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In vitro synthesis of infectious poliovirus RNA

(RNA-dependent RNA polymerase/picornavirus/SP6 polymerase)

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ABSTRACT **Replication of the infectious RNA genome of poliovirus is accomplished in cells by the viral RNA polymerase through negative-strand RNA intermediates. Full-length negative-strand poliovirus RNA was synthesized *in vitro* by transcription of infectious poliovirus cDNA with bacteriophage SP6 DNA-dependent RNA polymerase. When provided with this negative-strand RNA as template, the poliovirus RNA-dependent RNA polymerase synthesized full-length positive-strand molecules. The positive-strand RNAs synthesized *in vitro* were infectious when transfected into HeLa cells. In contrast, positive-strand copies of poliovirus RNA synthesized *in vitro* by SP6 polymerase, using a poliovirus cDNA template, were not infectious. Production of infectious positive-strand RNA by the poliovirus polymerase was not observed when magnesium or negative-strand RNA template was omitted from the reaction mixture. Infectivity of the product RNA was not destroyed by DNase treatment. The specific infectivity in HeLa cells of *in vitro*-synthesized positive-strand RNA was 4 × 10^4 plaque-forming units/μg of RNA.**

The poliovirus genome is an infectious single-stranded RNA molecule of 7.5 kilobases (kb) (1–4). In infected cells, the first step in replication of the viral genome is copying of the incoming, positive-strand RNA by the viral RNA polymerase to form negative-strand RNA (5). New viral positive strands are then produced by replication of the negative-strand templates. The *in vitro* synthesis of negative-strand RNA from positive-strand templates has been studied extensively, using purified components (6–10). However, the *in vitro* synthesis of positive-strand RNA has not been well characterized, because of the difficulty in obtaining negative-strand template.

The recent development of prokaryotic *in vitro* transcription systems employing bacteriophage DNA-dependent RNA polymerases has made it possible to produce large amounts of RNA transcripts of a defined polarity (11). We have used the *Salmonella* SP6 phage RNA polymerase to synthesize full-length negative-strand copies of poliovirus RNA, using cloned cDNA as a template. When used as a template for the poliovirus polymerase, the negative-strand RNAs are copied into full-length positive strands. It will now be possible to use these synthetic RNAs to study the *in vitro* replication of the viral negative strand.

We have found that viral positive-strand RNA synthesized *in vitro* from negative-strand templates is highly infectious when transfected into mammalian cells. This property will be useful for studying the poliovirus genome by mutagenesis of an infectious cDNA clone (12). Using the method reported here, it will be possible to synthesize viral positive-strand RNA *in vitro* using a specifically mutated cDNA as a template, transfect the positive-strand RNA into cells, and perform biochemical analyses directly on the transfected cells to identify the functional defects imparted by lethal or nonlethal mutations.

MATERIALS AND METHODS

SP6 Polymerase Templates. Construction of plasmids containing poliovirus-specific sequences for use as templates for *in vitro* synthesis of RNA by SP6 polymerase will be described in detail elsewhere. Briefly, *Sac* I oligonucleotide linkers were added to the ends of a full-length cDNA copy of the poliovirus type 1 Mahoney genome (pVR106; ref. 4). The cDNA was inserted in the SP6 transcription vector pSP64 (11) in both orientations with respect to the SP6 promoter. *In vitro* transcription of pSP64(PV5) and pVR123 will produce positive- and negative-strand RNAs, respectively. Plasmid pVR207 contains a full-length cDNA copy of the poliovirus type 2 Lansing genome (obtained from pVR204; ref. 13) inserted in the *Pst* I site of pSP64 so that positive-strand transcripts are generated by *in vitro* transcription. All three plasmids are infectious when transfected into HeLa cells (data not shown).

***In Vitro* Transcription.** RNA transcripts were produced from linear DNA templates by using SP6 polymerase as described (11). For transcription, pVR123 and pSP64(PV5) were digested with *Eco*RI and pVR207 was digested with *Sac* I. To produce capped transcripts, the cap analogue m^7GpppG (Pharmacia P-L Biochemicals) was included in the transcription reaction at 500 μM and the GTP concentration was reduced to 20 μM, as described (14).

Poliovirus Replicase Reactions. Poliovirus replicase was purified by phosphocellulose chromatography (6, 15). The phosphocellulose-purified replicase (fraction II) was further purified either by step elution (fraction III) or gradient elution (fraction IV) of proteins from poly(U)-Sepharose 4B (15). The standard 50-μl reaction mixture for negative-strand RNA-dependent replicase activity contained 50 mM Hepes (pH 8.0), 5 mM magnesium acetate, 4 mM dithiothreitol, 50 μM $ZnCl_2$, 10 μg of actinomycin D per ml, three unlabeled nucleoside triphosphates at 0.25 mM each, the nucleoside [α-^{32}P]triphosphate (10,000–20,000 cpm/pmol) at 5–10 μM, 250 ng of negative-strand RNA template, 2400 units of human placental RNase inhibitor/ml, and 4 μg of fraction III replicase. For host factor-dependent replicase reactions, fraction III replicase was replaced with 1 μg of fraction IV replicase and 0.05 μg of fraction VI host factor (16). Incubation was for 1 hr at 30°C. The RNA products were either precipitated with trichloroacetic acid for determination of acid-insoluble radioactivity on membrane filters or analyzed on methylmercuric hydroxide/agarose gels (17) after phenol extraction and ethanol precipitation. For synthesis of infectious poliovirus RNA on negative-strand template, reaction conditions were the same as described above except that all four ribonucleoside triphosphates (0.25 mM each) used were unlabeled.

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Abbreviations: kb, kilobase(s); pfu, plaque-forming unit(s).

Preparation of Double-Stranded Replicase Products. To prepare double-stranded replicase product for hybridization studies, the [^{32}P]UMP-labeled product of a 200-μl reaction mixture containing 2 μg of negative-strand RNA template and 20 μg of fraction III replicase was purified by phenol extraction and ethanol precipitation. The product was treated with pancreatic ribonuclease (125 μg/ml) in 0.3 ml NaCl/0.03 M sodium citrate for 30 min at 24°C, after which the enzyme was inactivated with 0.5% diethylpyrocarbonate for 30 min at 20°C. The remaining double-stranded RNA was isolated by phenol extraction and ethanol precipitation.

RNA Transfection. HeLa S3 cells (4×10^6) were plated in 6-cm plastic tissue culture dishes and transfected with RNA 24 hr later, using diethylaminoethyl (DEAE)-dextran as a facilitator (18). After transfection, the monolayers were covered with an agar overlay and plaques were counted 48–72 hr later as described (12).

RESULTS

Testing Infectivity of Poliovirus SP6 Transcripts. The low infectivity of poliovirus cDNA in HeLa cells (12) precludes the analysis of defects imparted by lethal mutations. Since poliovirus RNA is highly infectious (19), one solution to this problem would be to synthesize positive-strand poliovirus RNA *in vitro* from a cDNA template, using SP6 polymerase. To test this approach, plasmid pSP64(PV5) was constructed by inserting full-length poliovirus type 1 cDNA into an SP6 transcription vector (11) so that *in vitro* transcription with SP6 polymerase would produce a positive-strand RNA. Transcripts obtained with this DNA template were electrophoresed in 1.3% agarose gels containing methylmercuric hydroxide (Fig. 1, lane 1). Two distinct RNAs are visible, one that appears to be the same size as poliovirion RNA (7.5 kb, lane 6) and one of ≈0.8 kb. The 0.8-kb RNA is not observed when type 1 negative strands are synthesized *in vitro* (Fig. 1, lane 5) or when poliovirus type 2 positive-strand RNA is synthesized (lane 4). The small RNA is also observed when type 1 positive strands are synthesized with bacteriophage T7 RNA polymerase, using the T7 promoter (data not shown). The results of transcription experiments using plasmids containing different fragments of poliovirus cDNA indicate that the small RNA observed during synthesis of type 1 positive strands is produced by transcription termination within sequences near the 5′ end of poliovirus RNA (data not shown).

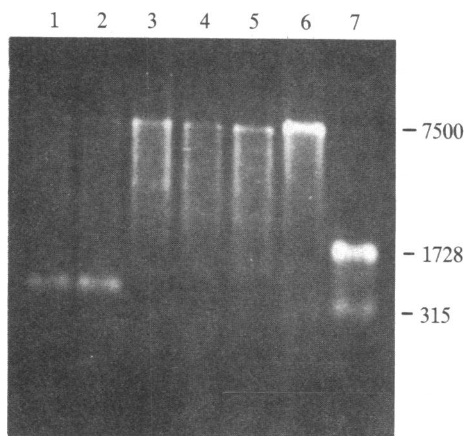

FIG. 1. Products of *in vitro* transcription of poliovirus cDNA-containing plasmids with SP6 polymerase. Transcripts were denatured with methylmercuric hydroxide, electrophoresed in a 1.3% agarose gel containing methylmercuric hydroxide, and stained with ethidium bromide. Lanes: 1, capped positive-strand poliovirus type 1 transcripts; 2, uncapped positive-strand poliovirus type 1 transcripts; 3, capped positive-strand poliovirus type 2 transcripts; 4, uncapped positive-strand poliovirus type 2 transcripts; 5, negative-strand poliovirus type 1 transcripts; 6, poliovirion RNA; lane 7, size markers produced by *in vitro* transcription, with T7 RNA polymerase, of pT7.0 (U.S. Biochemical, Cleveland, OH) that had been cleaved with *Ban* II, *Sal* I, and *Pvu* II to generate RNAs of 1728, 315, and 132 nucleotides (the smallest RNA is not visible).

The infectivity of the type 1 positive-strand SP6 transcript was tested by transfection of HeLa cells. No infectivity could be detected when up to 5 μg of total RNA transcripts were transfected into 4×10^6 cells. Under identical transfection conditions, 100 pg of poliovirion RNA produced ≈100 plaques. Lack of infectivity of the positive-strand SP6 transcript was not due to inhibition by the 0.8-kb termination product, since type 2 positive-strand SP6 transcripts, which do not contain this small RNA, were also not infectious. Furthermore, when type 1 positive-strand transcripts were mixed with virion RNA, the transfection efficiency of virion RNA was not changed (data not shown). It was possible that the positive-strand SP6 transcripts were not infectious because they were degraded within the cells. Since it has been shown that the presence of a 5′-terminal cap structure increases the stability of RNAs (20), reactions were performed in the presence of m^{7}GpppG to produce capped transcripts (14). Capped, full-length positive-strand type 1 and 2 RNAs were synthesized (Fig. 1, lanes 1 and 3), but these RNAs were not infectious in HeLa cells.

All poliovirus-specific RNAs produced by transcription of pSP64(PV5) and pVR123 with SP6 polymerase contain 190 and 82 bases of extra, nonviral sequences at their 5′ and 3′ ends, respectively. These extra sequences are derived from the multiple cloning site of the SP6 vector (11) and from oligo(dG)·oligo(dC) tails present at the termini of the poliovirus cDNA clones (4). It was possible that these extra sequences were interfering with the infectivity of the positive-strand template. New cDNA templates were constructed that did not contain oligo(dG)·oligo(dC) tails, but the positive-strand RNAs produced from these templates were not infectious (data not shown). Because it was technically difficult to remove the remaining nonviral sequences from the DNA templates, an alternative strategy for synthesizing infectious poliovirus RNA was employed.

Copying of SP6 Negative Strand RNA by Poliovirus Replicase. The strategy devised for the synthesis of positive-strand RNA is shown in Fig. 2. In the first step, negative-strand poliovirus RNA is synthesized from a DNA template using SP6 polymerase. After removal of the plasmid DNA, the negative-strand RNA is used as template for poliovirus replicase. If the viral replicase is able to initiate copying of the template specifically at polioviral sequences, then the positive-strand product might have the correct 5′ end required for infectivity.

To test this hypothesis, a full-length poliovirus negative-strand RNA was synthesized using SP6 polymerase and plasmid pVR123 DNA as template. This negative-strand RNA (Fig. 1, lane 5), which contained nonviral sequences at its ends, was used as a template for the synthesis of positive-strand RNA catalyzed by the poliovirus replicase. The poliovirus replicase synthesized acid-precipitable RNA products when supplied with the negative-strand template (Fig. 3). The reaction was almost linear up to an hour, after which RNA synthesis reached a plateau. The reaction was completely dependent on added template and since we used fraction III replicase, which already contained host factor (15), exogenous addition of host factor was not necessary for RNA-dependent RNA synthesis. A more purified poliovirus replicase preparation (fraction IV), however, was completely dependent on added host factor in the reaction for negative-strand-dependent RNA synthesis (Table 1). The host factor-dependent RNA synthesis was completely dependent on

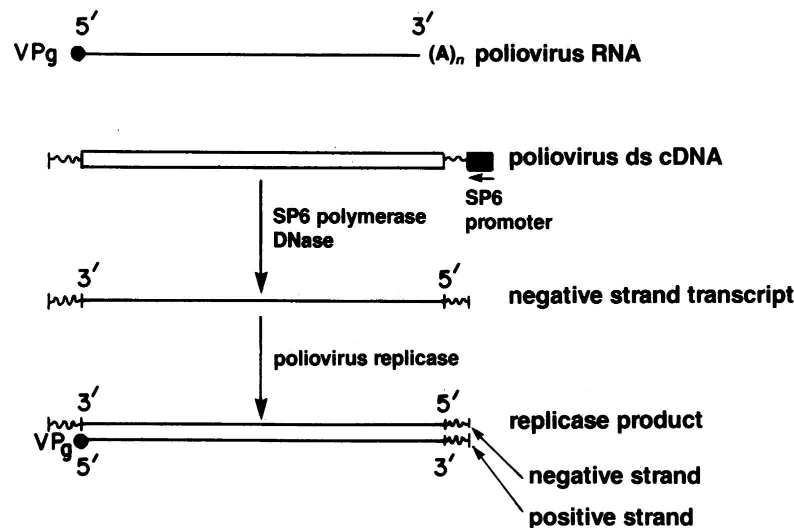

FIG. 2. Scheme for the *in vitro* synthesis of infectious poliovirus RNA. SP6 polymerase-directed transcription of cloned poliovirus cDNA in a vector containing the SP6 promoter produces negative-strand poliovirus RNA. The DNA template is destroyed by DNase treatment. Poliovirus replicase is used to copy the negative strand into a positive strand product that most likely remains hybridized to the template. The terminal structure of the positive strand transcript is not known but is shown here containing 5′ linked VPg (the 5′-terminal protein), initiating internally at poliovirus sequences, and copied to the end of the template. Curly lines represent nonviral sequences found at the ends of the cDNA and the RNA transcripts. ds, Double-stranded.

added magnesium and all four ribonucleoside triphosphates. Omission of zinc from the reaction mixture resulted in an ≈75% decrease in RNA synthesis. This result was expected since the activity of poliovirus replicase is stimulated by added zinc (8). Poliovirus replicase-catalyzed synthesis of negative-strand RNA from a plus-strand template is almost completely inhibited by 100 mM KCl (8). As can be seen in Table 1, the negative-strand-dependent RNA synthesis by the poliovirus replicase was also sensitive to KCl; about 25% of the replicase activity remained at 100 mM KCl.

Ninety-five percent of the product made by the fraction III replicase was ribonuclease-resistant (data not shown). To determine whether the product was a copy of the added negative-strand RNA, we isolated, denatured, and renatured the ribonuclease-resistant product either by itself or in the presence of negative-strand RNA, poliovirion RNA (positive strand), or a heterologous RNA (ribosomal RNA). Poliovirus negative-strand RNA could drive almost all of the product into a ribonuclease-resistant form, whereas the self-annealed product or the product annealed with poliovirion RNA or with rRNA showed only about 25% resistance to ribonuclease (Table 2). The high background of ribonuclease resistance in the self-annealed samples is most probably due to the presence of excess negative-strand RNA, used in the reactions as template. However, it was clear from the experiment that almost all of the *in vitro*-synthesized RNA was complementary to the negative-strand template.

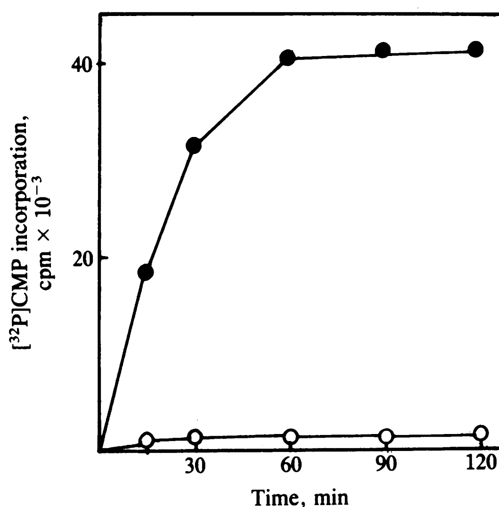

FIG. 3. Kinetics of negative-strand-RNA-dependent RNA synthesis catalyzed by fraction III replicase. Reaction mixture with (●) or without (○) 250 ng of negative-strand RNA and with 4 μg of replicase was incubated at 30°C, and at indicated times aliquots were removed and the amount of acid-insoluble radioactivity was determined.

The products of this replicase reaction ranged in size up to 7.5 kb (Fig. 4, lane 2). The largest replicase product comigrated with ^{32}P-labeled poliovirion RNA (lane 1) and the product made by copying poliovirion RNA with replicase (lane 3). Synthesis of these products was dependent upon the presence of magnesium (lane 4) and the presence of the SP6 negative-strand template (lane 6) in the reaction mixture. Despite the presence of terminal nonviral sequences in the template, it appears that poliovirus replicase is able to copy the negative-strand SP6 transcripts into a product that comigrates with full-length poliovirus RNA.

Infectivity of RNA Synthesized by Poliovirus Replicase. The RNA products synthesized by poliovirus replicase using negative-strand RNA templates were tested for infectivity by

Table 1. Characteristics of viral positive-strand synthesis *in vitro*

Omission or addition	[^{32}P]CMP incorporated, pmol
None	3.0
− Negative-strand RNA	0.08
− Replicase	0.1
− Host factor	0.035
− Mg^{2+}	0.1
− Three NTPs	0.18
− Zn^{2+}	0.8
+ 50 mM KCl	1.0
+ 100 mM KCl	0.7

Replicase reaction mixtures were as described in *Materials and Methods*, with fraction IV replicase and fraction VI host factor.

Table 2. Hybridization of replicase product to different RNAs

Added RNA	% ribonuclease-resistant
None	23
Poliovirion (positive strand)	26
Negative strand	98
rRNA	26

About 5 ng of double-stranded ^{32}P-labeled product of fraction III replicase copying negative-strand RNA template was prepared. The RNA pellet was dissolved in 20 μl of H_2O and boiled for 5 min in the presence of 80 ng of various "driver" RNAs. The mixture was adjusted to 0.5% $NaDodSO_4$/0.45 M NaCl/45 mM sodium citrate, and the RNAs were allowed to hybridize at 70°C for 1 hr. Samples were chilled on ice and split into equal portions; one portion was treated with 125 μg of pancreatic ribonuclease and 25 units of T_1 ribonuclease per ml. Acid-precipitable radioactivity was determined. Background radioactivity from a boiled but unannealed sample was subtracted (≈10%), and the percentage of labeled RNA resistant to ribonuclease was calculated.

transfection of HeLa cells (Table 3). When 1 ng of replicase product (amount based on negative-strand template) was transfected, 39 plaques were observed. Denaturation of the product RNA did not alter its infectivity, an expected result since poliovirus double-stranded RNA is infectious (21). Omission of magnesium or negative-strand template from the replicase reaction abolished infectivity of the product, indicating that the replicase preparations were not contaminated with infectious viral RNA. Furthermore, DNase treatment of the reaction product did not destroy infectivity, demonstrating the absence of DNA template carried over from the SP6 transcription. Under similar conditions, DNase destroyed the infectivity of 10 μg of the double-stranded DNA template pVR123 (data not shown). When capped and uncapped positive-strand RNAs were provided as templates for the viral replicase, up to full-length RNAs were synthesized as judged by agarose gel electrophoresis (data not shown). These negative-strand products were not infectious in HeLa cells (Table 3).

As shown in Table 3, ribonuclease treatment of the reaction product destroyed infectivity. This result was unexpected because most of the radioactivity incorporated into

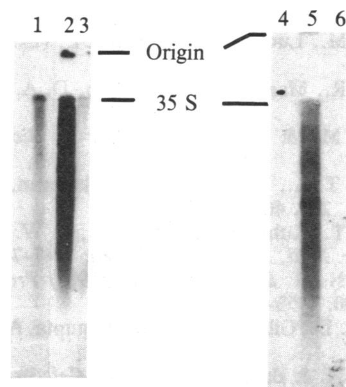

FIG. 4. Products of poliovirus replicase reactions. Reaction products were denatured with methylmercuric hydroxide and electrophoresed in a 1.3% agarose gel containing methylmercuric hydroxide. An autoradiograph of the gel is shown. Lanes: 1, poliovirion RNA labeled *in vivo* with [^{32}P]phosphate; 2, replicase product using as template negative-strand SP6-transcribed RNA; 3, replicase products using poliovirion RNA as template; 4–6, replicase products, using SP6-transcribed negative strand as template, without magnesium (lane 4), of the complete reaction mixture (lane 5), and without template (lane 6).

Table 3. Infectivity of RNA products obtained by *in vitro* copying of positive- and negative-strand SP6 RNA with poliovirus replicase

Template	Replicase reaction	pfu/ng[a]
Negative strand[b]	Complete	39
	Complete, boiled[c]	41
	Complete, DNase[d]	37
	$-Mg^{2+}$	0
	Complete, RNase[e]	0
None	−Template	0
Positive strand[f]		
Capped	Complete	0
Uncapped	Complete	0
Poliovirion RNA	None	940[g]
Poliovirion RNA + negative strand[h]	None, RNase	0.04[g]

[a]Plaques/ng of template RNA, unless otherwise noted.
[b]RNA templates produced by transcription of pVR123 with SP6 polymerase.
[c]Replicase product boiled and cooled before transfection.
[d]Replicase product (1 ng) treated with RNase-free DNase (Worthington DPRF, 50 μg/ml) before transfection.
[e]Replicase products treated with 40 pg of RNase A and 2 pg of RNase T_1 (11) before transfection.
[f]RNA templates produced by transcription of pSP64(PV5) with SP6 polymerase.
[g]Plaques/ng of poliovirion RNA.
[h]Virion RNA (100 ng) and negative-strand RNA (100 ng) hybridized (11) and digested with RNase (see e).

the replicase product was ribonuclease-resistant. To determine whether the negative-strand template was able to fully protect the infectivity of virion RNA from ribonuclease digestion, virion RNA was hybridized to negative-strand RNA produced by transcription of pVR123. The hybridization mixture was treated with ribonuclease and then tested for infectivity by transfection into HeLa cells. The infectivity of poliovirion RNA treated in this way was 0.04 plaque-forming unit (pfu)/ng, compared to 940 pfu/ng of untreated virion RNA (Table 3). This result indicates that *in vitro*-synthesized negative-strand RNA cannot fully protect positive-strand RNA from ribonuclease digestion.

DISCUSSION

The replication of poliovirus RNA has been studied extensively in defined *in vitro* systems (6–10). When provided with virion positive strands as a template, the viral replicase is able to synthesize full-length negative-strand RNA (8, 16, 22). The requirements and specificity of this reaction have been defined; based on these and other studies, models for *in vivo* replication of RNA have been proposed (23–25). Replication of the viral negative strand has not been examined extensively, because it is difficult to obtain reasonable quantities of negative-strand RNAs. Poliovirus negative-strand RNA must be purified from infected cells, where it is found only in the double-stranded replicative form and in the replicative intermediate (1). Here we show that it is possible to employ the bacteriophage SP6 RNA polymerase to direct the synthesis of full-length negative-strand RNA copies of poliovirus RNA. These negative-strand RNAs can be copied by the poliovirus replicase into an infectious, positive-strand RNA. These results show that the polymerase that copies poliovirus negative strands is the same one that copies positive strands.

The exact nature of the RNA product synthesized by poliovirus replicase when provided with the SP6-generated negative-strand template is not known. The negative-strand RNA contains extra, nonviral sequences at its termini (82 nucleotides at the 3′ end and 190 nucleotides at the 5′ end).

It is not possible to determine by size analysis of the product whether the replicase begins copying at the exact 3′ end of this template or whether it initiates internally at poliovirus-specific sequences. If the replicase initiated synthesis exactly at the 3′ end of the SP6 negative strand, then the 5′ end of the positive-strand product would contain an extra sequence. This product would probably be noninfectious, as we have shown for positive-strand SP6 transcripts that contain terminal nonviral sequences. Another difference between positive strands produced by replicase and those produced by SP6 polymerase might be the presence of the 5′-terminal protein, VPg. It is known that VPg is present on the products of *in vitro* replicase reactions (9, 26). However, VPg is not required for infectivity (27).

We do not know whether the replicase copies the entire negative-strand template or whether it terminates synthesis at the end of poliovirus-specific sequences in the 5′ end of the template. The sequence at the 3′ end of the positive-strand product might also be important for infectivity. Clearly, it will be necessary to determine the nucleotide sequences of the 5′ and 3′ ends of the positive-strand transcripts to resolve these questions.

It is not clear why the infectivity of the replicase product is destroyed by ribonuclease treatment (Table 3). However, the negative-strand template RNA is not able to fully protect the infectivity of virion RNA against ribonuclease digestion (Table 3). Perhaps the termini of the synthetic negative strands, which contain extra, nonviral sequences, do not properly hybridize with the ends of the positive-strand RNA. Subsequent cleavage of the positive-strand RNA near the termini would destroy infectivity. Since ribonuclease treatment of the negative-strand·virion RNA hybrid reduces infectivity by a factor of 10,000, a similar reduction in infectivity of the *in vitro* replicase products after ribonuclease treatment would drop infectivity below levels of detection.

The infectivity of *in vitro*-synthesized RNA reported here (4×10^4 pfu/μg) is calculated on the assumption that all the negative-strand template provided to the replicase is converted into positive-strand product. It is clear (Fig. 4, lanes 2 and 5) that only a small fraction of positive-strand product is full-length, and therefore its specific infectivity might be underestimated by a factor $\geq$10.

We have been studying functions of the poliovirus genome by mutagenizing infectious poliovirus cDNA and searching for viral mutants after transfecting the altered DNA into HeLa cells. A large proportion of the altered cDNAs are noninfectious (unpublished results), and it is not possible to study the defect in these cDNAs because the efficiency of transfection is too low. For example, a specific infectivity of 10^2 pfu/μg has been reported for poliovirus cDNA that has been placed under the control of the simian virus 40 promoter (28). At this level of infectivity, transfection of 10^6 HeLa cells with 10 μg of poliovirus cDNA would result in successful infection of only 10^3 cells (0.1%). Infectivity of 1.7×10^3 pfu/μg can be obtained when the same poliovirus cDNA-containing plasmid is transfected into COS cells (28). However, even if transfections were performed in COS cells, only about 1.7% of the cells could be successfully infected. These low levels of infectivity preclude the examination of virus-specific RNA and protein synthesis in transfected cells. In contrast, it has been reported that transfection of 10^6 cells with 10 μg of virion RNA results in infection of 60% of the cells (29). Using the method reported here, it should be possible to synthesize negative-strand RNA *in vitro* from mutated cDNA templates and then produce positive strands from the negative strands by using poliovirus replicase. The mutated positive strand RNA would then be used to transfect HeLa cells. Since a large fraction of cells will take up RNA, biochemical analyses could be performed directly on the transfected cells to determine the nature of the defect imparted by the original mutation. The ability to produce infectious RNA from a cDNA template expands the range of experimental manipulations that can be used to study the replication and genetics of poliovirus as well as other picornaviruses.

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