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Synchronous Replication of Poliovirus RNA: Initiation of Negative-Strand RNA Synthesis Requires the Guanidine-Inhibited Activity of Protein 2C

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We report that protein 2C, the putative nucleoside triphosphatase/helicase protein of poliovirus, is required for the initiation of negative-strand RNA synthesis. Preinitiation RNA replication complexes formed upon the translation of poliovirion RNA in HeLa S10 extracts containing 2 mM guanidine HCl, a reversible inhibitor of viral protein 2C. Upon incubation in reactions lacking guanidine, preinitiation RNA replication complexes synchronously initiated and elongated negative-strand RNA molecules, followed by the synchronous initiation and elongation of positive-strand RNA molecules. The immediate and exclusive synthesis of negative-strand RNA upon the removal of guanidine demonstrates that guanidine specifically blocks the initiation of negative-strand RNA synthesis. Readdition of guanidine HCl to reactions synchronously elongating nascent negative-strand RNA molecules did not prevent their continued elongation and completion. In fact, readdition of guanidine HCl to reactions containing preinitiation complexes elongating nascent negative-strand RNA molecules had no effect on subsequent positive-strand RNA synthesis initiation or elongation. Thus, the guanidine-inhibited function of viral protein 2C was not required for the elongation of negative-strand RNA molecules, the initiation of positive-strand RNA molecules, or the elongation of positive-strand RNA molecules. The guanidine-inhibited function of viral protein 2C is required only immediately before or during the initiation of negative-strand RNA synthesis. We suggest that guanidine may block an irreversible structural maturation of protein 2C and/or RNA replication complexes necessary for the initiation of RNA replication.

Poliovirus is the prototypic member of the *Picornaviridae* family of positive-strand RNA animal viruses. This family of viruses contains numerous pathogens that cause diverse maladies, including the common cold, myocarditis, meningitis, hepatitis, and poliomyelitis. Poliovirion RNA is about 7,500 nucleotides long and contains a 5′-terminal protein, VPg, and a 3′-terminal poly(A) sequence. The viral replication cycle begins by translation of the infecting virion RNA. By mechanisms not currently understood, virion RNA is eventually cleared of translating ribosomes and a switch is made from translation to RNA replication. The initiation of viral RNA replication involves the synthesis of negative-strand RNA by using the infecting virion RNA as a template. This step is followed by the asymmetric synthesis of positive-strand RNA on the newly made negative-strand RNA template. For theoretical purposes, RNA replication can be described to consist of four temporally sequential steps: (i) initiation of negative-strand RNA synthesis, (ii) elongation of negative-strand RNA, (iii) initiation of positive-strand RNA synthesis, and (iv) elongation of positive-strand RNA.

Poliovirus RNA replication is known to occur in membrane-associated complexes that contain replicative-intermediate RNA and newly synthesized virion RNA (9). The exact composition of the replication complexes remains unknown, as does the specific role of individual viral proteins in RNA replication. Only $3D^{Pol}$ has been identified to possess a clear function, the catalytic polymerization of viral RNA (13, 41–43). The three viral proteases ($2A^{Pro}$, $3C^{Pro}$, and $3CD^{Pro}$) are involved in the proteolytic processing of the viral polyprotein (20) and may possess additional functions that are required during translation and RNA replication (2–4, 21). Viral protein 3AB is a membrane-associated protein thought to be the immediate precursor of VPg at the site of RNA replication (15). VPg is covalently linked to the 5′ end of both negative- and positive-strand RNAs and to nascent molecules within replicative-intermediate RNA (1, 14, 24, 29). Amino acid motifs within protein 2C exhibit a high degree of homology with known nucleoside triphosphatase (NTPase)/helicase proteins (16–18). Viral protein 2C is a membrane-associated component of the viral RNA replication complex (9, 10) and possesses ATPase and GTPase activities (25, 34). Mutagenesis of conserved residues within the NTPase domain of protein 2C in an infectious cDNA clone of poliovirus yielded nonviable RNA transcripts (26). Based on these results, it was concluded that the NTPase activity of protein 2C is required for one or more steps in the replication of poliovirus RNA.

Guanidine HCl is a reversible inhibitor of poliovirus RNA replication at millimolar concentrations. It is generally believed to inhibit a required function of protein 2C because mutations that impart guanidine-resistant and guanidine-dependent phenotypes upon mutant viruses map in the 2C coding sequence (5, 31–33, 40). Although the inhibition of poliovirus RNA replication by guanidine HCl has been extensively studied, the specific function of protein 2C that is inhibited and the specific steps in the RNA replication cycle that are blocked by guanidine remain unknown. Theoretically, guanidine could cause a gain of function for protein 2C that inhibits viral RNA replication. This seems less likely, however, in the absence of any experimental evidence for this possibility. Normally, guanidine is described as an inhibitor of viral RNA replication and is believed to inhibit a specific function of protein 2C that is required for RNA replication. Guanidine does not inhibit the ATPase activity of purified protein 2C (34). In poliovirus-

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infected cells, the synthesis of single-stranded RNA appears to be more sensitive to guanidine inhibition than is the formation of double-stranded RNA (reviewed in reference 23). Based on the results of studies using crude RNA replication complexes in vitro, it was suggested that guanidine inhibits the initiation of viral RNA synthesis without inhibiting the elongation or release of completed positive-strand RNA molecules (39). Overall, the accumulated evidence from previous studies (reviewed in reference 23) suggests that guanidine inhibits a function of protein 2C that is required for the initiation of positive-strand RNA synthesis in membrane-bound RNA replication complexes.

In more recent studies, it has been demonstrated that infectious poliovirus can be efficiently synthesized in vitro by using HeLa S10 extracts that contain purified virion RNA (6, 7, 27). These extracts support the translation and replication of poliovirus RNA and the assembly of infectious virus in a temporally regulated fashion. Poliovirus preinitiation RNA replication complexes form in HeLa S10 translation-RNA replication reactions containing 2 mM guanidine HCl (6). We have used the preinitiation RNA replication complexes to investigate poliovirus RNA replication and have shown that a soluble cellular factor is required to initiate viral RNA synthesis (6). In this study, we report that poliovirus preinitiation RNA replication complexes synchronously replicate poliovirus RNA upon the removal of guanidine HCl. Four temporally sequential steps of viral RNA replication were observed: (i) initiation of negative-strand RNA synthesis, (ii) elongation of negative-strand RNA, (iii) initiation of positive-strand RNA synthesis, and (iv) elongation of positive-strand RNA (Fig. 1). As expected, RNA replication was asymmetric in these reactions, with positive strands being made in excess over negative strands. Although guanidine HCl inhibited the initiation of negative-strand RNA synthesis, it inhibited neither the elongation of nascent negative-strand RNA molecules nor the initiation or elongation of positive-strand RNA molecules. Based on these results, we conclude that the guanidine-inhibited activity of protein 2C is specifically required immediately before or during the initiation of negative-strand RNA synthesis.

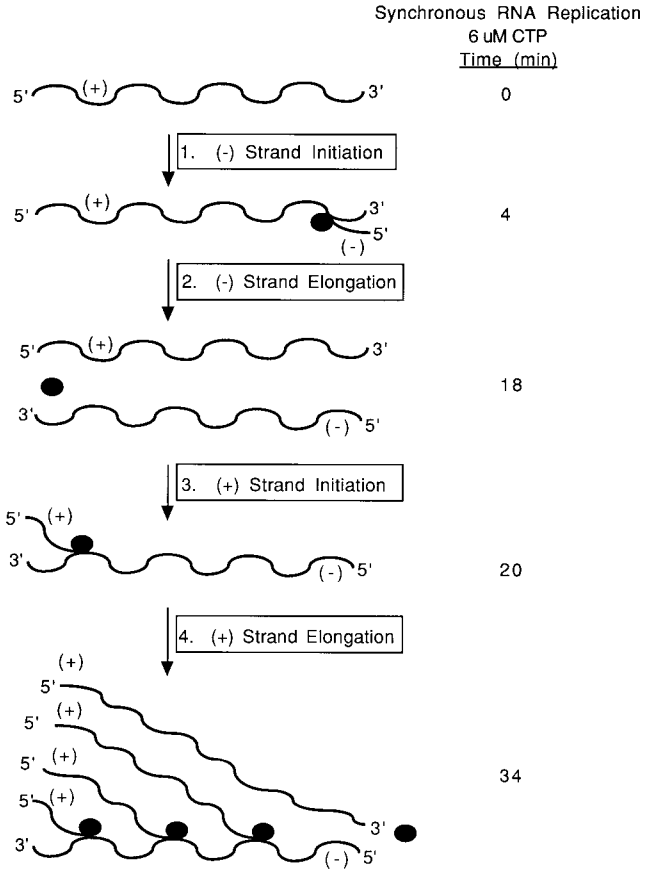

FIG. 1. Synchronous poliovirus RNA replication. Times correspond to data from Fig. 3.

MATERIALS AND METHODS

Cells and virus. HeLa S3 cells were maintained in suspension cultures at 37°C by daily passage at 2×10^5 to 4×10^5 cells per ml in Joklik's modified Eagle's medium (ICN Flow, Costa Mesa, Calif.) supplemented with 5% calf serum and 2% fetal clone serum (HyClone Laboratories, Logan, Utah). HeLa cells were collected by centrifugation and resuspended in fresh medium at 2×10^5 cells per ml 18 to 20 h prior to the preparation of the HeLa S10 extracts. Virus stocks were prepared by infecting cells with poliovirus type 1 (Mahoney strain) as described previously (44).

RNA preparation. Poliovirion RNA was purified from CsCl-banded virus as described previously (6).

Isolation of preinitiation RNA replication complexes. Preinitiation RNA replication complexes were formed during the translation of poliovirion RNA in HeLa S10 translation-replication reactions containing 2 mM guanidine HCl as described previously (6, 8). These reactions (50 μl, final volume) contained 25 μl of HeLa S10 extract, 10 μl of HeLa cell translation initiation factors, 5 μl of a 10× nucleotide reaction mix (10 mM ATP, 2.5 mM GTP, 2.5 mM CTP, 2.5 mM UTP, 600 mM KCH_3CO_2, 300 mM creatine phosphate, 4 mg of creatine kinase per ml, 155 mM HEPES KOH [pH 7.4]), 2 μl of 50 mM guanidine HCl, and 25 μg of poliovirion RNA per ml. Reactions were incubated at 34°C for 4 h to allow the preinitiation RNA replication complexes to form. Preinitiation RNA replication complexes were isolated from these reactions by centrifugation at 15,000 × *g* for 15 min at 4°C. The supernatant (S15) that contained soluble proteins and guanidine HCl was removed with a pipette and discarded. The pellet (P15) contained the preinitiation RNA replication complexes and was used in reaction mixtures to assay for viral RNA replication as described below.

RNA replication assays. RNA replication was measured by two different methods, designated methods 3 and 4 to be consistent with our previous publication (6).

(i) Method 3. RNA replication was assayed by labeling viral RNA synthesized in preinitiation RNA replication complexes resuspended in reactions containing [α-^{32}P]CTP as the labeled substrate, HeLa S10 extract, and HeLa translation initiation factors as previously described (method 3 in reference 6). The final concentration of CTP in these reactions was relatively high (~125 μM) due to the unlabeled CTP in the HeLa S10 extract.

(ii) Method 4. RNA replication was assayed by labeling viral RNA synthesized in preinitiation RNA replication complexes resuspended in reactions that did not contain added HeLa S10 extract or HeLa translation initiation factors. The pellet (P15) that contained the preinitiation RNA replication complexes was resuspended in 50-μl reactions containing 25 μl of S10 buffer [40 mM HEPES KOH (pH 7.4), 120 mM KCH_3CO_2, 5.5 mM $Mg(CH_3CO_2)_2$, 6 mM dithiothreitol, 10 mM KCl, 1 mM $CaCl_2$, 2 mM EGTA], 5 μl of 10× nucleotide reaction mix lacking CTP, and various amounts of [α-^{32}P]CTP at a final concentration of 5 μM as indicated. The reactions were incubated at 37°C for the indicated period of time, terminated by the addition of 350 μl of 0.5% sodium dodecyl sulfate (SDS) buffer (0.5% SDS, 10 mM Tris HCl [pH 7.5], 1 mM EDTA, 100 mM NaCl), extracted with phenol-chloroform-isoamyl alcohol, and ethanol precipitated. The reaction products were analyzed by electrophoresis in 1% CH_3HgOH-agarose gels as described previously (46). An RNA molecular weight ladder (Bethesda Research Laboratories) was used to determine the sizes of labeled RNA products. The gels were stained with ethidium bromide and photographed under UV light to visualize the RNA in each lane. The gels were then dried, and the radiolabeled RNA was detected by autoradiography.

One-dimensional RNase T_1 fingerprint analysis. ^{32}P-labeled viral RNA was denatured in 9 μl of CH_3HgOH buffer (50 mM CH_3HgOH, 50 mM H_3BO_3, 5 mM $Na_2B_4O_7 \cdot 10H_2O$, 10 mM Na_2SO_4, 1 mM Na_2EDTA [pH 8.2]) followed by the addition of 1 μl of 1 M β-mercaptoethanol and 1 μl of RNase T_1 (3 U/μl in 50 mM Tris [pH 7.5]–50% glycerol; Calbiochem Corporation). The labeled RNAs were digested with RNase T_1 at 37°C for 30 min, followed by the addition of 10 μl of 2× urea gel-loading buffer (18 M urea, 8.9 mM Tris base, 8.9 mM boric acid [pH 8.3], 0.2 mM EDTA, 20% [wt/vol] sucrose, 0.05% [wt/vol] bromophenol blue, 0.05% [wt/vol] xylene cyanol) and heating to 100°C for 3 min. The digested RNA was then analyzed by electrophoresis for 4.5 h at 25 W in a 0.4-mm-thick 20% polyacrylamide–7 M urea gel in TBE buffer (89 mM Tris base, 89 mM boric acid [pH 8.3], 2 mM EDTA). The positions of the labeled oligonucleotides in the gel were then detected by autoradiography. [^{32}P]CMP-labeled

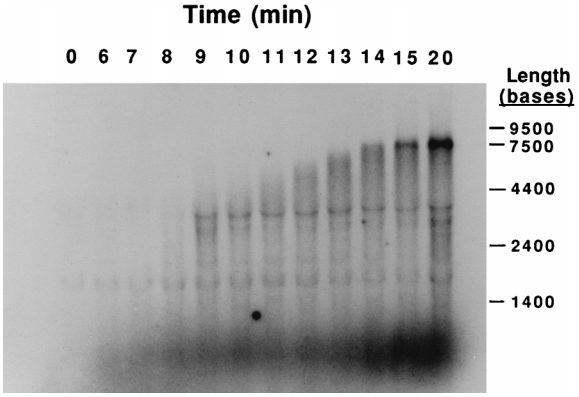

FIG. 2. Synchronous initiation and elongation of viral RNA within preinitiation complexes: low specific radioactivity. Preinitiation RNA replication complexes were isolated from a 700-μl HeLa S10 translation-replication reaction containing 2 mM guanidine HCl incubated at 34°C for 4.5 h. The preinitiation complexes were resuspended in a 700-μl reaction containing ~125 μM unlabeled CTP and 140 μCi of [α-^{32}P]CTP according to method 3 in Materials and Methods. The reaction was incubated at 37°C, and 50-μl aliquots were removed into 350 μl of SDS buffer at the indicated times, phenol extracted, and ethanol precipitated. The radiolabeled RNA products were separated after denaturation in CH_3HgOH by electrophoresis in 1% agarose and visualized by autoradiography. The positions of RNA size markers visualized by ethidium staining are indicated.

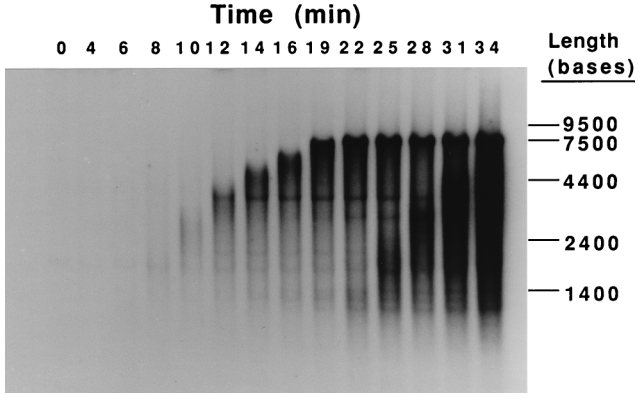

FIG. 3. Synchronous initiation and elongation of viral RNA within preinitiation complexes: high specific radioactivity. Preinitiation complexes were isolated from an 800-μl HeLa S10 translation-replication reaction containing 2 mM guanidine HCl incubated at 34°C for 4 h. The preinitiation complexes were resuspended in an 800-μl reaction containing 5 μM unlabeled CTP and 320 μCi of [α-^{32}P]CTP (final CTP concentration, 6 μM) as described for method 4 in Materials and Methods. The reaction was incubated at 37°C, and 50-μl aliquots were removed into 350 μl of SDS buffer at the indicated times, phenol extracted, and ethanol precipitated. The radiolabeled RNA products were separated after denaturation in CH_3HgOH by electrophoresis in 1% agarose and visualized by autoradiography. The positions of RNA size markers visualized by ethidium staining are indicated.

genome-length T7 polymerase transcripts of poliovirus cDNA clones were then digested with RNase T_1 and used as oligonucleotide markers specific for either positive- or negative-strand poliovirus RNA.

RESULTS

Replication of poliovirus RNA in preinitiation RNA replication complexes. Because guanidine is a reversible inhibitor of viral RNA replication and has no effect on viral protein synthesis, it was possible to isolate preinitiation RNA replication complexes from HeLa S10 reactions containing guanidine (6). Preinitiation RNA replication complexes synthesize labeled viral RNA and infectious virus when guanidine is removed (6). To characterize negative- and positive-strand RNA synthesis in these reactions, we used time course experiments to examine viral RNA made initially following the reversal of guanidine inhibition. When preinitiation RNA replication complexes were resuspended in fresh reactions containing HeLa S10 extract (method 3 in Materials and Methods), labeled nascent RNA products were observed from 9 to 13 min in these reactions (Fig. 2). These nascent RNA molecules were elongated at ~1,000 nucleotides/min and started to accumulate as a band of genome-length RNA by 15 min (Fig. 2). Based on an elongation rate of 1,000 nucleotides/min, genome-length negative-strand RNA should be synthesized in 7 to 8 min, followed by the synthesis of genome-length positive-strand RNA by 15 min. Because poliovirus RNA replication is highly asymmetric, the absence of any genome-length labeled viral RNA by 8 min in Fig. 2 may have resulted from our inability to detect very small amounts of labeled negative-strand RNA in this reaction. Therefore, we hypothesized that the wave of labeled RNA observed in Fig. 2 was elongating positive-strand molecules which utilized a negative-strand RNA template that was synthesized during the first 7 to 8 min of the reaction.

Synthesis of ^{32}P-labeled negative-strand RNA in vitro. To increase our ability to detect the synthesis of labeled negative-strand RNA, we modified the reaction conditions to increase the specific radioactivity of the [^{32}P]CTP substrate. In the experiment shown in Fig. 2, the preinitiation RNA replication complexes were resuspended in a fresh reaction mixture that contained HeLa S10 extract (method 3 in Materials and Methods). We estimated that the final CTP concentration in this reaction was ~125 μM due to the fact that some unlabeled CTP is present in the HeLa S10 extract. Previously, we showed that a soluble cellular factor present in HeLa S10 extracts is required to initiate viral RNA replication (6). Therefore, we routinely resuspended preinitiation RNA replication complexes in reaction mixes containing HeLa S10 extract. In subsequent experiments, however, we found that preinitiation RNA replication complexes isolated from translation reactions at 34°C were still active when resuspended in reactions without added HeLa S10 extract (8). Only when these complexes were prewashed in buffer to remove all of the soluble cellular factor was it necessary to add HeLa S10 extract to the RNA replication assays. Based on these observations, we modified our assay procedure and resuspended unwashed preinitiation RNA replication complexes in reaction mixes that did not contain added extract, and we reduced the total CTP concentration to 5 μM. The decrease in the CTP concentration resulted in about a 20-fold increase in the specific radioactivity of the [^{32}P]CTP substrate and increased the sensitivity of these reactions for detecting negative-strand synthesis. This change was also expected to decrease the chain elongation rate by about 50% because the apparent K_m of the poliovirus polymerase for CTP is about 6 μM (45). These modified reaction conditions (method 4 in Materials and Methods) were used in all subsequent experiments.

Synchronous initiation and sequential synthesis of negative- and positive-strand RNA in vitro. When preinitiation RNA replication complexes were resuspended in reaction mixes using the modified reaction conditions described above, radiolabeled RNAs about 4,300, 5,300, and 6,300 nucleotides long were observed at 12, 14, and 16 min, respectively (Fig. 3). The labeled nascent chains elongated at ~500 nucleotides/min and started to accumulate as a band of genome-length RNA by 19 min (Fig. 3). Beginning at 25 min, a second wave of labeled nascent RNA molecules was detected. Estimation of the size of

the largest RNA molecules synthesized at 25, 28, and 31 min suggested these nascent RNA chains were also elongating at ~500 nucleotides/min (Fig. 3). Based on the observed elongation rate and the size of the nascent chains that were detected at specific time points, we determined that genome-length RNA was synthesized at 18 and 36 min at the end of the first and second waves of RNA synthesis. RNA molecules 7,500 nucleotides long would take about 15 min to be synthesized from scratch at an elongation rate of 500 nucleotides/min. This prediction agrees reasonably well with the results in Fig. 3, where about 18 min was required to synthesize the first genome-length RNA molecules after the preinitiation complexes were resuspended in reaction buffer. This result indicated that the initiation of RNA synthesis in the preinitiation RNA replication complexes required about 3 min. This might be expected since some time is required for complexes that were resuspended at 0°C to reach the final reaction temperature of 37°C. In addition, guanidine blocks a specific function of protein 2C, and a few minutes may be required for 2C to regain activity and to catalyze any required biochemical steps prior to the initiation of RNA replication. Overall, it appears that there was about a 3-min delay in the initiation of RNA replication, a wave of negative-strand RNA synthesis that required about 15 min to synthesize genome-length molecules and then a second wave of positive-strand RNA synthesis that required another 15 to 18 min. The two waves of viral RNA synthesis were consistent with the theoretical view of RNA replication, with the first wave consisting of negative-strand RNA synthesis (initiation and elongation) and the second wave consisting of positive-strand RNA synthesis (initiation and elongation) (Fig. 1).

Polarity of RNA synthesized by preinitiation RNA replication complexes. The polarity of viral RNA synthesized by the preinitiation complexes was determined by using a one-dimensional RNase T_1 fingerprint technique (Fig. 4). T7 RNA polymerase transcripts of a poliovirus cDNA clone were digested with RNase T_1 and used to produce oligonucleotide ladders characteristic of negative- and positive-strand RNA following electrophoresis and autoradiography (Fig. 4, lanes 1 and 4). Labeled viral RNA synthesized during the initial 18 min of a reaction using conditions identical to those in Fig. 3 was analyzed after RNase T_1 digestion. The labeled T_1 oligonucleotides derived from this digestion aligned precisely with the negative-strand-specific oligonucleotides derived from the transcript RNA (lane 2). Labeled positive-strand-specific oligonucleotides were not detected in this lane. In contrast, the labeled T_1-specific oligonucleotides that were derived from a 36-min reaction aligned with the positive-strand-specific oligonucleotides (lane 3). Although small amounts of some negative-strand-specific oligonucleotides were detected (e.g., negative-strand-specific 20-mer in Fig. 4), it was apparent that the synthesis of positive-strand RNA greatly exceeded negative-strand synthesis during the second 18 min of the reaction. Therefore, the initial wave of viral RNA synthesized by the preinitiation complexes was exclusively negative-strand RNA, and the second wave of labeled RNA consisted of positive-strand molecules. From comparison of the intensities of the negative- and positive-strand-specific oligonucleotides from the 36-min reaction, it is clear that positive-strand RNA was synthesized in great excess in these reactions. This difference would have been even greater if the reactions were run for a longer period of time. The asymmetric nature of RNA replication is also clear in Fig. 3 when the first and second waves of RNA synthesis are compared. It is likely that the increased label and diffuse bands observed in the second wave of synthesis (e.g., lanes for 31 and 34 min in Fig. 3) resulted from the repeated initiation and elongation of nascent positive-strands as illustrated in Fig. 1.

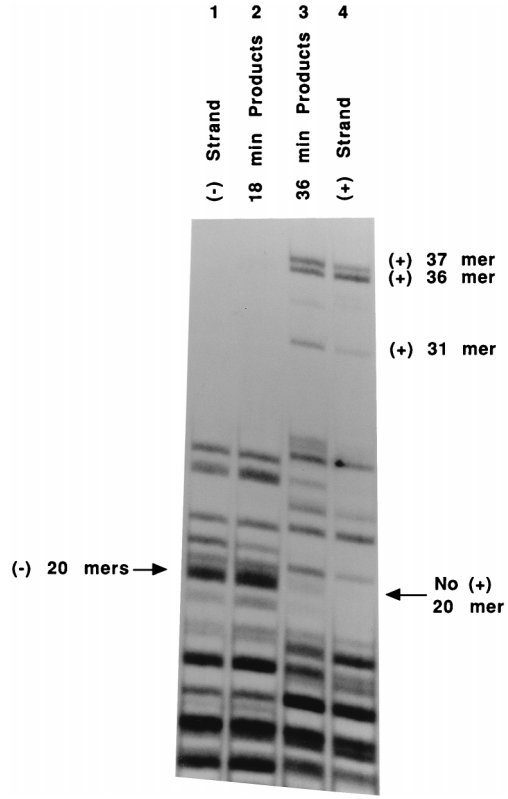

FIG. 4. Polarity of viral RNA synthesized by preinitiation complexes after the reversal of guanidine inhibition. Viral RNAs were radiolabeled in the following reactions before digestion with RNase T_1. Lane 1, poliovirus negative-strand RNA T7 transcribed from *Mlu*I-linearized plasmid pSM2612. Lane 2, preinitiation complexes were isolated from a 100-μl HeLa S10 translation-replication reaction containing 2 mM guanidine after incubation at 34°C for 4 h. The preinitiation complexes were resuspended in a 50-μl reaction containing 150 μCi of [α-^{32}P]CTP (7.5 μM CTP) and incubated at 37°C for 18 min as described for method 4 in Materials and Methods. Lane 3, preinitiation complexes were isolated from a 100-μl HeLa S10 translation-replication reaction containing 2 mM guanidine after incubation at 34°C for 4 h. The preinitiation complexes were resuspended in a 50-μl reaction containing 25 μCi of [α-^{32}P]CTP (7.5 μM CTP) and incubated at 37°C for 36 min. Lane 4, poliovirus positive-strand RNA transcribed from *Mlu*I-linearized plasmid pT7-Polio. The ^{32}P-labeled RNAs (17,000 cpm/reaction) were digested with RNase T_1 and fractionated by electrophoresis in a 20% polyacrylamide–7 M urea gel. The radiolabeled oligonucleotides were detected by autoradiography.

Guanidine inhibited negative-strand initiation but not elongation. To determine which steps of viral RNA replication are sensitive to guanidine inhibition, we added guanidine at various times to reactions synchronously replicating viral RNA (Fig. 5). In these experiments, we measured the cumulative amount of labeled viral RNA that was synthesized in a 50-min reaction. This was more than enough time to complete the synthesis of both genome-length negative- and positive-strand RNAs. When guanidine was added to reactions at various times between 1 and 14 min, we found that guanidine inhibited viral RNA replication only when added during the first 4 min of these reactions (Fig. 5, lanes 1 to 4). This time corresponded with the time at which the initiation of negative-strand RNA synthesis was occurring. This result indicated that guanidine inhibited the initiation of negative-strand RNA synthesis. In contrast, when guanidine was added to reactions between 6 and 14 min, the amount of labeled viral RNA synthesized was

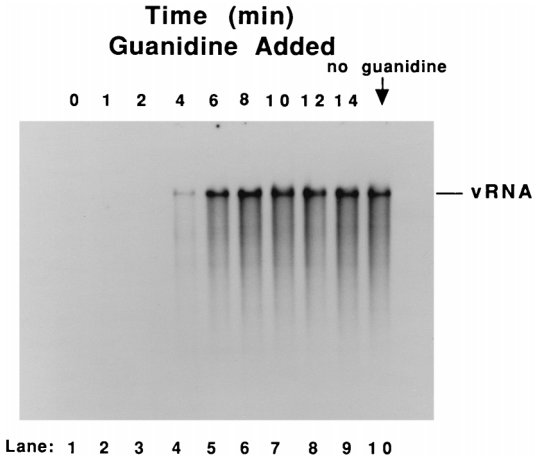

FIG. 5. Guanidine HCl inhibits RNA replication during the initiation of negative-strand RNA synthesis. Preinitiation complexes were resuspended in 50-μl reactions containing 20 μCi of [α-^{32}P]CTP (5 μM CTP) and incubated at 37°C as described for method 4 in Materials and Methods. At the indicated times, 2 μl of 50 mM guanidine HCl was added to the reactions, and incubation was continued. After 50 min of incubation, all reactions were terminated by solubilization in SDS buffer. The reaction products were phenol extracted, ethanol precipitated, denatured with CH_3HgOH, and fractionated by electrophoresis in 1% agarose. The gel was dried, and the radiolabeled RNAs were detected by autoradiography and quantitated by phosphorimaging. vRNA, poliovirus RNA.

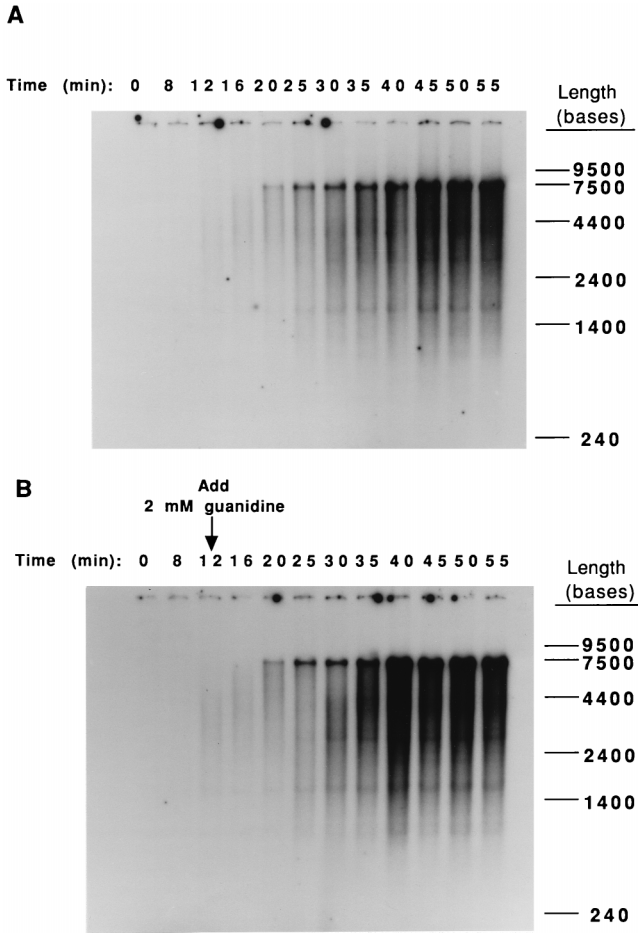

FIG. 6. Guanidine HCl does not inhibit the elongation of nascent negative-strand RNAs, nor does it inhibit the initiation and elongation of positive-strand RNAs. Preinitiation complexes were incubated in reactions as described for Fig. 3 (method 4 in Materials and Methods), and 50-μl aliquots were solubilized in SDS buffer at the indicated times. (A) Control reaction that lacked guanidine HCl; (B) reaction in which guanidine HCl was added to 2 mM at 12 min. The products were analyzed by agarose electrophoresis and autoradiography.

unaffected relative to a reaction in which guanidine was never added (compare lanes 5 to 9 with lane 10). This result indicates that guanidine did not inhibit negative-strand RNA elongation and did not inhibit positive-strand RNA initiation or elongation. Based on previous studies with guanidine (see the introduction), we anticipated that guanidine would have little effect on chain elongation, but we were surprised to find that guanidine did not block the initiation of positive-strand RNA synthesis.

Positive-strand RNA synthesis initiation and elongation were not affected by guanidine. To clearly demonstrate that guanidine did not inhibit any step of viral RNA replication beyond the initiation of negative-strand RNA synthesis, we used a time course experiment to monitor viral RNA replication in a reaction where guanidine was added at 12 min (Fig. 6B). This time was selected since it followed the initiation of negative-strand synthesis but preceded positive-strand synthesis. In the control reaction, viral RNA synthesis was measured in an identical reaction in the absence of added guanidine (Fig. 6A). The results from this experiment indicated that the addition of guanidine at 12 min had no detectable effect on viral RNA replication (Fig. 6). There was no observed inhibition in the elongation of nascent negative strands or in the initiation or elongation of positive-strand RNA (Fig. 6B).

RNA synthesized in the presence of guanidine (as in Fig. 6B) was analyzed by RNase T_1 fingerprinting to confirm that guanidine did not prevent positive-strand RNA synthesis. As expected, preinitiation complexes synthesized predominantly positive-strand RNA during a 50-min incubation in the absence of guanidine (Fig. 7, lane 3). Likewise, positive-strand RNA was the predominant product when guanidine was present from 8 to 50 min (lane 2). The only difference between the labeled RNAs made in the presence or absence of guanidine is a slight diminution in negative-strand RNA synthesis by guanidine as represented by the 20-mer region of the RNase T_1 fingerprint (compare lanes 2 and 3). Of special note is the presence of the VPg-linked T_1 oligonucleotide from positive-strand RNA, VPgUUAAAACAG (lanes 2 and 3). This oligonucleotide represents the very 5′ end of positive-strand RNA. The presence of this VPg-linked oligonucleotide demonstrates conclusively that guanidine did not prevent the authentic initiation and synthesis of positive-strand RNA.

DISCUSSION

Two significant points regarding the replication of poliovirus RNA are reported in this paper. (i) Poliovirus preinitiation RNA replication complexes synchronously replicate poliovirus RNA. This provides an ideal assay to study the four major steps of the poliovirus RNA replication cycle (Fig. 1). (ii) Using this assay, we showed that guanidine HCl specifically inhibits the initiation of negative-strand RNA synthesis. Therefore, the guanidine-inhibited function of protein 2C is not directly required for either positive-strand RNA synthesis initiation or nascent RNA chain elongation. This finding identifies one important difference between the biochemical activities required for negative- and positive-strand RNA synthesis.

Poliovirus RNA replication takes place in replication complexes which are located on the cytoplasmic surface of virus-induced membranous vesicles (12). By expressing recombinant

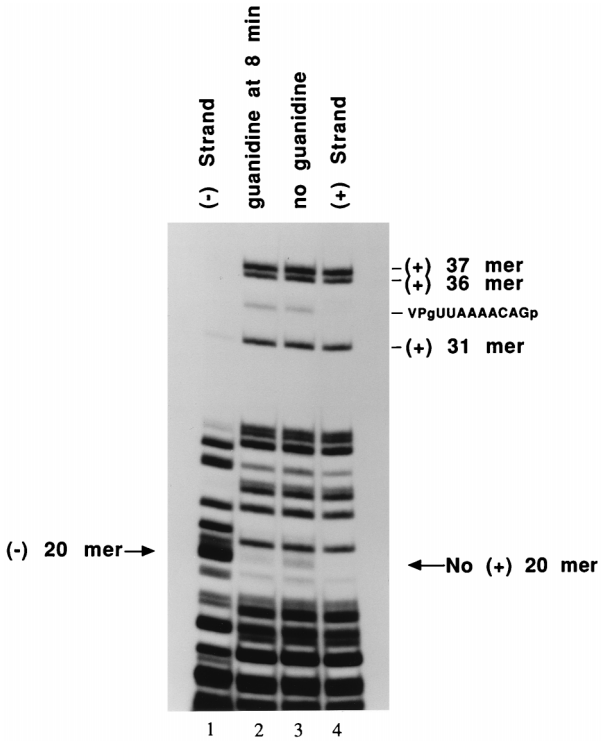

FIG. 7. Polarity of viral RNA synthesized in the presence of guanidine HCl. Viral RNAs were radiolabeled in the following reactions before digestion with RNase T_1. Lane 1, poliovirus negative-strand RNA transcribed from *Mlu*I-linearized plasmid pSM2612. Lane 2, preinitiation complexes were resuspended in 50-μl reactions containing 100 μCi of [α-^{32}P]CTP (5 μM CTP) and incubated at 37°C as described for method 4 in Materials and Methods. Guanidine HCl was added to 2 mM after 8 min of incubation, and incubation was continued for a total of 50 min. Lane 3, preinitiation complexes were resuspended in a 50-μl reaction containing 100 μCi of [α-^{32}P]CTP (5 μM CTP) and incubated at 37°C for 50 min. Lane 4, poliovirus positive-strand RNA transcribed from *Mlu*I-linearized plasmid pT7-Polio. The ^{32}P-labeled RNAs (40,000 cpm/reaction) were digested with RNase T_1 and fractionated after denaturation in a 20% polyacrylamide–7 M urea gel as described in Materials and Methods. Radiolabeled oligonucleotides were detected by autoradiography.

P2 proteins, it was shown that 2C and 2BC are responsible for vesicle induction in poliovirus-infected cells (11). These vesicles have membranes of multiorganelle origin including the rough endoplasmic reticulum, Golgi complex, and lysosomes (37). When isolated from infected cells, the virus-induced vesicles form rosettes which appear to surround the replication complex, as visualized by electron microscopy (12). Replication complexes associated with the rosettes, as well as individual vesicles, are active in RNA replication assays in vitro (12). Both the initiation and elongation of positive-strand RNA can be detected in these reactions (9, 10, 38). Although not detected because of a large background of positive-strand RNA synthesis, it is possible that negative-strand synthesis was also occurring in these reactions. An obvious limitation in using replication complexes isolated from infected cells is that essentially all steps of RNA replication are occurring simultaneously. Consequently, it is very difficult to characterize the individual steps in the replication cycle.

Synchronous RNA replication. To investigate each step in the RNA replication cycle, it would be advantageous to experimentally synchronize RNA replication such that the individual steps of RNA replication could be made to occur as temporally separate and sequential events. We demonstrate in this report that poliovirus preinitiation RNA replication complexes, formed in the presence of a reversible inhibitor of viral RNA replication (i.e., guanidine), synchronously replicate viral RNA upon incubation in appropriate reactions lacking guanidine. The initiation of negative-strand RNA synthesis occurs simultaneously within the population of preinitiation RNA replication complexes. Nascent negative-strand RNA molecules are then elongated synchronously such that a wave of radiolabeled elongating RNA molecules can be detected over time following their separation by electrophoresis and visualization by autoradiography (Fig. 3). Following the synchronous accumulation of genome-length negative-strand RNA molecules, a second wave of nascent molecules of positive polarity was synthesized (Fig. 3 and 4). Thus, preinitiation RNA replication complexes synthesized negative- and positive-strand RNA in a temporally separate and sequential fashion. Under the reaction conditions used for Fig. 3, negative-strand RNA synthesis occurred between 0 and 18 min whereas positive-strand RNA synthesis occurred between 18 and 36 min. By not adding HeLa S10 extract to these reactions, we were able to decrease the concentration of unlabeled CTP and increase the specific radioactivity of the labeled substrate to make detection of negative-strand RNA molecules possible. Therefore, for the first time, it is now possible to directly follow the synthesis of labeled negative-strand RNA molecules in poliovirus RNA replication complexes. Being able to directly quantitate the amount and size of nascent negative-strand RNA molecules as a function of time has obvious advantages over PCR amplification procedures that were used in previous studies (27). Synchronous RNA replication assays, such as those described in this report, provide an ideal experimental system to study the specific mode of action of inhibitors of poliovirus RNA replication. Any inhibitor of poliovirus RNA replication can now be characterized more precisely than has been the case in the past. In addition, viral RNA transcripts containing either conditional or lethal mutations can be characterized in these assays to determine which step(s) of RNA replication is affected by each mutation. Finally, the role of cellular proteins in viral RNA replication can be better characterized by using synchronous RNA replication assays.

Guanidine inhibits only the initiation of negative-strand RNA synthesis. We used the synchronous replication of poliovirus RNA by preinitiation RNA replication complexes to determine what step(s) of RNA replication is inhibited by guanidine. By adding guanidine HCl at various times to reactions synchronously replicating poliovirus RNA, we showed that the guanidine-sensitive step of RNA replication is the initiation of negative-strand RNA synthesis (Fig. 5 to 7). Guanidine completely inhibited both negative- and positive-strand RNA synthesis when added to reactions during the first 2 min of incubation (Fig. 5). In this case, positive-strand RNA synthesis was prevented only as a consequence of the lack of negative-strand RNA templates. When guanidine HCl was added to reactions at times after the initiation of negative-strand RNA synthesis but well before the completion of genome-length negative strand RNAs, we observed no inhibition in the elongation of nascent negative-strand RNA molecules (Fig. 6). In addition, we found that guanidine HCl did not inhibit the initiation or the elongation of positive-strand RNA molecules (Fig. 6). Therefore, the only guanidine-inhibited step of poliovirus RNA replication is the initiation of negative-strand RNA synthesis. Guanidine inhibits only positive-strand RNA synthesis as a consequence of previously inhibiting negative-strand RNA synthesis. Considering the previous reports that guanidine appears to inhibit the initiation of positive-strand RNA synthesis in poliovirus-infected cells (see the introduction), it now appears that this may be due to a primary defect in negative-

strand RNA synthesis. This observation suggests that individual negative-strand RNA molecules are used as template RNAs for the initiation of positive-strand RNA synthesis for only a limited period of time in vivo. Once the synthesis of new negative-strand RNA molecules is blocked by addition of guanidine, the inhibition of positive-strand RNA synthesis would be observed as a secondary effect. Although it is possible that guanidine can inhibit viral RNA replication by different mechanisms in vitro and in vivo, we do not believe that this is significant factor in this case. For technical reasons, it is difficult to actually differentiate in infected cells whether a drug produces a primary defect in negative- or positive-strand RNA synthesis. Therefore, we believe that the results of our in vitro experiments are generally consistent with the results of previous studies as explained above and that guanidine HCl prevents positive-strand RNA synthesis only as a consequence of previously blocking the initiation of negative-strand RNA synthesis.

Role of protein 2C in RNA replication. Protein 2C, the target of guanidine inhibition (5, 31–33, 40), is a putative RNA helicase. Putative RNA helicase proteins are encoded by most single-stranded positive-polarity viruses with genomes longer than 5.8 kb (19). RNA helicase proteins are thought to be necessary to unwind double-stranded RNA structures during RNA replication (19). All known helicase proteins exhibit NTPase activity (19). Poliovirus protein 2C contains an NTP-binding domain characteristic of helicase proteins (16–19), and purified protein 2C exhibits ATPase and GTPase activities (25, 36). Guanidine, however, does not inhibit the ATPase activity of purified 2C (34) even though amino acid residues associated with the guanidine-sensitive phenotype of this protein are found proximal to the conserved amino acid motifs within the NTP-binding domain (40). Attempts to demonstrate RNA helicase activity with purified 2C have failed (34); however, 2C may require other viral or cellular proteins to exhibit helicase activity. Protein 2C also contains an RNA-binding domain (35), and 2C-containing proteins (i.e., P2, 2BC, and 2C) can be UV cross-linked to viral RNA within infected cells (10). Guanidine, however, does not prevent the UV cross-linking of 2C proteins to viral RNA (10). An amphipathic helix within the amino-terminal portion of 2C is required for viability (28) and may impart membrane affinity to protein 2C. Proteins 2C and 2BC have been shown to induce membranous alterations and the formation of membranous vesicles within the cytoplasm of cells (11), similar to those observed in virus-infected cells. Guanidine is reported to prevent 2C-containing proteins from binding to membranous vesicles and to dissociate multivesicular RNA replication complexes (10). Yet, we find that pelleted preinitiation complexes formed in the presence of guanidine contain all of the components necessary for RNA replication, including protein 2C (6). Overall, it remains unclear what specific function of protein 2C is inhibited by guanidine, but the results of this study suggest that macromolecular structures and/or enzymatic activities that are affected by guanidine are required either directly or indirectly for the initiation of negative-strand RNA synthesis.

Our current results demonstrate that the guanidine-inhibited function of protein 2C is required during the initiation of negative-strand RNA synthesis and that subsequent steps in RNA replication (i.e., elongation of negative strands, initiation of positive strands, and elongation of positive strands) are unaffected by guanidine. These results do not preclude the possible role of protein 2C in these subsequent steps of RNA replication. One possibility is that protein 2C is a multifunctional protein, with only one activity being inhibited by guanidine. Another possibility is that guanidine prevents an irreversible maturation step of protein 2C, such as oligomerization (40), required for the conversion of preinitiation RNA replication complexes into active RNA replication complexes. Once activated, the RNA replication complexes would be resistant to subsequent inhibition by guanidine. A mutation in the NTPase domain of 2C prevents the formation of tubular membrane structures (11), supporting the notion that normal 2C activities may involve structural maturation. Guanidine may prevent this maturation step. Interestingly, individual membranous vesicles from RNA replication complexes have extensive tubulation on their surface associated with RNA synthesis (12). Additional information is required to further characterize the specific roles of protein 2C in RNA replication.

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