RNA Duplex Unwinding Activity of Poliovirus RNA-Dependent RNA Polymerase 3Dpol

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Received 28 December 1992/Accepted 13 February 1993

The ability of highly purified preparations of poliovirus RNA-dependent RNA polymerase, 3Dpol, to unwind RNA duplex structures was examined during a chain elongation reaction in vitro. Using an antisense RNA prehybridized to an RNA template, we show that poliovirus polymerase can elongate through a highly stable RNA duplex of over 1,000 bp. Radiolabeled antisense RNA was displaced from the template during the reaction, and product RNAs which were equal in length to the template strand were synthesized. Unwinding did not occur in the absence of chain elongation and did not require hydrolysis of the γ-phosphate of ATP. The rate of elongation through the duplex region was comparable to the rate of elongation on the single-stranded region of the template. Parallel experiments conducted with avian myeloblastosis virus reverse transcriptase showed that this enzyme was not able to unwind the RNA duplex, suggesting that strand displacement by poliovirus 3Dpol is not a property shared by all polymerases.

Poliovirus, the prototypic member of the Picornaviridae family, has a single-stranded RNA genome of positive polarity approximately 7,500 nucleotides (nt) long. The genome is covalently linked to a small viral protein, VPg, at its 5′ terminus (9, 21) and includes a genetically encoded poly(A) segment at its 3′ terminus (49). Soon after entry of the virus into a host cell, the genome is released into the cytoplasm and serves as a mRNA to direct translation of a single large polypeptide which is subsequently processed by viral proteases into mature proteins. They include structural proteins from the P1 region of the genome and nonstructural proteins from the P2 and P3 regions which function in the proteolytic maturation process of viral proteins and in viral genome replication (for reviews, see references 18 and 43).

As a first step in viral replication, the genomic RNA is copied to produce a complementary RNA of negative polarity. This reaction is catalyzed by the viral RNA-dependent RNA polymerase, 3Dpol, an enzyme belonging to a group of enzymes found only in RNA viruses. Subsequently, this newly synthesized RNA serves as a template to direct synthesis of daughter genomic RNAs, thereby completing a round of replication. Despite long-term efforts in several laboratories, little is known about the biochemical mechanism of RNA-dependent RNA synthesis (for a review, see reference 36). In virus-infected cells, RNA synthesis occurs in replication complexes associated with virus-induced membrane vesicles of rough endoplasmic reticulum origin (2, 5). Replication complexes consist of heterogeneous, multi-stranded structures called replicative intermediates (RI) (4, 11, 25) in association with an only partially characterized set of RNA replication proteins. RI contains a core RNA molecule of genome length and various numbers of complementary strands of nascent RNA of various lengths. The core structures of RI, when examined after isolation from virus-infected cells and deproteinization, appear to contain significant segments of duplex RNA on the basis of their resistance to digestion by RNase and electron microscopic studies (38, 42). However, RNA cross-linking studies suggest that, in vivo, RI appears to be predominantly single stranded with nascent strands duplexed with their templates only near the replication fork (38). 3Dpol is the only protein required for elongation of RNA chains in vitro (24, 47); however, current models propose that, in vivo, 3Dpol must function in concert with other viral and/or cellular proteins to accomplish the complete reaction. These additional proteins have been suggested to function in a variety of activities thought to compose parts of the RNA replication reaction. For example, P2-derived proteins (2C or its precursor 2BC) have been proposed to be involved in the formation and maintenance of replication complex-associated membranous structures as well as in anchoring replication complexes to these structures (3). The involvement of these proteins in RNA replication has been confirmed by genetic studies analyzing several mutants of 2B (17) and 2C (22, 27, 32, 45). In addition, VPg (3B) or its precursor (3AB) has been proposed to function as a primer for the initiation of RNA polymerization (29, 44).

In order for an RNA strand to serve as template for multiple rounds of product RNA synthesis, the RNA replication machinery should either prevent formation of extensive base pairing between the template RNA and the nascent complementary strand or contain a helicase activity which is able to unwind RNA duplex subsequent to its formation. Such a mechanism would also be necessary to release newly synthesized RNAs to serve as templates for subsequent rounds of replication, translation, or packaging. Dmitrieva et al. (7) reported that an RNA helicase activity involved in the synthesis of encephalomyocarditis virus (EMCV)-specific single-stranded RNA was observed in relatively crude replication complexes isolated from virus-infected cells. More recently, they reported an ATP- and RNA elongation-dependent RNA helicase activity of either viral or cellular origin.

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which initially copurified with the EMCV RNA polymerase but was lost upon further purification (8). Identification of the viral 2C protein as a potential nucleoside triphosphate (NTP) binding protein by computer-assisted sequence analysis (12-14), in conjunction with the demonstrated implication of 2C in viral RNA replication, has led to the suggestion that this protein performs the RNA helicase activity required for the RNA replication reaction. At present, however, no direct biochemical assay of 2C as an RNA helicase has been reported.

Although genetic studies have provided some hints of the roles of some viral proteins in RNA replication, the molecular mechanism of the reaction has not yet been established. The enzyme that catalyzes RNA chain elongation, 3D\text{pol}, has been purified both from virus-infected cells (23, 50) and from bacteria (30, 33) or insect cells (31) expressing the recombinant protein, and its properties are beginning to be analyzed (39). As part of our long-term studies of the 3D polymerase protein and its functional activities, we examined the RNA duplex unwinding ability of highly purified recombinant 3D\text{pol} during chain elongation in vitro. We report here that this enzyme can unwind a long stretch of RNA duplex so as to displace strands for continued polymerization of nascent chains and that the unwinding reaction proceeds in an elongation-dependent, ATP-independent manner requiring no additional proteins.

**MATERIALS AND METHODS**

**Construction of plasmids.** The construction of the plasmids used to generate RNAs to serve as substrates for 3D\text{pol} unwinding activity assay is diagrammed in Fig. 1. Methods were as described by Sambrook et al. (41). pPOV-3 (16), which contains the 3'-terminal segment of poliovirus cDNA was digested with BamHI and SalI restriction endonucleases. The fragment containing the poliovirus sequence (nt 4600 through 3' terminus) was gel purified and subsequently ligated into pGEM-4 (Promega) which had been digested with BamHI and SalI and dephosphorylated with calf intestinal phosphatase. The resulting plasmid, pPV-2C*3D, was used to generate a template and a primer RNA. pPV-2C*3D was digested with BglII and SphI, the ends were filled in with Klenow enzyme, and the plasmid was religated to generate pPV-2C*3C*. This plasmid contains poliovirus cDNA sequence from nt 4600 to 5601 and was used to generate an antisense RNA.

**RNA transcription.** RNA transcription was performed with reagents from Promega according to the manufacturer's suggested conditions with some minor modifications. Template RNA (positive polarity) was transcribed from pPV-2C*3D with SP6 RNA polymerase subsequent to digestion of the plasmid with SphI. The primer RNA and the antisense RNA (negative polarity) were transcribed from pPV-2C*3D and pPV-2C*3C*, respectively, with T7 RNA polymerase subsequent to digestion of the plasmids with SalI and EcoRI, respectively. To generate $^{32}$P-labeled antisense RNA, [α-32P]UTP (Amersham) was added to the transcription reaction to a final specific activity of 12 mcg of UTP per mmol. In addition, the concentration of unlabeled UTP was lowered by 10-fold to 0.1 mM, while the concentrations of the other nucleotides were kept at 1 mM. Subsequent to transcription at 37°C for 2 h, the DNA was digested with RQ1 D Nase (Promega) at 37°C for 30 min. RNA was extracted with phenol-chloroform and was precipitated with ethanol with 2 M ammonium acetate as a salt to minimize coprecipitation of unincorporated nucleotides. The pellet was resuspended in TE buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA) and stored at -20°C.

**RNA substrate preparation.** RNA substrates were formed by incubating mixtures of RNAs at 65°C for 30 min in TE buffer containing 100 mM NaCl. Approximately equal molar amounts of all three RNAs were derived by experimentally titrating the hybridization reaction so that the mobility of all detectable single-stranded template RNA was shifted to that of duplex RNA on an agarose gel. No excess primer or antisense RNAs were apparent after staining with ethidium bromide. RNA substrates were subsequently stored at -20°C. To prepare RNA substrates without detectable levels of nucleotide contamination, these RNA preparations were subjected to a second ethanol precipitation with 2 M ammonium acetate.

To examine the degree of base pairing between the template strand and either the antisense strand or the primer, RNA was treated with RNase A (10 μg/ml) at 30°C for 30 min in TE buffer containing 300 mM NaCl, and the RNase-
resistant duplexes were analyzed by electrophoresis in an agarose gel.

**Purification of poliovirus polymerase 3D.** Poliovirus RNA polymerase was expressed in *Escherichia coli* harboring the plasmid, pEXC-3D, at 30°C, and crude sonicates were prepared as described by Rothstein et al. (40). The lysates were clarified by centrifugation at 100,000 × g, and polymerase was purified from the supernatant (S-100), basically as described previously (31). The purification protocol included 0 to 40% saturation ammonium sulfate precipitation, phosphocellulose (Whatman), Mono-Q (Pharmacia LKB Biotechnology), and Phenyl-Superose (Pharmacia LKB) column chromatography. As a final step, the enzyme was bound and eluted from a poly(U)-Sepharose 4B (Pharmacia LKB) column (1.5 by 8 cm) in 50 mM Tris-HCl (pH 8.0)–5 mM β-mercaptoethanol–0.1% Nonidet P-40–10% glycerol, with a 100-ml gradient from 0.05 M KCl to 0.4 M KCl.

For all purification steps the presence of poliovirus polymerase was monitored by assaying poly(U) polymerase activity on a poly(A) template as described by Hey et al. (16). For the final purification step, polymerase purity was evaluated by analysis of column fractions in a sodium dodecyl sulfate (SDS)–10% polyacrylamide gel and detection of protein by silver staining (28).

**Poliovirus polymerase and AMV RT assays, and product analysis.** Approximately 0.25 μg of template RNA and 30 ng of purified poliovirus polymerase were incubated at 30°C for 1 h under the following conditions unless noted otherwise: 50 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; pH 8.0), 0.5 mM each NTP (Boehringer Mannheim), 4 mM dithiothreitol, 3 mM magnesium acetate, 0.06 mM ZnCl₂, 4 μg of actinomycin D per ml, and 0.8 U of RNasin (Promega) per μl. In some experiments, 5'-adenylylimidodiphosphate (AMP-PNP; Boehringer Mannheim) was used at 0.5 mM. In experiments in which de novo-synthesized RNA was labeled, [α-32P]UTP was added to a final specific activity of 4 mCi/mmoll, and approximately 1 μg of RNA template was used. Polymerase reactions with avian myeloblastosis virus (AMV) reverse transcriptase (RT; Promega) were performed according to the manufacturer’s suggested conditions with either 1 or 0.75 mM deoxyribonucleotides at either 30 or 42°C. Product DNA was labeled with [α-32P]dATP (1.3 mCi/mmoll; Amersham). Reaction products were analyzed on 1% agarose gels under either nondenaturing or glyoxal-denaturing conditions as described by Sambrook et al. (41).

**RESULTS**

**RNA substrates for RNA duplex unwinding assays.** To examine RNA duplex unwinding activity of poliovirus RNA-dependent RNA polymerase, we used the strategy previously developed by Dmitrieva et al. (8), applied to a different set of RNA substrates. The assay involves a polymerization reaction with an RNA template prehybridized to a segment of complementary, antisense RNA. The antisense RNA serves as an obstacle for a polymerase. RNA duplex unwinding activity allows displacement of the antisense RNA from the template RNA. The length of the resulting polymerized product is equal to that of the template RNA if unwinding occurs; a shorter product, equal to the length of the unhybridized segment of the template, results if the antisense RNA is not unwound and displaced.

Figure 2 shows a diagram of the template RNA (positive polarity), a template-specific primer RNA (negative polarity), and the antisense RNA (negative polarity), synthesized to produce substrates for the RNA duplex unwinding assay. The corresponding regions of the poliovirus genome are indicated at the top of the figure. The template RNA is approximately 3,022 nt long, including 2,841 nt of poliovirus RNA sequences, approximately 50 nt of poly(A) tail, plus 30 and 101 nt of vector sequences at the 5' and 3' ends of the RNA, respectively. The primer RNA is 278 nt long, containing 106 nt of poliovirus RNA sequences, approximately 50 nt of poly(U) tail, and 122 nt of vector sequences. The antisense RNA is 1,074 nt long, including 1,006 nt of poliovirus RNA sequences, and 21 and 20 nt of vector sequences at the 5' and 3' ends of the RNA, respectively. Because there are 21 nt of vector sequence at the 5' terminus of the antisense RNA which do not hybridize with the template, the duplex formed between the template and the antisense RNA is only 1,026 bp.

The template-specific primer RNA was designed to anneal to the 3' end of the template and to extend slightly beyond it, since it contained a short segment (21 nt) of noncomplementary RNA at its 5' terminus. This prevented the template from forming a hairpin turn at its own 3' terminus which would allow self-primed RNA synthesis to occur. We have observed that any template containing a heterogeneous sequence at its 3' terminus supports primer-independent synthesis that produces a snapback product approximately twice the length of the template (see below). RNAs which are polyadenylated at their 3' termini do not self-prime; the presence of even as few as six heterogeneous nucleotides following the poly(A) tail, however, results in self-primed RNA synthesis by the poliovirus RNA polymerase (data not shown).

The transcripts shown in Fig. 2 were annealed in various combinations to produce substrates for the RNA duplex unwinding assay. A schematic diagram and an ethidium bromide-stained gel of these substrates are shown in Fig. 3. Substrate 1 (lane 1) is the RNA template alone. Incubation of the template together with primer (substrate 2) resulted in a detectable shift in its mobility (lane 2), indicating the formation of a partial duplex. The larger duplex region formed between the template and antisense RNA (substrate 3) caused an even greater mobility shift (lane 3). Substrate 4 is composed of template, primer, and antisense, and its mobility was retarded even more (lane 4). Substrates 5 and 6 (lanes 5 and 6) are the same as 3 and 4 except that the antisense RNA was labeled with 32P (indicated by a thicker line in the diagram in Fig. 3). These were prepared to allow direct measurement of the displacement of the antisense RNA.
during the unwinding reaction. Treatment of substrate 6 with RNase A under conditions of high salt specific for the digestion of single-stranded RNAs generated a sharp, RNase-resistant, radiolabeled band of approximately 1,026 bp, indicating stable base pairing between the template and the antisense RNAs (data not shown). In addition, an ethidium bromide-stained band of approximately 300 bp was observed, representing the template-primer duplex region (data not shown).

**Poliovirus RNA polymerase.** We have previously described the purification and characterization of the poliovirus RNA-dependent RNA polymerase from clones of E. coli harboring the plasmid pExc-3D (31). This plasmid encodes the poliovirus 3CD protein driven by the trp promoter (37). Induction of expression of 3CD results in autocatalytic cleavage by the 3C protease sequence to generate the 3D polymerase protein, identical to that produced after infection of cells with poliovirus. SDS-polyacrylamide gel electrophoresis (PAGE) analysis of the purified protein used in these studies is shown in Fig. 4. No contaminating proteins were detected by silver staining.

**Poliovirus polymerase can elongate through a large segment of RNA duplex.** To determine whether an antisense RNA hybridized to the template presented a stable obstacle to the poliovirus polymerase, the RNA substrates described in Fig. 3 were utilized in a standard polymerase elongation assay containing [32P]UTP, and the products were examined on a denaturing agarose gel. Figure 5 shows autoradiograms of the reaction products. When the template alone (substrate 1), with no primer and no antisense RNA, was presented as a substrate to the purified poliovirus RNA polymerase, a predominant product of approximately 6,000 nt was made (lane 1). This product is approximately twice the length of the template and indicated that this template could self-prime, presumably by creating a hairpin structure at the 3' terminus that allowed the synthesis of a snapback RNA in which the newly synthesized polynucleotide was covalently attached to the template. Annealing the antisense RNA to this template (substrate 3) had no effect on the self-primer capacity of the template and, more importantly, had no effect on the length of the product (Fig. 5, lane 2), suggesting that the antisense RNA was unwound and displaced from the template allowing polymerization of new product RNA complementary to the 5' end of the template. Addition of a primer to these substrates (to generate substrates 2 and 4) produced the products shown in Fig. 5, lanes 3 and 4. Some self-priming still occurred, most likely from excess templates not hybridized to primer. This small amount of unhybridized template would not have been detectable by ethidium bromide staining in Fig. 3. The major product, however, was about 3,000 nt in length, equal to the length of the template. Again, no difference in product length was observed whether or not antisense RNA was annealed to the template. These data demonstrate that the poliovirus polymerase elongated through the region of 1,000 bp presented by the template-antisense duplex region and suggest that the enzyme could unwind the duplex. Two small bands migrating near and below 1,000 nt were observed in lanes 2 and 4 (Fig. 5). These products appeared when antisense RNA in the absence of template was presented as substrates (data not shown), but the exact nature of these bands has not been investigated.

Parallel experiments were conducted with AMV RT (Fig. 6), which has been previously shown to be unable to displace complementary RNA from an RNA template (6). The same template-primer substrates, in the presence and absence of antisense RNA (substrates 2 and 4) were used to direct DNA synthesis with AMV RT at 30°C. As a control, lane 1 (Fig. 6) shows the predominantly 3,000-nt product of RNA synthesized by the poliovirus polymerase with substrate 2, as seen before in Fig. 5. Small amounts of the self-primed, dimer-length product are also visible. RT produced a full-length cDNA of the same 3,000-nt size (Fig. 6, lane 2); in addition, another product just over 2,000 nt in length is apparent, most
was observed unwind the antisense to a size the in activity unwinding to enzyme in activity the result allowed the RNA through affect the not data not formed strand, AMV to secondary length, evidently on an phoresis a RNA seen 42°C the purified also poliovirus that the result was shown; the Fig. 5. RNA synthesis by poliovirus polymerase. RNA substrates 1, 3, 2, and 4 (schematically illustrated in the box) were used in a 3Dpol polymerization assay in the presence of [32P]UTP. RNA products were denatured with glyoxal and subjected to electrophoresis on an agarose gel, in lanes 1 to 4. An autoradiogram of the dried gel is shown. The antisense RNA (lanes A; ~1,000 nt) and the template RNA (not shown; ~3,000 nt) were used as mobility markers. The approximate length of the 6,000-nt product was estimated from its mobility relative to the markers.

FIG. 5. RNA synthesis by poliovirus polymerase. RNA substrates 1, 3, 2, and 4 (schematically illustrated in the box) were used in a 3Dpol polymerization assay in the presence of [32P]UTP. RNA products were denatured with glyoxal and subjected to electrophoresis on an agarose gel, in lanes 1 to 4. An autoradiogram of the dried gel is shown. The antisense RNA (lanes A; ~1,000 nt) and the template RNA (not shown; ~3,000 nt) were used as mobility markers. The approximate length of the 6,000-nt product was estimated from its mobility relative to the markers.

likely the result of a pause (strong stop) by the enzyme due to secondary structure present in the template RNA. When the AMV RT was presented with substrate 4, which contained hybridized antisense RNA, the enzyme was apparently unable to unwind the duplex and displace the antisense strand, and synthesis terminated at the block. This resulted in a predominant cDNA product of approximately 2,000 nt in length, as seen in Fig. 6, lane 3. A shorter product of about 1,000 nt was also detected, presumably due to a RT pause or the antisense RNA as described above (Fig. 5, lanes 2 and 4). Identical results were obtained when reactions were performed at 42°C (the normal reaction condition for AMV RT; data not shown), suggesting that the reduced temperature was not the reason for the inability of AMV RT to elongate through the RNA duplex region.

Addition of poliovirus polymerase to the RT reaction did not affect the length of the cDNA product (Fig. 6, lane 4). This result allowed us to exclude the possibility that the RNA unwinding observed in the poliovirus polymerase-catalyzed reaction was due to a contaminating helicase activity in the purified enzyme preparation. If a contaminant was unwinding the duplex so as to enable the poliovirus enzyme to elongate to the end of the template, then its unwinding activity would also have allowed RT to synthesize a full-length product. Thus, the unwinding activity observed in the poliovirus polymerase reaction was inherent in the enzyme itself.

Displacement of antisense RNA from the template. In order to demonstrate directly whether poliovirus polymerase can unwind the antisense RNA from the template, we performed an antisense displacement assay. In this assay, we used the substrate (Fig. 3, substrate 6) containing primer and radio-labeled antisense RNA, in a polymerization assay with unlabeled nucleotides. The rationale for this assay is that the antisense RNA, when unwound from the template by an advancing polymerase, would be displaced from the template, and the radioactivity in the substrate would disappear with time. Concomitantly, a corresponding signal would appear as free antisense RNA. Samples were removed from a polymerization reaction at various times during the course of the assay, and the products were analyzed on a nondenaturing agarose gel. Figure 7A shows an autoradiogram of the gel. Reaction products from 0, 15, 30, 60, 90, and 120 min of incubation are shown in lanes 1 to 6, respectively. As expected, the radioactivity in the substrate gradually decreased, accompanied by an accumulation of a band comigrating with the antisense RNA. The kinetics of antisense displacement correlated with the kinetics of RNA polymerization in the same reaction. The displaced product increased linearly with time during the first 60 min but reached a plateau thereafter. This shows directly that the antisense strand was unwound and released from the template. Omission of the poliovirus polymerase from the reaction incubated for 120 min resulted in a stable RNA duplex, with no displacement of the antisense RNA (lane 7). In addition to free antisense RNA, a minor product that migrated more slowly than the substrate appeared during the course of the reaction, but not in reactions in which the polymerase was
omitted. This product comigrated with the fully duplexed final product of the polymerization reaction, and thus it appears to be an intermediate in which the polymerase has synthesized up to the 5' terminus of the antisense RNA. During the polymerization reactions, not all of the templates were transcribed. In general, approximately 50% of the templates were converted to product during 120 min of reaction. Somewhat less than 50% of the antisense RNA was recovered as a discrete band on the gel; some of the antisense RNA occurs as duplex intermediate as described above, and it is possible that some might be degraded.

The antisense RNA migrates as two bands in a non-denaturing agarose gel (Fig. 7A, lane A). The distribution of RNA in each band was concentration dependent with higher concentration favoring the more slowly migrating band. Heating the sample immediately prior to electrophoresis reduced the proportion which migrated more slowly. The upper band thus appears to be a loosely base-paired dimer; it migrates just above a 1,018-bp DNA marker and it was sensitive to digestion with RNase A under salt conditions in which stable duplexes were resistant and single-stranded RNAs were digested (data not shown).

Antisense displacement was also examined after reverse transcription of the same substrate with AMV RT (Fig. 7B). Lane 1 shows that although the amount of label from the substrate band decreased, no antisense RNA was released during the reverse transcription. Instead, a band of approximately 1,000 bp was generated presumably by the RNase H activity of RT which hydrolyzed the RNA strand of the RNA-DNA duplex formed after cDNA synthesis, thereby releasing the remaining template-antisense RNA duplex. The same product profile was observed when poliovirus polymerase was added to the RT (lane 2), also suggesting that the poliovirus polymerase preparation does not have a contaminating RNA helicase. Lane 3 shows poliovirus polymerase alone under DNA synthesis reaction conditions for RT as a negative control. A similar pattern was attained from reaction in which neither RT nor poliovirus polymerase was present (data not shown).

Unwinding depends on chain elongation but not ATP hydrolysis. To determine whether duplex unwinding required RNA polymerization, strand displacement was measured with substrate 6 (Fig. 3) under conditions which precluded RNA synthesis. When all ribonucleotides were omitted from the reaction, no unwinding occurred (Fig. 8, lane 1). Supplementation with all four ribonucleotides restored unwinding and antisense displacement (lane 2), but addition of only three did not (lanes 3 to 6). The absence of unwinding activity in these reactions additionally supports the previous conclusion that contaminating helicase activity was not present in our purified enzyme preparation. Some activity was observed in reactions which lacked only ATP (lane 3); this was likely due to minor contaminations of ATP in the mixture of CTP, GTP, and UTP. Reducing the ATP concentration in the reaction from 500 to 25 nM eliminated the low level of activity observed in the absence of ATP (data not shown). Ribonucleotide titrations showed that RNA polymerization and duplex unwinding occurred, albeit less efficiently, with ATP concentrations as low as 5 nM, which is 100-fold lower than the standard reaction conditions. Thus, only low levels of ATP contamination in the other NTP preparations at 500 μM could supply sufficient ATP for some activity to be observed. Under the conditions of limiting reaction, a smear of intermediate RNA products were seen to accumulate above the template band (lane 3, brackets).

The experiments described above demonstrated that hydrolysis of the γ-phosphate of ATP was required in addition to chain elongation, a nonhydrolyzable analog of...
ATP, AMP-PNP was substituted for ATP in the reaction. No unwinding occurred in the presence of AMP-PNP alone (Fig. 8, lane 7); however, full activity was restored when CTP, GTP, and UTP (lane 8) or when all four NTPs (lane 9) were added to the AMP-PNP reaction (compare to lane 2). Unwinding assays done at 25 μM NTP to exclude the possibility that the unwinding activity was due to minor contaminants of ATP in CTP, GTP, UTP, and AMP-PNP preparations also revealed that AMP-PNP could substitute for ATP during unwinding activity (data not shown). These experiments show that while RNA chain elongation is required for RNA duplex unwinding activity, hydrolysis of ATP is not required.

**DISCUSSION**

Many viruses that infect bacterial, animal, or plant cells contain their genetic information in a single-stranded RNA genome of positive polarity. These viruses encode an RNA-dependent RNA polymerase which catalyzes the unique reaction of RNA synthesis from a single-stranded RNA template. Replication of viral RNA takes place in two stages: synthesis of a complementary (negative polarity) RNA with the viral genome as template, and then synthesis of progeny virus genomic RNA with the negative-strand RNA as template. In the case of poliovirus RNA replication, RNA synthesis occurs via partially double-stranded structures called replicative intermediates. To prevent accumulation of dead end products in which newly synthesized RNA strands are extensively hybridized to their templates, the nascent strand must be prevented from base pairing with the template or be displaced by an unwinding activity. Unwinding of double-stranded DNA or RNA is an essential process in many cellular functions, including replication, recombination, repair, transcription, mRNA splicing, and initiation of translation of mRNAs. This task is generally accomplished by NTP-dependent DNA or RNA helicases which translocate along one strand of duplex DNA or RNA coupled to the hydrolysis of ATP, resulting in unwinding of the duplex.

In this study we investigated the RNA duplex unwinding ability of poliovirus RNA-dependent RNA polymerase, 3Dpol, during a chain elongation reaction in vitro. We showed that poliovirus polymerase can elongate through a highly stable RNA duplex of over 1,000 bp. This was achieved by unwinding of the duplex and displacing the antisense RNA from the template. The unwinding activity is an inherent property of the enzyme. It did not occur in the absence of concomitant RNA chain elongation, and it did not require hydrolysis of the γ-phosphate of ATP. Therefore, the activity exhibited by poliovirus polymerase is different from those of true DNA or RNA helicases which must harvest the energy from hydrolysis of nucleotides in order to drive the reaction (10). Since RNA duplex unwinding activity of 3Dpol is dependent on elongation, it is implied that the direction of unwinding occurs from 3' to 5' with respect to the template strand. On the basis of our observation that only very small amounts of reaction intermediates accumulated during the course of the antisense displacement assay (Fig. 7A), the rate of elongation through the duplex region does not appear to differ much from that occurring on the single-stranded region of the template. This conclusion is supported by the fact that antisense RNAs were displaced within 15 min of the reaction (Fig. 7A, lane 2), which is consistent with the elongation rate reported in previous studies (31). Although RNA duplex unwinding by 3Dpol occurs quite efficiently, the role, if any, of this activity in RNA replication in vivo has not been demonstrated.

It was previously reported that partially purified EMCV RNA polymerase preparations from virus-infected cell extracts were able to displace complementary RNA from a template in a reaction that was both elongation and ATP dependent (8). This unwinding activity was not considered to be an inherent property of the EMCV polymerase since the activity was lost upon further purification of the enzyme. No restoration of the unwinding activity, however, was achieved by reconstituting different fractions from the purification procedure. Therefore, one possible explanation for the apparent discrepancy with the findings reported in this study could be that the unwinding, but not the elongation property of the EMCV polymerase was inactivated during purification of the enzyme. Alternatively, the properties of the poliovirus and EMCV polymerases may be inherently different. For example, EMCV polymerase may be less processive than the poliovirus enzyme and therefore may be unable to elongate through a duplex region of the template. To circumvent this problem, EMCV may have evolved to utilize an RNA helicase (either viral or cellular) as part of its replication machinery.

Most positive-stranded RNA viruses of both plants and animals encode a protein which contains nucleotide binding motifs and which appears to be involved in viral RNA replication (12–14). These proteins appear to be homologous to the 2C proteins of the picornaviruses, and they have been postulated to function as NTP-dependent RNA helicases. The cylindrical inclusion (CI) protein of plum pox potyvirus (a positive-strand RNA plant virus), which contains the NTP binding motif, has been demonstrated to have a nucleic acid-stimulated ATPase activity and an ATP-dependent RNA helicase activity (19, 20). Although CI protein has been postulated to be involved in RNA replication, the exact biochemical function(s) of this protein has not been elucidated. From site-directed mutagenesis studies (27, 45), the nucleotide binding motifs of poliovirus 2C have been suggested to be important in viral RNA replication. At present, however, no biochemical evidence that 2C is an ATP-dependent RNA helicase has been demonstrated.

Hayes and Buck (15) have reported previously that purified RNA-dependent RNA polymerase of cucumber mosaic virus catalyzes the complete replication of cucumber mosaic virus genomic RNA (i.e., synthesis of progeny plus strand from a plus-strand template provided in the reaction). Cucumber mosaic virus polymerase contains two virus-encoded polypeptides (1a and 2a) and one host polypeptide. On the basis of the observation that the 1a subunit of the polymerase contains sequence motifs characteristic of nucleic acid helicases, the investigators suggested that the complete replication of viral RNA was possible because of a helicase activity present in the purified polymerase. They also suggested that 2C has an analogous function in the process of poliovirus RNA replication. To date, no in vitro system has been developed which can catalyze the complete replication of poliovirus RNA. Since we have shown that duplex RNA does not impose any noticeable resistance to chain elongation by 3Dpol, we suggest that the inability to reconstitute an in vitro system which can catalyze the complete replication reaction is likely because of problems in initiation rather than elongation.

The observation that the poliovirus polymerase by itself can unwind an RNA duplex during the elongation reaction does not exclude the possibility that 2C is an RNA helicase. The molecular mechanism of initiation of RNA synthesis is
still unknown; for example, it has not been determined what components are involved, what the order of reactions is, or how the synthesis of negative strands differs from that of the positive strands. Since 3DPol is the only protein known to be required and sufficient for the elongation reaction in vitro, it is possible that 2C is involved in RNA unwinding during the initiation of RNA synthesis and 3DPol unwinds RNA duplex during chain elongation.

The RNA duplex unwinding activity exhibited by poliovirus polymerase during chain elongation does not appear to be a common feature shared by all polymerases, since AMV RT did not unwind the same substrate used for poliovirus polymerase. Different polymerase systems appear to have evolved alternate mechanisms to solve the problem of duplex formation between the template and the nascent strand during nucleic acid synthesis. Several reports have shown that the bacteriophage φ6, which has three segments of double-stranded RNA as its genome, transcribes semiconservatively such that one of the parental strands of each duplex is displaced by a newly made strand (46, 48). This scheme appears to be quite similar to that of the elongation reaction by poliovirus polymerase described in this study in which nascent strands form duplex structures with the template while displacing the prehybridized antisense RNA. It is not known whether the φ6 polymerase is sufficient for this strand displacement or requires another auxiliary protein(s). In the case of the transcription reaction catalyzed by eukaryotic RNA polymerase II, a nascent RNA strand is prevented from hybridizing to the template DNA by the polymerase because of the enzyme’s ability to bind the nascent transcript (35). According to the model, the nascent RNA enters a product binding site on the protein surface while the transcription bubble advances along with the polymerase which thereby prevents formation of product-template duplex. In the case of bacteriophage φ6, the template RNA rather than the polymerase is responsible for the prevention of duplex RNA. According to the proposed mechanism, both the template strand and the nascent RNA product are displaced from intermolecular base pair interactions in favor of the formation of local intramolecular base pairing (26, 34). This hypothesis has been supported by the observation that RNAs that form less stable intramolecular structures have a greater tendency to form extended RNA duplexes during replication, resulting in reduced synthesis of new RNA strands (1).

As expected from previous data (6), RT did not elongate through the region of the template which was hybridized to a complementary RNA strand. During a retrovirus replication cycle, a complementary DNA strand is synthesized from the viral genomic RNA, generating an RNA-DNA duplex. Subsequently, the RNA strand of the duplex is digested by the RNase H activity of RT, and genomic DNA is synthesized. RT can unwind and displace RNase H hydrolysis products from a DNA template (6), although it was unable to unwind RNA-RNA duplex. Thus, separation of product from template occurs by the combined action of RNase H and an unwinding-like activity of RT.

This study demonstrates that poliovirus RNA polymerase possesses an unwinding activity which displaces extensively base-paired segments of RNA from its template preceding progression of a replication fork, allowing base pairing of the nascent strand to the template. The role of this activity during replication of poliovirus RNA is unknown; it may remove secondary structure in the template or it may unwind duplex segments of different strands of RNA. Clearly, the release of template from product RNA strands to allow further rounds of synthesis appears to be a requirement for the replication reaction.

ACKNOWLEDGMENTS

This work was supported by grant AI 17386 from the National Institutes of Health. T.M.D. was a recipient of a short-term Fogarty International Fellowship.

REFERENCES


