Proc. Natl. Acad. Sci. USA
Vol. 83, pp. 2330–2334, April 1986
Biochemistry

Synthesis of infectious poliovirus RNA by purified T7 RNA polymerase

(poliovirus cDNA/T7 promoter)

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Communicated by Richard B. Setlow, December 9, 1985

ABSTRACT **Plasmids containing the entire cDNA sequence of poliovirus type 1 (Mahoney strain) under control of a promoter for T7 RNA polymerase have been constructed. Purified T7 RNA polymerase efficiently transcribes the entire poliovirus cDNA in either direction to produce full-length poliovirus RNA [(+)RNA] or its complement [(−)RNA]. The (+)RNA produced initially had 60 nucleotides on the 5′ side of the poliovirus RNA sequence, including a string of 18 consecutive guanine residues generated in the original cloning and an additional 626 nucleotides of pBR322 sequence beyond the poly(A) tract at the 3′ end. Such RNA, while much more infectious than the plasmid DNA, is only about 0.1% as infectious as RNA isolated from the virus. Subsequently, a T7 promoter was placed only 2 base pairs ahead of the poliovirus sequence, so that T7 RNA polymerase synthesizes poliovirus RNA with only 2 additional guanine residues at the 5′ end and no more than seven nucleotides past the poly(A) tract at the 3′ end. Such RNA has much higher specific infectivity, about 5% that of RNA isolated from the virus. The ability to make infectious poliovirus RNA efficiently from cloned DNA makes it possible to apply techniques of *in vitro* mutagenesis to the analysis of poliovirus functions and the construction of novel and perhaps useful derivatives of poliovirus. A source of variant RNAs should also allow detailed study of the synthesis and processing of poliovirus proteins *in vitro*.**

RNA isolated from poliovirus can initiate infection of cultured mammalian cells (1). Virion RNA normally has a small protein, VPg, covalently attached to its 5′ end, but VPg is not needed for infectivity of polio RNA (2, 3). Unfortunately, techniques that would allow *in vitro* mutagenesis of large RNA molecules without introducing lethal damage are not well developed, and it has not been possible to generate useful variants of poliovirus by chemical alteration of the virion RNA. Recently, however, complete cDNA clones of poliovirus type 1 RNA have been made (4–6), and the DNA of such clones is infectious. This makes it possible to apply the wide variety of techniques available for *in vitro* mutagenesis of DNA to the study of poliovirus.

The mechanism by which cells produce infectious poliovirus from cDNA clones is not known. Presumably, the cloned DNA is transcribed in the cell nucleus to produce RNAs that are transported to the cytoplasm, where they serve as mRNAs for protein synthesis and template for replication. However, during a normal infection poliovirus RNA does not enter the nucleus and is not spliced, but it carries adventitious splicing and polyadenylylation signals that could be recognized in the nuclear transcripts produced from the cDNA (R. J. Kuhn and E.W., unpublished results). Splicing or polyadenylylation at such sites might be expected to inactivate most of the nuclear transcripts. Furthermore, the poliovirus RNA produced from available cDNA vectors would be embedded in longer primary transcripts that might have to be processed to produce biologically active molecules. Therefore, it is not surprising that the efficiency of infection by cDNA clones is very low. The efficiency can be increased by up to three orders of magnitude if the plasmid is capable of replicating (5), but at best it remains two or three orders of magnitude lower than that of virion RNA.

If infectious RNA could be produced by transcription of a polio cDNA clone *in vitro*, the ease of manipulation of DNA could be combined with the efficiency of infection of RNA to systematically explore the biological function of the poliovirus genome *in vivo* and *in vitro*. T7 RNA polymerase is ideal for this because it is highly selective for its own promoters (7), it efficiently makes complete transcripts of very long DNAs from a variety of sources (unpublished results), and it is easily prepared from the cloned gene (8). We have now placed the polio cDNA under control of a T7 promoter and have shown that purified T7 RNA polymerase can produce large amounts of infectious poliovirus RNA. A similar approach, using *Escherichia coli* RNA polymerase, has been successfully applied to brome mosaic virus by Alquist *et al.* (9).

MATERIALS AND METHODS

Bacteria, Plasmids, and Cloning Procedures. *Escherichia coli* strains C600 (10) and HMS174 (11) were used for selection and propagation of recombinant plasmids. Plasmid pPV16, kindly provided by Bert L. Semler, has the full-length poliovirus cDNA, on an *Eco*RI fragment from pDS303, inserted in the counterclockwise orientation in the *Eco*RI site of pNT4 (5). Plasmids pAR2192 and pAR2369 are derivatives of pBR322 that contain the *ϕ10* promoter for T7 RNA polymerase (see ref. 12 for nucleotide sequences of T7 promoters and DNA fragments). The construction of these plasmids will be described in detail elsewhere. Briefly, pAR2192 contains a fragment of T7 DNA (nucleotides −23 to +26 relative to the RNA start site of *ϕ10*) cloned in the *Bam*HI site of pBR322 so that transcription from *ϕ10* is directed counterclockwise. This plasmid contains single *Bam*HI and *Eco*RI sites immediately downstream of the promoter-containing fragment, in the sequence CGGGATC-CGAATTC, and the DNA normally found between these sites in pBR322 has been eliminated. Plasmid pAR2369 also contains the *ϕ10* promoter in the *Bam*HI site of pBR322, in this case nucleotides −26 to +2 relative to the start site. A *Bam*HI site lies immediately upstream of the promoter-containing fragment; immediately downstream, the AGG at the RNA start site of *ϕ10* (−1 to +2) is half of a *Stu* I site

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Abbreviation: pfu, plaque forming unit.
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(AGGCCT), which is followed immediately by a *Bam*HI site in the sequence AGGCCTGGATCC. This is the only *Stu* I site in the plasmid. *Stu* I cuts at the center of its recognition sequence, leaving a blunt end AGG at position −1 to +2. DNA fragments inserted at this site should be transcribed by T7 RNA polymerase to produce RNAs having only an additional pair of guanines at their 5′ end.

Standard techniques were used to clone DNA fragments and identify recombinant clones (13). For large-scale purification, plasmid DNA was prepared by a lysis procedure involving $NaDodSO_4$ (14) and further purified by differential precipitation of RNA with 2 M LiCl and by chromatography on Sephacryl S1000 (Pharmacia).

Enzymes and Synthetic DNAs. Restriction endonucleases and enzymes used in cloning DNA were obtained from New England Biolabs. T7 RNA polymerase was produced from the cloned gene (8) and purified by an improved method to be described elsewhere. Synthetic DNAs were synthesized using β-cyanoethyl phosphoramidites (American BioNuclear, Emeryville, CA) and a Microsyn 1450 DNA synthesizer (Systec, Minneapolis, MN).

***In Vitro* Transcription.** Reaction mixtures contained 20 mM sodium phosphate, pH 7.7, 8 mM $MgCl_2$, 10 mM dithiothreitol, 4 mM spermidine·HCl, 50 mM NaCl, and 0.4–1 mM each ATP, CTP, GTP, and UTP. Template DNAs (20 μg/ml) were linearized by cutting with an appropriate restriction enzyme followed by phenol extraction and ethanol precipitation. Synthesis was initiated by addition of T7 RNA polymerase (1–5 μg/ml), and reactions were incubated for 30 min at 37°C.

In most transfection experiments, samples of the entire reaction mixture were added to cells. Where RNA was purified away from template DNA before transfection, this was done by treating the reaction mixture with DNase I, followed by phenol extraction and several precipitations with 70% ethanol in the presence of 2.5 M ammonium acetate.

Cell Culture, Viruses, and RNA Transfections. Poliovirus type 1 (Mahoney strain) was grown in HeLa S3 cell suspensions and virion RNA was purified as described (15). Monolayers of HeLa R19 cells grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% (vol/vol) fetal bovine serum were used for titration of virus and for transfection experiments. Transfections were carried out essentially as described by Koch (1). RNA was diluted in HBSS buffer (5 g of Hepes, 8 g of NaCl, 0.37 g of KCl, 0.125 g of $Na_2HPO_4·2H_2O$, and 1 g of dextrose per liter, pH 7.05), mixed with an equal volume of DEAE-dextran (Pharmacia) (1 mg/ml in HBSS buffer), and placed at 0°C for 30 min. Recipient monolayer cells, 90–100% confluent, were washed once with DMEM and then incubated for 30 min at room temperature with 0.2 ml of the RNA–DEAE-dextran mixture per 35-mm plate. Plaques were allowed to develop by incubating the plates at 37°C in DMEM containing 2% (vol/vol) fetal bovine serum or under a semisolid overlay containing the same medium plus 50 mM $MgCl_2$ and 0.9% Noble agar (Difco).

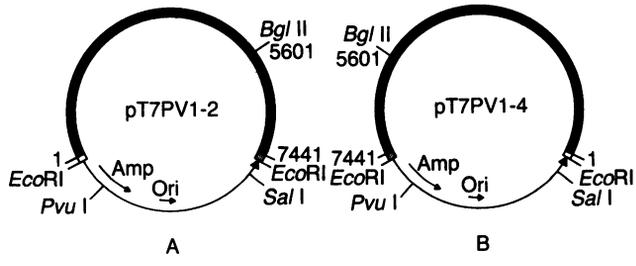

FIG. 1. Plasmids containing full-length poliovirus cDNA and the T7 *ϕ10* promoter. Closed triangle, T7 *ϕ10* promoter; closed area, poliovirus cDNA; open area, poly(dG/dC); dotted area, poly(A). Transcription by purified T7 RNA polymerase produces polio (−)RNA from pT7PV-2 and (+)RNA from pT7PV1-4.

RESULTS

Purified T7 RNA Polymerase Efficiently Transcribes Polio DNA. To examine the ability of purified T7 RNA polymerase to transcribe the poliovirus genome, we placed a full-length cDNA copy of polio RNA in a plasmid under control of the *ϕ10* promoter for T7 RNA polymerase. Initially, we inserted the appropriate *Eco*RI fragment from pPV16 into the *Eco*RI site of pAR2192. This *Eco*RI fragment contains the entire nucleotide sequence of poliovirus RNA preceded by 18 consecutive guanine residues and ending in a poly(A) tract of 84 residues (5). The fragment was cloned in both orientations relative to *ϕ10*: transcription of pT7PV1-2 produces (−)RNA and transcription of pT7PV1-4 produces (+)RNA, having the polarity of virus RNA (Fig. 1).

These plasmid DNAs were linearized by cutting with *Pvu* I or *Sal* I, restriction enzymes that cut uniquely within the vector but not within the polio DNA, and transcribed with purified T7 RNA polymerase. Large amounts of RNA of the sizes expected for runoff transcripts from *ϕ10* were produced from both pT7PV1-2 and pT7PV1-4 DNA (Fig. 2 *A* and *C*). RNAs ending at the *Pvu* I or *Sal* I sites would be about 8217 or 11,301 nucleotides long: from 5′ to 3′, the (+)RNA would be expected to contain 26 nucleotides of T7 sequence, 16 nucleotides of linker sequence, 18 consecutive guanine residues, 7525 nucleotides of polio sequence (7441 nucleotides of the heteropolymeric chain plus 84 consecutive adenine residues), 2 nucleotides of linker sequence, and 630 or 3714 nucleotides of pBR322 sequence. The (−)RNA would be the same length but would contain 84 consecutive uridine residues on the 5′ side of the polio (−)RNA sequence and 18

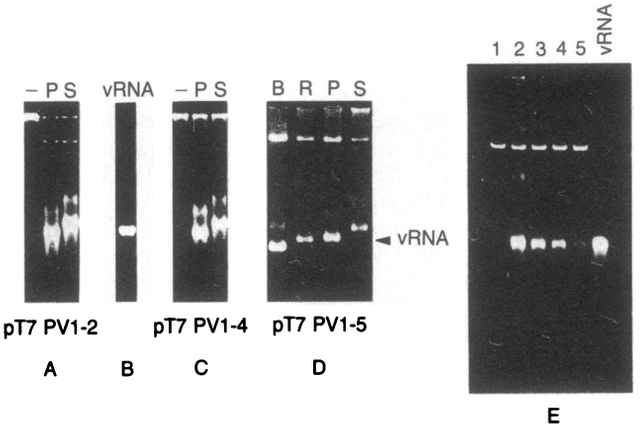

FIG. 2. RNAs transcribed from poliovirus cDNA clones by purified T7 RNA polymerase. Reactions were stopped by adding EDTA, and portions of the reaction mixture were analyzed by agarose gel electrophoresis essentially as described (8). Templates were (*A*) pT7PV1-2, intact (lane −), cut with *Pvu* I (lane P), or cut with *Sal* I (lane S); (*C*) pT7PV1-4, intact (lane −), cut with *Pvu* I (lane P), or cut with *Sal* I (lane S); (*D*) pT7PV1-5 cut with *Bgl* II (lane B), *Eco*RI (lane R), *Pvu* I (lane P), or *Sal* I (lane S); (*E*) pT7PV1-5 cut with *Eco*RI (see Figs. 1 and 4 for the locations of restriction sites). Polio RNA isolated from the virion (vRNA) is shown in *B* and *E*. The vRNA in *B* was electrophoresed under the same conditions as the RNAs in *A* and *C*. (E) Lanes 1–5: no enzyme, 10, 5, 2.5, or 1.25 μg of T7 RNA polymerase/ml, respectively. Samples containing 2.5 μl of reaction mixture were analyzed along with 1 μg of virion RNA. The band appearing in the no enzyme control (lane 1) is the template DNA. Template DNA is also apparent along with the RNA in the reaction mixtures containing linear pT7PV-2 and pT7PV1-5 but not in reactions containing circular template or pT7PV1-4, where it appears to be in an aggregate at the origin.

Table 1. Infectivity of *in vitro* transcripts

Template DNA	Site at 3′ end	Polio strand	Input RNA	pfu/μg	Experiments, no.
			Virion RNA	2.1×10^6	6
pT7PV1-2	*Pvu* I	(−)	Reaction mixture	<10	2
pT7PV1-2	*Sal* I	(−)	Reaction mixture	<10	2
pT7PV1-4	*Pvu* I	(+)	Reaction mixture	1.3×10^3	4
			Purified RNA	223	3
			Reaction mixture + RNase	<10	1
pT7PV1-4	*Sal* I	(+)	Reaction mixture	1.6×10^3	3
pT7PV1-4	*Bgl* II	(+)	Reaction mixture	<10	2
pT7PV1-5	*Eco*RI	(+)	Reaction mixture	1.0×10^5	2

Appropriate dilutions of the transcription reaction mixtures were used to transfect HeLa R19 cell monolayers. The plates were stained with neutral red 3 days after transfection. Concentrations of purified RNAs were determined by absorbance; concentrations of RNAs in reaction mixtures were estimated by comparing to known quantities of virion RNA subjected to electrophoresis in the same agarose gel. Virion RNA was purified from poliovirus type 1 (Mahoney) as described (15). Infectivities of the plasmid DNAs were less than 10 pfu/μg of DNA; in certain plates one plaque was observed.

consecutive cytosine residues on the 3′ side. Clearly, T7 RNA polymerase is capable of transcribing full-length polio DNA efficiently in either direction.

Polio RNA Produced by Transcription Is Infectious. Plasmid pPV16 DNA, the source of the polio DNA in pT7PV1-2 and pT7PV1-4, has a specific infectivity of 10–20 pfu/μg of DNA when tested on COS cells, but little and sometimes no detectable infectivity on HeLa cells. Plasmid pT7PV1-2 and pT7PV1-4 DNAs both had infectivities similar to pPV16 DNA, indicating that no rearrangements inactivated the polio DNA during construction of these plasmids.

HeLa cells were used to test the infectivity of transcripts produced from pT7PV1-2 and pT7PV1-4 DNAs by T7 RNA polymerase without further purification of the RNA, because any background from infective plasmid DNA would be minimal in these cells (Table 1). Template DNA was linearized by cutting at either the *Pvu* I site or the *Sal* I site (see Fig. 1). Control RNA prepared from virions had a specific infectivity averaging around 2×10^6 pfu/μg. As expected, template DNA itself had very low levels of infectivity, and (−)RNA produced from pT7PV1-2, showed no detectable infectivity. However, RNA produced from pT7PV1-4, which should contain full-length polio (+)RNA embedded in unrelated RNA, was infectious at a level of $1–2 \times 10^3$ pfu/μg, whether template DNA was linearized at the *Pvu* I or the *Sal* I site. This is at least two orders of magnitude higher than the background level of DNA infectivity, but still only 0.1% the level obtained with virion RNA. Further controls (Table 1) showed that the infectivity produced by T7 RNA polymerase from pT7PV1-4 DNA is lost upon treatment with RNase, and that RNA purified away from the plasmid DNA is also infectious, although some infectivity was lost during purification.

These results show that purified T7 RNA polymerase can produce infectious polio RNA from cloned poliovirus cDNA. The specific infectivity is much higher than that of the cDNA clones but remains well below that of RNA isolated from virions. It seemed likely that the additional nucleotides at either end of the polio RNA sequence might be a major reason the RNA produced *in vitro* has lower specific activity than virion RNA. Therefore, we constructed a plasmid from which T7 RNA polymerase should be able to produce polio RNA having only two additional guanine residues at the 5′ end and at most seven additional nucleotides beyond the poly(A) tract at the 3′ end.

Synthesis of Polio RNA with Minimal Additional Sequences. Almost all extra nucleotides at the 3′ end of the cloned polio DNA can be eliminated by cutting at the *Eco*RI site at the end of the poly(A) tract. The main problem was to eliminate the excess nucleotides at the 5′ end. We did this in three steps: (*i*) a small fragment containing the beginning of the polio cDNA was isolated and the nucleotides ahead of the polio sequence were removed; (*ii*) the *ϕ10* promoter was joined to this fragment so that the first nucleotide of the polio sequence is the third nucleotide transcribed by T7 RNA polymerase; and (*iii*) full-length polio DNA was reconstituted by joining this fragment to the rest of the polio cDNA. The specific steps were as follows (see Figs. 3 and 4).

An *Eco*RI–*Bam*HI fragment containing the poly(G) tract

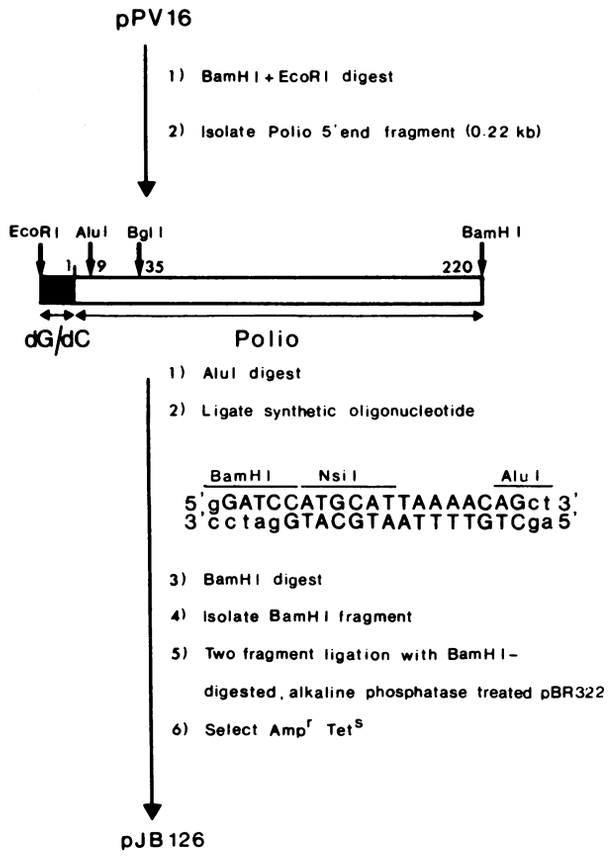

FIG. 3. Removal of the poly(dG/dC) tract from the end of polio cDNA. The nucleotide sequence of the synthetic DNA duplex is shown in capital letters; lower case letters have been added to show the restriction sites.

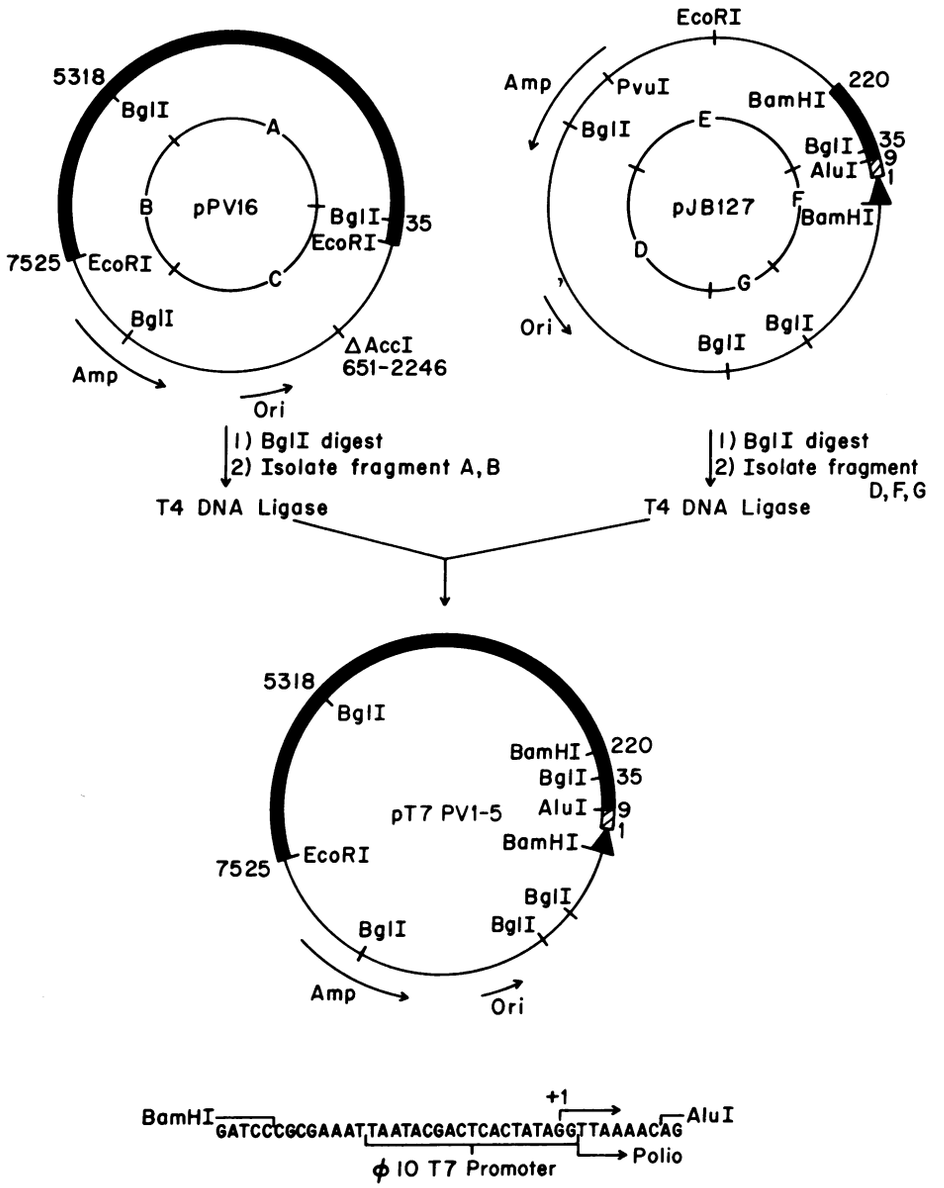

FIG. 4. Construction of poliovirus cDNA clone pT7PV1-5. At the bottom is indicated the sequence of the *ϕ10* promoter immediately preceding the poliovirus sequence. Transcripts are thought to be initiated with pppGGUUAAAA.

and the first 220 base pairs of poliovirus cDNA was isolated from pPV16 and digested with *Alu* I. To this digest was added a synthetic DNA duplex that, upon ligation to the *Alu* I–*Bam*HI fragment, would reconstruct nucleotides 1–9 of the polio cDNA and place restriction sites for *Bam*HI and *Nsi* I immediately ahead of the polio sequence. After ligation with T4 DNA ligase, the mixture was cut with *Bam*HI, and the desired fragment was purified and cloned into the *Bam*HI site of pBR322 to produce pJB126 (Fig. 3).

Plasmid pJB126 was cut at its unique *Nsi* I site, and the resulting 3′ extension was eliminated by digestion with Klenow fragment of *E. coli* DNA polymerase to produce a blunt-ended fragment beginning at the first nucleotide of polio DNA. The DNA extending from this site to the *Pvu* I site was ligated to the large *Pvu* I–*Stu* I fragment of pAR2369 to create plasmid pJB127, in which the first nucleotide of the polio sequence is located two nucleotides past the RNA start site of the *ϕ10* promoter (Fig. 4).

The complete polio DNA sequence was reconstituted by taking advantage of the cutting specificity of *Bgl* I, which cuts after the seventh nucleotide in the sequence GCCNNNNNGGC. As it happens, the five different fragments that must be ligated to reconstitute the desired plasmid will uniquely assemble in the correct orientation, and the plasmid was made simply by ligating the mixture of five fragments (Fig. 4). Restriction analysis and limited DNA sequencing verified that the desired plasmid, designated pT7PV1-5, had been obtained. Moreover, pT7PV1-5 DNA was infectious on COS cells at the level of pPV16 DNA (data not shown).

When plasmid pT7PV1-5 is linearized by cutting at the unique restriction sites *Bgl* II, *Eco*RI, *Pvu* I, or *Sal* I, transcription by T7 RNA polymerase produced RNAs of the expected lengths (Fig. 2*D*). Transcription of the *Eco*RI-linearized plasmid with varying amounts of polymerase is shown in Fig. 2*E*. The product RNA should be poliovirus RNA preceded by two guanine residues and ending with 3–7 nucleotides contributed by the *Eco*RI linker. Indeed, the *in vitro* product comigrates with virion RNA and no evidence of degradation or premature termination is detectable (Fig. 2*E*). The specific infectivity of the RNA was found to be about 10^5 pfu/μg, about 5% that of virion RNA (Table 1). As will be detailed elsewhere, viruses isolated from the plaques were

indistinguishable from the parental poliovirus type 1 (Mahoney) when tested with a number of Mahoney-specific or type 1 Sabin-specific monoclonal antibodies. Moreover, the virus-induced protein synthesis showed a pattern characteristic of the parental virus isolate. Finally, primer extension experiments, using a synthetic oligodeoxynucleotide and viral RNA, showed that the 5′ termini of RNA of parental virus and that of recovered virus are identical. The transcripts originating from pT7PV1-5 had a terminus that was extended by one to two bases as expected (data not shown).

DISCUSSION

The ability to produce large amounts of infectious poliovirus RNA *in vitro* from cDNA clones will greatly facilitate studies of poliovirus function and may also have practical advantages in the production of seed virus for vaccines. The cloned gene for T7 RNA polymerase provides a convenient and ample source of this enzyme (8), so virtually unlimited amounts of infectious or variant polio RNA can be produced in a highly reproducible manner. And the well-developed *in vitro* mutagenesis and recombinant DNA technology should allow construction of a wide variety of well-defined polio variants.

The minimum complete polio RNA we have produced so far *in vitro* is predicted to contain two guanine residues ahead of the polio sequence and three to seven nucleotides specified by the *Eco*RI linker after the poly(A) tract at the 3′ end (the exact number depending on where the runoff transcripts end). It may be possible to use appropriate *in vitro* RNAs to determine the mechanism by which the normal poliovirus RNA is generated from RNAs that have different numbers and sequences of additional nucleotides before and/or after the polio sequence. We have already determined that the virion RNA derived from the minimum *in vitro* RNA has a normal 5′ end. Perhaps replication of the polio RNA begins with a VPgpU(U) primer, which can prime initiation internally to generate the normal end, as proposed by Omata *et al.* (6).

The *in vitro* polio RNAs should prove very useful in the study of translation of polio RNA and processing of the polyprotein. Indeed, the transcripts having 60 additional nucleotides at the 5′ end are efficiently translated in a reticulocyte lysate to produce a protein pattern indistinguishable from that obtained with virion RNA (ref. 16; unpublished results in collaboration with G. Drugeon and A.-L. Haenni, Institut Jacques Monod, Université Paris 7). T7 RNA polymerase is capable of initiating transcripts with synthetic eukaryotic mRNA cap sequences (unpublished results), as has already been reported for the similar SP6 RNA polymerase (17). Although polio RNA is not normally capped during infection, capping of the *in vitro* RNA might be expected to improve the stability and translational efficiency of these RNAs in the *in vitro* systems and perhaps *in vivo* as well.

The techniques we have used here can in principle be applied to produce intact RNA for any RNA virus. Whether it will be possible to synthesize RNA without additional residues at the 5′ or 3′ ends and whether RNAs with additional residues will be infectious, will depend on individual peculiarities. However, it seems likely that T7 RNA polymerase will be able to produce large amounts of complete RNA from almost any DNA that is linked to it and that such RNA can be useful for defining viral functions both *in vivo* and *in vitro*.

Note Added in Proof. Synthesis of infectious rhinovirus RNA by SP6 polymerase has been achieved by S. Mizutani and R. J. Colonno (18).

We thank J. Robert Putnak for performing the initial infectivity tests of clones pT7PV1-2 and pT7PV1-4; Bert L. Semler for providing clone pPV16; Vincent Racaniello for a probe of an oligodeoxynucleotide primer; Richard Kuhn for discussions; and Marc Girard for his interest and support. S.v.d.W. was a visitor under the auspices of an exchange program between the Université 7, Paris, and State University of New York at Stony Brook. This work was supported by grants of the National Institute of Allergy and Infectious Diseases (AI15122) and the National Cancer Institute (CA28146) to E.W.; the research carried out at Brookhaven National Laboratory was sponsored by the U.S. Department of Energy.

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