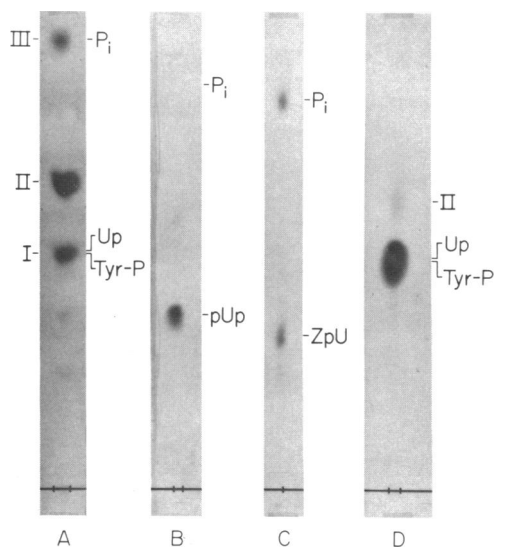

FIG. 3. Paper electrophoresis at pH 3.5 of degradation products of VPg-[^{32}P]pUp. (*A*) HCl hydrolysate of VPg-[^{32}P]pUp on Whatman 3 MM paper; (*B*) digest of spot II (of *A*) with snake venom exonuclease on DEAE-paper; (*C*) digest of spot II (designated as ZpUp) with bacterial alkaline phosphatase on Whatman 3 MM paper; (*D*) digest of spot II with micrococcal nuclease on Whatman 3 MM paper.

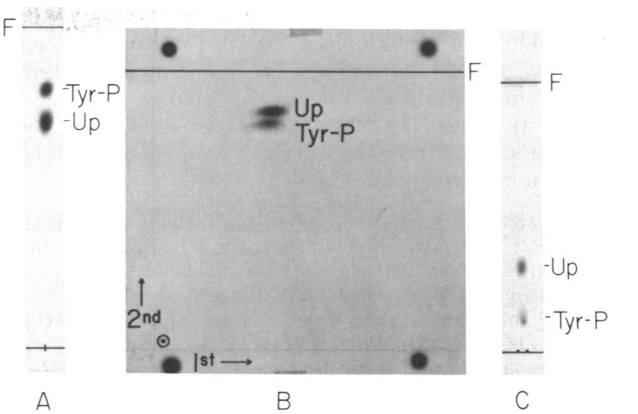

FIG. 4. Separation of Tyr-*P* and Up by thin-layer chromatography. The radioactive spot that comigrated with Tyr-*P* and Up after micrococcal nuclease digestion (Fig. 3*D*) was eluted. Aliquots were applied together with the appropriate markers to cellulose plates (*A* and *B*) and a silica gel plate (*C*) that were subsequently developed with (*A*) system A, (*B*) system B, and (*C*) system C. F indicates the position of the solvent front. After autoradiography, the spots of plates *A* and *B* were cut out and their radioactivities were determined. (*A*) Tyr-*P*, 375 cpm, and Up, 391 cpm; (*B*) Tyr-*P*, 325 cpm, and Up, 352 cpm.

DISCUSSION

The data presented here unambiguously establish that the genome-linked protein of PV1 is bound to the RNA via a phosphodiester between tyrosine and uridylic acid.§

There is no precedent for such a linkage between protein and polyribonucleotide. O^4-(5′-Adenylyl)tyrosine residues however, have been identified in the *Escherichia coli* enzyme glutamine synthetase and O^4-(5′-uridylyl)tyrosine has been found in a regulatory protein that modulates the enzymatic activity of *E. coli* glutamine synthetase (33, 35). Interestingly, the phosphodiester of nucleotidyl-*O*-tyrosine can be considered to be an "energy-rich" phosphate bond with a standard free energy of hydrolysis of approximately −9.6 kcal/mol (40 kJ/mol) (35).

The 5′-terminal nucleoside of poliovirus type 2 (PV2) and encephalomyocarditis virus (EMC) RNA is also uridine, and it is covalently linked to a small protein (ref. 7; A. Babich, A. Nomoto, and E. Wimmer, unpublished data). In addition, in minus strand RNA, VPg is bound to the 5′-terminal uridine of the 5′ poly(U) segment (4). We consider it likely that the protein-RNA linkage in all these RNAs is O^4-(5′-uridylyl)tyrosine because the bonds in (EMC)VPg-RNA, (PV2)VPg-RNA, and (minus strand)VPg-RNA have the same chemical properties as the bond in (PV1)VPg-RNA and can be cleaved with snake venom 3′-exonuclease (ref. 7; A. Babich, A. Nomoto, and E. Wimmer, unpublished data). Whether all picornavirus genomes are protein-linked via a nucleotidyl-*O*-tyrosine bond remains to be seen. This question is also unanswered for the single-stranded RNA genome of the plant virus cowpea mosaic virus, which also has a small protein covalently linked to its 5′ end (G. Bruening, personal communication).

Many covalent interactions between DNA and proteins have recently been established: (*i*) those in which the protein is permanently linked to DNA as in adenovirus DNA (36) or *Bacillus subtilis* phage ϕ29 DNA (37) and (*ii*) those in which the protein appears to be linked only transiently to DNA. The latter interaction has been shown for the relaxation complexes of plasmid ColE1 (38), for the ω protein of *E. coli* (39), for a

§ Ambros and Baltimore have informed us that they have arrived at the same conclusion by using a slightly different approach (34).

"nicking and closing" enzyme from rat liver cells (40), and also for protein A of bacteriophage ϕX174 (41) and a protein of simian virus 40 (42). In no case has the nature of the linkage between protein and DNA been elucidated.

The formation of the VPg-RNA complex, its cleavage to yield viral mRNA, and the biological significance of these processes remain unresolved problems.

We thank Drs. Seymour S. Cohen and Richard I. Gumport for stimulating discussions, Dr. Waldo E. Cohn for a critical reading of the manuscript, Nancy Reich for excellent technical assistance, and Sandi Donaldson for the preparation of the manuscript. This work was supported in part by Grant CA-16879 awarded by the National Cancer Institute and Grant AI-15122 awarded by the National Institute of Allergy and Infectious Diseases, Department of Health, Education and Welfare. P.G.R. is a member of the Molecular Biology Program of SUNY at Stony Brook and recipient of a predoctoral traineeship from the National Institutes of Health, Grant CM-07212.

1. Lee, Y. F., Nomoto, A. & Wimmer, E. (1976) *Prog. Nucl. Acid Res. Mol. Biol.* **19,** 89–95.
2. Lee, Y. F., Nomoto, A., Detjen, B. M. & Wimmer, E. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 59–63.
3. Flanegan, J. B., Pettersson, R. F., Ambros, V., Hewlett, M. J. & Baltimore, D. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 961–965.
4. Nomoto, A., Detjen, B. M., Pozzatti, R. & Wimmer, E. (1977) *Nature (London)* **268,** 208–213.
5. Sangar, D., Rowlands, D., Harris, T. J. R. & Brown, F. (1977) *Nature (London)* **268,** 648–650.
6. Hruby, D. E. & Roberts, W. K. (1978) *J. Virol.* **25,** 413–415.
7. Golini, F., Nomoto, A. & Wimmer, E. (1978) *Virology,* **89,** 112–118.
8. Pettersson, R. F., Flanegan, J. B., Rose, J. K. & Baltimore, D. (1977) *Nature (London)* 270–272.
9. Nomoto, A., Kitamura, N., Golini, F. & Wimmer, E. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 5345–5349.
10. Nomoto, A., Lee, Y. F. & Wimmer, E. (1976) *Proc. Natl. Acad. Sci. USA* **73,** 375–380.
11. Plimmer, R. H. A. (1941) *Biochem. J.* **35,** 461–469.
12. Mitchell, H. K. & Lunan, K. D. (1964) *Arch. Biochem. Biophys.* **106,** 219–222.
13. Greenwood, F. C., Hunter, W. M. & Glover, J. S. (1963) *Biochem. J.* **89,** 114–123.
14. Bray, D. & Brownlee, S. (1973) *Anal. Biochem.* **55,** 213–221.
15. Thorell, J. I. & Johansson, B. G. (1971) *Biochim. Biophys. Acta* **251,** 363–369.
16. Bolton, A. E. & Hunter, W. M. (1973) *Biochem. J.* **133,** 529–539.
17. Cole, C., Smoler, D., Wimmer, E. & Baltimore, D. (1971) *J. Virol.* **7,** 478–485.
18. Laemmli, U. K. (1970) *Nature (London)* **277,** 680–685.
19. Bonner, W. M. & Laskey, R. A. (1974) *Eur. J. Biochem.* **46,** 83–88.
20. Feldmann, H. & Falter, H. (1971) *Eur. J. Biochem.* **18,** 573–581.
21. Nishimura, S. (1972) *Prog. Nucl. Acid Res. Mol. Biol.* **12,** 49–85.
22. Wimmer, E. & Reichmann, M. E. (1969) *Nature (London)* **221,** 1122–1126.
23. Krystal, G., Winn, P., Millward, S. & Sakuma, S. (1975) *Virology* **64,** 505–512.
24. Gumport, R. I. & Lehman, I. R. (1971) *Proc. Natl. Acad. Sci. USA* **68,** 2559–2563.
25. Bogdanov, A. A., Antonovich, E. G., Terganova, G. V. & Prokof'ev, M. A. (1962) *Biokhimiya* **27,** 1054–1060.
26. Yuodka, B. A., Sasnauskene, S. & Lioranchaite, L. (1973) *Zhurnal Organicheskoi Khimii* **9,** 1630–1634.
27. Mandel, B. (1964) *Virology* **22,** 360–367.
28. Carthew, P. & Martin, S. J. (1974) *J. Gen. Virol.* **24,** 525–534.
29. Rowlands, D. J., Shirley, M. W., Sangar, D. V. & Brown, F. (1975) *J. Gen. Virol.* **29,** 223–234.
30. Lonberg-Holm, K. & Butterworth, B. E. (1976) *Virology* **71,** 207–216.
31. Lund, G. A., Ziola, B. R., Salmi, A. & Scraba, D. G. (1977) *Virology* **78,** 35–44.
32. Talbot, P., Rowlands, D. J., Burroughs, J. N., Sangar, D. V. & Brown, F. (1973) *J. Gen. Virol.* **19,** 369–380.
33. Adler, S. P., Purich, D. & Stadtman, E. R. (1975) *J. Biol. Chem.* **250,** 6264–6272.
34. Ambros, V. & Baltimore, D. (1978) *J. Biol. Chem.* **253,** 5263–5266.
35. Holzer, H. & Wohlhueter, R. (1972) *Adv. Enzyme Regul.* **10,** 121–132.
36. Rekosh, D. M. K., Russell, W. C., Bellet, A. J. D. & Robinson, A. J. (1977) *Cell* **11,** 283–295.
37. Salas, M., Mellado, R. P., Viñuela, E. & Sogo, J. M. (1978) *J. Mol. Biol.* **119,** 269–291.
38. Guiney, D. G. & Helinski, D. R. (1975) *J. Biol. Chem.* **250,** 8796–8803.
39. Depew, R. E., Liu, L. F. & Wang, J. C. (1978) *J. Biol. Chem.* **253,** 511–518.
40. Champoux, J. J. (1978) *J. Mol. Biol.* **118,** 441–446.
41. Eisenberg, S., Griffith, J. & Kornberg, A. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 3198–3202.
42. Kasamatsu, H. & Wu, M. (1976) *Proc. Natl. Acad. Sci. USA* **73,** 1945–1949.