Poliovirus Snapback Double-Stranded RNA Isolated from Infected HeLa Cells Is Deficient in Poly(A)

OLIVER C. RICHARDS,^{1,2*} TIM D. HEY,^{2†} AND ELLIE EHRENFELD^{1,2}

Departments of Biochemistry¹ and Cellular, Viral, and Molecular Biology,² University of Utah School of Medicine, Salt Lake City, Utah 84132

Received 20 January 1987/Accepted 6 April 1987

A portion of poliovirus double-stranded RNA (25 to 50%) isolated from infected HeLa cells contains hairpin loops at one end of the duplex structure. These structures rapidly reformed double-stranded molecules after denaturation and appeared as molecules of up to two times genome length upon electrophoresis in denaturing agarose gels. A second form of poliovirus double-stranded RNA was readily denaturable into genome length strands. When the hairpin RNA was treated with S1 nuclease, subsequent denaturation resulted in formation of strands of up to genome length. Hairpin molecules contained very little, if any, poly(A) sequences, suggesting that the hairpin forms after nucleolytic removal of the 3' end of plus-strand templates. We conclude that the hairpin double-stranded RNA found in infected cells is likely generated by intracellular nicking and self-priming and that it does not represent an intermediate in the process of RNA replication.

The biochemical mechanism of poliovirus RNA replication has not been defined. Although it is generally agreed that the RNA chain elongation activity is contained in the virally encoded polypeptide 3D, the mechanism by which synthesis of new chains is initiated remains unknown and under some controversy. Equally unclear and likely related to the RNA chain initiation reaction is the process by which the small viral protein, VPg, becomes covalently linked to the 5' ends of viral RNA molecules.

Attempts during the last decade to develop RNA replication systems *in vitro* have led to the isolation of several host factor preparations that are currently under study in different laboratories as potential components of the RNA chain initiation mechanism (3, 6, 8-10, 14, 21, 22). An activity that uridylyates VPg to form VPgpUpU has been detected in a crude, membranous fraction that has RNA-synthetic activity *in vitro* (19). At least some VPgpUpU molecules can be elongated to longer polynucleotides (18), and these findings have supported the suggestion that the uridylyated protein serves directly as a primer for synthesis of new chains. This model is consistent with earlier results that identified a host protein that supported synthesis of full-length complementary RNA *in vitro* by the viral polymerase 3D (7, 13). At least some fraction of this product RNA was shown to be linked to VPg sequences, which must have been an inadvertent contaminant in the polymerase preparation.

A second model was proposed by Young et al. (22), whose host factor preparation, in conjunction with the viral polymerase, produces products *in vitro* that are elongated directly onto the RNA template to yield a population of snapback hairpin molecules that, upon denaturation, extend up to twice the length of the template. This model requires that the hairpin subsequently be cleaved, perhaps coupled with VPg addition to the newly generated 5' ends. Alternatively, linkage of VPg to product RNA could be explained by nonspecific cleavage of VPg-linked template RNAs, followed by addition of nucleotides to the generated 3' end of cleaved template molecules. A biochemical mechanism for

host factor-dependent hairpin formation was proposed by Andrews et al. (4), who demonstrated a terminal uridylyl transferase activity in their host factor preparation that was suggested to add a sufficient number of uridylate residues to the 3' end of the polyadenylated poliovirus RNA template so as to generate a self-hybridizing and therefore self-priming substrate for the polymerase. As evidence in support of the latter model, it was reported that snapback dimer length RNA molecules could be isolated from poliovirus-infected cells (22) and encephalomyocarditis virus-infected cells (16), thus raising the possibility that these snapback structures represent true intermediates of the replicative reaction occurring *in vivo*.

In our laboratory, a similar purified polymerase-host factor reaction that produces heterogeneous products of up to dimer length *in vitro* has been studied and reported (11). It was demonstrated, however, that hairpin formation occurred by host factor-induced endonucleolytic cleavage of the template at different sites so as to produce a population of self-priming molecules resulting from nicks introduced in or adjacent to hairpins that constitute part of the RNA secondary structure. The elongated template molecules had lost their original 3' ends before elongation, and templates whose original 3' ends were oxidized such that chain elongation could not occur nevertheless served equally well as templates after incubation with the host factor. We concluded that the *in vitro* reaction that we had studied that produced template-elongated products of up to apparent dimer length bore no relevance to the biological mechanism of poliovirus RNA replication. In support of these observations, Lubinski et al. (12) have reported the synthesis of hairpin double-stranded (ds) products from subgenomic templates of poliovirus RNA by viral polymerase *in vitro*, with some products apparently initiating from internal template sites.

In this report, we describe the analysis of snapback hairpin RNAs isolated from poliovirus-infected HeLa cells. We show that these molecules do not contain the poly(A) tract or greatly reduced lengths of poly(A) tracts normally found at the 3' ends of poliovirus plus-strand RNA, and thus they are generated by nucleolytic scission and elongation of

* Corresponding author.

† Present address: Section of Genetics, Cornell University, Ithaca, NY 14853.

FIG. 1. Rate zonal sucrose sedimentation of poliovirus ds RNA. (A) The profile of denatured polio ds RNA (●) is shown, as well as that of poliovirus ds RNA without methylmercury hydroxide treatment (△) centrifuged in a separate tube. Fractions 9 to 15 and 19 to 24 were combined separately, and RNA was collected by ethanol precipitation and centrifugation to serve as the source of non-snapback ds RNA strands and snapback RNA, respectively. (B) Snapback RNA from A was again denatured with methylmercury hydroxide and sedimented in a sucrose gradient. The positions of HeLa cell rRNAs sedimented in a separate gradient are indicated.

resulting self-priming molecules, just as in our *in vitro* system.

Polioviral RNA was labeled with ^{32}P in the presence of actinomycin D (2 $\mu\text{g}/\text{ml}$) between 1.5 and 8 h postinfection of HeLa cells with poliovirus, and ds RNA was purified from the cytoplasm of these cells, as described previously (15). The RNA was treated with 15 mM methylmercury hydroxide at 25°C for 30 min, immediately layered onto a 10 to 30% sucrose gradient (0.1 M LiCl, 0.01 M Tris hydrochloride [pH 7.5], 2 mM EDTA, 0.1% sodium dodecyl sulfate), and centrifuged in a Beckman SW41 rotor at 26,000 rpm (3°C for 16.25 h). Fractions were pumped from the bottom of the tube and assayed for radioactivity. Figure 1A shows a sedimentation profile of this ds RNA with its characteristic 20S peak (△). Most of this material was denatured in 15 mM methylmercury hydroxide just before sedimentation through a rate zonal sucrose gradient (Fig. 1, ●). Some of this RNA displayed a peak of 35S, characteristic of genome length plus and minus strands. Approximately 30 to 40% of this preparation, however, sedimented at its original sedimentation coefficient of 20S by virtue of snapback intramolecular renaturation. In different preparations isolated over a period of 2 years, the fraction of snapback RNA in the total ds RNA population varied between 25 and 50%. That this population of 20S molecules was not the result of incomplete denaturation during the initial denaturation is shown by collection of the 20S peak in Fig. 1A by ethanol precipitation, denaturation with methylmercury hydroxide again, and centrifugation in a rate zonal sucrose gradient (Fig. 1B). Almost the entire RNA population sedimented with a peak at 20S, confirming that this RNA represents snapback molecules.

If the 20S peak of RNA represents snapback dimer length strands, then these molecules should be cleaved by the single-strand-specific S1 nuclease into genome length strands by virtue of the susceptibility of the hairpin segment to cleavage. Figure 2 shows an autoradiograph of various fractions obtained during purification of poliovirus ds RNA from infected cells. These fractions were analyzed in a methylmercury hydroxide-agarose gel (5). All RNA samples were incubated in S1 buffer (0.03 M sodium acetate [pH 4.5],

0.3 M NaCl, 3 mM ZnCl_2 , 10 μg of alkali-digested salmon sperm DNA per ml) at 46°C for 30 min, but the sample in lane e also contained 1 U of S1 nuclease (Pharmacia). Samples were extracted with chloroform-phenol, precipitated with ethanol, dissolved in 0.055 M sodium borate (pH 8.1)–0.01 M Na_2SO_4 –1 mM EDTA, incubated with 10 mM methylmercury hydroxide at 25°C for 15 min, and electrophoresed in a 0.9% agarose-methylmercury hydroxide gel. After electrophoresis, the gel was fixed in 10% acetic acid–10 mM cysteine, dehydrated with methanol, and dried on a water aspirator before autoradiography. Lane c shows the pattern of denatured snapback RNA, displaying two distinct bands including approximately dimer length strands (15,000 nucleotides) and a major band of RNA between dimer and genome length strands. Additional discrete bands of various lengths are also visible on the original autoradiogram. Lane d displays the RNA profile after S1 nuclease digestion in which dimer length strands and the other major and minor species are converted to genome length strands (7,500 nucleotides) and additional species of less than genome length. Thus, the population of snapback RNAs in infected cells is composed predominantly of less than dimer length molecules which are reduced to their apparent monomer lengths after digestion with S1 nuclease. Lane b shows that nonsnapback ds RNA contains strands which comigrate with poliovirus RNA (lane a).

Since snapback molecules of various lengths are generated in infected cells, the question arose of how the complementary strands were primed. The hairpin RNA molecules generated *in vitro* had lost the poly(A) tract originally present on the 3' ends of the poliovirus RNA templates because of nicking at internal sites (11). We reasoned, therefore, that the population of snapback molecules generated *in vivo*, which includes predominantly strands of less than dimer length, may also have lost their poly(A) se-

FIG. 2. Autoradiograph of a methylmercury hydroxide-agarose gel of [^{32}P]RNAs from poliovirus-infected HeLa cells. Lanes: a, single-stranded poliovirus RNA adsorbed by oligo(dT)-cellulose; b, nonsnapback ds RNA; c, snapback RNA; d, snapback RNA digested with S1 nuclease. The number on the left indicates nucleotides in genome-length strands.

FIG. 3. High-voltage electrophoresis of nucleotides from poly(A) tracts of poliovirus RNAs. Lanes: a, marker mononucleotides; b, poliovirus RNA 3' end labeled with [^{32}P]pCp and RNA ligase (14, 2,000 cpm); c, single-stranded, poly(A) $^{+}$ poliovirus RNA labeled in vivo with ^{32}P , 700 cpm; d, snapback ds RNA labeled in vivo with ^{32}P , 150 cpm; e, nonsnapback ds RNA labeled in vivo with ^{32}P , 400 cpm. The positions of nucleotides are indicated on the left, and numbers above the nucleotide spots are counts per minute obtained in a scintillation spectrometer.

quences before self-priming. To determine whether the snapback RNA shown in Fig. 1A contained poly(A), the gradient-purified material was cut with S1 nuclease, heated to 104°C, cooled, and digested with RNases A and T1 (1). These enzymes together catalyze hydrolysis at all internucleotide linkages except ApN and thus leave any poly(A) tract undigested. For comparison, the single strands recovered after denaturation of ds RNA (Fig. 1A, fractions 9 to 14), as well as single-stranded RNA isolated by cellulose chromatography from the original nucleic acid extraction of infected cells, were also collected and digested with RNases A and T1. Each sample was subjected to gel filtration on Sephadex G-100 to remove the small nucleotide digestion products from any poly(A) sequences. The efficiency of this method of analysis was monitored by performing the same analysis on virion RNA 3' end labeled with [^{32}P]pCp and T4 RNA ligase (Fig. 3, lane b). Snapback RNA was initially digested with S1 nuclease as for Fig. 2. All samples for lanes b to e were digested with RNase T1 (1 U/ μg of RNA) and RNase A (2×10^{-3} $\mu\text{g}/\mu\text{g}$ of RNA) in 7 M urea-0.02 M citrate (pH 5.0)-1 mM EDTA at 50°C for 30 min; treated with pronase (1 mg/ml) in 0.2% sodium dodecyl sulfate at 37°C for 5 min; extracted with phenol-chloroform; and precipitated with ethanol. These precipitates were dissolved in 0.2 M LiCl-0.03 M Tris hydrochloride (pH 7.5)-2 mM EDTA-0.1% sodium dodecyl sulfate and passed through a Sephadex G-100 column (0.7 by 27 cm) in the same buffer to obtain the excluded material [poly(A) tracts], which was again collected by ethanol precipitation. All samples were then hydrolyzed in 10% piperidine in sealed capillaries at 90°C for 90 min, spotted on Whatman 3 MM filter paper sheets, and supplemented with a mixture of nonradioactive ribonucleoside 3'-monophosphates. Electrophoresis was performed in pH 3.5 buffer (5% glacial acetic acid, 0.5% pyridine, 1 mM EDTA) at 3,000 V and 90 mA for 50 min. The paper was dried and autoradiographed. Afterward, nucleotide spots

were visualized with UV light, cut out, and counted for radioactivity. It was immediately evident that the RNase digestion products from the snapback RNA sample contained a much smaller proportion of Sephadex-excluded material than did any of the other samples. Radioactivity in the excluded material from digested single-stranded RNA and nonsnapback ds RNA was 0.8 and 0.9%, respectively, of the total radioactivity in the undigested samples, consistent with previous estimates of the poly(A) content of these species (17, 20). Analysis of similar amounts of radiolabeled snapback ds RNA, however, yielded barely detectable excluded material (0.1%) from the Sephadex column. This was a preliminary indication that the poly(A) content of this sample was unusually low. RNase-resistant, poly(A) sequences were recovered from intracellular single-stranded RNA (Fig. 3, lane c) and single strands recovered after denaturation of ds RNA (lane e), but very little, if any, was recovered from snapback RNAs (lane d). Quantitation of the autoradiogram was done by cutting out nucleotide regions from each lane (indicated with nonradioactive nucleotide markers) and counting them in a scintillation spectrometer. Radioactivity (counts per minute) is indicated above each nucleotide location. The low level of radioactivity in the putative poly(A) material from snapback RNA accentuates any possible contribution from contaminating material acquired during sample preparation. Such contamination may have derived from poly(A) tracts in the nonsnapback ds RNA which overlapped snapback RNA in the initial sucrose gradient fractionation. Thus, even the very low level of poly(A) material found in the snapback RNA may be from contamination, and the snapback RNA may have no poly(A) tracts.

The lengths of poly(A) tract were estimated for ^{32}P -labeled single-stranded, poly(A) $^{+}$ poliovirus RNA and for nonsnapback ds RNA by sizing in a 12% polyacrylamide-7 M urea gel (data not shown). The average lengths of these tracts were estimated at 70 and 140 nucleotides, respectively. These values agree well with data reported previously (2, 17, 20), suggesting that at least a portion of poliovirus ds RNA has poly(A) tracts considerably longer than virion RNA.

The above results demonstrate that the snapback RNA molecules present in poliovirus-infected HeLa cells lack (or have greatly reduced levels) of poly(A) sequences normally present at the 3' ends of plus strands and probably represent hairpin structures synthesized from incomplete templates. We suggest that these molecules are likely generated in vivo by extension from a nicked template that formed self-priming structures similar to what we observed in vitro after incubation of virion RNA templates with a host factor preparation that caused endonucleolytic cuts. If these snapback RNAs represented intermediates in the normal RNA replication reaction, they would be expected to retain a full complement of viral RNA sequences, including the poly(A) tract. In light of the results reported here, we conclude that viral hairpin ds RNA does not represent an intermediate in the viral RNA replication reaction.

We thank Jami Adamson for typing the manuscript.

This research was supported by Public Health Service grant no. AI-17386 from the National Institutes of Health.

LITERATURE CITED

- Ahlquist, P., R. Dasgupta, D. S. Shih, D. Zimmermann, and P. Kaesberg. 1979. Two-step binding of eukaryotic ribosomes to brome mosaic virus RNA 3. *Nature (London)* **281**:277-282.
- Ahlquist, P., and P. Kaesberg. 1979. Determination of the length distribution of poly(A) at the 3' terminus of the virion RNAs of

- EMC virus, poliovirus, rhinovirus, RAV-61 and CPMV and of mouse globin mRNA. *Nucleic Acids Res.* 7:1195-1204.
3. **Andrews, N. C., and D. Baltimore.** 1986. Purification of a terminal uridylyl transferase that acts as host factor in the *in vitro* poliovirus replicase reaction. *Proc. Natl. Acad. Sci. USA* 83:221-225.
 4. **Andrews, N. C., D. Levin, and D. Baltimore.** 1985. Poliovirus replicase stimulation by terminal uridylyl transferase. *J. Biol. Chem.* 260:7628-7635.
 5. **Bailey, J. M., and N. Davidson.** 1976. Methylmercury as reversible denaturing agent for agarose gel electrophoresis. *Anal. Biochem.* 70:75-85.
 6. **Baron, M. H., and D. Baltimore.** 1982. Purification and properties of a host cell protein required for poliovirus RNA replication *in vitro*. *J. Biol. Chem.* 257:12351-12358.
 7. **Baron, M. H., and D. Baltimore.** 1982. *In vitro* copying of viral positive strand RNA by poliovirus replicase. *J. Biol. Chem.* 257:12359-12366.
 8. **Dasgupta, A.** 1983. Purification of host factor required for *in vitro* transcription of poliovirus RNA. *Virology* 128:245-251.
 9. **Dasgupta, A., P. Zabel, and D. Baltimore.** 1980. Dependence of the activity of the poliovirus replicase on a host cell protein. *Cell* 19:423-429.
 10. **Hey, T. D., O. C. Richards, and E. Ehrenfeld.** 1986. Synthesis of plus- and minus-strand RNA from poliovirion RNA template *in vitro*. *J. Virol.* 58:790-796.
 11. **Hey, T. D., O. C. Richards, and E. Ehrenfeld.** 1987. Host factor-induced template modification during synthesis of poliovirus RNA *in vitro*. *J. Virol.* 61:802-811.
 12. **Lubinski, J. M., G. Kaplan, V. R. Racaniello, and A. Dasgupta.** 1986. Mechanism of *in vitro* synthesis of covalently linked dimeric RNA molecules by the poliovirus replicase. *J. Virol.* 58:459-467.
 13. **Morrow, C. D., and A. Dasgupta.** 1983. Antibody to a synthetic nonapeptide corresponding to the NH₂ terminus of poliovirus genome-linked protein VPg reacts with native VPg and inhibits *in vitro* replication of poliovirus RNA. *J. Virol.* 48:429-439.
 14. **Morrow, C. D., G. F. Gibbons, and A. Dasgupta.** 1985. The host protein required for *in vitro* replication of poliovirus is a protein kinase that phosphorylates eukaryotic initiation factor-2. *Cell* 40:913-921.
 15. **Richards, O. C., and E. Ehrenfeld.** 1980. Heterogeneity of the 3' end of minus-strand RNA in the poliovirus replicative form. *J. Virol.* 36:387-394.
 16. **Senkevich, T. G., I. M. Cumakov, G. V. Lipskaya, and V. I. Agol.** 1980. Palindrome-like dimers of double-stranded RNA of encephalomyocarditis virus. *Virology* 102:339-348.
 17. **Spector, D. H., and D. Baltimore.** 1975. Polyadenylic acid on poliovirus RNA. II. Poly(A) on intracellular RNAs. *J. Virol.* 15:1418-1431.
 18. **Takeda, N., R. J. Kuhn, C.-F. Yang, T. Takegami, and E. Wimmer.** 1986. Initiation of poliovirus plus-strand RNA synthesis in a membrane complex of infected HeLa cells. *J. Virol.* 60:43-53.
 19. **Takegami, T., R. J. Kuhn, C. W. Anderson, and E. Wimmer.** 1983. Membrane-dependent uridylylation of the genome-linked protein VPg of poliovirus. *Proc. Natl. Acad. Sci. USA* 80:7447-7451.
 20. **Yogo, Y., and E. Wimmer.** 1973. Poly(A) and poly(U) in poliovirus double stranded RNA. *Nature (London) New Biol.* 242:171-174.
 21. **Young, D. C., B. M. Dunn, G. J. Tobin, and J. B. Flanagan.** 1986. Anti-VPg antibody precipitation of product RNA synthesized *in vitro* by the poliovirus polymerase and host factor is mediated by VPg on the poliovirion RNA template. *J. Virol.* 58:715-723.
 22. **Young, D. C., D. M. Tuschall, and J. B. Flanagan.** 1985. Poliovirus RNA-dependent RNA polymerase and host cell protein synthesize product RNA twice the size of poliovirion RNA *in vitro*. *J. Virol.* 54:256-264.