JOURNAL OF VIROLOGY, Jan. 1989, p. 467–470
0022-538X/89/010467-04$02.00/0

Vol. 63, No. 1

Role of 3′-End Sequences in Infectivity of Poliovirus Transcripts Made In Vitro

PETER SARNOW

Department of Biochemistry, Biophysics and Genetics, and Department of Microbiology and Immunology, University of Colorado Health Sciences Center, Denver, Colorado 80262

Received 11 August 1988/Accepted 6 October 1988

It has been shown by van der Werf et al. (S. van der Werf, J. Bradley, E. Wimmer, F. W. Studier, and J. Dunn, Proc. Natl. Acad. Sci. USA 83:2330–2334, 1986) that in vitro synthesis of poliovirus RNA by T7 RNA polymerase gives rise to infectious RNA molecules; however, these molecules are only 5% as infectious as RNA isolated from virions. A plasmid, T7D-polio, was constructed that allows the in vitro synthesis of full-length RNA molecules with two additional guanine residues at the 5′ end. However, T7D-polio differed from the construct of van der Werf et al. in that RNA transcribed from T7D-polio has an authentic 3′ end, ending with only a polyadenine nucleotide sequence. Transfection of these RNA molecules into mammalian cells produced wild-type poliovirus with an efficiency similar to that of virion RNA. The use of this vector in the characterization of viral mutants in vivo and in vitro is discussed.

Recombinant DNA technology has proven to be fruitful in the application of genetics to the study of RNA viruses. The discovery that infectious cDNA copies of RNA viruses can be obtained (14, 16) has allowed the use of site-directed mutagenesis techniques in the construction of viral mutants (3, 8, 15). In addition, in vitro transcription of cloned cDNA of animal (13, 21), insect (5), and plant (1, 11, 17) RNA viruses has produced infectious RNA molecules in several instances. The production of infectious poliovirus by in vitro transcription of a cDNA copy has been described recently (21). The in vitro-made RNA contained two additional nucleotides at the 5′ end as well as seven nucleotides 3′ of the poly(A) tract; these RNA molecules were 5% as infectious as poliovirion RNA. We report here the construction of a vector that can be transcribed by T7 RNA polymerase to yield RNA molecules bearing authentic polyadenylated 3′ end that are as infectious as virion RNA.

The construction of plasmid T7-polio, used to transcribe viral RNA with only two additional guanine nucleotides at the 5′ end is shown in Fig. 1. Plasmid p5′polio was constructed by subcloning a *Sac*I-*Pst*I fragment from pSV2-polio (3) into a pGem3 vector (Promega Biotec, Madison, Wis.). This plasmid can be transcribed by T7 RNA polymerase to yield viral molecules 1,859 nucleotides in length which contain a *Sac*I linker and 50 guanine residues at the 5′ end. A synthetic oligodeoxynucleotide (5′CTGTTTTAACCT ATAGTG3′) was used to introduce, by site-directed mutagenesis, the nucleotide deletion needed to juxtapose the T7 promoter and poliovirus cDNA sequences (4, 7, 22) (see Fig. 1). The recombinant clone bearing the T7 promoter element next to poliovirus sequences was isolated and digested with *Bgl*I, and the small *Bgl*I fragment was isolated and ligated to the two large *Bgl*I fragments obtained from digestion of pSV2-polio. The resulting plasmid was termed T7-polio (see Fig. 1). The expected nucleotide sequence at the junction of the T7 promoter and the 5′ end of poliovirus was verified by DNA sequencing (10). Upon linearization of T7-polio DNA with *Eco*RI and in vitro transcription with T7 RNA polymerase, RNA molecules were synthesized which had two extra guanine residues at their 5′ ends before the first nucleotide of the poliovirus genome and 17 nucleotides at their 3′ ends after a stretch of 12 adenine residues. The concentration of full-length RNA molecules was determined by comparing known amounts of virion RNA with various dilutions of in vitro-made RNA in an ethidium bromide-stained agarose gel. These RNA molecules were introduced into human HeLa cells by DEAE-dextran transfection (19), and an infectivity of 3.0×10^4 PFU/μg of RNA was observed in dilution experiments; in parallel experiments, virion RNA displayed an infectivity of 1.5×10^6 PFU/μg (see Table 1). Capping of the in vitro-made transcripts had no effect on the infectivity (not shown). These results are in accord with the findings of van der Werf et al. (21), who constructed a plasmid similar to T7-polio by different methods.

In an attempt to produce viral RNA molecules that are closer in sequence to virion RNA we constructed T7D-polio. We used the strategy outlined in Fig. 1 to insert a unique *Mlu*I restriction endonuclease site betwen the viral poly(A) sequences at the 3′ end and the poly(C) sequences which were derived from the cDNA cloning of the infectious viral RNA (14). Briefly, the oligodeoxynucleotide (5′AAAAA AACGCGTCCCCC3′) was annealed to gapped duplexes prepared by annealing T7-polio cut with *Pvu*I (see Fig. 1) to T7-polio cut with *Hin*dIII (position 6516) and *Eco*RI (position 7498). Gapped duplexes were repaired with the Klenow enzyme, ligated with T4 DNA ligase, and transformed into *Escherichia coli* (4, 7, 9, 22). Bacterial colonies were screened with the radiolabeled oligodeoxynucleotide, and a positive clone, T7D-polio, was identified. Again, the presence of the sequences surrounding the expected introduced *Mlu*I restriction endonuclease site was verified by DNA sequencing (10). The restriction endonuclease *Mlu*I leaves staggered ends in digested DNA with a GCGC-5′ overhang. This single-stranded overhang can be removed by treatment of *Mlu*I-digested DNA with exonuclease VII (2), leaving a DNA template whose coding strand terminates in 12 adenine residues. Thus, T7D-polio DNA can be transcribed by T7 RNA polymerase to produce viral RNA molecules that contain two extra guanine residues at their 5′ ends and oligoadenine tails at their 3′ ends. These 3′ sequences can then be extended in vitro to form long polyadenine tails by the action of poly(A) polymerase (18).

Figure 2 shows the nucleotide sequences of the T7D-polio cDNA and in vitro transcripts. The 5′ ends contain the two

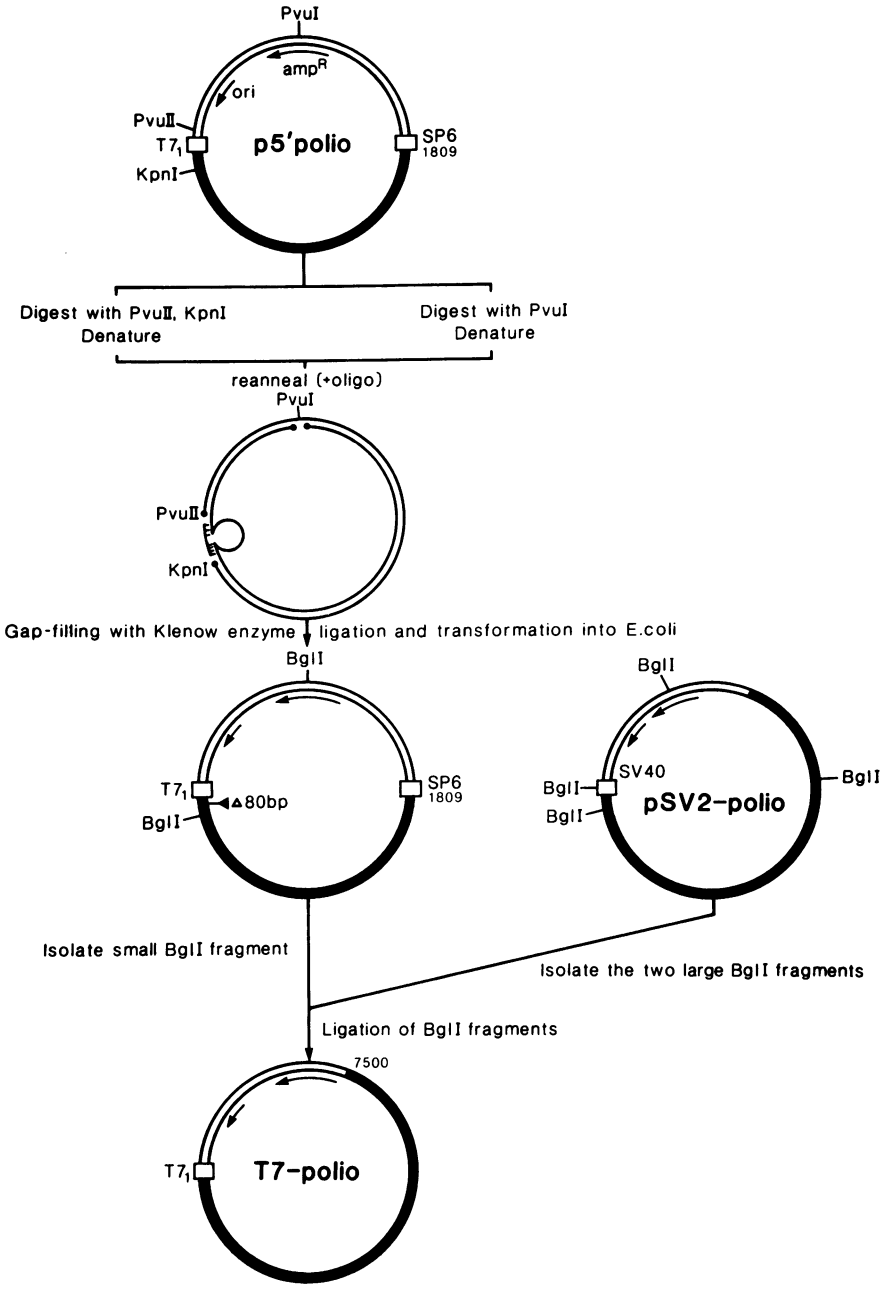

FIG. 1. Construction of plasmid T7-polio. The blackened lines represent poliovirus cDNA sequences, and the blank lines denote pBR322 sequences. The promoter elements for simian virus 40, T7, and SP6 RNA polymerases are diagrammed as open boxes. The numbers next to the circles represent positions in the poliovirus genome. Restriction endonuclease sites for *Bgl*I, *Kpn*I, *Pvu*I, and *Pvu*II are indicated.

extra guanine nucleotides, and the 3′ ends contain either CGCG nucleotides derived from the *Mlu*I site (a and b) or adenosine residues (c and d) which were transcribed from an exonuclease VII-treated linearized template (2). In another experiment, extra adenine nucleotides were added in vitro by poly(A) polymerase (b and d).

Transcripts were made in vitro and analyzed by agarose gel electrophoresis (Fig. 3). Lanes 1 and 2 contain virion RNA (0.1 and 0.01 μg, respectively), lane 3 shows RNA molecules made in vitro containing 12 adenine residues at their 3′ ends, and lane 4 displays RNA molecules that have been extended by poly(A) polymerase. The poly(A) polymerase reaction was carried out by incubating 5 μg of RNA transcript with 10 U of poly(A) polymerase (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) and 0.25 mM ATP in 30 mM Tris hydrochloride (pH 8)–10 mM $MgCl_2$–2.5 mM $MnCl_2$–250 mM NaCl. The upper band in lanes 3 and 4 is the linearized template cDNA which was not removed. The RNA produced in vitro by transcription of T7D-polio DNA displays electrophoretic mobility similar to that of virion RNA (lane 3). As expected, RNA that has been polyadenylated in vitro migrated somewhat more slowly in the gel (lane 4). A more detailed electrophoretic analysis (now shown) revealed that the polyadenylated RNA is

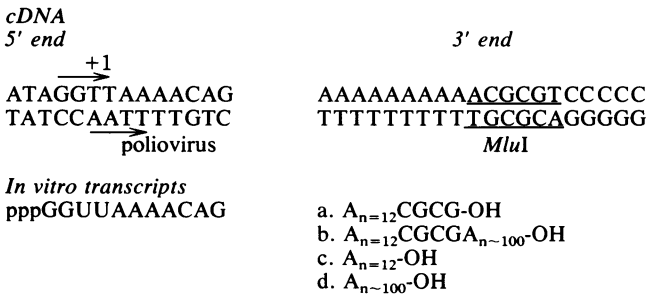

FIG. 2. Nucleotide sequences of template cDNAs and in vitro transcripts.

TABLE 1. Infectivity of in vitro transcripts

Nucleotide sequence of transcript	Infectivity (PFU/μg)[a]
pppGGUUAA...$A_{n=12}C_{n=17}$	3.0×10^4
pppGGUUAA...$A_{n=12}$CGCG	2.0×10^5
pppGGUUAA...$A_{n=12}$CGCG$A_{n\sim100}$	1.5×10^6
pppGGUUAA...$A_{n=12}$	1.5×10^5
pppGGUUAA...$A_{n\sim100}$	1.5×10^6
Virion RNA	1.5×10^6

[a] The numbers for the infectivities are averaged from five independent experiments.

heterogeneous in length, with an average length of 100 adenine residues at the 3' end.

Next, we transfected the in vitro-made RNA transcripts into HeLa cells and compared their infectivities with that of virion RNA (Table 1). This was done by transfecting cells with various dilutions of the in vitro-synthesized RNA molecules, using a DEAE-dextran transfection protocol (19) followed by agar overlay of transfected cells. Infectious center assays were also performed to estimate the extent of infection of transfected cells. From this finding, it was estimated that as many as 40 to 60% of the cells could be transfected with polyadenylated RNA made in vitro.

All of our transcripts have identical 5' ends but contain different nucleotide sequences at their 3' ends; RNA molecules containing 17 cytosine nucleotides were 2% as infectious as virion RNA, whereas RNA molecules with only 4 nucleotide residues after the adenine tail showed an increased infectivity to 10% of the virion RNA. If the latter transcripts were polyadenylated in vitro, infectivities comparable to that of virion RNA can be obtained. Similarly, while transcripts terminating with 12 adenine nucleotides were 10% as infectious as virion RNA, RNA polyadenylated in vitro was as infectious as virion RNA. It is interesting that an extra 4 nucleotides at the end of the adenine (12 A's) tract did not diminish the infectivity of the RNA, while 17 cytosine residues did. Although the 3'-terminal sequences of these RNA molecules have not been determined directly, it has been shown that T7 RNA polymerase continues transcription of single-stranded template extensions (12). Therefore, we presume that the 3'-terminal sequences of these RNAs are as shown in Table 1.

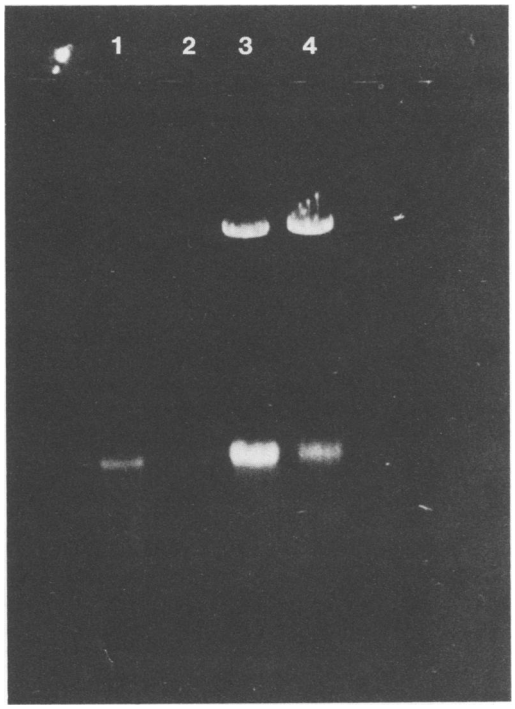

FIG. 3. Analysis of in vitro-made viral transcripts by agarose gel electrophoresis. T7D DNA was linearized with *Mlu*I and treated with exonuclease VII, and transcripts were made in vitro by T7 RNA polymerase. The products were analyzed immediately by electrophoresis in a 1% agarose gel (21). Lanes: 1 and 2, 0.1 and 0.01 μg virion RNA, respectively; 3, transcription products of *Mlu*I-linearized, exonuclease VII-treated T7D plasmid; 4, RNA molecules shown in lane 3 after in vitro polyadenylation with ATP and poly(A) polymerase.

These results show that long heteropolymeric nucleotide sequences at the 3' end of poliovirus RNA decreased the infectivities of the RNA molecules, whereas long homopolymeric adenine sequences increased RNA infectivity. The lengths of the adenine sequences were important to the yield of highly infectious RNA. From earlier studies, it is known that adenine sequences shorter than 20 nucleotides reduce the infectivity of virion RNA 20-fold (20). Therefore, we speculate that this is the sole reason why the pppGGUUAA. . .$A_{n=12}$CGCG and pppGGUUAA. . .$A_{n=12}$ transcripts displayed low infectivities. It could be that RNA molecules with short polyadenine tails are unstable or unfavorable templates for translation or initiation of minus-strand RNA synthesis.

The capacity to produce poliovirus RNA in vitro that is identical to virion RNA at the 3' end, bears only two extra guanine nucleotides at the 5'end, and is infectious as virion RNA is a useful tool to perform several kinds of studies. First, these templates can be used to explore the role of nucleotide sequences in the 3'-noncoding region and the length of the poly(A) tail in the initiation of minus-strand RNA synthesis in vitro and in vivo. Second, these RNA molecules are ideal substrates for in vitro translation studies. Third, a new attempt can be made to introduce lethal mutants, made by in vitro site-directed mutagenesis methods, into mammalian cells and to study their phenotypes in a single infectious cycle, an approach which has been difficult due to the low infectivities of cDNA clones and infectious transcripts. Very recently, however, Kaplan and Racaniello (6) presented evidence that RNA produced in vitro from noninfectious poliovirus cDNAs bearing deletions in the capsid region could be studied in a single cycle of infection. Increasing the specific infectivity of RNA transcripts made in vitro may extend this experimental approach to the study of mutants defective in replication as well.

I am very grateful to David Baltimore, in whose laboratory these studies were initiated, for his support. I thank Karla Kirkegaard for many valuable suggestions throughout the course of this work and Sandi Jacobson, Dennis Macejak, and Lyle Najita for comments on the manuscript.

This study was supported by Public Health Service grants AI-25105 and BRSG-05357 from the National Institutes of Health.

LITERATURE CITED

1. **Ahlquist, P., R. French, M. Janda, and L. S. Loesch-Fries.** 1984. Multicomponent RNA plant virus infection derived from cloned viral cDNA. Proc. Natl. Acad. Sci. USA **81**:7066–7070.
2. **Berk, A. J., and P. A. Sharp.** 1978. Structure of the adenovirus 2 early mRNAs. Cell **14**:695–711.
3. **Bernstein, H. D., N. Sonenberg, and D. Baltimore.** 1985. Poliovirus mutant that does not selectively inhibit host cell protein synthesis. Mol. Cell. Biol. **5**:2913–2923.
4. **Dalbadie-McFarland, G., L. W. Cohen, A. D. Riggs, C. Morin, K. Itakura, and J. H. Richards.** 1982. Oligonucleotide-directed mutagenesis as a general and powerful method for studies of protein function. Proc. Natl. Acad. Sci. USA **79**:6409–6413.
5. **Dasmahapatra, B., R. Dasgupta, K. Saunders, B. Selling, T. Gallagher, and P. Kaesberg.** 1986. Infectious RNA derived by transcription from cloned cDNA copies of the genomic RNA of an insect virus. Proc. Natl. Acad. Sci. USA **83**:63–66.
6. **Kaplan, G., and V. R. Racaniello.** 1988. Construction and characterization of poliovirus subgenomic replicons. J. Virol. **62**:1687–1696.
7. **Kirkegaard, K., and J. C. Wang.** 1985. Bacterial DNA topoisomerase can relax positively supercoiled DNA containing a single-stranded loop. J. Mol. Biol. **185**:625–637.
8. **Kuhn, R. J., H. Tada, M. F. Ypma-Wong, J. Dunn, B. L. Semler, and E. Wimmer.** 1988. Construction of a "mutagenesis cartridge" for poliovirus genome-linked viral protein: isolation and characterization of viable and nonviable mutants. Proc. Natl. Acad. Sci. USA **85**:519–523.
9. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
10. **Maxam, A. M. and W. Gilbert.** 1977. A new method for sequencing DNA. Proc. Natl. Acad. Sci. USA **74**:560–565.
11. **Meshi, T., M. Ishikawa, F. Motoyoshi, K. Semba, and Y. Okada.** 1986. In vitro transcription of infectious RNAs from full-length cDNAs of tobacco mosaic virus. Proc. Natl. Acad. Sci. USA **83**:5043–5047.
12. **Milligan, J. F., D. R. Groebe, G. W. Witherell, and O. C. Uhlenbeck.** 1987. Oligoribonucleotide synthesis using T7 RNA polymerase and synthetic DNA templates. Nucleic Acids Res. **15**:8783–8798.
13. **Mizutani, S., and R. J. Colonno.** 1985. In vitro synthesis of an infectious RNA from cDNA clones of human rhinovirus type 14. J. Virol. **56**:628–632.
14. **Racaniello, V. R., and D. Baltimore.** 1981. Cloned poliovirus complementary DNA is infectious in mammalian cells. Science **214**:916–919.
15. **Sarnow, P., H. D. Bernstein, and D. Baltimore.** 1986. A poliovirus temperature-sensitive mutant located in a noncoding region of the genome. Proc. Natl. Acad. Sci. USA **83**:7571–7575.
16. **Semler, B. L., A. J. Dorner, and E. Wimmer.** 1984. Production of infectious poliovirus from cloned cDNA is dramatically increased by SV40 transcription and replication signals. Nucleic Acids Res. **12**:5123–5141.
17. **Simon, A. E., and S. H. Howell.** 1987. Synthesis in vitro of infectious RNA copies of the virulent satellite of turnip crinkle virus. Virology **156**:146–152.
18. **Sippel, A.** 1973. Purification and characterization of adenosine triphosphate: ribonucleic acid adenyltransferase from Escherichia Coli. Eur. J. Biochem. **37**:31–40.
19. **Somapayrac, L. M., and K. J. Danna.** 1981. Efficient infection of monkey cells with DNA of simian virus 40. Proc. Natl. Acad. Sci. USA **78**:7575–7578.
20. **Spector, D. H., and D. Baltimore.** 1974. Requirement of 3'-terminal poly (adenylic acid) for the infectivity of poliovirus RNA. Proc. Natl. Acad. Sci. USA **71**:2983–2987.
21. **van der Werf, S., J. Bradley, E. Wimmer, F. W. Studier, and J. Dunn.** 1986. Synthesis of infectious poliovirus RNA by purified T7 RNA polymerase. Proc. Natl. Acad. Sci. USA **83**:2330–2334.
22. **Wood, W. I., J. Gitschier, L. A. Lasky, and R. M. Lawn.** 1985. Base composition-independent hybridization in tetramethylammonium chloride: a method for oligonucleotide screening of highly complex gene libraries. Proc. Natl. Acad. Sci. USA **82**: 1585–1588.