JOURNAL OF VIROLOGY, Sept. 1994, p. 5811–5818
0022-538X/94/$04.00+0

Vol. 68, No. 9

Identification of Terminal Adenylyl Transferase Activity of the Poliovirus Polymerase 3D^{pol}

KRISTI L. NEUFELD,[1,2] JOSÉ M. GALARZA,[3] OLIVER C. RICHARDS,[2] DONALD F. SUMMERS,[3,4] AND ELLIE EHRENFELD[2]*

Department of Cellular, Viral and Molecular Biology, University of Utah School of Medicine, Salt Lake City, Utah 84132,[1] and Departments of Molecular Biology and Biochemistry,[2] Microbiology and Molecular Genetics,[3] and Medicine,[4] University of California, Irvine, California 92717

Received 8 February 1994/Accepted 9 June 1994

A terminal adenylyl transferase (TATase) activity has been identified in preparations of purified poliovirus RNA-dependent RNA polymerase (3D^{pol}). Highly purified 3D^{pol} is capable of adding [^{32}P]AMP to the 3′ ends of chemically synthesized 12-nucleotide (nt)-long RNAs. The purified 52-kDa polypeptide, isolated after sodium dodecyl sulfate-polyacrylamide gel electrophoresis and renatured, retained the TATase activity. Two 3D^{pol} mutants, purified from *Escherichia coli* expression systems, displayed no detectable polymerase activity and were unable to catalyze TATase activity. Likewise, extracts from the parental *E. coli* strain that harbored no expression plasmid were unable to catalyze formation of the TATase products. With the RNA oligonucleotide 5′-CCUGCUUUUGCA-3′ used as an acceptor, the products formed by wild-type 3D^{pol} were 9 and 18 nt longer than the 12-nt oligomer. GTP, CTP, and UTP did not serve as substrates for transfer to this RNA, either by themselves or when all deoxynucleoside triphosphates were present in the reaction. Results from kinetic and stoichiometric analyses suggest that the reaction is catalytic and shows substrate and enzyme dependence. The 3′-terminal 13 nt of poliovirus minus-strand RNA also served as an acceptor for TATase activity, raising the possibility that this activity functions in poliovirus RNA replication. The efficiency of utilization and the nature of the products formed during the reaction were dependent on the acceptor RNA.

Poliovirus is an icosahedral virus with a positive-sense RNA genome approximately 7,500 nucleotides (nt) long. The viral genome encodes an RNA-dependent RNA polymerase, 3D^{pol}, which is capable of elongating RNA strands in a primer- and template-dependent manner (17, 49, 50). The enzyme manifests strong nucleotide-binding activity (40) and is able to unwind duplex RNA as it elongates (15). During poliovirus RNA replication, the genomic plus-strand RNA serves as template for synthesis of minus-strand RNA. Minus strands in turn serve as templates for the synthesis of more plus-strand RNA. However, the protein requirement, protein functions, and the mechanism of initiation of RNA replication remain poorly understood. RNA strand initiation must occur in such a way that the integrity of the terminal sequences is maintained. The small viral protein, VPg, is covalently attached to the 5′ end of all nascent strands of poliovirus RNA. At least two models that account for these observations have been proposed (reviewed in references 26 and 37). In one model, the viral protein VPg is uridylylated, possibly by 3D^{pol} (48). This nucleotidyl protein then can be elongated into new RNA strands by 3D^{pol} (8, 16, 18, 28, 31, 45, 48, 51). In a second model, uridylate (U) residues are added to the 3′-terminal adenylate residue of poliovirus RNA, possibly by a host terminal uridylyl transferase (TUTase) (4, 5). The added U residues allow the template to fold back and self-prime synthesis of new RNA strands by 3D^{pol}. Endonucleolytic scission between template and product strands would be accompanied by VPg addition, perhaps in an RNA-catalyzed reaction (47).

The two models are not necessarily mutually exclusive. Minus- and plus-strand RNAs might be initiated by different mechanisms; therefore, one model could describe minus-strand RNA synthesis, and the other could describe plus-strand synthesis. Several independent studies have demonstrated differential effects on synthesis of plus and minus strands by either mutations or other perturbations (2, 18, 24, 35), suggesting that different mechanisms or requirements may operate during synthesis of the two strands.

Because of the integral role that 3D^{pol} plays in viral RNA replication, we have been involved in a long-term examination of this protein and its biochemical properties. Application of knowledge gained from such studies to proposed models might bring us closer to an understanding of the mechanism of RNA replication. Since many other RNA and DNA polymerases have been shown to possess multiple enzymatic activities, it would not be surprising if poliovirus 3D^{pol} also has several distinct activities (33, 34, 46). For example, human immunodeficiency virus reverse transcriptase, like many retroviral reverse transcriptases, possesses both a DNA chain elongation activity and the ability to hydrolyze RNA when it is in duplex with DNA (RNase H activity) (34). These two activities are catalyzed by two separate domains of the reverse transcriptase molecule. The poliovirus polymerase protein also has at least two functions. In addition to RNA-dependent RNA polymerase activity, 3D^{pol} protein sequences contribute to 3CD protease activity. Mutational studies of the polymerase protein revealed that the RNA polymerase activity and the protein processing activity did not reside in distinct regions along the linear polypeptide (13). Further mutational studies, combined with three-dimensional X-ray crystallographic analysis of the polymerase, may yet reveal distinct domains responsible for the various polymerase activities. Other suggested activities of 3D^{pol} include RNA unwinding (15), uridylylation of VPg (48), interaction with cellular proteins and poliovirus RNA near the 5′ terminus of the positive strand (1), and association with membrane structures (14).

* Corresponding author. Mailing address: Department of Molecular Biology and Biochemistry, University of California, Irvine, CA 92717. Phone: (714) 725-3264. Fax: (714) 856-8551.

In the course of performing standard polymerase assays using small RNA oligomers to prime the reaction, we observed an activity of the highly purified polymerase that had not been described previously. Upon further examination, it was determined that $3D^{Pol}$ was able to add multiple adenylate (A) residues to the 3′ terminus of RNA in a nontemplated manner. We have classified this activity as a terminal adenylyl transferase (TATase) activity, although $3D^{Pol}$ has properties that distinguish it from TATases reported in the literature. It is proposed that this novel activity could function in poliovirus RNA replication.

MATERIALS AND METHODS

Synthesis of RNA 12- and 13-mers. Oligoribonucleotides were synthesized at the core facility of the University of Utah, using solid-phase methodology and phosphoramidate chemistry. Oligoribonucleotides were 12 or 13 nt long, and their sequences are shown in Table 1.

TABLE 1. RNA oligomers used in the terminal nucleotidyl transferase assay

Oligomer	Sequence (5′→3′)	^{32}P-NTP	Product sizes (nt)	Relative intensity (%)
1-A	CCUGCUUUUGCA	A	21, 30	100
		G		
		C		
		U		
PV	AGAGCUGUUUUAA	A	16, 19	50
		G		
		C		
		U		

Nucleotide transfer reactions. Transfer reactions were carried out in a mixture containing 25 μl of 50 mM *N*-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES; pH 7.2), 4 mM dithiothreitol, 3 mM magnesium acetate, 0.06 mM $ZnCl_2$, actinomycin D (4 μg/ml), 4 U of RNAsin (Promega Biotec, Madison, Wis.), 0.5 mM UTP, CTP, and GTP (or otherwise as indicated), 24 μM ATP, 20 μCi of [α-^{32}P]ATP, 500 ng (125 pmol) of oligoribonucleotide acceptor (measured by A_{260}, assuming an extinction coefficient of 25), and 740 fmol of $3D^{Pol}$ purified from *Escherichia coli* expressing the enzyme from a recombinant cDNA (38). The reaction mixtures were incubated at 32°C for 40 min unless otherwise specified. After phenol-chloroform extraction, samples were precipitated with ethanol in the presence of yeast tRNA as a carrier. Precipitated material was washed twice with 70% ethanol and resuspended in 8 μl of loading buffer containing 80% formamide, 10% glycerol, 1 mM EDTA, and 0.025% bromphenol blue, and xylene cyanol. Samples were loaded on a 10% polyacrylamide–7 M urea gel, which was run at 1,000 V until the bromphenol blue dye migrated one-half to three-fourths the length of the gel. Gels were dried and exposed to Kodak X-Omat AR-5 film (Eastman Kodak Co., Rochester, N.Y.). When CTP, GTP, or UTP was the labeling nucleotide, reactions contained 24 μM this nucleotide, 20 μCi of ^{32}P-labeled nucleoside triphosphate (NTP) (3,000 Ci/mmol) and 500 μM each unlabeled NTP.

Poliovirus polymerase purification. Polymerase was purified through the phenyl-Superose column step from *E. coli* containing the plasmid pEXC-3D, pEXC-3D-μ7256, or pEXC-3D-μ6707 as described previously (32). Polymerase 3D-149 was purified through the Mono Q chromatography step from *E. coli* harboring the plasmid pEXC-3D-μ6432. Polymerase concentrations were determined by poly(A)-dependent poly(U) polymerase assay and comparative silver staining as described previously (32), or by Western blotting (immunoblotting) and silver staining for polymerase mutants that had no activity. For one experiment, dialyzed wild-type polymerase was boiled for 3 min in gel sample buffer and resolved by electrophoresis in a sodium dodecyl sulfate (SDS)–10% polyacrylamide gel. A Westran membrane (Schleicher & Schuell) was prepared for protein transfer by rinsing with 100% methanol followed by transfer buffer (20 mM Tris-HCl [pH 8.0], 150 mM glycine). Protein was transferred at 4°C for 2 h at 60 V from the gel to the membrane in transfer buffer. Following transfer, the membrane was incubated with 0.2% amido black for 15 min to stain the protein. The membrane was destained in water until the protein could be visualized. The stained region was excised and cut into small pieces to fit in an Eppendorf tube, and 250 μl of elution buffer (100 mM Tris-HCl [pH 9.0], 2% SDS, 1% Triton X-100, 150 mM NaCl) was added for 1 h at room temperature. The liquid was collected, and the membrane pieces were rinsed with 250 μl of elution buffer. Proteins were precipitated from the combined eluates with 4 volumes of acetone and incubated for 30 min in a dry ice-ethanol bath. The precipitated proteins were pelleted by centrifugation in a microcentrifuge at 14,000 rpm for 20 min. The pellet was washed with 80% acetone in buffer A (50 mM Tris [pH 8.0], 0.1% Nonidet P-40, 10% glycerol, 5 mM β-mercaptoethanol, 10 mM KCl). The pellet was resuspended in 20 μl of 6 M guanidine-HCl in buffer A and incubated for 15 min at room temperature to completely dissolve the pellet, and then the guanidine concentration was lowered by the addition of 80 μl of buffer A. The protein was renatured by dialysis against buffer A for 12 h at 4°C.

RESULTS

Identification of TATase activity in preparations of poliovirus polymerase. The RNA-dependent RNA polymerase isolated from poliovirus-infected cells has been reported to be both template and primer dependent (49, 50). In the course of carrying out RNA synthesis assays with purified poliovirus polymerase using various template and primer RNAs, we observed incorporation of labeled nucleotide in reactions that contained certain primer RNAs (12-nt oligomers) in the absence of template. Figure 1 shows the results of a typical experiment in which the small RNA primer, 5′-CCUGCU UUUGCA-3′ (oligomer 1-A in Table 1), was incubated, in the absence of template, with [α-^{32}P]ATP and purified poliovirus polymerase ($3D^{Pol}$), using buffer conditions optimized for polymerase activity. Following a 40-min incubation at 32°C, products were separated on a 7 M urea–10% polyacrylamide sequencing gel, which was then dried and autoradiographed. The major labeled products of the complete reaction (Fig. 1, lane 2) appear to be 21 and 30 nt long when compared against a 13-nt size standard (lane 1) and a ladder of nucleotide markers shown to the left. (To the extent that gel mobility may be affected by sequence or the presence or absence of terminal phosphates, the precise lengths of the products are estimates.) The products are most likely the result of the addition of labeled AMP to the 3′ terminus of the 12-mer RNA (oligomer 1-A). Two products of the same mobility were produced in a reaction that also included unlabeled GTP, UTP, and CTP, each at 500 μM (lane 3). It was occasionally observed that the 30-nt product was somewhat more abundant when all four

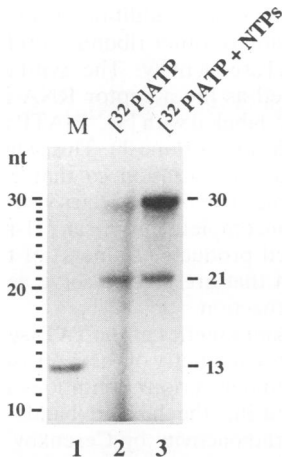

FIG. 1. Identification of TATase activity in preparations of poliovirus polymerase. Purified 3D^{pol} was assayed for TATase activity by using RNA oligomer 1A (Table 1) as the substrate and [α-^{32}P]ATP to label products. Following incubation for 40 min at 32°C with standard buffer and salt conditions for polymerase activity, product RNA was separated from protein by phenol-chloroform extraction and ethanol precipitation. Products were resolved in a 10% polyacrylamide–7 M urea gel as detailed in Materials and Methods. Lane 2 contains the products from a reaction in which [α-^{32}P]ATP is the only NTP present. In lane 3, all NTPs were included in the reaction, but only ATP was labeled. The mobility of an oligonucleotide ladder, created by end labeling a mixed population of dephosphorylated poly(A) with [^{32}P]pCp and T4 RNA ligase, is marked on the left. A 13-nt-long synthetic RNA (oligomer PV; Table 1), labeled with [γ-^{32}P]ATP by using polynucleotide kinase, served as the 13-nt marker (M; lane 1).

nucleotides were present in the reaction (lane 3). When either 3D^{pol} or oligomer 1-A was eliminated from the reaction, no products were formed (data not shown).

Association of TATase activity with poliovirus polymerase. The 3D^{pol} used in these experiments was purified by subcellular fractionation, fractional ammonium sulfate precipitation, phosphocellulose ion-exchange chromatography, Mono Q fast protein liquid chromatography (FPLC), and phenyl-Superose FPLC. It migrates in SDS-polyacrylamide gel electrophoresis (PAGE) as a 52-kDa polypeptide and appears to be >98% pure by silver staining (Fig. 2, lane 2). Low levels of contaminating proteins are present, which could potentially account

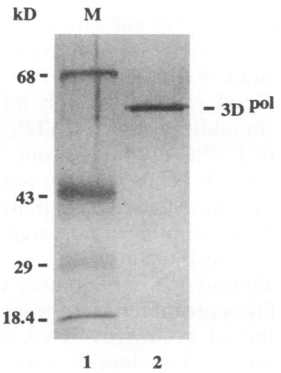

FIG. 2. SDS-PAGE of 3D^{pol}. Purified 3D^{pol} was analyzed on an SDS–10% polyacrylamide gel and visualized by silver staining (lane 2). Molecular mass standards (M) were resolved on the same gel and are shown in lane 1.

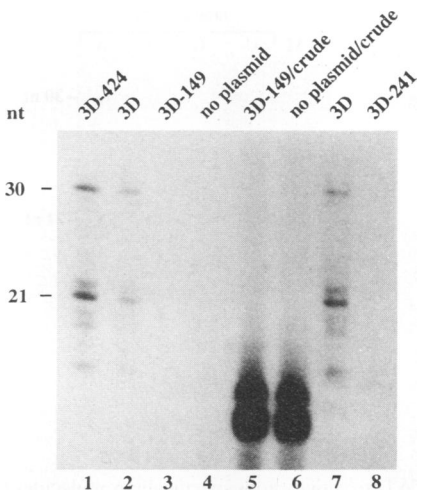

FIG. 3. TATase activity is catalyzed by poliovirus 3D^{pol}. Various preparations were tested for TATase activity as described for Fig. 1, lane 3. Polymerase was purified through the phenyl-Superose step unless otherwise indicated. Reactions were catalyzed by 3D-424 (lane 1), wild-type 3D (lanes 2 and 7), or 3D-149 purified through the Mono Q chromatography step (lane 3) or only by ammonium sulfate fractionation (lane 5), *E. coli* containing no plasmid purified through the Mono Q chromatography step (lane 4) or only by ammonium sulfate fractionation (lane 6), or 3D-241 (lane 8).

for the observed TATase activity. To eliminate this possibility, 3D^{pol} was purified away from proteins with different mobilities by separation on SDS–10% polyacrylamide gels. The polypeptide was transferred from the gel to a Westran membrane, eluted from the membrane, precipitated, and renatured after being dissolved in 6 M guanidine. When 3D^{pol} purified in this way was used in the template-independent reaction shown in Fig. 1, lane 2, products of identical mobilities were formed (data not shown), thus providing evidence that the observed activity can be attributed to 3D^{pol}.

Genetic evidence that the terminal adenylyl transfer was catalyzed by 3D^{pol} and not a contaminating bacterial enzyme was obtained by examining TATase activity in bacteria transformed with plasmids encoding mutant 3D proteins. pEXC-3D-μ6432 and pEXC-3D-μ6707 direct expression of polymerase proteins with an Ile insertion following amino acids 149 (3D-149) and 241 (3D-241), respectively (13). Neither protein displayed detectable polymerase activity or GTP-binding ability in vitro (13, 40). Purification of 3D-149 through the Mono Q chromatography step and of 3D-241 through the phenyl-Superose step was performed by monitoring fractions by immunoblot, and the samples were subsequently assayed for TATase activity. Both preparations showed no activity (Fig. 3, lanes 3 and 8) when compared with wild-type 3D^{pol} purified in parallel with 3D-241 (lane 7). Since contaminating bacterial proteins present in those fractions containing wild-type polymerase protein are equally likely to be present in fractions containing the mutant polymerase proteins, the absence of TATase activity in both of these preparations is due to the mutations in the 3D proteins.

Another mutant 3D^{pol} protein, 3D-424, which contained a substitution of His for Asn at position 424, was expressed from *E. coli* containing pEXC-3D-μ7256 (12) and purified through the phenyl-Superose step. This mutant protein had levels of polymerase activity similar to those of the wild-type enzyme when expressed and assayed at 37°C in vitro (not shown) (12),

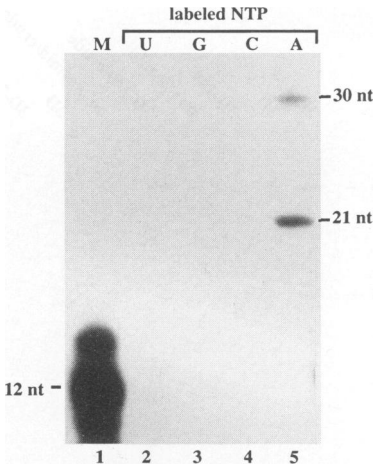

FIG. 4. TATase activity adds specifically A molecules to the 3′ ends of acceptor RNA. TATase assays were performed as described for Fig. 1, lane 3, except that the indicated NTPs were substituted for the [α-^{32}P]ATP. All four NTPs were present in each reaction, and the ^{32}P-labeled NTP was UTP (lane 2), GTP (lane 3), CTP (lane 4), or ATP (lane 5). The acceptor RNA, oligomer 1-A, was labeled by using polynucleotide kinase and served as the 12-nt marker (M) in lane 1.

and the TATase activities were nearly identical as well (Fig. 3, lanes 2 and 1).

Finally, the *E. coli* host strain, C600, was tested for TATase activity at early and late stages of purification. Crude preparations from *E. coli* C600 that had been subjected only to ammonium sulfate fractionation catalyzed synthesis of ^{32}P-labeled products when incubated with oligomer 1-A and [^{32}P]ATP under conditions that supported TATase activity (Fig. 3, lane 6). The products of this reaction, however, were of a different size than products formed by wild-type 3D^{pol} (lanes 2 and 7). Whereas 3D^{pol} catalyzed formation of major products that were 21 and 30 nt long, bacterial protein(s) catalyzed synthesis of two major products approximately 14 and 15 nt long. When products were separated on a gel that allowed resolution of larger fragments, several minor but distinct products that ranged in size from 60 to 100 nt were visible. The same products were formed by similar crude preparations from C600 harboring pEXC-3D-μ6432, which programmed expression of the inactive 3D-149 (Fig. 3, lane 5). Further purification through the Mono Q stage of C600 alone or C600 expressing 3D-149 resulted in protein preparations that possessed no TATase activity (lanes 4 and 3). This result indicates that although *E. coli* contains a protein(s) capable of adenylate transfer, this activity is distinct from the TATase activity catalyzed by 3D^{pol}, and in fact, this activity is purified from 3D^{pol} during the purification regimen. From these results, we conclude that the TATase activity described in this report is catalyzed by poliovirus 3D^{pol}.

NTP specificity of TATase activity of poliovirus polymerase. Providing only the single nucleotide [α-^{32}P]UTP, [α-^{32}P]GTP, or [α-^{32}P]CTP in the terminal nucleotidyl transferase assay resulted in no product formation (data not shown). In addition, [α-^{32}P]UTP, [α-^{32}P]GTP, or [α-^{32}P]CTP was substituted for [α-^{32}P]ATP in reaction mixtures which contained the remaining three unlabeled NTPs and oligomer 1-A (Fig. 4, lanes 2 to 5). No products were formed when the labeled nucleotide was other than ATP, indicating that only A residues were added to the RNA 12-mer. Even lengthy exposures of the gel to film did not reveal incorporation of other nucleotides into the products. Because of the specific addition of A residues to oligomer 1-A, to the exclusion of other ribonucleotides, we refer to this activity as a TATase activity. The synthetic RNA 12-mer, oligomer 1-A, used as the acceptor RNA in the experiments described thus far, labeled with [γ-^{32}P]ATP and polynucleotide kinase, is shown in Fig. 4 (lane 1). This sample was loaded on the gel at a high concentration so that even minor species would be detectable. The other species visible in this lane most likely represent incomplete products (11-mers and 10-mers, etc.) and branched products (13-mers) of the RNA oligomer synthesis reaction that are able to serve as substrates for the phosphorylation reaction.

Stoichiometry and kinetics of the TATase reaction. To study the kinetics and stoichiometry of the TATase activity, reactions were performed under various conditions and products were quantitated by cutting the labeled bands from the gel and determining the radioactivity by Cerenkov counting. The cumulative AMP incorporation into both of the products is presented in Fig. 5.

The rate of [α-^{32}P]AMP incorporation into products was linear for at least 80 min in a reaction containing 500 ng (125 pmol) of oligomer 1-A and 40 ng (740 fmol) of 3D^{pol} (Fig. 5A). In other experiments, linear incorporation occurred for as long as it was measured, with the longest incubation time being 180 min (data not shown). From data presented in Fig. 5B, it was determined that after 40 min of incubation, there was 3 fmol of 21-mer product for every 1 fmol of 30-mer product.

Product formation was directly proportional to the concentration of 3D^{pol} in the reaction (Fig. 5C). At the highest polymerase concentration tested (80 ng [1.5 pmol]/25-μl reaction) in a reaction that contained 500 ng of oligomer 1-A, approximately 1 of 320 molecules of 12-mer RNA was terminally adenylylated after 40 min, assuming processivity. This estimate assumes that 3D^{pol} adds either 9 or 18 adenylate residues to an acceptor molecule with a relative ratio of 3:1, as calculated from Fig. 5B. Since incorporation of AMP was linear over time up to at least 180 min, we can extrapolate that 1 of every 72 molecules of 12-mer substrate would be adenylylated by 180 min.

At a constant concentration of enzyme (40 ng of 3D^{pol}/25 μl), the reaction rate increased with increasing oligomer 1-A concentration until a maximum velocity was reached, using 500 ng of oligomer 1-A RNA (Fig. 5D). From these data, V_{max} and K_m for the RNA substrate were calculated to be 3 pmol of AMP incorporated/40 min and 10^{-6} M, respectively.

Sequence requirements of substrate for TATase activity. The importance of the 3′-terminal nucleotide of the acceptor RNA was evaluated by testing a series of oligonucleotides with the same sequence except for substitution of the A residue at the 3′ terminus of oligomer 1-A with other nucleotides. Each acceptor RNA tested was able to serve as substrate in a reaction that contained ATP as the only nucleotide (data not shown). However, in addition to [^{32}P]ATP, the same acceptor RNAs could utilize [^{32}P]GTP, and in one case [^{32}P]CTP, at lower efficiency when all NTPs were present in the reaction. The possibility of intermolecular base pairing between RNA oligomers leading to a potentially templated reaction confused the interpretation of these results.

The fact that different RNA oligomers were used as substrates for this reaction prompted us to test whether the 3′ end of the minus strand of poliovirus RNA could serve as an acceptor for nontemplated nucleotide addition. When a 13-nt oligomer RNA with the sequence corresponding to the 3′ end of poliovirus minus-strand RNA (oligomer PV; Table 1) was used as a substrate in the reaction with [^{32}P]ATP and unlabeled CTP, GTP, and UTP, two products, 16 and 19 nt in

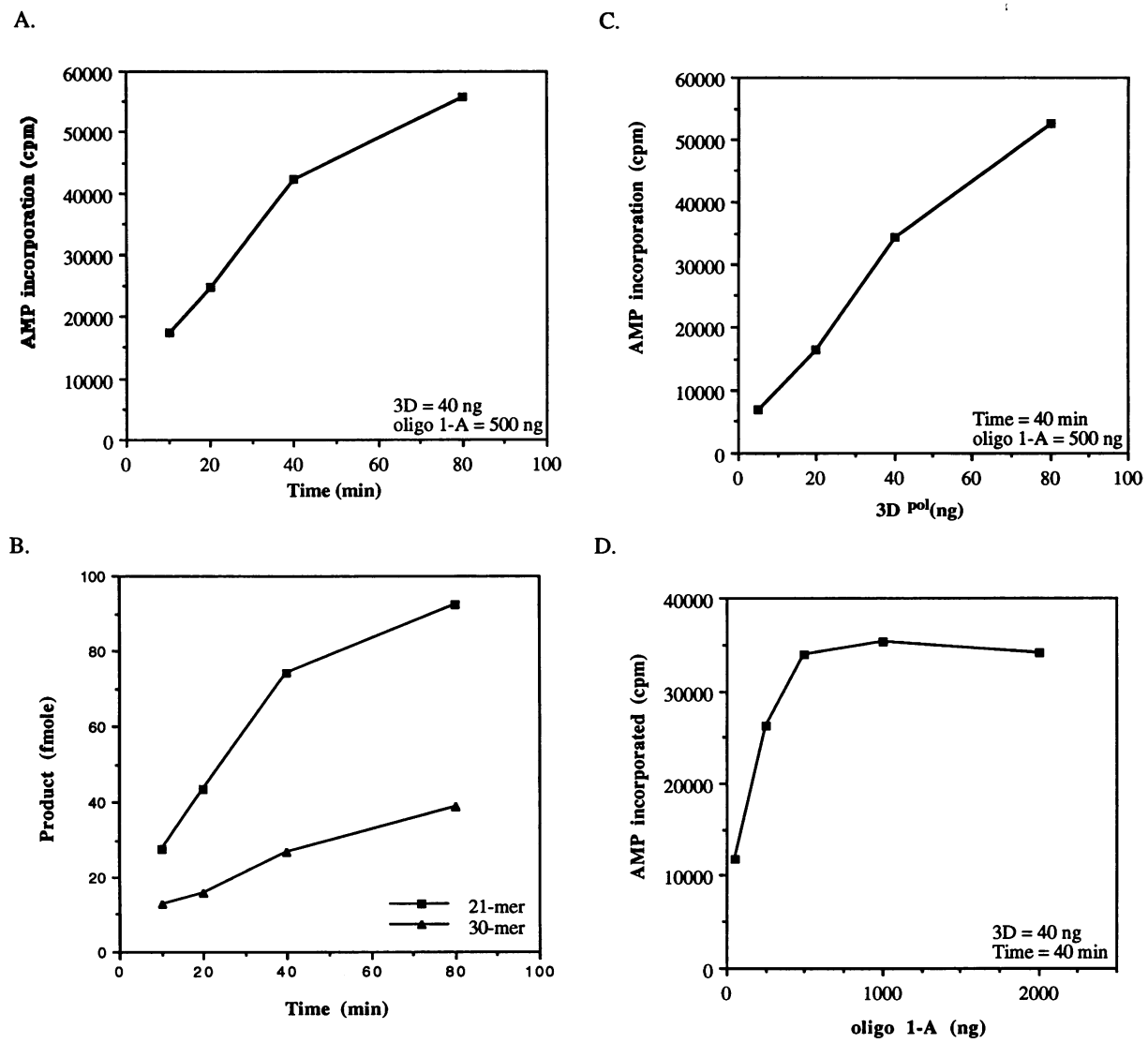

FIG. 5. Kinetic parameters of the TATase reaction. Reactions were performed under various conditions described below and included 0.5 mM UTP, CTP, and GTP, 25 μM ATP, and 20 μCi of [α-^{32}P]ATP. One third of the reaction was used for denaturing PAGE (10% polyacrylamide gel). Labeled products were excised from the resolving gel and counted by the method of Cerenkov. (A) Radioactivity for the sum of the 21-mer and 31-mer products accumulated over 80 min, using 40 ng of 3D^{pol} and 500 ng of oligomer 1-A (Table 1) in the reaction. (B) The two major products plotted separately as femtomoles of 21- and 30-nt product accumulated over time. One femtomole of 21-mer is equal to 660 cpm, and 1 fmol of 30-mer is equal to 1,320 cpm, assuming Cerenkov counting efficiency of 50%. In panel C, the oligomer 1-A concentration was kept constant (500 ng per reaction) but the amount of 3D^{pol} was varied from 5 to 80 ng. Total counts were plotted as in panel A. In panel D, the 3D^{pol} concentration was kept constant (40 ng per reaction) and the amount of oligomer 1-A was varied from 50 to 2,000 ng (12.5 to 500 pmol).

length, were formed (Fig. 6, lane 1). The same RNA oligomer labeled with [γ-^{32}P]ATP by using polynucleotide kinase was used as a 13-nt marker (lane 2). In a different assay that contained [^{32}P]ATP in the absence of other NTPs, two products, 16 and 19 nt long, were formed, suggesting that the enzyme adds blocks of three AMPs twice rather than blocks of nine AMPs twice as seen with oligomer 1-A. Kinetic analysis of the two products revealed formation profiles similar to those of the two products formed with oligomer 1-A (data not shown). In one experiment in which [^{32}P]ATP was replaced with either [^{32}P]GTP or [^{32}P]UTP no products were formed (Table 1). These results support the hypothesis that the poliovirus 3′-terminal 13 residues of minus-strand RNA can serve as

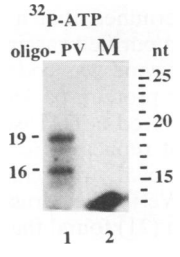

FIG. 6. TATase activity when acceptor RNA with poliovirus sequence is used. A TATase assay was performed as described for Fig. 3, using labeled ATP and including the other three unlabeled NTPs in the reaction. Synthetic RNA used as the acceptor molecule was oligomer PV (lane 1). The 13-nt marker (M) is shown in lane 2.

acceptor for a terminal adenylyl transfer by the poliovirus $3D^{Pol}$.

To analyze acceptor RNA requirement in more detail, poly(A) and oligo$(dT)_{10}$ were tested for their abilities to serve as acceptors for the TATase reaction. When poly(A) was used in a reaction with all NTPs present but only [^{32}P]ATP labeled, $3D^{Pol}$ was able to add label to the poly(A) (data not shown). In contrast, when oligo$(dT)_{10}$ was used as the acceptor under the same conditions, $3D^{Pol}$ was unable to add labeled AMP to it, even though oligo$(dT)_{10}$ can be used to prime an in vitro polymerase reaction with poly(A) as template (data not shown).

DISCUSSION

Poliovirus $3D^{Pol}$ specifically added adenylate residues to oligomer 1-A or PV. We conclude, therefore, that this enzyme manifests specific TATase activity with certain acceptor RNAs. The activity differs from activities of previously reported TATases and poly(A) polymerases. One of the unique features of $3D^{Pol}$ TATase activity is that a specific number of residues is added to a given acceptor RNA. In the case of oligomer 1-A, the major products formed were always 9 and 18 nt longer than the substrate. Only very low levels of intermediate-size products were detected during the course of the reaction. Intermediate-size products would be expected if the addition of A residues was as slow as suggested by the overall rate. This observation indicates that the polymerase rapidly added nine adenylate residues to oligomer 1-A once TATase activity was initiated. The rate-limiting step is likely acceptor RNA recognition or initiation rather than the elongation step. It is unclear how $3D^{Pol}$ counts terminally added nucleotides. The observation that changing the RNA substrate from 5′-CCUGCUU UUGCA-3′ (oligomer 1-A) to 5′-AGAGCUGUUUUAA-3′ (oligomer PV) altered the number of AMP residues added suggests that nucleotide counting is somehow influenced by acceptor RNA. Another example of acceptor RNA influencing the number of nucleotides added comes from studies of the TUTase purified from rabbit reticulocytes (5). With poly(C) as the acceptor RNA, an average of 19 U residues were added by the TUTase; with poly(A), 4 U residues were added; and with oligo(U), 30 to 40 U residues were added. In a subsequent study using the same TUTase, 5 U residues, on average, were added to poliovirion RNA (4). These products, however, were quite heterogeneous; therefore, the number of residues added represents an average rather than a block addition. This is true of products from poly(A) and poly(U) polymerases reported in the literature as well (23, 41).

Copurification of TUTase or TATase activity with a viral replicase has been described several times in the literature, leading to the hypothesis that cellular enzymes are recruited by the virus and serve some function in viral genome replication. Brown et al. (11) determined that a terminal riboadenylate transferase is present in purified vaccinia virion cores. It is not known whether this enzyme was coded by the vaccinia virus DNA or was a cellular protein, packaged in the viral capsid. Zabel et al. (55) discovered a TUTase as a cellular contaminant in preparations of replicase from the plus-strand RNA virus cowpea mosaic virus. During characterization of subcellular fractions from West Nile virus-infected BHK-21/W12 cells, Grun and Brinton (21) found that a cellular TATase and a cellular TUTase cofractionated with the viral RNA-dependent RNA polymerase. West Nile virus, like poliovirus, has a single-stranded RNA genome of positive polarity. It belongs to the family *flaviviridae*. Grun and Brinton determined that the cellular TUTase and TATase did not add a detectable level of radiolabeled nucleotide to any form of West Nile virus RNA in vitro and therefore concluded that these enzymes play no specific role in flavivirus RNA synthesis.

If the poliovirus TATase activity functions during viral RNA replication, some of the viral RNA products might reflect a nontemplated A addition. The terminal sequences have been determined for all three types of viral RNA found in poliovirus-infected cells: replicative intermediate (RI), replicative form (RF), and single stranded. RI RNAs are heterogeneous RNA molecules, partially single and partially double stranded (7, 10, 19, 20, 39), whereas RF RNAs are double stranded (6, 8, 26, 30). The poly(A) sequence at the 3′ terminus of poliovirus genomic RNA has been shown to average 89 nt in length (42, 52). Because of the poly(U) stretch found at the 5′ termini of minus strands of RI and RF RNAs, it is generally believed that the majority of the poly(A) tract on plus-strand RNA is added in a template-dependent manner (27, 42, 44, 52). RI poly(U) tracts were found to be 50 to 200 nt long when measured 3 h postinfection (42, 54). However, in RF RNA, the 5′ termini of the minus strand had a poly(U) stretch of 60 to 80 nt and a poly(A) tract at the 3′ termini of the complementary strand that extended approximately 90 nt beyond the poly(U) tract (27, 52). In addition, Richards and Ehrenfeld (36) found that the 3′ termini of minus-strand RNA from RF RNA had extra A residues compared with the corresponding plus strand. Spector and Baltimore (43) reported that in crude membrane fractions from poliovirus-infected HeLa cells, poly(A) could be added to both newly synthesized and preexisting poliovirus RNA molecules of all types in vitro. These observations raise the question, how are these nontemplated A residues added to the poliovirus RNA? One possibility is that AMP residues are added to 3′ ends by a cellular poly(A) synthetase. The results presented in this report suggest an alternative possibility that the poliovirus polymerase is able to add the nontemplated A residues by virtue of its TATase activity.

The function of these additional A residues is unclear. They could be added to plus-strand poliovirus RNA for much the same reason that poly(A) tails are added to mRNA, to protect the message and stimulate translation. The fact that A residues are added to the 3′ terminus of minus-strand RNA points to a possible function other than translation. Although the function of extra A residues on minus-strand RNA could merely be to protect the terminal sequences from degradation, it is possible that added A residues play a role in initiation of plus-strand RNA synthesis.

The mechanism by which the synthesis of plus-strand RNA is initiated is not known. Figure 7 illustrates a model that incorporates a role for a TATase activity to add poly(A) residues at the 3′ end of minus-strand RNA during the initiation of plus-strand RNA synthesis. In the model, synthesis of minus-strand RNA is completed in a template-dependent manner (Fig. 7I). After addition of the last six nucleotides, UUUUAA, $3D^{Pol}$ adds three to six extra A residues by virtue of its TATase activity (Fig. 7II). These extra A residues could then form a hairpin structure to base pair with the four U residues, priming synthesis of plus-strand RNA (Fig. 7III). VPg, already uridylylated to form VPg-pUpU, would serve as the acceptor in a transesterification reaction occurring between the extra A residues that are looped out and the A residues base paired to U residues (Fig. 7IV). This would result in the linking of VPg-pUpU to the string of four A residues and the liberation of a free 3′ adenylate tail attached to the minus-strand RNA (Fig. 7V).

At the present time, there are insufficient biological or biochemical data to support any specific model for poliovirus RNA synthesis. The involvement of a TATase activity as

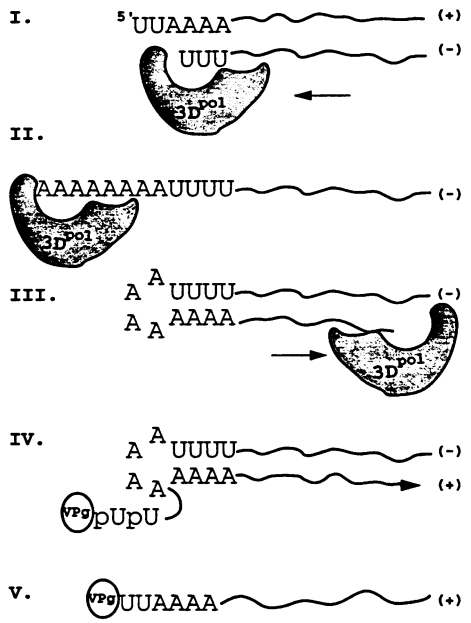

FIG. 7. A possible role for TATase activity during poliovirus plus-strand RNA initiation and synthesis. (I) Poliovirus 3D^{pol} completes synthesis of minus-strand RNA in a template-dependent manner. (II) Following addition of the last templated nucleotide, 3D^{pol} adds three to six extra A residues by virtue of its TATase activity. (III) The 3′-terminal A residues fold back and base pair with the U residues in the minus-strand RNA, priming synthesis of plus-strand RNA by 3D^{pol}. (IV) Uridylylated VPg is transferred to the plus-strand RNA between the extra A residues on the template and the base-paired product strand. (V) Following transesterification, the protein VPg is linked to the nascent plus-strand RNA.

suggested here is supported by data from Harmon et al. (22), who found that the 5′-terminal two U residues of the poliovirus plus-strand RNA sequence are not required for infectivity. Transcripts produced from poliovirus cDNA lacking the 5′-terminal two nucleotides produced viable virus when transfected into cells. Recovered virus had regained the missing terminal U residues. A similar result was found with coxsackievirus B3, another member of the picornavirus family (25). In the model shown in Fig. 7, if the first two U residues were missing in the plus-strand poliovirus RNA, all A residues could be added by virtue of the TATase activity of 3D^{pol}, rather than having the first two added in a template-dependent manner. Lubinski et al. (29) found that when primed with oligo(U), preparations of 3D^{pol} were able to catalyze synthesis of an RNA product twice the length of virion RNA template. The implication of this result is that in vitro, 3D^{pol} can synthesize minus-strand RNA in a primer- and template-dependent manner and that once that has occurred, the minus-strand RNA can fold back to prime elongation of a plus strand of RNA. This is consistent with the model presented here. The model is also compatible with data presented by Andino et al. (1–3). These investigators described a ribonucleoprotein complex which forms at the 5′ end of plus-strand RNA. Disruption of this complex selectively affected positive-strand RNA accumulation, leading to the suggestion that the nucleoprotein complex functions to localize replication proteins to the site of plus-strand RNA initiation (1, 2). In our model, if the 5′ end of plus-strand RNA folds into a cloverleaf-like structure after serving as template for the synthesis of minus-strand RNA, the 3′ terminus of the nascent minus strand is liberated. This could be a necessary prerequisite for 3D^{pol} terminal nucleotidyl transferase to adenylylate the 3′ end of the minus-strand RNA (Fig. 7II).

ACKNOWLEDGMENTS

We thank Robert Shackman for synthesis of the RNA oligomers and Susan Baker for technical assistance.

This work was supported in part by Public Health Service grants AI17386 and AI12316 from the National Institutes of Health. Additional support for K.L.N. came from Public Health Service grant CA-09602 from the National Cancer Institute.

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