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Sequence of picornavirus RNAs containing a radioiodinated 5′-linked peptide reveals a conserved 5′ sequence

(poliovirus/coxsackie B virus/RNA sequence/genome-linked virion protein)

MARTINEZ J. HEWLETT AND ROBERT Z. FLORKIEWICZ

Department of Cellular and Developmental Biology, University of Arizona, Tucson, Arizona 85721

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ABSTRACT **Virion RNA (vRNA) from poliovirus type 1 (PV1), poliovirus type 2 (PV2), and coxsackie virus B1 (Cox B1) were treated with proteinase K to remove all but a small peptide of the covalently attached 5′ genome-linked virion protein (VPg). The peptide on these RNA molecules was then treated with Bolton–Hunter ^{125}I reagent, which iodinates primary amine groups, in order to obtain specific 5′-terminal radioactive labeling. Sequences of ^{125}I-labeled vRNAs were determined by using a set of base-specific RNases and a partial alkaline hydrolysis "ladder." The first 20 positions of these RNAs show a remarkable conservation of sequence. The initial 10 nucleotides are identical in PV1, PV2, and Cox B1, with the sequence VPg-pU-U-A-A-A-A-C-A-G-C-. The next 10 nucleotides show a one-base difference between PV1 and PV2 and 50% homology between PV1 and Cox B1. This conserved 5′ region may provide a recognition site for interaction between the viral mRNA and the host translation system.**

The translation of picornavirus mRNA within the infected host cell is unusual because these viral RNAs are not capped and methylated at the 5′ terminus (1–4). In fact, the virion RNA (vRNA) of several picornaviruses contains a small genome-linked protein (VPg) attached covalently to the 5′ terminus via a bond between a phosphate and a tyrosine residue (5–10). Polyribosome-associated vRNA of poliovirus type 1 (PV1) does not contain VPg but instead terminates with pUp (1–3). The presence of VPg on PV1 has been used to select a RNase T1-resistant oligonucleotide and therefore to obtain the 5′-terminal sequence (VPg)-pU-U-A-A-A-A-C-A-G (6).

We have specifically labeled the 5′ terminus of vRNAs by iodination (using Bolton–Hunter ^{125}I reagent*) of a proteinase K-resistant core of VPg attached to the RNA molecule (11) and have used these molecules to obtain sequence information. We report here a comparison of the 5′-terminal sequence of PV1 (Mahoney), poliovirus type 2 (PV2), and coxsackie virus B1 (Cox B1). We find a remarkable conservation of sequence within the initial 20 nucleotides. Because picornavirus infection results in a specific inhibition of host protein synthesis and a preferential translation of viral proteins, this sequence suggests that a common 5′ structure in the viral mRNA may be required for ribosomal recognition.

MATERIALS AND METHODS

Growth of Cells and Viruses. HeLa S3 cells were grown in suspension culture as described (12). The growth and purification of PV1 in these cells was by methods published elsewhere (13). PV2 and Cox B1 were obtained from J. Holland (University of California, San Diego). Virus was grown in HeLa cells as described for PV1. PV2 and Cox B1 were purified by lysing infected cells with 0.01 M NaCl/0.01 M Tris·HCl, pH 7.4, containing 1% Nonidet P-40 and pelleting virus from the cytoplasm in the absence of sodium dodecyl sulfate (NaDodSO$_4$). Virus was purified by isopycnic banding in CsCl.

Purification and Proteinase K Treatment of vRNA. Intact vRNA from each virus was obtained by acid/NaDodSO$_4$ lysis (14) and sedimentation through 15–30% sucrose gradients containing 0.5% NaDodSO$_4$ (13); 35S material was recovered by ethanol precipitation at −20°C. vRNA was treated with proteinase K (EM Laboratories, Elmsford, NY) at 37°C for 1 hr as described (6). RNA was recovered from this digestion by NaDodSO$_4$/phenol extraction (13) and ethanol precipitation.

5′-Radioiodination of vRNA. Proteinase K-treated vRNA (100 μg) was dissolved in 50 μl of 0.1 M borate buffer at pH 8.5 and heated to 65°C for 1 min. The solution was then transferred to a plastic microcentrifuge tube containing 0.5–1.0 mCi (1 Ci = 3.7×10^{10} becquerels) of Bolton–Hunter ^{125}I reagent (Amersham) at 4°C. After 20 min, 250 μl of 0.2 M glycine/0.1 M borate, pH 8.5, was added and the reaction was allowed to continue for 5 min. The reaction mixtures were made 0.4 M in sodium acetate and 0.5% in NaDodSO$_4$, and the RNA was recovered by ethanol precipitation. The RNA was then layered onto a 15–30% sucrose gradient containing 0.5% NaDodSO$_4$ (13), and 35S material was recovered and ethanol precipitated.

^{125}I-Labeled vRNA (^{125}I-vRNA) was completely digested with RNase T1 (Calbiochem) by dissolving 1 μg of RNA in 5 μl of the enzyme solution (100 units/ml) in 10 mM Tris/1 mM EDTA, pH 7.5, and incubating at 37°C for 30 min. The products were separated on cellulose acetate by ionophoresis at pH 3.5 as described (6).

RNA Sequence Analysis. ^{125}I-vRNA sequence analysis was performed essentially as described by Donis-Keller *et al.* (15) and by Simoncsits *et al.* (16). A complete set of RNA fragments (ladder) was obtained by dissolving 2–3 μg of RNA in 5 μl of bicarbonate buffer (pH 9.0) and heating at 90°C for 15 min (15). *Physarum* RNase [(A+U)-specific; the enzyme was a kind gift from W. Gilbert and H. Donis-Keller] and RNase T1 (G-specific, Calbiochem) were used as described (15). RNase U2 (A-specific, Calbiochem) was used as reported by Simoncsits *et al.* (16). All samples were electrophoresed, along with bromphenol blue and xylene cyanol blue as marker dyes, in 20% acrylamide gels containing 7 M urea with a Tris borate buffer system at pH 8.3 (15).

Autoradiography of cellulose acetate and acrylamide gels

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Abbreviations: PV1, poliovirus type 1; PV2, poliovirus type 2; Cox B1, coxsackie virus B1; NaDodSO$_4$, sodium dodecyl sulfate; vRNA, virion RNA; VPg, genome-linked virion protein.

* Bolton–Hunter ^{125}I reagent is iodinated 3-(4-hydroxyphenyl)propionic acid *N*-hydroxysuccinimide ester. This reagent reacts with primary amine groups to form an amide bond with the iodinated acyl moiety (11). We use the term "iodination" to describe the results of this reaction.

was with Kodak XR-2 film and a Dupont Cronex II intensifying screen at −70°C.

RESULTS

Specific Labeling of 5′ Terminal VPg Peptide. Bolton–Hunter reagent specifically iodinates primary amine groups (11), and this reaction has been used to iodinate the terminal covalently linked protein of adenovirus (17) and the VPg on PV1 (8). In preliminary experiments we also found that the vRNA of PV1 reacts with Bolton–Hunter ^{125}I reagent at pH 8.5 such that only VPg is labeled and no nucleotides are iodinated (data not shown). However, the size of VPg is such that its presence dominates the electrophoretic mobility of small oligonucleotides and therefore these molecules cannot provide sequence information.

When vRNA is treated with proteinase K at 37°C for 1 hr, a fragment of VPg (two or three amino acids) remains covalently attached to the RNA (6). Proteinase K-treated RNA was treated with Bolton–Hunter ^{125}I reagent at pH 8.5, and intact molecules were isolated by sedimentation through 15–30% sucrose gradients (Fig. 1). The specific activity of RNA labeled in this manner is about 10^4 cpm/μg of RNA.

PV1 ^{125}I-RNA, pooled as indicated in Fig. 1, was recovered after ethanol precipitation and completely digested with RNase T1. Ionophoresis of the products of this reaction on cellulose acetate at pH 3.5 (Fig. 2) revealed a single labeled band migrating faster than the blue dye. When PV1 RNA uniformly labeled with ^{32}P is digested with RNase T1 and ionophoresed in this manner, a dense smear of oligonucleotides is seen in the same region (6). This suggests that only one oligonucleotide is iodinated. The mobility of this ^{125}I-labeled RNase T1-resistant oligonucleotide is much faster than that of the RNase T1 fragment containing the intact protein (6), consistent with the removal of most of VPg with proteinase K. This result indicates that the iodinated peptide does not greatly retard the mobility of the oligonucleotide.

PV2 and Cox B1 vRNAs were treated with proteinase K and iodinated as described above. In both cases, the recovered RNA yielded single iodinated RNase T1-resistant oligonucleotides (data not shown). This is consistent with the presence of a VPg-like protein on PV2 and Cox B1 and also indicates the presence of a proteinase K-resistant core that remains covalently attached to the RNA.

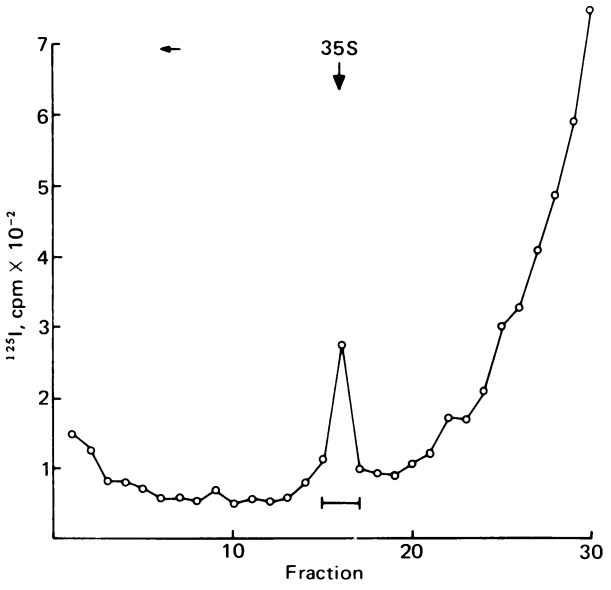

FIG. 1. NaDodSO$_4$/sucrose gradient purification of ^{125}I-labeled, proteinase K-treated PV1. RNA was layered onto a 15–30% sucrose gradient containing 0.5% NaDodSO$_4$ and centrifuged in a Beckman SW 27 rotor at 21,000 rpm and 21°C for 16 hr. Arrow, position of ^{32}P-labeled PV1 RNA sedimented in a parallel gradient. Fractions indicated by the bar were pooled, and the RNA in them was recovered by two successive ethanol precipitations.

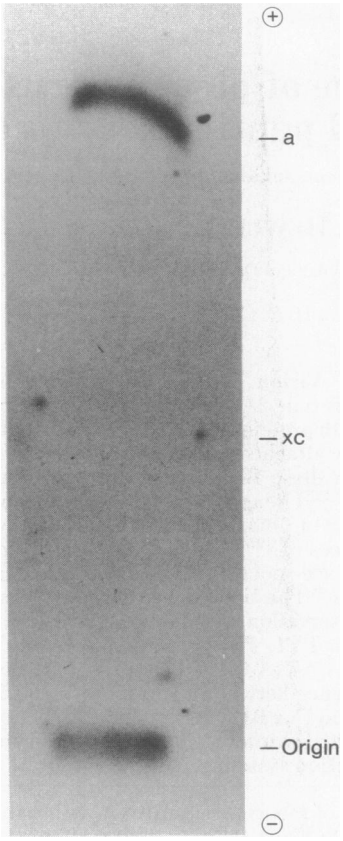

FIG. 2. Cellulose acetate ionophoresis of a limit RNase T1 digest of PV1 ^{125}I-RNA. The total digest was applied to a cellulose acetate strip (3 × 56 cm) and ionophoresis was carried out at pH 3.5 for 30 min at 5000 V. XC, position of marker xylene cyanol blue; a, position of the iodinated 5′ RNase T1 oligonucleotide.

Derivation of PV1 RNA Sequence. ^{125}I-RNA was partially digested with RNase T1, RNase U2, or a mixture of the *Physarum* RNases according to published procedures (15, 16). In addition, a "ladder" digest was prepared by partial alkaline hydrolysis (15). Fig. 3 shows an autoradiogram from a set of digests of PV1 ^{125}I-RNA.

We begin by comparing the known sequence of the protein-linked RNase T1 oligonucleotide from PV1 (6) with the pattern in the gel in Fig. 3. The first G residue [position 9 in the previous data (6)] is clear in the T1 digest and we have used this as an orientation point. We can identify the C residue at position 7 (missing band in A+U; no band in G) and the run of four As at positions 3–6 (bands in A+U and A). Our results do not clearly show the A residue at position 8 and this assignment is made from previous data (6).

Interpretation of nucleotide positions before position 3 in our gel is difficult because there are more bands present in the ladder and overdigested A+U lanes than are consistent with our earlier results (6). These bands could be an artifact of the iodination technique. However, we have found that mononucleotide produced by complete digestion of ^{125}I-labeled RNA with a mixture of RNases T1, T2, and A migrates as a single band in this gel system (data not shown). Another possibility is that the bands are real and represent additional nucleotides (Us)

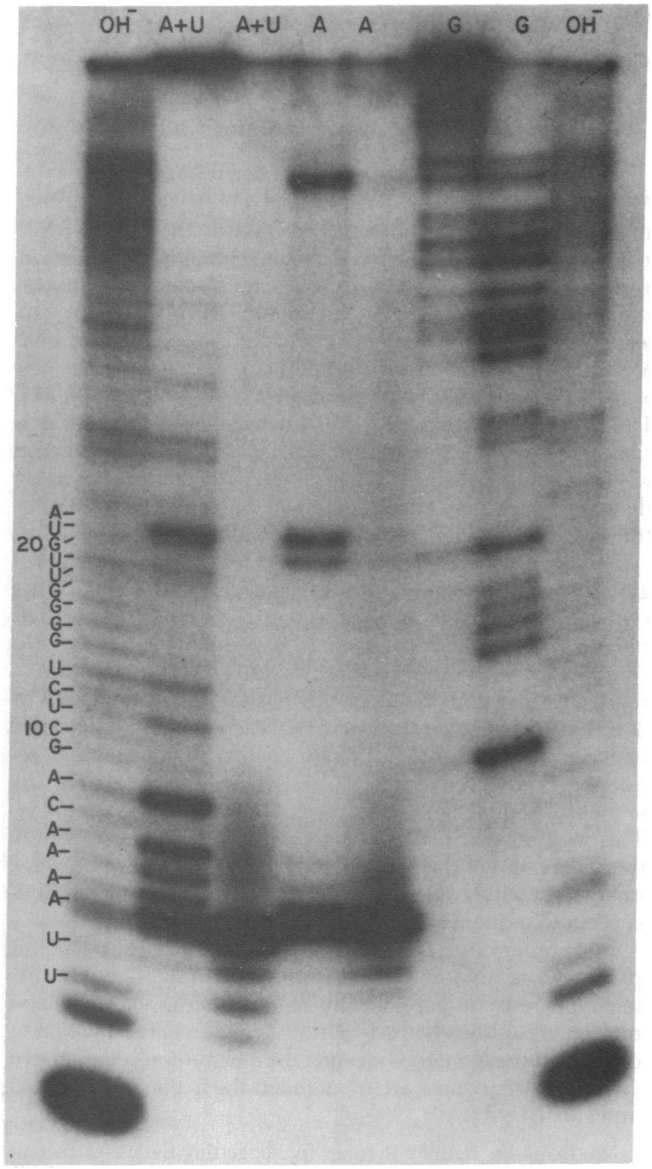

FIG. 3. Sequence analysis of 5′-labeled PV1 ^{125}I-RNA. Partial digests and acrylamide gel electrophoresis are described in *Materials and Methods* and in previous publications (10, 11). Lanes OH⁻ are ladders (2 μg of RNA). Lanes A+U are partial digests with *Physarum* RNase using undiluted and 1:10 diluted enzyme (units not specified; 2 μg of RNA). Lanes A are digests with RNase U2, using 0.02 or 0.002 unit per reaction (1 μg of RNA). Lanes G are digests with RNase T1, using 0.1 or 0.01 unit per reaction (1 μg of RNA). Electrophoresis was through a 20% acrylamide gel containing 7 M urea. The intense spot at the bottom of the OH⁻ lanes is free iodine released during the alkaline hydrolysis.

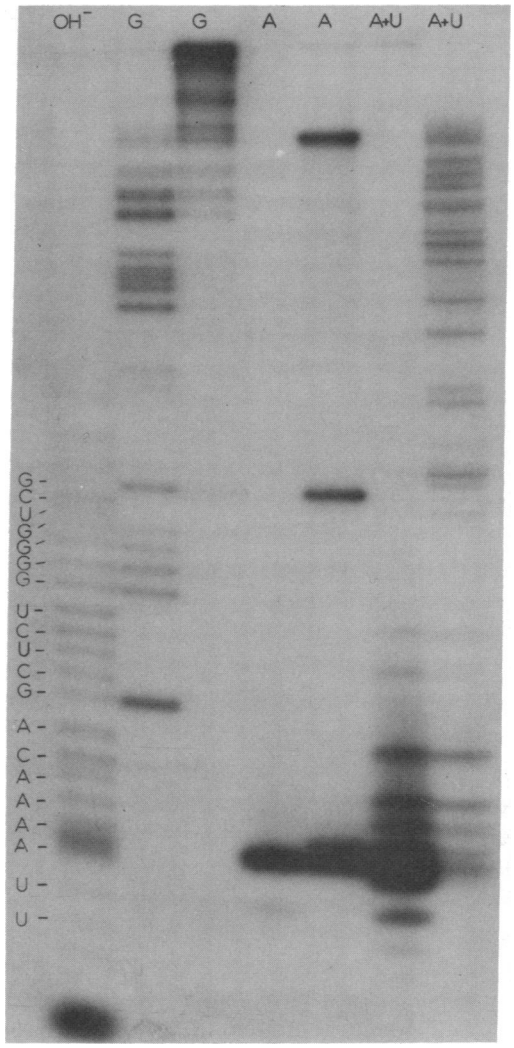

FIG. 4. Sequence analysis of 5′-labeled PV2 ^{125}I-RNA. RNase digestion and electrophoresis were as described in Fig. 3.

before the run of As. Previous data for vRNA (6) and for polyribosome-associated RNA (18) are consistent with only two Us in the terminal T1 oligonucleotide. We think the most likely explanation is that the extra bands are artifacts of partial alkaline hydrolysis or overdigestion by the *Physarum* enzymes. Alkaline hydrolysis yields a mixture of products that have cyclic phosphate and 2′ (or 3′) phosphate termini and that will have different mobilities for mono- or dinucleotides in this gel system (16). Overdigestion with *Physarum* RNase (A+U) may result in a similar situation. Therefore, we have assigned (VPg)-pU-U as the terminal sequence.

The sequence after position 9 can now be easily read and the entire structure thus far determined is (VPg)-pU-U-A-A-A-A-C-A-G-C-U-C-U-G-G-G-G-U-U-G-U-A. G residues are most clear in the display of Fig. 3, starting with the G at position 9 and including the distinctive run of Gs at 14–17. C residues are also easily read as bands absent in the A+U, G, and A digests. Thus, the C at 10 is distinguished from the G at 9 because there is *no* band at 10 except in the ladder, whereas at 9 there is a dark band in the G digest. The deduced sequence was in agreement after three separate determinations. Beyond position 22 we were not able to assign sequence with any confidence, because of the broad bands produced on autoradiographs using ^{125}I and intensifying screens.

RNA Sequence for PV2 and Cox B1. There is a striking similarity of the gel patterns for positions 1–20 in PV1 (Fig. 3), PV2 (Fig. 4), and Cox B1 (Fig. 5). A notable difference is the change in the run of four Gs at 14–17 in PV1 and PV2 to G-U-G-G-G in Cox B1. However, the sequence diverges significantly, especially in the position of G residues, at positions beyond 20. These data confirm that the three RNAs are different in overall sequence, which is also supported by fingerprints of each RNA species (data not shown). The nucleotide at position 20 is interpreted as G in both PV2 and Cox B1 on the basis of the band in the RNase T1 digest, the distinct "G-shift" in the ladder, and the faint band in the *Physarum* RNase digest (Figs. 4 and 5). Fig. 6 presents the sequence data in a comparative form.

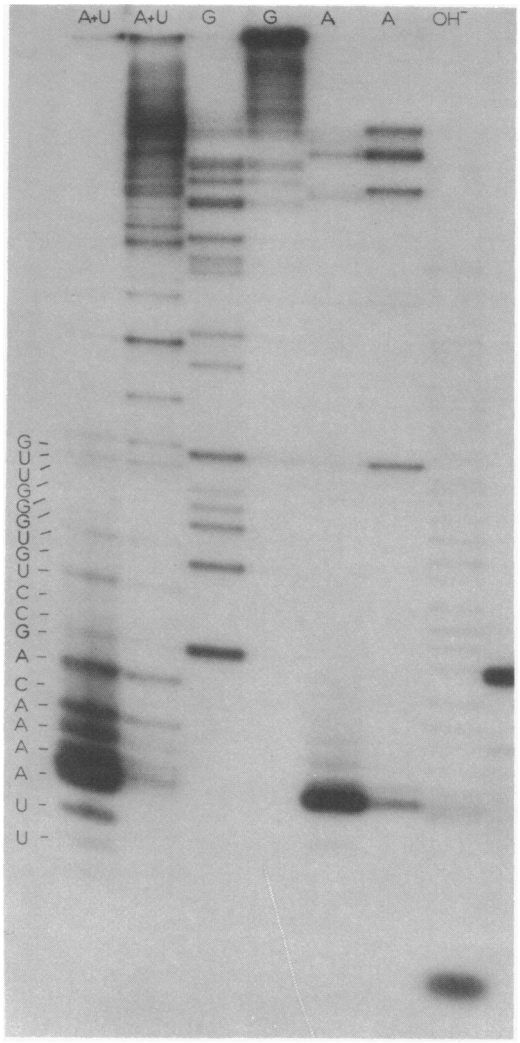

FIG. 5. Sequence analysis of 5′-labeled Cox B1 ^{125}I-RNA. Conditions were as described in Fig. 3.

DISCUSSION

The use of Bolton–Hunter reagent has proved to be of unique advantage for specific 5′-labeling of VPg or fragments of VPg on picornavirus RNA. Rothberg *et al.* (8) have reported that chloramine-T and lactoperoxidase iodinations do not result in labeling of VPg on PV1, presumably because the hydroxyl group of the only tyrosine residue within the molecule is covalently linked to the RNA and not available for reaction. The presence of the proteinase K-resistant peptide does not affect the separation of small oligonucleotides. In addition, iodination with Bolton–Hunter reagent does not result in the labeling of any nucleotide positions and therefore allows the production of "nested" sets of fragments. Therefore, we have developed a method for direct sequence determination of picornavirus RNA and other RNAs with similar terminal proteins.

The 5′-terminal sequences shown in Fig. 6 reveal a remarkable conservation. Young has reported (19) that the sequence relatedness by hybridization between PV1 and PV2 is 36% and between PV1 and Cox B4 it is 5%. The picornaviruses share a similar mode of replication, and common viral functions are expected. However, this conservation of sequence occurs in an apparently nontranslated region of the genome and has been maintained in spite of a strong overall sequence divergence among these viruses.

Picornavirus mRNA is not capped as are other eukaryotic

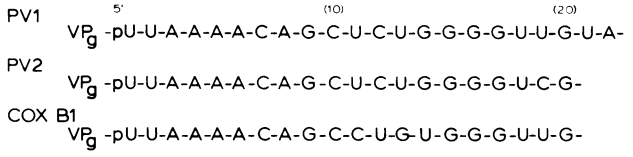

FIG. 6. Sequences at 5′ end of PV1, PV2, and Cox B1. We use the symbol VPg for each of the 5′ proteins without implying identity.

mRNAs, and a common 5′ sequence may be the basis of RNA structural properties involved in ribosome binding and initiation of protein synthesis. Poliovirus infection results in the inactivation of the host initiation factor eIF-4B, involved in cap recognition (20), and leads to a block in the initiation of host protein synthesis. Interaction with the cap structure may be one of several events occurring during eukaryotic initiation (4, 21). The conserved region may function in lieu of the cap as a ribosome attachment signal in picornavirus mRNA. Because we have not found an AUG within the sequences we describe, these sequences may not be contained with an initiation region for a viral protein.

This conserved sequence could also function to define a region at the 3′ end of the minus strand that interacts with the replication enzyme(s) of the virus. It has been proposed that picornavirus replication *in vivo* is primed by a VPg-pUp complex (5, 6). A conserved region of the minus strand may represent a recognition site for VPg or the viral polymerase. The 3′ sequences of several picornaviruses, including PV1, have been reported (22, 23). Although conservation of some 3′ sequences is observed among several viruses, we do not see any sequences complementary to the 5′ sequences described above. This suggests that identical conserved sites are not present in the 3′ sequence of the plus and minus vRNA strands. Therefore, interaction with viral replicase is not a likely function of the 5′ conserved sequence.

In summary, we report a method for specifically labeling picornavirus RNA at the 5′ terminus by iodination of a proteinase K-resistant peptide with Bolton–Hunter reagent. These molecules can then be subjected to sequence determination by direct enzymatic methods, giving the structures reported above. Conserved sequences are observed at the 5′ end of PV1, PV2, and Cox B1 vRNA.

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