Polyadenylic Acid at the 3′-Terminus of Poliovirus RNA*

(chemical labeling/enzymatic fragmentation/Millipore-filter binding/column chromatography/gel electrophoresis)

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ABSTRACT Poliovirus RNA that has been derivatized at the 3′-end with NaIO₃-NaBH₄ yields, after hydrolysis with alkali or RNase T₂, predominantly labeled residues of modified adenosine; no labeled nucleoside derivative is produced by digestion with RNase A or RNase T₁. The 3′-terminal bases of the RNA are, therefore, ...ApA₀₃. Hydrolyzates of poliovirus [³²P]RNA, after exhaustive digestion with RNase T₁ or RNase A, contain, besides internal oligonucleotides, polynucleotides resistant to further action of ribonucleases T₁ and A, respectively; these polynucleotides were isolated by membrane-filter binding or ion-exchange chromatography. The sequence of the T₁-resistant polynucleotide was determined to be (Ap)₄A₀₃, that of the RNase A-resistant polynucleotide was GpGp(Ap)₄A₀₃. The chain length (α) of the polyadenylic acid, as analyzed by different methods, averages 89 nucleotides. Gel electrophoresis revealed heterogeneity of the size of poly(A). Poliovirus RNA, when labeled in vitro at the 3′-end, contains 3′-Hpoly(A); when labeled in vivo with [³²P]H₂PO₄, it contains [³²P]H(Ap)₄A₀₃. The data establish that ...YpGpGp(Ap)₄A₀₃ is the 3′-terminal sequence of poliovirus RNA, Type 1 (Mahoney). Since this mammalian virus reproduces in the cell cytoplasm, these observations may modify prior interpretations of the function of polyadenylate ends on messenger RNAs.

Terminal nucleotide sequences of mammalian messenger RNAs have become of particular interest recently due to the finding that untranslated polyadenyl acid linked to the messenger might play a role in the processing of nuclear precurso RNA (1-5). During infection of suitable cells, the single-stranded RNA of poliovirus serves as template in viral RNA replication and also as mRNA for virus-specific protein synthesis (6). Thus, poliovirus RNA is a convenient source of homogeneous mRNA for sequence studies with respect to structure-function relationships in mammalian protein synthesis. Since poliovirus multiplies in the cytoplasm with no nuclear functions of the mammalian host cell involved, (6), structural differences found between the viral RNA and host-cell mRNA might also elucidate mechanisms relevant to their different pathways of biosynthesis: nuclear transcription and cytoplasmic replication.

We have been studying the 3′-terminal nucleotide sequence of poliovirus RNA to try to identify the terminal oligonucleotide produced by RNase T₁ digestion by chemical labeling methods (7, 8) or by specific isolation procedures (9). These methods have successfully been used in sequence studies of bacteriophage RNA (9, 10) and of plant virus RNA (8); however, when applied to poliovirus RNA, they did not yield a terminal oligonucleotide of a chain length commonly found in viral RNA (3-15 nucleotides long). As will be shown below, end-group analysis identified the 3′-terminal bases of poliovirus RNA as ...A₀₃, which sequence differs from the ...CCC₀₃ or the ...CCC₀₃ termini found in other single-stranded virus RNAs. Armstrong et al. reported recently that poly(A) is part of the ribonucleic acids of polio and EEE virus (11). In this communication, we present evidence that ...YpGpGp(Ap)₄A₀₃ is the 3′-terminus of poliovirus RNA, the indicated number of adenylic acid residues being the average of a population of shorter and longer poly(A) termini. No poly(A)-containing sequences other than the 3′-terminal have been detected in the ribonucleic acid of poliovirus, Type 1 (Mahoney).

MATERIALS AND METHODS

NaBH₄ (2-12 Ci/mmol) was purchased from Amersham/Searle; snake venom diesterase and pancreatic ribonuclease (RNase A) were obtained from Sigma Chemical Co. and Worthington Biochemical; RNase T₁, RNase T₂, and yeast tRNA from Calbiochem.; [³²P]adenosine (20 Ci/mmol) from Schwarz/Mann; carrier-free [³²P]H₂PO₄ from New England Nuclear; polyadenylic acid from Miles Laboratories; pre-coated thin-layer chromatography plates (Silica Gel F-254) from Brinkmann Instruments, Inc., and Membrane filters (Millipore, HAWG 02500, HA 0.45 μm) from Millipore Corp.

Purification of Poliovirus and Poliovirus RNA. Suspension cultures of HeLa cells grown in minimal essential medium (Joklik modified, Grand Island Biological Co., Grand Island, N.Y.) were infected with 30-50 plaque-forming units of poliovirus, Type 1 (Mahoney) per cell; the virus was isolated 7 hr after infection (12). The preparation of poliovirus labeled with [³²P]adenosine was similar, except that the culture medium contained 5 μg/ml of actinomycin D and 50 μCi/ml of [³²P]adenosine. Poliovirus was purified twice in sucrose gradients containing sodium dodecyl sulfate; in some experiments, the virus was purified further in CsCl gradients. [³²P]-Labeled poliovirus was isolated from spinner cultures of HeLa cells grown in a phosphate-free medium containing

Abbreviations: Nucleic acids and their constituents are abbreviated as recommended by the IUPAC-IUB Commission [J. Mol. Biol. 55, 299 (1971)]; N′, hydroxy methyl diethylen glycol derivative of a nucleoside; [RNase A]poly(N), RNase A-resistant polynucleotide derived from poliovirus RNA; [T₁]poly(N), RNase T₁-resistant polynucleotide derived from poliovirus RNA.

This is paper II in the series, "Sequence Studies of Poliovirus RNA." An abstract of this work appeared in Bacteriol. Proc. 236 (1972).
Polyacrylamide Gel Electrophoresis. Poly(A)-containing fragments were analyzed by electrophoresis in 10% polyacrylamide-0.25% ethylene diacrylate gels (15, 16) in phosphate buffer containing 2.2 M formaldehyde (17) or in Tris-HCl–phosphate–dodecyl sulfate (13). Commercial yeast tRNA and formaldehyde-denatured yeast tRNA [heated at 63° for 15 min in 2.2 M formaldehyde–0.1 M phosphate (pH 7.5) (17)] were used as markers. The gels were sliced (13) or scanned at 280 nm in a Gilford model 2410-S linear transport attached to a Gilford model 2400-S Spectrophotometer. At this wave length, the background was considerably lower than at 260 nm.

Analytical Procedures and Radioactive Counting. Column chromatography, high-voltage paper electrophoresis, methods for counting radioactive samples, and procedures for autoradiography were described (8, 13). Thin-layer chromatograms were developed in the first dimension with either isopropanol–concentrated ammonia–water 7:1:2; Solvent I, or n-butyl alcohol–n-propyl alcohol–concentrated ammonia–water 65:5:10:20; Solvent II, and in the second dimension with water saturated n-butyl alcohol (Solvent III) (8).

RESULTS

Identification of the 3' Terminal Bases. Of the methods described in the literature to identify the 3'-terminal base of high molecular weight RNA, the most sensitive is that of Rajbhandary (7). The 3'-terminal nucleoside (N) is converted into a tritium-labeled hydroxymethyl diethyleneglycol derivative (N') by oxidation with periodate, followed by reduction with NaBH₄. We have labeled poliovirus RNA with periodate–boroxydride, degraded the derivative (in the presence of carrier tRNA) with KOH, RNase A, RNase T₁, or RNase T₂, and separated the products by two-dimensional thin-layer chromatography in the presence of appropriate markers (8).

As can be seen from Table 1, the majority of tritium label incorporated into the end-group specific derivative (N') after alkaline hydrolysis or digestion with RNase T₂ was associated with A'. The results were identical when two different solvents were used for the two-dimensional thin-layer chromatography; thus, adenosine is the 3'-terminal nucleoside of poliovirus RNA.

If the penultimate base at the 3'-end were a pyrimidine residue, hydrolysis with RNase A should also give rise to labeled A', since RNase A cleaves the phosphodiester linkage between a 3'-terminal adenyl hydroxymethyl diethyleneglycol glycol and a pyrimidine nucleoside (19). No counts travel with any of the N' marker compounds after exhaustive RNase A digestion (Table 1). The same result was obtained with RNase T₁. Thus, neither a pyrimidine nucleoside nor guanosine can be the second nucleoside from the 3'-end. The 3'-terminal sequence of poliovirus RNA is, therefore, ... ApAOh...

The overall yields obtained with poliovirus RNA by this technique are low. Decreased yields have been observed as the size of the derivatized RNA increases (see ref 20 and literature cited therein). Theoretically, two equivalents of trithion should be incorporated per terminal nucleoside; calculated on this
basis, our yields of A' were never higher than 27.5%. The possibility that periodate oxidation was blocked by a phosphate ester on the 3'-terminal hydroxyl group in a fraction of the viral RNA can be excluded (see below). Low yields for derivatization of high molecular weight RNA have been reported (19-23), but high yields have also been observed (24, 25). There is no obvious reason for the differences in yield, since the labeling conditions in all these studies are very similar.

Isolation of Poly(A)-like Polynucleotides. Poliovirus [32P]-RNA, when exhaustively digested with RNase A or with RNase T1, yields polynucleotides, designated as [RNase A]poly(N) or [T1]poly(N), respectively, that are resistant to further action of the same enzymes. Both of these polynucleotides can be separated from other digestion products by Millipore filter binding (2). However, small amounts of oligonucleotides (3-8%) remain as contaminants in the polynucleotides, even after two cycles of Millipore binding and extraction. The contaminants can be removed by DEAE-Sephadex chromatography. As can be seen from Fig. 1, [RNase A]poly(N) and [T1]poly(N) elute at the same position, relative to marker tRNA, indicating that the chain length of the fragments is similar. The steep gradient of NaCl concentration used for elution, however, does not allow one to decide whether the material in each peak is necessarily homogeneous.

To assure that the polynucleotides were originally covalently linked to the viral genome, and not simply contaminants arising from aggregation, we incubated [32P]RNA with excess unlabeled poly(A) (11) in 85% dimethyl sulfoxide at 37∞C; under these conditions, double-stranded structures are denatured (26). Intact poliovirus RNA, with a sedimentation constant of 35 S, was recovered by sedimentation through sucrose gradients. RNase T1 digestion of this RNA yielded the same amount of [T1]poly(N) as native viral RNA.

Nucleotide Sequences of the Poly(A)-like Polynucleotides. Since [RNase A]poly(N) and [T1]poly(N) bind to Millipore filter at high salt concentrations (2), and are resistant to RNase A and RNase T1, respectively, their main component is probably adenylic acid. Poly(A)-like polynucleotides, labeled with 32P, were isolated by Millipore filter binding and treated with alkali; the hydrolysates were analyzed by paper electrophoresis. More than 90% of the counts were associated with Ap. Various amounts of labeled Gp, Up, and Cp, however, were also observed, due to contaminants (see above). For sequence studies, the adenine-rich fragments were further purified on DEAE-Sephadex columns, and digested with specific enzymes; the products were analyzed by paper electrophoresis. The results (Table 2) can be summarized as follows: (i) [T1]Poly(N): digestion with RNase T2 yields only labeled Ap; digestion with snake venom diesterase yields only Pa; treatment with RNase A leaves [T1]poly(N) unchanged. Thus, [T1]poly(N) is (Ap)ApAp. (ii) [RNase A]-poly(N): digestion with RNase T2 yields labeled Gp and Ap, in a molar ratio of 1:44; digestion with RNase T1 yields poly(A) plus the same amount of Gp as obtained with RNase T2. Hydrolysis with snake venom diesterase yields Pa and Gp, the amount of Gp being half of that found after endonucleolytic digestion with RNase T1 or RNase T2. Thus [RNase A]-poly(N) is GpGp(Ap)Ap. The chain length (n) of the poly(A) sequence is 89 ± 6, as calculated from the base ratio obtained from analyses of the fragments with different enzymes (Table 2). As will be shown below, n indicates only an average chain length of the fragments.

The fact that [T1]poly(N) and [RNase A]poly(N) do not contain Gp and Yp, respectively, suggests that these fragments are derived from the 3'-terminus of poliovirus RNA. This conclusion was verified by the following experiments:

Table 1. Analysis of hydrolysates of [32P]-RNA from poliovirus

<table>
<thead>
<tr>
<th>Marker compounds</th>
<th>A'</th>
<th>C'</th>
<th>U'</th>
<th>G'</th>
</tr>
</thead>
<tbody>
<tr>
<td>[32P]-RNA hydrolyzed with:</td>
<td>%†</td>
<td>%†</td>
<td>%‡</td>
<td>%‡</td>
</tr>
<tr>
<td>KOH*</td>
<td>71 ± 6.5</td>
<td>21 ± 6.0</td>
<td>15 ± 5.6</td>
<td>4 ± 3.4</td>
</tr>
<tr>
<td>RNase T2†</td>
<td>74 ± 7.0</td>
<td>14 ± 1.7</td>
<td>7 ± 6.9</td>
<td>1.1 ± 1.5</td>
</tr>
<tr>
<td>RNase A*</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>RNase T1†</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

* Average of 4 independent determinations.
† Average of 2 independent determinations.
‡ Percentage of theoretical yield. 
§ Percentage of theoretical yield if N' were the 3'-terminal base. The calculation was based on a molecular weight of poliovirus RNA of 2.8 × 106 (18), and upon the assumption that each terminal derivative carries 2 tritium atoms.
100 pmol of poliovirus RNA were labeled at the 3'-end as described above, and then combined with 1.6 × 10^6 cpm of poliovirus [32P]RNA and digested with RNase T1. The poly(A)-like polynucleotide was purified by two membrane binding steps, and analyzed on a DEAE-Sephadex column in 7 M urea (Fig. 2). [T1]poly(N) was labeled with tritium, although the background in the column fractions is high due to exchangeable radioactivity. The adenine-rich fragment, recovered from the column fractions, was analyzed for the 3'-terminal base by alkaline hydrolysis and subsequent thin-layer chromatography. In two independent experiments, 98.5 ± 0.7% of the counts coincided with A', the rest of the counts (1.1%) were found with G'. We have also labeled poliovirus RNA with [3H]adenosine in vivo. The tritium-labeled [T1]poly(N) obtained from this RNA yielded, upon digestion with alkali, [3H]Ap and [3H]A. The mole ratio of the nucleotide (internal) to the nucleoside (3'-terminal) gave a chain length for the fragment comparable to that described above (unpublished results).

The results presented here establish that YpGpGp(Ap)¼ACH is the 3'-terminal sequence of poliovirus RNA.

The Content of Poly(A) in Poliovirus RNA. Poliovirus RNA consists of 7500 nucleotides (18), 1.19% of which would be represented as 3'-terminal polyadenylic acid if the homopolynucleotide were indeed 99 nucleotides long. In order to verify the average chain length of the poly(A), we measured the radioactivity associated with the terminal fragment after enzymatic digestion of [32P]RNA. The Millipore-binding technique was not adequate for these studies, because the binding of poly(A) from a hydrolysate of [32P]RNA is not quantitative (60–80%). Furthermore, the bound homopolynucleotide is contaminated with internal fragments; thus, synthetic [3H]poly(A) could not be used as an internal standard. Hydrolysates of [32P]RNA from digests with RNase A–RNase T1 were, therefore, applied directly to a DEAE-Sephadex column, which was equilibrated with 0.3 M NaCl–5 mM Tris–HCl (pH 7.5)–7 M urea. The largest internal isothiop produced by digestion of poliovirus RNA with RNase A–RNase T1 is seven nucleotides long (Yogo and Wimmer, unpublished results). Thus, at 0.3 M NaCl the internal oligonucleotides should not bind to the column and can be removed by extensive washing with buffer. The 3'-terminal poly(A) was eluted from DEAE-Sephadex with a steep gradient of NaCl, and its identity was confirmed by analysis of its alkaline hydrolysate. The amount of poly(A) contained in about 2 × 10^6 cpm of poliovirus RNA (3 experiments) was 1.14 ± 0.02%. This value is in agreement with the content of poly(A) in poliovirus RNA as calculated from sequence analyses, suggesting that each RNA molecule may be terminated with polyadenylic acid. This is supported by the finding that even in the presence of a 4-fold excess of tRNA, all intact viral RNA is retained on a Millipore filter after filtration in 0.5 M KCl (G. Brawerman, personal communication, and our own results); however, in the presence of a large excess of tRNA (that is, under the conditions used for the binding of the terminal fragments) only 8% of the viral RNA binds to the filter.

The structure of the adenine-rich fragments and the content of poly(A) indicate that poliovirus RNA does not contain internal polyadenylic acid sequences.

Gel Electrophoresis of the Terminal Poly(A). Electrophoresis of the 3'-terminal fragment of poliovirus RNA in polyacrylamide gels did not yield a single peak of poly(A), as has been described for poly(A)-rich fragments found in cellular RNA (3) or in adenovirus-specific RNA (8). The [RNase A]poly(N) fragment of poliovirus RNA is distributed (Fig. 3) over a wide region of the gel, indicating different chain lengths of the poly(A) termini. Yeast tRNA and tRNA fully denatured with formaldehyde (17) were electrophoresed on parallel gels, as marker compounds, in the presence of 2.2 M formaldehyde. Under these conditions, more than half of the labeled [RNase A]poly(N) migrates slower than formaldehyde-treated tRNA; the pattern was nearly identical when [T1]poly(N) was analyzed. The wide distribution of terminal fragments through the gels was reproducibly observed when purified [RNase A]poly(N) or [T1]poly(N) were electrophoresed, or when the RNase A–RNase T1 digest of [32P]RNA was applied directly to the gels. Furthermore, reduction of the amount of RNase A and RNase T1 used for the standard digests, by 80%, or enzymatic hydrolysis in 0.3 M KCl—at which salt concentration poly(A) is protected from endonucleolytic cleavage (27)—did not produce a single peak of poly(A) after gel electrophoresis. Thus, we conclude that the 3'-
terminal poly(A) in poliovirus RNA is heterogeneous in chain length.

DISCUSSION

Adenine-rich polyribonucleotides that vary in length from 40 to 200 nucleotides occur in the cytoplasm of rat liver cells (28), in the nucleus of Ehrlich ascites cells (29), or in the virion or reovirus (30). Polyadenylic acid is part of the nucleotide sequence of vaccinia virus-specific mRNA (31), HeLa polyribosomal RNA (3, 31), rabbit reticuloocyte mRNA (32), ascites cell polyribosomal RNA (2), and adenovirus-specific RNA (5). The biological function of these homopolyribonucleotides remains obscure, although evidence has been presented that may link the A-rich segments with the biosynthesis of mRNA and its transport to the cytoplasm (4). Polyadenylic acid, however, must have also functions other than the regulation of synthesis of nuclear RNAs, since it is located at the 3'-terminus of cellular polyribosomal RNAs (33), as well as of RNAs that are synthesized in the cytoplasm (see ref. 30, and the results presented in this paper). Thus, polyadenylic acid might play a role in the translation of mammalian mRNA; or it could be involved in the release mechanisms of nascent strands from a replication complex in viral RNA synthesis.

We are currently trying to analyze the complementary (negative) strands for polyuridylic acid. Failure to find poly(U) in the negative strands would indicate post-transcriptional addition of poly(A) to the viral RNA, a mechanism that could explain the heterogeneity of the termini in size.

Untranslated nucleotide sequences at the 3'-end of the viral genome have been identified in coliphage Qβ RNA (34) and in coliphage R17 RNA (35). These termini are thought to play a role in the highly specific recognition process between coliphage RNA and its replicase (34, 35). It is not known whether the homopolyribonucleotide at the 3'-end of poliovirus RNA is translated into polylysine. However, the sequence of polyadenylic acid described here can hardly be part of a specific replicase recognition site, since other cytoplasmic RNAs of the host cell are also terminated with poly(A). Thus, nucleotide sequences preceding the poly(A) and/or internal portions of poliovirus RNA must function as recognition signals. Alternatively, specificity of the viral replicase for the homologous viral genome may not exist in mammals. In this case, to prevent template activity of cytoplasmic RNAs in viral replication, the site of viral RNA synthesis would have to be separated from cellular components. In poliovirus replication, the segregation of the RNA synthesizing machine might be realized in the formation of a "replicative complex" (6).

Finally, we would like to emphasize the basic difference between the 3'-terminal structure of single-stranded RNAs in plant viruses and coliphages on the one hand, and in mammalian viruses on the other hand. All plant virus and coliphage RNAs that have been analyzed are terminated with an identical tetrancleotide ... CCCC at (20, 10), or, in a single case, with ... CCC at (8, 36). The function of the common terminus, which is preceded by a virus-specific untranslated sequence of nucleotides, is obscure. It is known only that the terminal adenylate in bacteriophage R17 and Qβ is not necessary for the infectivity of the viral RNA (24, 25). Such a ... CCCA tetrancleotide has not been detected at the 3'-end of mammalian virus RNA. In fact, RNA from viruses with multipartite single-stranded RNA genomes carry a uridine residue at the 3'-end (37–39), while poliovirus RNA, as we report here, is terminated with poly(A).

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<table>
<thead>
<tr>
<th>Digestion with</th>
<th>Nucleotides produced</th>
<th>% of total radioactivity in [RNase A]-poly(N)</th>
<th>[T1]-poly(N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNase T2</td>
<td>Ap</td>
<td>97.5 ± 0.1 (3)*</td>
<td>99.7 ± 0.1 (2)*</td>
</tr>
<tr>
<td></td>
<td>Gp</td>
<td>2.3 ± 0.2</td>
<td>0.1 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>Up</td>
<td>0.1 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>Cp</td>
<td>0.2 ± 0.1</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>Snake venom</td>
<td>pA</td>
<td>98.7 ± 0.1</td>
<td>99.7 ± 0.1</td>
</tr>
<tr>
<td>diesterase (2)*</td>
<td>pG</td>
<td>1.2 ± 0.2</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>pU</td>
<td>0.1 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>pC</td>
<td>0.1 ± 0.0</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>RNase T1 for</td>
<td>Ap</td>
<td>0.1 ± 0.0</td>
<td>0.1 ± 0.0</td>
</tr>
<tr>
<td>[RNase A]-poly(N) (3)*</td>
<td>Up</td>
<td>0.1 ± 0.1</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>RNase A for</td>
<td>Cp</td>
<td>0.0 ± 0.1</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>[T1]-poly(N) (1)*</td>
<td>Poly(A)</td>
<td>97.4 ± 0.4</td>
<td>99.9 ± 0.0</td>
</tr>
</tbody>
</table>

* n, the number of experiments, is given in parentheses. Errors are standard deviations.