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A convenient sequencing method for 5' protein-linked RNAs

Akio Nomoto and Nobumasa Imura

Department of Public Health, School of Pharmaceutical Sciences, Kitasato University, 9-1, Shirokane 5-chome, Minato-ku, Tokyo 108, Japan

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ABSTRACT

A convenient nucleotide sequencing method for 5'end protein-linked RNAs was developed. Genome of LSc, 2ab poliovirus, which has a protein (VPg) covalently linked to the 5'terminus, was labelled with ^{125}I Bolton and Hunter reagent after proteinase K treatment. No sign of labelling of nucleotide moiety in the genome with the reagent was detected. A labelled oligo peptide-linked ribonuclease T1 fragment was obtained from the 5'end of the genome. Analysis of the complex by two dimensional gel electrophoresis after partial alkali digestion or by the nucleotide sequencing method of Donis-Keller et al. (1) revealed that LSc, 2ab strain genome had an identical 5'end structure to that of Mahoney strain genome, that is, VPg-pUpUpApApApApCpApGp. Our results have shown that this labelling method is useful for analysis of 5'end sequence of RNAs linked to protein at the 5'termini.

INTRODUCTION

In picornavirus infected cells the viral genomic RNA is translated into a large polypeptide that is processed during the translation into the viral specific proteins required for genome replication and for construction of virion particle (2). Considering that the large precursor peptide has a molecular weight of more than 220,000 (2), and the genome has approximately 7,800 nucleotides in its molecule (3), the ribosome binding site on the viral mRNA for the initiation of protein synthesis must locate close to 5'end of the RNA. On the other hand, it is well known that infection by picornavirus results in extensive inhibition of host cell protein synthesis at the initiation step and only virus-specific protein synthesis can be seen by 3-4 hr after infection (4, 5). Furthermore, translation of vesicular stomatitis virus (VSV) mRNA is prevented when

VSV-infected cells are superinfected with poliovirus (6, 7). these selective inhibition of translation was recently elucidated by Rose et al. (8) who reported the specific inactivation of an initiation factor (eIF-4B) by poliovirus infection, which factor was indicated to interact with the 5'cap structure on mRNA (9). The mechanism for ribosome binding to poliovirus mRNA to form initiation complex, however, is still obscure. It is very possible that a nucleotide sequence required for the ribosome binding exists in the region close to 5'end of the genomic RNA.

Among animal viruses, picornavirus and calicivirus appear to have a protein covalently attached to the 5'-termini of their genomes (10-16). Available evidences suggest that the genome-linked protein (VPg) is encoded by the viral genome (13) and that VPg might be involved in RNA replication of poliovirus possibly in the initiation of RNA synthesis (11, 12).

We here show that labelling the peptide on the 5'end of RNA with ^{125}I Bolton and Hunter reagent (17) is useful for sequencing 5'end protein linked RNA.

MATERIALS AND METHODS

Virus and RNA

Spinner cultured HeLa S3 cells ($4x10^6$ cells/ml) were infected with 50-100 plaque-forming units per cell of LSc, 2ab strain of type 1 poliovirus. Seven and a half hours after infection, a cytoplasmic extract was prepared by homogenization with a Dounce Homogenizer. The virus was pelleted from the cytoplasmic extract after addition of NaCl to 0.3M, EDTA to 0.005M, and sarcosyl to 1% (18), and sedimented twice through a 36 ml 15-30% sucrose gradient containing 0.3M NaCl, 0.01M Tris-HCl (pH 7.5), 0.001M EDTA and 1% sarcosyl at 24°C for 2.5 hr at 27,000 rpm in Spinco SW 27 rotor. Fractions containing 150 S virion particles were extracted three times with phenol/chloroform, then 35 S virion RNA was purified as previously described (16).

Proteinase K treatment of the genome

Approximately 200 µg of genomic RNA was treated with 0.2 mg/ml proteinase K (Boehringer Mannheim GmbH) at 37°C for 1 hr in a buffer solution containing 0.1 M NaCl, 0.01 M Tris-HCl (pH 7.5), 0.001 M EDTA, and 0.5% SDS (19). RNA with short oligopeptide at the 5'end was recovered from the mixture by phenol/chloroform extraction.

Labelling the peptide on RNA with ^{125}I Bolton and Hunter reagent

For labelling the peptide on genomic RNA, 35 S RNA was further purified, after the treatment with proteinase K followed by phenol/chloroform extraction, by sedimentation through 15-30% sucrose density gradient containing 0.1 M NaCl, 0.01 M Tris-HCl (pH 7.5), 0.001 M EDTA and 0.5% SDS at 24°C for 4.75 hr at 40,000 rpm in Spinco SW 41 rotor. Virion RNA fractions were then applied to oligo dT cellulose column in a binding buffer containing 0.5 M NaCl, 0.01 M Tris-HCl (pH 7.5), 0.001 M EDTA and 0.1% SDS. The eluate from the column with an elution buffer containing 0.01 M Tris-HCl (pH 7.5), 0.001 M EDTA, and 0.1% SDS was pooled. The RNA was precipitated five times with ethanol in the presence of 0.1 M NaCl at -20°C, and used for labelling.

About 100 µg of RNA was labelled with ^{125}I Bolton and Hunter reagent (NEN) in 25 µl of a buffer solution containing 0.1 M Na-borate (pH 8.5), 0.001 M EDTA, and 0.1% lithium dodecyl sulphate at 4°C for 15 hr as previously described (16, 20). After the reaction, the iodinated peptide-RNA complex was purified by gel filtration with 5 ml of Sephadex G-25 followed by sedimentation through a 15-30% sucrose density gradient as described above.

Ribonuclease digestion or alkali treatment

Digestion of RNA with RNase T1 (Sankyo) or with a mixture of RNase T1 and RNase A (Miles) was carried out as previously described (16). Partial digestion with RNase U2 (Sankyo) or limited alkaline hydrolysis for determinating positions of adenines in RNA was taken place according to the method of

Donis-Keller et al. (1)

For the "wandering spot" analysis (21), an aliquot of 5'end derived RNase T1 fragment was incubated at 37°C in 20 ul 0.1 M NaOH. Five µl, 10 µl, and 5 µl aliquots were withdrawn at incubation times of 10 min, 20 min, and 30 min respectively. The reaction was terminated by adding HCl at a final concentration of 0.2 M. Samples were pooled, incubated at 37°C for 3 min to cleave cyclic phosphate linkages, added with 2 volumes of 9 M urea and 1 drop of glycerol, then applied to two dimensional gel electrophoresis.

Polyacrylamide gel electrophoresis

Laemmli's buffer system (22) was employed for 14 % polyacrlamide gel electrophoresis in the presence of 4 M urea as previously described (16). Two dimensional polyacrylamide gel electrophoresis was carried out according to the method of Lee et al. (3, 16) for the purification of 5'end derived RNase T1 resistant oligonucleotide and for the "wandering spot" analysis of the oligonucleotide. Mapping adenines in RNA was done on 20% polyacrylamide gel with 7 M urea according to the method of Donis-Keller et al. (1).

Column chromatography

^{125}I-labelled peptide-RNA, after digestion with RNase T1 or with a mixture of RNase T1 and RNase A, was analysed by ion exchange column chromatography in the presence of 7 M urea on DEAE-Sephadex A-25 at pH 7.5 (12).

RESULTS

Labelling peptide on virion RNA with ^{125}I

LSc, 2ab strain of type 1 poliovirus has a small basic protein (VPg) covalently attached to the 5'terminus of the genome and VPg-linked nucleotide is pUp, just like its wild type strain (Mahoney strain) (16). Because of the existance of VPg at the 5'termini, it seemed to be impossible to label 5'end of the genome with phosphorous-32 by polynucleotide kinase. Accordingly, we made an effort to labell VPg with ^{125}I in vitro.

Standard chloramine T method was tried and found to be unsuccessful (A. Nomoto, unpublished results), probably because only one tyrosine residue is contained in VPg (23). Recently, it has been shown that VPg can be iodinated to high specific activity in vitro, using N-succinimidyl 3,4-hydroxy, 5-^{125}I-iodo-phenyl-propionate (Bolton and Hunter reagent) (20).

To confirm the incorporation of ^{125}I into VPg, 35 S virion RNA of LSc, 2ab strain was labelled with Bolton and Hunter reagent and digested with a mixture of RNase T1 and RNase A or with RNase T1 alone. The digests were analysed by electrophoresis on 14% polyacrylamide gel in Laemmli's buffer system containing 4 M urea (Fig. 1, a, b,c).

As shown in Fig. 1., only one distinct band appeared on each track of b and c, suggesting that RNA moiety of the genome was not labelled by the reaction with the reagent. The labelled 35 S RNA could not enter the gel and stayed at the origin (Fig. 1. a). VPg-pUp on track b of Fig. 1. has been confirmed to co-migrate with ^{32}P-labelled VPg-pUp on a polyacrylamide gel under the same conditions as those of Fig. 1 (16). The distinct band on track c of Fig. 1., therefore, should be VPg containing RNase T1 resistant oligonucleotide. It was almost impossible, however, to sequence the RNase T1 fragment thus obtained by the method described in the text, because of the fairly large size of VPg and the adhesiveness of VPg (10).

VPg was then digested with proteinase K, prior to labelling with Bolton and Hunter reagent, and the similar experiments were carried out (Fig. 1. d, e, f). It has been suggested that proteinase K leaves a stub still attached to the RNA under the digestion condition used (16). Therefore, the band in the track f of Fig. 1. must be a short peptide containing RNase T1 fragment which migrated faster than VPg-pUp. The digest with a mixture of RNase T1 and RNase A run off the gel under these conditions (Fig. 1. e). The ^{125}I-labelled short peptide containing RNase T1 fragment, indicated as $(aa)_n$-pUp$(Np)_n$Gp in Fig. 1., was investigated further to examine the usefulness of the labelling method for sequencing RNA.

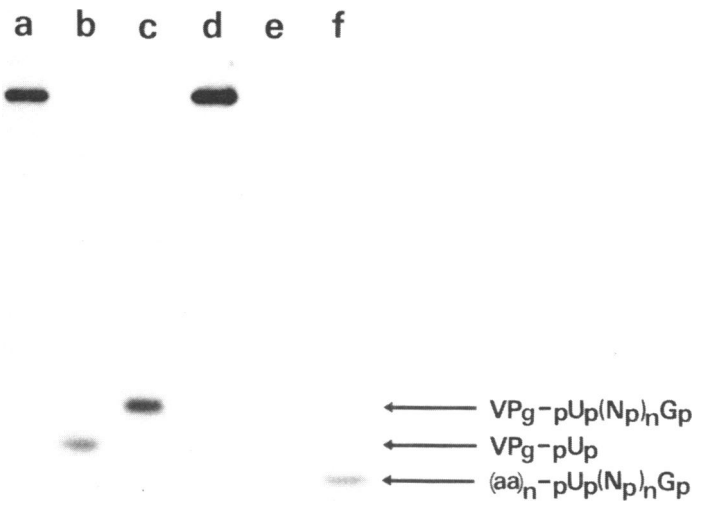

Figure 1. Analysis of RNase digests of ^{125}I-labelled virion RNA by polyacrylamide gel electrophoresis. Virion RNA of poliovirus LSc, 2ab strain was labelled with ^{125}I by Bolton and Hunter reagent before (a, b, and c) or after (d, e, and f) proteinase K digestion under the conditions described in MATERIALS and METHODS. The ^{125}I-labelled virion RNA was then digested with a mixture of RNase T1 and RNase A (b and e) or with RNase T1 alone (c and f). Samples for track a and d were ^{125}I-labelled 35 S RNAs which were not treated with RNase. The digests were applied to 4 M urea containing polyacrylamide gel electrophoresis in Laemmli's buffer system. After electrophoresis at 250 V for 3 hr, the gel was dried on filter paper and radioactive components were visualized by autoradiography.

Characterization and Purification of ^{125}I labelled RNase T1 fragment derived from 5'end of the genome.

To know an approximate length of the RNase T1 fragment, ^{125}I-labelled virion 35 S RNA linked to short peptide at the 5'end was analysed by ion exchange column chromatography with 7 M urea containing DEAE-Sephadex A-25 at pH 7.5 after digestion with RNase T1 or with a mixture of RNase T1 and RNase A (Fig.2). As shown in Fig. 2., ^{125}I-labelled RNase T1 fragment eluted from the column with optical marker oligonucleotides whose

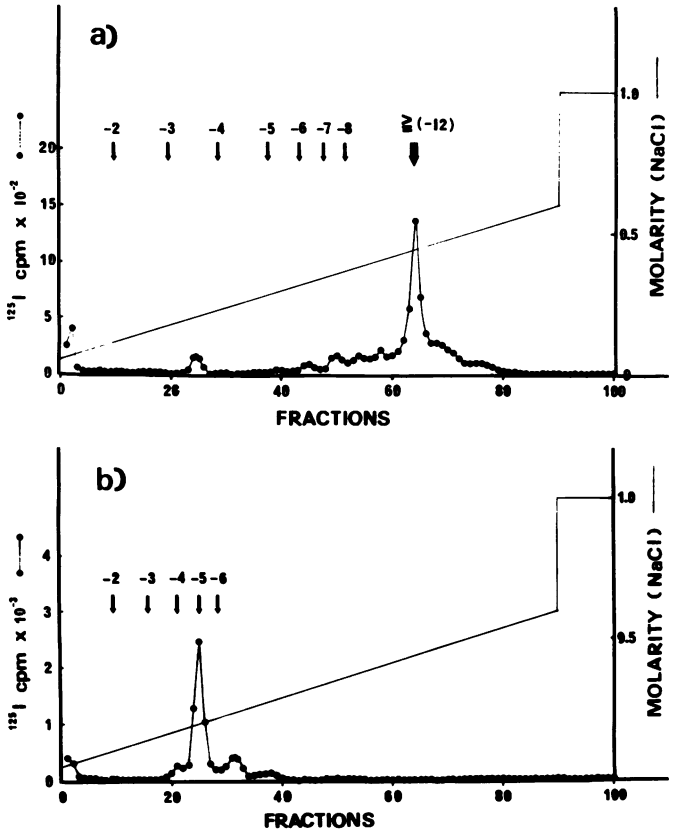

Figure 2. Analysis of ^{125}I-labelled compound in virion RNA after treatment with RNases by ion exchange column chromatography. Virion RNA, after proteinase K digestion , was labelled with ^{125}I by Bolton and Hunter reagent described as in legend of Fig. 1. d, e, f. a) The labelled RNA was digested with RNase T1 and analysed ion exchange column chromatography with DEAE-Sephadex A-25 in the presence of 7 M urea. Before application, the samples were mixed with RNase T1 digests of cold yeast RNAs as optical markers. The elution positions of the marker oligonucleotides were indicated by arrows. b) The ^{125}I-labelled virion RNA was digested with a mixture of RNase T1 and RNase A, and analysed as described in a).

minus charge seemed to be more than 12, and $(aa)_n$-pUp eluted with optical markers of tetranucleotides, suggesting that the RNase T1 fragment contains 8 nucleotides or more. In order to remove some impurities observed in Fig. 1. f. and Fig. 2. a.,

the digests of labelled virion RNA with RNase T1 were applied to two dimensional gel electrophoresis (Fig.3). Two spots (indicated by A and B in Fig. 3) were observed after visualization by autoradiography. There are three possible explanations for this phenomenon as follows: 1) A is the true RNase T1 fragment and B is derived from A by losing phosphate from the 3'terminal guanine residue. 2) A and B are the same peptide-RNA complex but the reaction with Bolton and Hunter reagent is not sufficient, resulting that A has more negative net charge than B. Based on the reaction mechanism of the reagent (17), it can be easily supposed that net charge of labelled peptide-RNA complex depends how many the reagent react with amino residues in peptide. 3) Digestion of VPg on RNA with proteinase K yields two peptide-RNA complex refering to amino acid content.

The possibility 1) was not the case, because "wandering spot" analysis of A showed a virtually identical pattern to that of B(data not shown, see Fig.4), which clearly indicated that both RNase T1 fragments extracted from spot A and spot B have an identical nucleotide moieties. To exclude the possibility 2), the ratio of RNA to Bolton and Hunter reagent was lowered to

Fig. 3. Analysis and purification of ^{125}I-labelled RNase T1 resistant oligonucleotide by two dimensional gel electrophoresis.
The sample was prepared as described in legends of Fig. 1. f. and Fig. 2. a. The position of bromphenol blue which co-migrated with the sample was indicated by dotted circle.

one-fifth of standard ratio described in the text and the labelled products were analysed by a similar procedure to that of Fig. 3. Autoradiogram thus obtained was exactly like Fig. 3. (data not shown), suggesting that the reaction with the reagent was sufficient. Finally, the condition of digestion reaction with proteinase K was investigated to test the possibility 3). When much more exzyme and longer reaction time were employed for the reaction, spot A was denser than spot B on autoradiogram (data not shown). These results indicated that RNase T1 fragment in spot A has a shorter peptide than that in spot B because of different extent of digestion with proteinase K.

Sequence of peptide linked RNase T1 fragment from 5'end of LSc, 2ab genome.

RNase T1 fragment, purified by two dimensional gel electrophoresis shown in Fig.3 , was treated with alkali and the partially digested products were analysed by two dimensional gel electrophoresis. Autoradiogram of the "wandering spot" analysis of spot A was shown in Fig. 4. As discussed previously, oligonucleotides were separated mainly by their charges at pH 3.3 on the first gel, and chain length on the second gel. Based on the principle of the gel electrophoresis (16), a shift of the spot on the gel caused by an additional nucleotide is settled by the charge of the last nucleotide added to the 3'end on the first gel. Partial alkaline hydrolysis yielded two forms of each oligonucleotide; one with a cyclic phosphodiester and the other with a phosphomonoester terminus. Amount of each oligonucleotide with a cyclic terminal phosphate, indicated by dotted circle in Fig. 4., was greatly reduced by acid treatment after partial alkaline hydrolysis. The sequence could not be read off only by the "wandering spot" analysis because the A shift was not clearly distinguished from the C shift. When the wandering spot analysis was carried out after alkaline phosphatase treatment of the fragment, the G shift observed in the ninth spot disappeared and the spot indicated by arrow in Fig. 4. was the only one ninth spot (data not shown), suggesting that the spot must be derived from the RNase T1 fragment by losing terminal phosphate, probably by contaminated phospho-

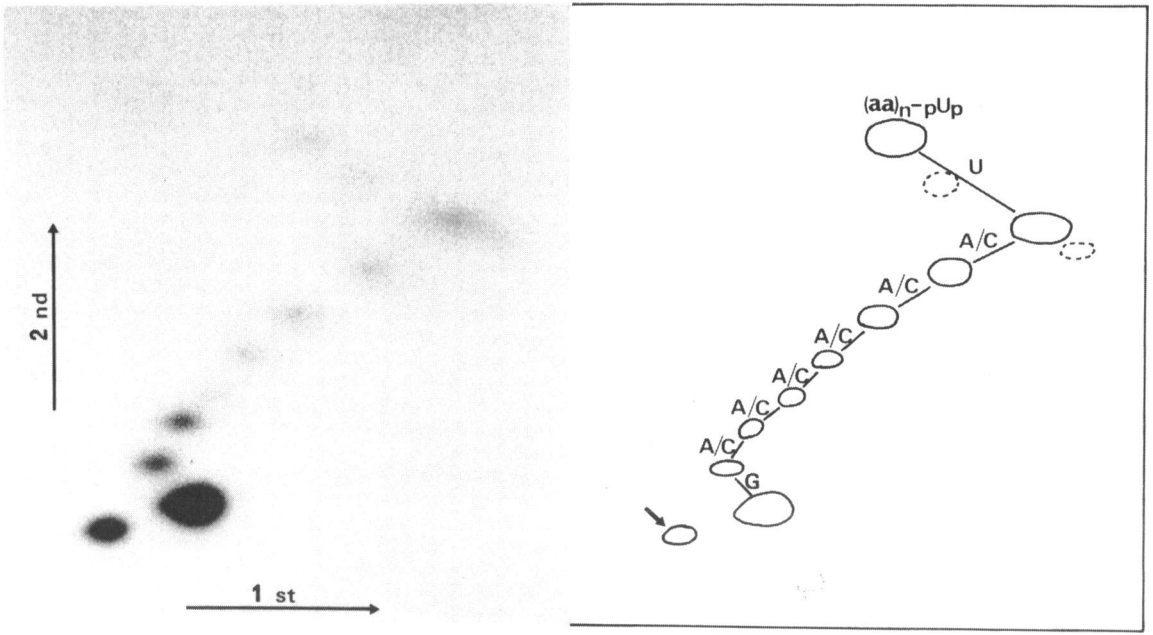

Figure 4. "Wandering spot" analysis of ^{125}I-labelled RNase T1 fragment by two dimensional gel electrophoresis. ^{125}I-labelled RNase T1 fragment, extracted from the area containing spot A on the gel shown in Fig. 3., was partially hydrolysed with alkaline described in MATERIALS AND METHODS and analysed by two dimensional gel electrophoresis. Oligonucleotides with cyclic terminal phosphates were indicated by dotted circles. The spot indicated by an arrow was ^{125}I-labelled RNase T1 fragment without 3'-terminal phosphate on it as mentioned in the text.

monoesterase like activity. A similar spot was also observed in Fig. 5. (see legend to Fig. 5).

To distinguish between A and C residues, the RNase T1 fragment was further analysed by nucleotide sequencing method developed by Donis-Keller et al. As shown in Fig. 5., adenine residues in the RNase T1 fragment were able to be mapped. We thus showed that the genome of poliovirus LSc, 2ab strain had the same 5'end structure as that of Mahoney strain, that is,

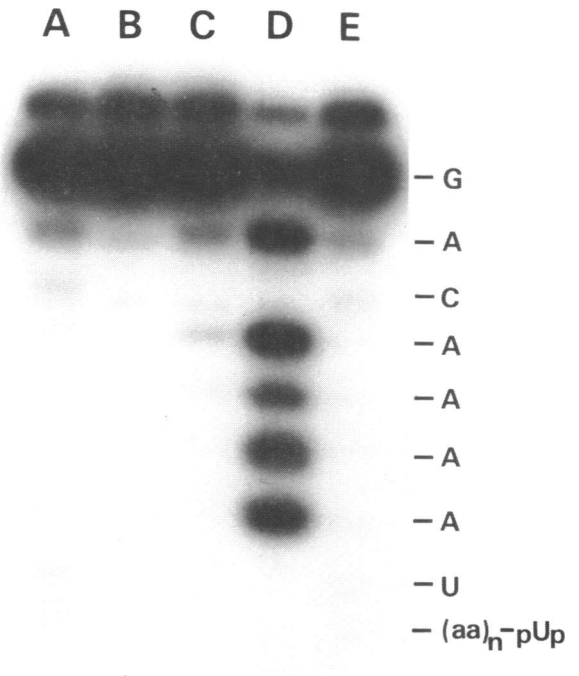

Figure 5. Mapping positions of adenine residues in the RNase T1 fragment. RNase U2 (C,D) or alkali A,E) limiting treatments were carried out according to the RNA sequencing method developed by Donis-Keller et al.. Undigested RNase T1 fragment was applied on track B. RNase U2/RNA ratio used in the experiments was 4×10^{-2} (C) or 4×10^{-1} (D). The reaction time with alkaline was 15 min (E) or 30 min (A). The bands at the top of the autoradiogram were found to be ^{125}I-labelled RNase T1 fragment which lost 3'-terminal phosphate, since alkaline phosphatase treatment of control sample shown in track B resulted in one band which co-migraged with the top bands of Fig. 5. (data not shown).

VPg-pUpUpApApApApCpApGp.

DISCUSSION

The 5'end labelling method to determine the sequence of picornavirus virion RNA, so far, was not successful since the covalently linked protein interfered polynucleotide kinase mediated incorporation of ^{32}P into the 5'end of the RNA. This problem is now solved by the method of labelling 5'end peptide with Bolton and Hunter reagent, which had been predicted by Harris et al. (20).

Selectively labelling the peptide on 5'end of RNA with Bolton and Hunter reagent, after proteinase K treatment of VPg-RNA, made it possible to determine the nucleotide sequence at the 5'end of poliovirus LSc, 2ab strain genome. Preliminary experiments show that this labelling method is also useful for determination of RNA sequence of 40-50 nucleotides from the 5'end when the condition for proteinase K digestion was selected (A. Nomoto, unpublished results). These results suggest the possibilities that 5'end RNA sequences of many protein linked RNAs are to be determined, that is, picornavirus (10-14, 16), calicivirus (15), and cowpea mosaic virus (24) RNAs. A similar RNA sequencing method has been developed by M. J. Hewlett who applied the method to several enterovirus genomes (M. J. Hewlett, personal communication).

Which amino acids of VPg left in peptide on 5'end of the RNA, after proteinase K digestion, was not known. It is interesting, however, that labelled $(aa)_n$-pUp obtained by our standard condition for proteinase K digestion eluted from the column with marker oligonucleotides of tetranucleotides which have a negative charge of -5 at pH 7.5, which indicated labelled peptide moiety has a net charge of -2, suggesting that the peptide moiety contains at least one acidic amino acid.

The linkage between VPg and RNA, in the case of poliovirus Mahoney strain, was determined as O^4-(5-uridylyl)tyrosine (23) which was very stable with alkali or acid treatment. The linkage of LSc, 2ab strain genome was thoroughly resistant to alkali or acid treatment under the conditions used in this study, suggesting the possibility that both strains of poliovirus

have the same linkage between VPg and RNA.

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