Volume 8 Number 16 1980 Nucleic Acids Research

The structure of the covalent linkage between proteins and RNA in encephalomyocarditis virus

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Received 25 March 1980

ABSTRACT

Two proteins, VPgA and VPgB, are covalently bound to the virion RNA of encephalomyocarditis (EMC) virus. Their molecular weights, as determined by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate are 10,000 and 8,000, respectively. A study of nucleotide-peptides isolated from VPg-RNA compound has shown that VPgA is bound to the 5'-terminal nucleotide of RNA by a phosphodiester bond. The 5'-terminal nucleotide of RNA is uridylic acid. It is the hydroxy group of the Tyr residue of VPgA that is involved in the formation of the linkage with RNA. VPgB-RNA seems to be similar to VPgA-RNA both in the structure of the RNA-protein linkage and localization of VPgB on RNA.

INTRODUCTION

The genomes of picornaviruses are 3'-polyadenylated single-stranded plus chains of RNA. Virion RNAs of picornaviruses (i.e. of poliovirus type I, EMC virus, foot-and-mouth disease virus and mengo virus) are covalently bound with low molecular weight virus-specific proteins (VPg) /1-8/. In the case of poliovirus, the linkage between VPg and RNA involves the 5'-terminal uridylic acid of RNA and the hydroxy group of the tyrosine residue of polio-VPg /9,10/. VPg is linked not only to the virion RNA of poliovirus, but also to the minus chain of RNA and to nascent RNA strands of the polio replicative intermediate /11,12/. Viral mRNA, however, lacks VPg /13,14/.

The function of VPg is being investigated now. For elucidating this function and for understanding the mechanism of formation and hydrolysis of the linkage between the protein and RNA, the structure of the VPg-RNA compound should be studied. This paper describes our data pertaining to the chemical

nature of the linkage between the two VPg proteins and EMC virion RNA.

MATERIALS AND METHODS

Viral RNA

^{32}P-labelled EMC virion RNA was prepared from purified EMC virus grown in Krebs II ascites cells in the presence of ^{32}P-orthophosphate as described previously /5/. The specific radioactivity of EMC virion ^{32}P-RNA was $3 \cdot 10^4$–10^5 cpm/µg RNA.

Enzymatic digestions

Limit digestion of RNA with RNases A (Worthington), T_1 and T_2 (Sankyo) was performed as described in /15/. The conditions for micrococcal nuclease (Sigma) hydrolysis have been published /9/. The reaction conditions for carboxypeptidase A (Sigma) and leucineaminopeptidase (Serva) were as described in /16/. Pronase (Calbiochem) digestion was in 0.5 ml 0.01 M Tris-HCl (pH 7.5) at an enzyme concentration of 0.1 mg/ml for 15 h at 37°C. Bacterial alkaline phosphatase (Worthington) hydrolysis was carried out for I h at 37°C using 60 µg/ml of the enzyme in 50 µl of 0.03 M Tris-HCl (pH 7.5). To inactivate the phosphatase, the sample was heated at 100°C for 5 min in the presence of I mM EDTA. Snake venom phosphodiesterase (Worthington) digestion was performed for 2 h at 37°C with 40 µg/ml of the enzyme in 50 µl of 0.03 M Tris-HCl (pH 8.0), 0.01 M $MgCl_2$.

Plastic tubes and pipettes pretreated with a solution of bovine serum albumin (I mg/ml) for 5 min at 60°C were used with the VPg-containing compounds.

Acid hydrolysis of nucleotide-peptides

Nucleotide-peptides were hydrolysed *in vacuo* with 250 µl of 4 M HCl for 6 h at 105°C. The hydrolysate was evaporated and dissolved in water.

Polyacrylamide gel (PAG) electrophoresis

PAG electrophoresis (12.5% acrylamide) in 0.1% sodium dodecyl sulfate (SDS), 8 M urea was as described in /17/. Cytochrome c (M.W. 12,300) and insulin (M.W. 5,700) were used as marker proteins. ^{32}P-containing material was eluted from PAG with

a solution of pronase (0.1 mg/ml).

Paper electrophoresis

Electrophoretic separations were performed in ammonia/formic acid buffer (pH 3.5) or acetic acid/formic acid buffer (pH 1.7) on Whatman 3LM paper, the voltage gradient being 60-90 v/cm. If a sample was contaminated with salts or urea, the ^{32}P-containing fractions were rerun without additional treatment. The resolved substances were eluted from the paper with water.

Thin-layer chromatography

Thin-layer chromatography was carried out on cellulose plates (Eastman Kodak) in solvent systems: (A) first dimension, isobutyric acid/concentrated ammonia/water (66:1:33, v/v/v); second dimension, i-propanol/concentrated HCl/water (68:17:17, v/v/v). (B) first dimension, isobutyric acid/0.5 M ammonia (5:3, v/v); second dimension, isopropanol/concentrated HCl/water (70:15:15, v/v/v). The position of nucleotide markers was determined by UV absorbance; O-phosphoamino acid markers were detected by ninhydrin staining.

The synthesis of marker O-phosphotyrosine from L-tyrosine was performed as in /18/. The product was identified by spectrophotometric titration and electrophoretically before and after treatment with bacterial alkaline phosphatase.

Detection of radioactivity

^{32}P-radioactivity in PAG was detected by autoradiography. ^{32}P-(or ^{3}H-)radioactivity on Whatman paper or thin-layer plates was determined by measuring the radioactivity of strips in a toluene scintillator.

RESULTS

Digestion of EMC RNA and fractionation of the hydrolysate

EMC virion ^{32}P-RNA isolated by three phenol deproteinizations of EMC virus was digested with a mixture of nucleases A, T_I and T_2 and the products were separated by paper electrophoresis at pH 3.5. Along with ^{32}P-mononucleotides, part of the ^{32}P-containing material was recovered as a diffuse spot migra-

ting toward the cathode, thus being positively charged at pH 3.5. This compound had a low electrophoretic mobility (-0.2 relative to xylene cyanol (XC)) and adsorbed firmly on the surfaces. Therefore, PAG electrophoresis in the presence of SDS was used for the analysis of hydrolysate. In our previous experiments the results were identical regardless of whether the RNA hydrolysate or the substance positively charged at pH 3.5 and isolated by paper electrophoresis was subjected to PAG electrophoresis - one ^{32}P-containing zone was detected in PAG /5/. Fig. 1 shows the results of PAG electrophoresis of the RNA hydrolysate at a higher voltage gradient (20 v/cm, 5 h). Two ^{32}P-containing zones were detected in PAG, the mobilities of which corresponded to these of proteins with molecular weights of 10,000 and 8,000. The ratio of radioactivity in these zones varied in different experiments. Up to 20% of the radioactivity detected in these two zones were detained at the origin. Treatment of RNA with alkaline phosphatase prior to RNA digestion, as well as treatment of the RNA hydrolysate with β-mercaptoethanol (1%, 60°C, 10 min),did not alter the electrophoretic pattern.

A compound migrating toward the cathode was recovered when combined ^{32}P-material was isolated from PAG and analyzed by paper electrophoresis (data not shown). The results of pronase treatment of this compound are shown in Fig. 2. At least 70% of the extracted ^{32}P-labelled compound proved to be sensitive to pronase treatment. The amount of liberated free phosphate varied in several experiments, probably owing to inspecific adsorption of P_i which in most cases was insignificant.

The above data suggest that the products of hydrolysis of EMC virion RNA contain proteins with molecular weights of 10,000 and 8,000, which we designate as VPgA and VPgB, respectively. These proteins are linked to a product of complete RNA digestion, i.e. nucleoside phosphate, nucleoside diphosphate or dinucleotide, depending on the location of VPg's within the viral genome and the type of linkage. Treatment of these compounds with pronase yielded nucleotide-peptides I, II and III (see Fig. 2).

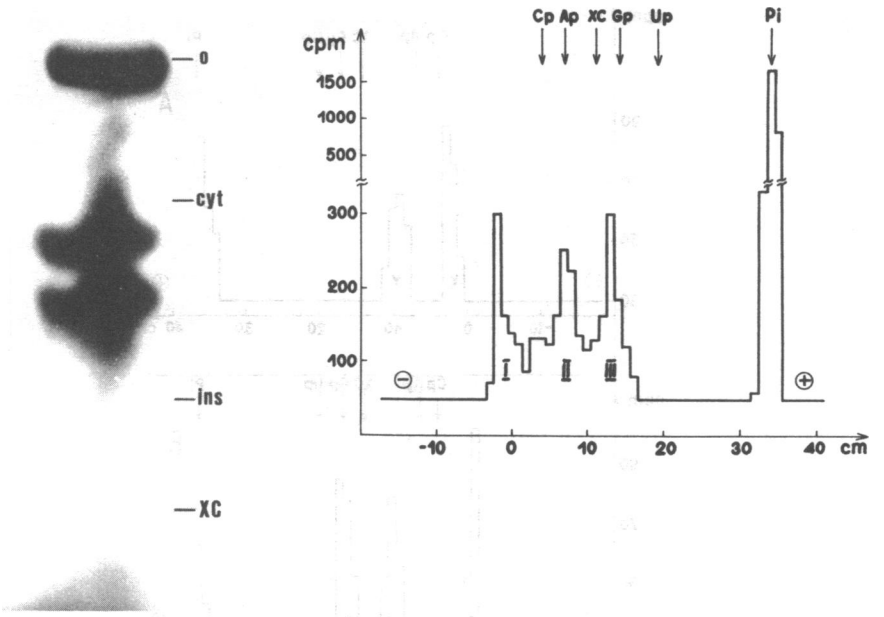

Fig. 1. PAG electrophoresis of EMC virion ^{32}P-RNA hydrolysed with a mixture of RNases. The positions of cytochrome c, insulin, XC and the origin are indicated.

Fig. 2. Paper electrophoresis at pH 3.5 of pronase-treated ^{32}P-zones, isolated from PAG. I-III, the respective nucleotide-peptides. Arrows here and in other figures indicate the positions of the markers. O-place of sample application.

Identification of VPg-linked nucleotide

Nucleotide-peptides I, II and III (Fig. 2) were treated with bacterial alkaline phosphatase and pronase and analyzed by paper electrophoresis at pH 3.5. The results are presented in Fig. 3. Nucleotide-peptide I did not markedly change its electrophoretic mobility after this and all subsequent treatments; this may be accounted for both by the insensitivity of substance I or by inactivation of the enzymes, as the material in the site of sample application was contaminated with urea extracted from PAG.

Successive alkaline phosphatase and pronase treatment yielded nucleotide-peptides X and Y (from nucleotide-peptide II) and nucleotide-peptides Y and Z (from nucleotide-peptide III). In both cases about half of the ^{32}P-radioactivity was

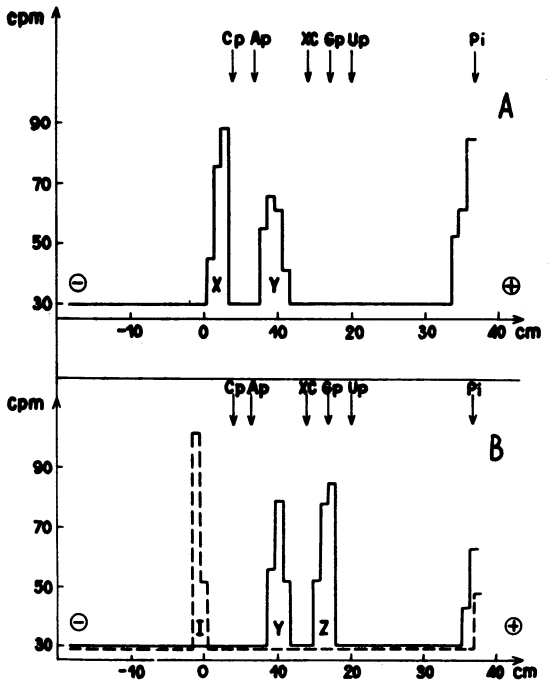

Fig. 3. Paper electrophoresis at pH 3.5 of nucleotide-peptides treated with bacterial alkaline phosphatase and pronase. A, nucleotide-peptide II. B, nucleotide-peptide I (- - - -) and nucleotide-peptide III (———). 700 cpm and 640 cpm of treated nucleotide-peptide II and III, respectively, were applied.

released from nucleotide-peptides II and III in the form of inorganic phosphate. The nucleotide-peptides II and III isolated as described above proved to be completely resistant to 0.1 M NaOH (37°C, 1.5 h) and 4 M NH_2OH (pH 5.0, 37°C, 1.5 h). Resistance to mild alkali treatment and liberation of the half of the radioactivity as free phosphate after complete bacterial phosphatase digestion testifies to the possible participation of the phosphate group of the nucleotide in the linkage with VPg, as it will be proved further.

To identify the nucleotide linked with peptides, nucleotide-peptides I, X, Y and Z were treated with snake venom phosphodiesterase and analyzed by paper electrophoresis at pH 3.5. The whole of nucleotide-peptide X and one-third of nucleotide-

peptide Y were converted to a compound with the electrophoretic mobility close to that of Up (Fig. 4). Nucleotide-peptide Z was also sensitive to phosphodiesterase treatment, but we failed to isolate the products of its hydrolysis. Phosphodiesterase-resistant portion of nucleotide-peptide Y proved insensitive to the subsequent treatment with I M HCl (37°C, 1 h).

A compound with the electrophoretic mobility close to that of uridylic acid was identified by two-dimensional thin-layer chromatography on cellulose plates using solvent system A. Both in the case of nucleotide-peptide X and nucleotide-peptide Y, snake venom phosphodiesterase treatment liberated uridine-5'-phosphate. The same results were obtained with EMC virion RNA labelled with ^{3}H-uridine by us /6/ and with ^{32}P-labelled RNA by Golini et al. /4/.

The hydrolytic properties of the nucleotide-peptides testify to the fact that their nucleic acid and protein components

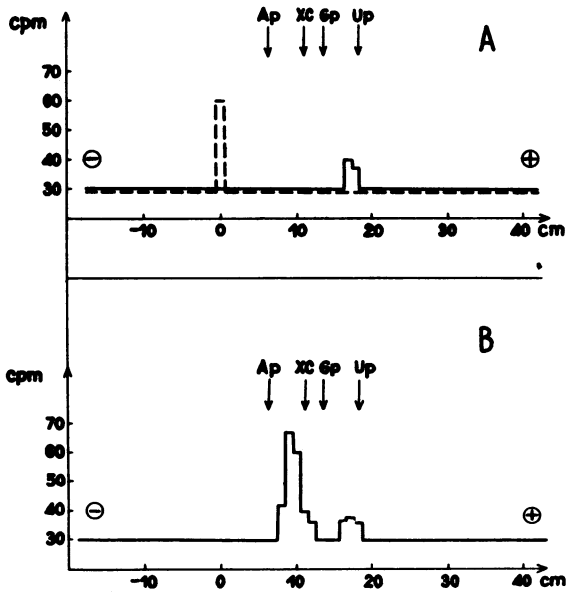

Fig. 4. Paper electrophoresis at pH 3.5 of nucleotide-peptides treated with snake venom phosphodiesterase. A, nucleotide-peptide I (- - - -) and nucleotide-peptide X (———); B, nucleotide-peptide Y.

are linked via a phosphodiester bond. From the snake venom phosphodiesterase specificity one may infer that the VPg-RNA linkage is localized at the 5'-terminal nucleotide of RNA, which is then uridylic acid. The VPg-RNA linkage in EMC virus can therefore be presented as VPg-pUp...

Identification of the amino acid participating in the formation of VPg-RNA linkage

VPg proteins are bound to EMC virion RNA via a phosphodiester bond. Therefore the amino acids involved in the formation of this bond might be serine, threonine and tyrosine. Phosphodiesters of uridylic acid and of these hydroxyamino acids are sufficiently stable under the conditions used for nucleotide-peptide isolation and in the above mentioned conditions of treatment with HCl and NH_2OH /19,20/. The stability of the uridylyl-peptide linkage in 0.1 M NaOH can be accounted for by both the stability of the phosphodiesters in serine and threonine di- and tripeptides /21/ and by the stability of the tyrosine derivative of uridylic acid /20/.

Successive digestion of uridylated VPgA and VPgB with endo- and exopeptidases (pronase, carboxypeptidase A, leucinaminopeptidase) yielded several nucleotide-peptides (data not shown). For more complete hydrolysis of the peptide fragments, HCl treatment was used.

A zone of PAC corresponding to VPgA-pUp (see Fig. 1) was treated with pronase. The extracted nucleotide-peptides were hydrolysed with 4 M HCl and analyzed by paper electrophoresis at pH 3.5 (Fig. 5). In addition to the products of incomplete acid hydrolysis that were also presented prior to HCl treatment (see Fig. 2), a ^{32}P-containing material with a higher electrophoretic mobility was detected. These products (fractions II-24, Fig. 5) were eluted with water and subjected to paper electrophoresis at pH 1.7.

Paper electrophoresis at pH 1.7 allowed us to separate phosphohydroxyamino acids, i.e. O-phosphoserine (pSer), O-phosphothreonine (pThr) and O-phosphotyrosine (pTyr) from uridylic acid (Fig. 6). In addition to a very low amount of uridylic acid, uridine-3',5'-diphosphate and, possibly, O-phosphotyrosine, a compound containing more than 90% of ^{32}P-radioactivity was re-

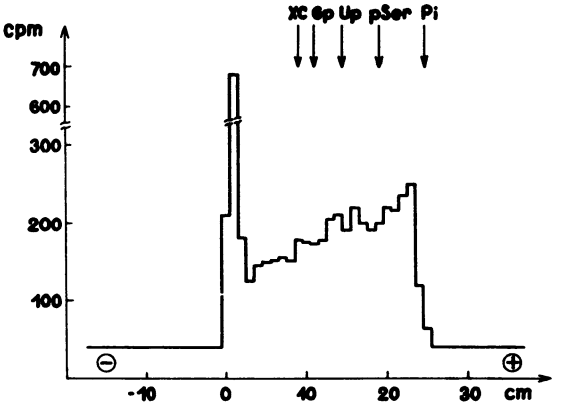

Fig. 5. Paper electrophoresis of VPgA-pUp hydrolysed with 4 M HCl for 6 h at 105°C.

covered with the mobility at pH 1.7 close to that of P_i. However, it became obvious after the electrophoresis of this compound at pH 3.5 that it consisted of two components, P_i and compound "C" (Fig. 7). Treatment of this compound with micrococcal nuclease yielded Up and pTyr in equal amounts, as was shown by paper electrophoresis at pH 1.7 (Fig. 8). Micrococcal nuclease digestion was incomplete, which is probably due to a low rate of hydrolysis of this substrate. In addition, O-phosphotyrosine was identified by two-dimensional thin-layer chromatography on cellulose plates (solvent system B).

The low yield of pTyr is caused by significant hydrolysis of phosphomono- and diester bonds in pTyr derivatives during

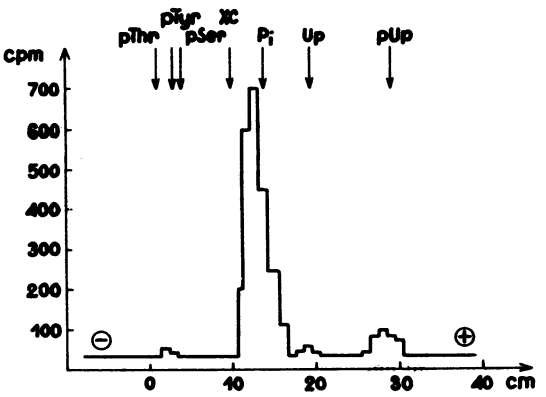

Fig. 6. Paper electrophoresis at pH 1.7 of fractions II-24 (Fig.5).

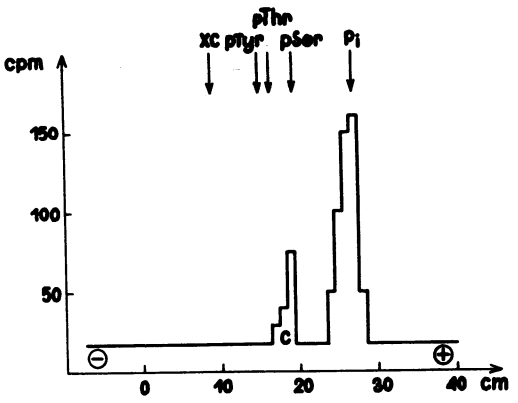

Fig. 7. Paper electrophoresis at pH 3.5 of fractions II-I6 (Fig. 6).

acid treatment and by marked losses of the analyzed material after successive separations.

The susceptibility of the bond between the nucleotide and the amino acid to micrococcal nuclease, as well as generation of Up and O-phosphohydroxyamino acid as the result of such treatment, are in complete agreement with our previous conclusions about the protein nature of VPg and the phosphodiester linkage between VPg and 5'-terminal uridylic acid in RNA /5/.

The above data show that the linking group between VPgA and EMC genomic RNA is Tyr-O-pUp (compound "C" in Figs 7 and 8). Previously, when studying a preparation that contained both VPgA and VPgB, we succeeded in identifying O-phosphotyrosine

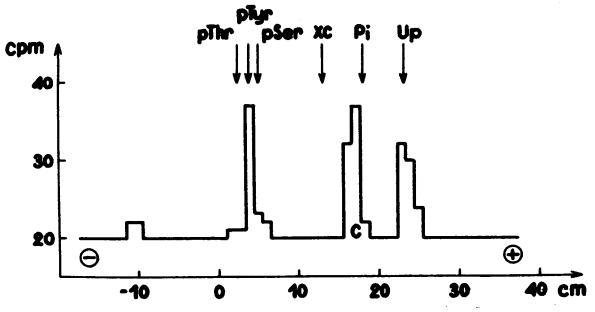

Fig. 8. Paper electrophoresis at pH 1.7 of compound "C" hydrolysed with micrococcal nuclease.

in the HCl-hydrolysate of the nucleotide-peptides as the only O-phosphorylated hydroxyamino acid /22/. This prompts us to the conclusion that the linkage between VPgB and EMC virion RNA has the same structure as VPgA-RNA linkage.

DISCUSSION

The nature of the linkage between VPg proteins and EMC RNA

Two proteins, VPgA and VPgB, are covalently bound to virion RNA of EMC virus. The conclusion about the protein nature of VPgA and VPgB was made on the basis of the following data: the compounds linked with the 5'-end nucleotide of EMC RNA are positively charged at pH 3.5; on being subjected to PAG electrophoresis, they display the mobilities that correspond to those of proteins with molecular weights of 10,000 and 8,000; they are sensitive to pronase treatment, and, finally, VPgA (and probably VPgB) contains a hydroxyamino acid that is covalently bound to the nucleotide. However, labelling of VPg with ^{14}C-amino acids (Val, Leu, Phe, 1 mCi each), and ^{3}H-Tyr (5 mCi) did not give in our hands a sufficient incorporation of the radioactivity in VPg.

The conclusion about the covalent nature of the linkage between RNA and proteins was based on the isolation of nucleotide-peptides from the preparation of virion RNA by enzymatic treatments; on susceptibility of the linkage between the nucleic and protein components to snake venom phosphodiesterase and micrococcal nuclease treatments; on the hydrolytic stability of these compounds in mild alkali and acid media. These results prove that at least VPgA is linked to the EMC virion RNA by phosphodiester bond between the hydroxy group of tyrosine residue and the 5'-phosphate of the terminal uridylic acid.

It is most likely that VPgB is linked with the 5'-end of EMC RNA in the similar way, as the only nucleotide detected in the nucleotide-peptides isolated from the RNA preparation containing both VPg proteins, is uridylic acid, and the only derivative of the amino acid is O-phosphotyrosine /22/. In addition, the nucleotide derivatives of VPgA and VPgB were equal-

ly stable in 0.3 M NaOH (37°C, 16 h) and nucleotide-peptides isolated from them had similar electrophoretic mobilities at pH 3.5.

It is interesting that the VPgA/VPgB ratio varies (0.7 to 2.8), whereas the total amount of protein (VPgA + VPgB) found experimentally remains constant. This result can be explained on the basis of our suggestion that a part of EMC virion RNA molecules contains VPgB protein instead of VPgA on the 5'-end of RNA.

Virus-specific proteins associated with genome of picornaviruses (VPg)

The structure of the covalent linkage of VPg with RNA is established now for two picornaviruses, i.e. for poliovirus (type I) /9,10/ and for EMC virus. In both cases the tyrosine residue of VPg is linked via a hydroxy group with the phosphate group of 5'-terminal uridylic acid of the respective virion RNA. Polio- and EMC-VPg are virus-specific low molecular weight proteins. EMC-VPg has the highest molecular weight among VPg proteins of picornaviruses /4/. Varying the conditions of PAG electrophoresis for VPg-pUp, we found out that two, rather than one VPg, i.e. VPgA and VPgB, are bound to EMC virion RNA. Both VPg's have a single polypeptide chain.

It would be interesting to compare VPgA and VPgB. There are two possibilities: VPgA and VPgB could be related or unrelated proteins. In the former case VPgB could be formed as a result of termination of translation at the termination codon, preceding that of VPgA, or else, as a result of post-translational cleavage. The latter hypothesis seems to be quite realistic, as the processing of EMC proteins is well established /23/. Then there will be two possibilities, i.e. processing of free protein or processing of the protein covalently bound to RNA (or nucleotide).

The function of VPg proteins remains obscure. It seems to us that the idea that VPg plays the role of primer for initiating synthesis of viral RNA /24/, deserves special attention. It can be speculated that both VPg proteins are formed as a result of processing of uridylated viral replicase. The fact that

the structure of covalent VPg-RNA linkage in EMC virus and poliovirus is identical (except that in EMC virus two VPg forms are found) prompts one to the conclusion that picornaviruses have a common mechanism for the formation and hydrolysis of VPg-RNA bond.

ACKNOWLEDGMENTS

We are grateful to Prof. V.I. Agol for critical reading of the manuscript and helpful discussions. We wish to thank Mrs. T. Kheifets for the translation of the manuscript. Our thanks are also due to the department of isotopes for technical assistance.

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