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Poliovirus RNA-Dependent RNA Polymerase Synthesizes Full-Length Copies of Poliovirion RNA, Cellular mRNA, and Several Plant Virus RNAs In Vitro

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The poliovirus RNA-dependent RNA polymerase was active on synthetic homopolymeric RNA templates as well as on every natural RNA tested. The polymerase copied polyadenylate · oligouridylate [oligo(U)], polycytidylate · oligoinosinate, and polyinosinate · oligocytidylate templates to about the same extent. The observed activity on polyuridylate · oligoadenylate was about fourfold less. Full-length copies of both poliovirion RNA and a wide variety of other polyadenylated RNAs were synthesized by the polymerase in the presence of oligo(U). Polymerase elongation rates on poliovirion RNA and a heterologous RNA (squash mosaic virus RNA) were about the same. Changes in the Mg^{2+} concentration affected the elongation rates on both RNAs to the same extent. With two non-polyadenylated RNAs (tobacco mosaic virus RNA and brome mosaic virus RNA3), the results were different. The purified polymerase synthesized a subgenomic-sized product RNA on brome mosaic virus RNA3 in the presence of oligo(U). This product RNA appeared to initiate on oligo(U) hybridized to an internal oligoadenylate sequence in brome mosaic virus RNA3. No oligo(U)-primed product was synthesized on tobacco mosaic virus RNA. When partially purified polymerase was used in place of the completely purified enzyme, some oligo(U)-independent activity was observed on the brome mosaic virus and tobacco mosaic virus RNAs. The size of the product RNA from these reactions suggested that at least some of the product RNA was full-sized and covalently linked to the template RNA. Thus, the polymerase was found to copy many different types of RNA and to make full-length copies of the RNAs tested.

A poliovirus RNA-dependent RNA polymerase was isolated as a soluble and template-dependent enzyme from the cytoplasm of infected cells (5, 8, 9, 11). A single virus-specific protein, designated p63 (also NCVP4 [20] and P3-4b [23]), was found to copurify with the soluble polymerase as well as with the endogenous RNA replication complex (9, 25). Similar results have also been obtained with the foot-and-mouth disease virus RNA polymerase (18). In addition, several RNA-negative mutations have been shown to map in the foot-and-mouth disease virus RNA polymerase protein, p56a, which appears to be the foot-and-mouth disease virus equivalent to the poliovirus polymerase protein, p63 (19).

Our previous characterization of the in vitro product RNA synthesized by the poliovirus polymerase was centered on reactions which contained its native template, poliovirion RNA (26). Virion RNA is a single-stranded RNA molecule of positive polarity which has a 3'-terminal polyadenylate [poly(A)] sequence and a 5'-terminal covalently attached protein, VPg (10, 17). In reactions that required all four ribonucleoside triphosphates, Mg^{2+}, and oligouridylate [oligo(U)], the purified polymerase was shown to synthesize genome-sized product RNA that was heteropolymeric, of negative polarity, and covalently attached to the oligo(U) primer (26). The amount, size distribution, and rate of synthesis of the product RNA were dependent on the Mg^{2+} concentration, pH, and temperature of the in vitro reaction (26). Under optimal conditions, predominately genome-sized product RNA was synthesized by the purified polymerase at an elongation rate of about 1,200 nucleotides per min.

Dasgupta et al. (5) found that the poliovirus polymerase was active on several different heteropolymeric RNAs in addition to poliovirion RNA. They found that highly purified preparations of the polymerase were active on several poly(A)-containing RNAs when oligo(U) or a host-coded factor isolated from uninfected cells was added to the in vitro reaction mixture (6). In

addition, they reported that poly(A) · oligo(U) was the only primed homopolymer that was copied by the purified polymerase.

In this study, we have examined the ability of the purified polymerase to copy RNA templates other than poliovirion RNA and poly(A) and have characterized the size of the resulting product RNA after denaturation with CH_3HgOH. We found that the polymerase was able to copy all homopolymeric and heteropolymeric templates tested. Under the appropriate reaction conditions, the polymerase was shown to synthesize full-sized product RNA when a variety of polyadenylated heteropolymeric RNAs were tested. Some polymerase activity was also detected on two non-polyadenylated RNAs, and the size of the product RNAs from these reactions suggested that the product RNA was covalently linked to the template RNA.

MATERIALS AND METHODS

Virus and cell culture. Suspension cultures of HeLa S3 cells in Joklik's modified Eagle medium (GIBCO) supplemented with 7% calf serum were maintained at 3 $\times 10^5$ to 6 $\times 10^5$ cells per ml. Cells were infected with poliovirus type 1 (Mahoney strain) as previously described (27).

Polymerase purification. Cytoplasmic extracts of infected cells were prepared at 5 h postinfection with a Dounce homogenizer. A high-speed (200,000 $\times$ *g*) supernatant was prepared from a cytoplasmic extract, fractionated with ammonium sulfate, and chromatographed on phosphocellulose, Sephacryl S-200, and hydroxylapatite as described previously (25). Polymerase activity was assayed at each purification step with poly(A) · oligo(U) (25). The purified polymerase was dialyzed against 50 mM Tris-hydrochloride (pH 8.0)–50% glycerol–0.1 M KCl–2 mM dithiothreitol and stored at −70°C. The purity of the polymerase was analyzed by electrophoresis on a 10% polyacrylamide gel containing sodium dodecyl sulfate (SDS) (16). Protein bands were detected by a silver staining technique (21). p63 was the major protein band present and represented about 80% of the total protein detected in the gel. The concentration of p63 present in the purified polymerase was estimated by scanning the gel with a Joyce-Loebl microdensitometer and comparing the peak area for the polymerase with those for known amounts of protein markers in the same gel. The estimated concentration for p63 in the purified enzyme was about 7 μg/ml.

In some experiments, partially purified polymerase was used. In this case, the enzyme was taken after the phosphocellulose step. The peak fraction of activity that eluted from the phosphocellulose column was divided into small aliquots that were stored at −70°C.

Polymerase reaction conditions. In reactions containing homopolymeric templates, the reaction mixture (60 μl, final volume) contained 4 μl of purified polymerase (about 35 ng of total protein), 2.5 μg of polyribonucleotide template, 1.25 μg of complementary oligoribonucleotide primer, the appropriate ^{3}H-labeled ribonucleoside triphosphate at 13 or 50 μM, 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2′-ethanesulfonic acid), pH 8.0, 3 mM magnesium acetate, and 10 mM dithiothreitol. The homopolymeric templates and primers were prepared for use as described below. The labeled ribonucleoside triphosphates used in the reactions were [5-^{3}H]CTP (24 Ci/mmol; Amersham Corp.), [5,6-^{3}H]UTP (25 Ci/mmol; ICN), [8-^{3}H]GTP (10 Ci/mmol; ICN), and [2,8-^{3}H]ATP (26 Ci/mmol; ICN). The reactions were run for 30 min at 30°C and stopped by adding 1 ml of 7% trichloroacetic acid, 2% sodium pyrophosphate, and 100 μg of carrier RNA. Labeled product RNA was collected on membrane filters (GN-6, 0.45 μm; Gelman Sciences, Inc.) and counted in 5 ml of Aquasol-2 scintillation fluid (New England Nuclear Corp.) with a Beckman LS7500 liquid scintillation counter.

In reactions for which heteropolymeric templates were used, the reaction conditions were modified as follows. The reaction mixture (30 μl, final volume) contained 2 μl of purified polymerase (about 18 ng of total protein); 2 μg of template RNA; 0.05 μg of oligo(U)$_{20}$; 15 μCi of [α-^{32}P]GTP (410 Ci/mmol; Amersham); 0.85 mM each ATP, CTP, and UTP; 42 mM HEPES (pH 8.0); 3 mM magnesium acetate; and 8 mM dithiothreitol. The reactions were normally run for 1 h at 30°C. At the end of the reaction, the incorporation of [^{32}P]GMP into product RNA was measured by placing a 5-μl sample of each reaction mixture into trichloroacetic acid and filtering as described above. The remaining portion of the reaction mixture was adjusted to 0.4 M sodium acetate, and three volumes of cold 95% ethanol were added to precipitate the labeled product RNA, which was collected by centrifugation at 12,000 $\times$ *g* for 5 min. The product RNA was analyzed by CH_3HgOH-agarose gel electrophoresis within 6 h after the reaction. Nuclease degradation was avoided by treatment of solutions and reaction tubes with diethylpyrocarbonate as described previously (26).

Preparation of homopolymeric templates and primers. The polyribonucleotides poly(A), polyinosinate [poly(I)], polycytidylate [poly(C)], and polyuridylate [poly(U)] were resuspended at 1 mg/ml in sterile water and used without additional treatment. The oligoribonucleotides oligoadenylate [oligo(A)$_{10-20}$], oligoinosinate [oligo(I)$_{10-20}$], oligocytidylate [oligo(C)$_{10-20}$], and oligo(U)$_{10-20}$ were resuspended in 10 mM Tris-hydrochloride (pH 8.0)–120 mM NaCl at 2 mg/ml (as determined by absorbance at 260 nm) and treated with 2 U (as defined by Bethesda Research Laboratories) of bacterial alkaline phosphatase in a total volume of 25 μl for 1 h at 37°C. SDS was added to the solution to a final concentration of 1%, and the proteins were removed by extraction with phenol-chloroform-isoamyl alcohol (25:24:1) as described previously (24). The aqueous phase was adjusted to 0.4 M sodium acetate, three volumes of cold 95% ethanol were added, and the oligoribonucleotide was collected by centrifugation. The oligoribonucleotide precipitate was resuspended in sterile water at 1 mg/ml and stored at −20°C. To ensure as much uniformity as possible, all homopolymers were obtained from the same source (Collaborative Research, Inc.).

Purification of plant virus RNAs. Squash mosaic virus (SqMV) (ATCC PV36) was propagated in zucchini squash (*Circubita pepo*) and purified by a modification of the method of Nelson and Knuhtsen (22) developed by Hiebert and Purcifull (15). After acid

clarification of the tissue homogenate at pH 5.0, virions were precipitated with polyethylene glycol. The middle and bottom nucleoproteins were separated by centrifugation on a CsCl density gradient, and the virions were dissociated by adding an equal volume of a solution containing 200 mM ammonium carbonate (pH 9.0), 2 mM EDTA, and 2% SDS (3). The dissociated virions were centrifuged on a sucrose gradient, and the middle- and bottom-component RNAs were isolated from the 26S and 34S peaks on the gradient (15).

Cowpea severe mosaic virus (CpSMV) (Arkansas isolate, ATCC PV273) was cultured in cowpea plants (*Vigna unguiculata* L. Walp. var. "Knuckle Purple Hull"). CpSMV was purified by a procedure similar to that described for SqMV (15), except for the use of sodium phosphate buffer (pH 7.0) during homogenization and a 0.7 volume of chloroform–*n*-butanol (1:1) for clarification. The middle- and bottom-component RNAs were obtained from 26S and 34S peaks on a sucrose gradient as described above for SqMV.

Tobacco etch virus (ATCC PV69) was cultured for 3 to 8 weeks in *Nicotiana tabacum* and then purified as described previously (7). Filtered leaf tissue homogenate was clarified with Triton X-100, and the virus was precipitated with polyethylene glycol. The virus was purified on a CsCl density gradient. The virions were lysed and centrifuged on a sucrose gradient, and the RNA was obtained from a 39S peak off the gradient (7).

Tobacco mosaic virus (TMV) (common strain, ATCC PV135) was cultured in *Nicotiana tabacum*. TMV was purified as described previously (12), except for a modification in the clarification step, in which a 0.6 volume of chloroform–*n*-butanol (1:1) was used instead of 8% *n*-butanol. The virions were lysed, and the RNA was isolated from a 32.5S peak after centrifugation on a sucrose gradient.

Brome mosaic virus (BMV) (ATCC PV47), obtained from M. K. Brakke, University of Nebraska, Lincoln, was harvested from infected Moore barley (*Hordeum vulgare*) approximately 1.5 weeks after inoculation. After acid clarification of the homogenate at pH 4.7, the virus was concentrated by polyethylene glycol precipitation and two cycles of differential centrifugation. BMV RNA3 was obtained from a 14S peak after centrifugation of dissociated virions on a sucrose density gradient as described above for SqMV.

Purification of polyadenylated RNA from HeLa cells. Polyadenylated RNA was isolated from cytoplasmic extracts of uninfected HeLa cells. The cells (6.5×10^8) were collected by centrifugation and resuspended in ice-cold 1% Nonidet P-40–10 mM Tris-hydrochloride (pH 8.0)–10 mM NaCl. The nuclei and other cellular debris were removed by centrifugation at $5,000 \times g$ for 5 min, and the resulting cytoplasmic extract was made 1% in SDS and extracted three times with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) and three times with chloroform-isoamyl alcohol (24:1). The RNA was precipitated from the aqueous phase with ethanol, resuspended in high salt buffer, bound to an oligodeoxythymidylate-cellulose column, and eluted from the column in low salt buffer as described previously (14). The polyadenylated RNA was precipitated in ethanol and stored at −20°C.

Preparation of poliovirion RNA. Poliovirion RNA was isolated from virions purified by CsCl equilibrium density gradient centrifugation (4). The virus was isolated from a cytoplasmic extract of infected HeLa cells by centrifugation and suspended in 1 ml of 10 mM Tris-hydrochloride (pH 7.5)–0.1 M NaCl–0.001 M EDTA–0.5% SDS, 1 ml of 10% Brij 35, plus a sufficient amount of CsCl and the above buffer to give an average density of 1.34 g/cm^3 in a total volume of 9 ml. The virus was banded in the gradient by centrifugation at 40,000 rpm in a Beckman 50 Ti rotor for 15 h at 22°C. The band of virus made visible by light scattering was removed from the center of the gradient, and the virus was collected by centrifugation. The virions were disrupted, and the 35S viral RNA was purified by centrifugation on a sucrose gradient as described previously (11).

Agarose gel electrophoresis. The size of the labeled product RNA was analyzed by electrophoresis in 1% agarose (high gelling temperature agarose; Miles Laboratories, Inc.) gels containing 5 mM CH_3HgOH (Alpha Products), using the method of Bailey and Davidson (2) and following the protocol previously described (26). The product RNA was denatured for 10 min at 25°C in 15 mM CH_3HgOH before electrophoresis to ensure complete denaturation of the labeled product RNA. The samples were layered on the gel and electrophoresed at 110 V for about 2.5 h. At the end of the run, the gel was soaked for 30 min in 0.5 M ammonium acetate containing 2 μg of ethidium bromide per ml, photographed, and dried. The dried gel was exposed to Du Pont Cronex 4 X-ray film, using a Dupont Lightning-Plus enhancing screen.

RESULTS

Polymerase activity on homopolymeric templates. The ability of the purified poliovirus RNA polymerase to copy various types of RNA was initially characterized by measuring its activity on different primed homopolymers. The activity of the polymerase on poly(U), poly(C), and poly(I) was measured relative to its known activity on poly(A). Activity on each template was assayed in the presence of a complementary primer and the appropriate ^{3}H-labeled ribonucleoside triphosphate. About the same level of polymerase activity was found with each template tested, except with poly(U), with which the activity was at least fourfold lower (Table 1). These results indicated that the polymerase was able to copy each of the primed homopolymers and that it showed no particular preference for poly(A). This apparent lack of specificity for poly(A) was further tested by measuring enzyme activity on both poly(A) and poly(C) templates at different enzyme concentrations (Table 2). With both templates, the observed activity was about the same and appeared to increase as an exponential function of enzyme concentration. We have no direct explanation for the nonlinearity of this reaction except to suggest that a multisubunit form of the enzyme that copied the homopolymeric RNAs more efficiently may have formed at the higher enzyme concentrations. In any event, it was clear that equivalent levels of activity were observed with both RNAs

TABLE 1. Polymerase activity on homopolymer template primers

Template	Primer	Labeled substrate	Substrate concn (μM)	[^{3}H]NMP incorporated (pmol)
Poly(A)	Oligo(U)	[^{3}H]UTP	13	0.5
			50	0.8
Poly(U)	Oligo(A)	[^{3}H]ATP	13	0.1
			50	0.2
Poly(C)	Oligo(I)	[^{3}H]GTP	13	0.3
			50	0.6
Poly(I)	Oligo(C)	[^{3}H]CTP	13	0.6
			50	1.0

and that the polymerase did not show any specificity for poly(A) under the conditions used in this study.

Polymerase activity on cellular mRNAs. The poliovirus RNA polymerase appears to specifically copy viral RNA in the cytoplasm of infected cells. It was therefore of interest to determine whether the purified polymerase showed any specificity for poliovirion RNA relative to HeLa mRNA in the oligo(U)-primed reaction in vitro. When poliovirion RNA was added to the in vitro reaction, a significant amount of full-sized product RNA was synthesized by the polymerase in a 1-h reaction (26; Fig. 1B, lane 1; Fig. 2B, lane 1). When HeLa cytoplasmic polyadenylated RNA was used in the reaction, a large amount of labeled product RNA that was heterogeneous in size was also synthesized by the polymerase (Fig. 1B, lane 3). The majority of the product RNA was smaller than an 18S rRNA marker, as was the majority of the template RNA that was detected by ethidium bromide staining (Fig. 1B, lane 3; Fig. 1A, lane 3). Because of the heterogeneous size of the template RNA, it was not possible to determine the size of the product RNA relative to the template RNA being copied,

TABLE 2. Activity on poly(C) and poly(A) templates as function of enzyme concentration

Polymerase concn (μg/ml)	Template	Primer	Labeled substrate	[^{3}H]NMP incorporated (pmol)
0.3	Poly(A)	Oligo(U)	[^{3}H]UTP	0.3
1.3				3.9
2.7				24
0.3	Poly(C)	Oligo(I)	[^{3}H]GTP	0.3
1.3				3.9
2.7				23

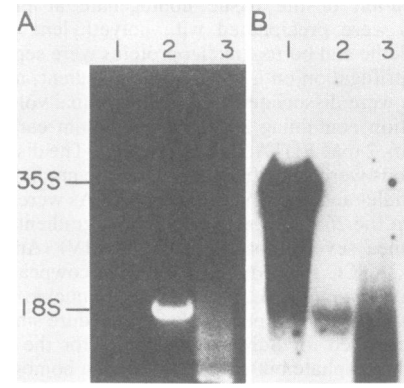

FIG. 1. Electrophoresis of product RNA synthesized by the poliovirus polymerase on polyadenlyated RNA from uninfected HeLa cells. Product RNA was synthesized in reactions that contained [α-^{32}P]GTP, oligo(U), and the phosphocellulose-purified polymerase. The product RNA was denatured in 15 mM CH_3HgOH and electrophoresed on a 1% agarose gel containing 5 mM CH_3HgOH. (A) Photograph of the gel stained with ethidium bromide. Lane 1, RNA recovered from reaction containing poliovirion RNA template; lane 2, labeled 18S rRNA marker; lane 3, RNA recovered from reaction containing HeLa polyadenylated RNA template. (B) Autoradiogram of the same gel.

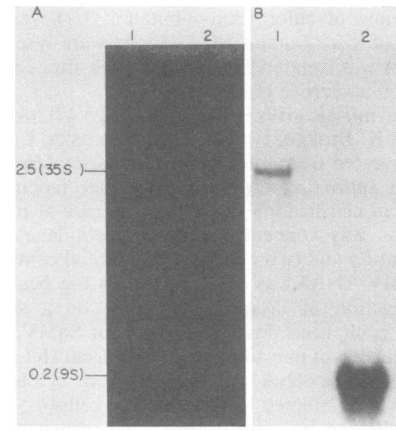

FIG. 2. Electrophoresis of product RNA synthesized by the poliovirus polymerase on rabbit globin mRNA. Product RNA was synthesized in vitro in reactions that contained [α-^{32}P]GTP, oligo(U), and the completely purified polymerase. The product RNA was denatured and electrophoresed on a CH_3HgOH agarose gel as described in the legend to Fig. 1. (A) Photograph of the gel stained with ethidium bromide. Lane 1, RNA recovered from reaction containing poliovirion RNA template; lane 2, RNA recovered from reaction containing rabbit globin mRNA template. (B) Autoradiogram of the same gel. The molecular weights ($\times 10^{-6}$) and the sedimentation coefficients of the RNAs are shown on the left side of the photograph in (A).

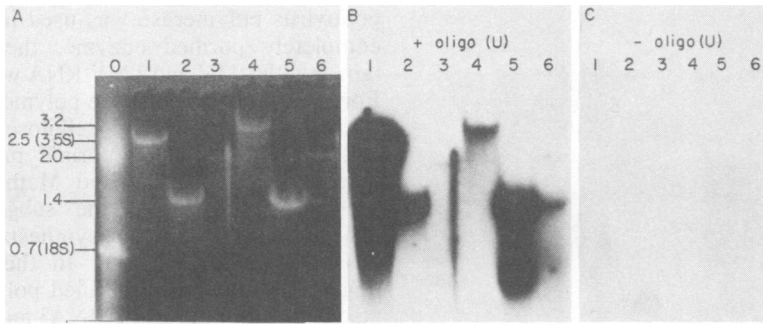

FIG. 3. Characterization of product RNA synthesized by the polymerase on several polyadenylated plant virus RNAs. Product RNA was synthesized in the presence of [α-^{32}P]GTP, the completely purified polymerase, and oligo(U) where indicated. The product RNA was denatured and electrophoresed on agarose gels containing CH_3HgOH as described in the legend to Fig. 1. (A) Photograph of the gel stained with ethidium bromide. Lane 0, HeLa cytoplasmic RNA marker. The remaining lanes contained the RNA recovered from reactions containing poliovirion RNA (lane 1), SqMV middle-component RNA (lane 2), SqMV bottom-component RNA (lane 3), tobacco etch virus RNA (lane 4), CpSMV middle-component RNA (lane 5), and CpSMV bottom-component RNA (lane 6). The CpSMV bottom-component RNA was apparently contaminated with some CpSMV middle-component RNA. (B) Autoradiogram of the same gel. Product RNA was synthesized in the presence of oligo(U). (C) Autoradiogram of a similar gel containing RNA recovered from the same reactions in which oligo(U) was not added. The molecular weights ($\times 10^{-6}$) of the various RNAs are shown on the left side of (A).

but it was clear that the polymerase showed little specificity for virion RNA in this reaction. To determine whether the polymerase would synthesize a complete copy of a cellular mRNA, rabbit globin mRNA ($M_r = 2 \times 10^5$) was used as a template. In this case, it was clear that a significant amount of the labeled product RNA from a 1-h reaction was full-sized (Fig. 2). Thus, the polymerase efficiently copied HeLa mRNA and synthesized full-sized copies of globin mRNA in vitro.

Polyadenylated plant virus RNA templates. To characterize the product RNA synthesized by the polymerase on other polyadenylated RNAs, we used virion RNAs from three plant viruses, tobacco etch virus, CpSMV, and SqMV. All three RNAs contain a 3′-terminal poly(A) sequence and a 5′-terminal covalently linked protein similar to VPg (7, 15, 22). The poliovirus polymerase was active on each of the plant virus RNAs tested in the presence of added oligo(U) (Fig. 3). As with poliovirion RNA, the purified polymerase did not synthesize detectable amounts of product RNA in the absence of oligo(U) (Fig. 3C). In the presence of oligo(U), some full-sized product RNA was synthesized on each plant virus RNA (Fig. 3B). The polymerase appeared to show some preference for poliovirion RNA, since the largest amount of labeled product RNA was recovered from reactions containing poliovirion RNA. The polymerase also appeared to show some specificity for SqMV middle-component RNA ($M_r = 1.4 \times 10^6$) over SqMV bottom-component RNA ($M_r = 2.0 \times 10^6$) (Fig. 3B, lanes 2 and 3). This was also observed for the middle- and bottom-component RNAs from CpSMV (Fig. 3B, lanes 5 and 6). Although some template specificity was observed, the polymerase was able to make complete copies of each template tested.

Elongation rates on poliovirion and SqMV RNA. The above results showed that the polymerase would efficiently copy several different polyadenylated RNAs. To determine whether the polymerase would copy a heterologous RNA and poliovirion RNA at the same elongation rate, we measured the elongation rate for the polymerase reaction with poliovirion RNA and SqMV middle-component RNA. The size of the product RNA was determined as a function of time for the reactions with both templates (Fig. 4). Elongation rates were determined by taking the slope of the line obtained by plotting the size of the largest product RNA present at each time point (see Fig. 4 and reference 26 for additional details). The elongation rates obtained were similar for both RNAs (Table 3). These results suggested that the elongation rates for the in vitro polymerase reaction were independent of the template used. The changes in the elongation rates caused by changes in the Mg^{2+} concentration also appeared to be independent of the template used (Table 3).

Non-polyadenylated RNA templates. To examine the activity of the polymerase on RNAs not containing a 3′-terminal poly(A) sequence, we used virion RNA from TMV ($M_r = 2.1 \times 10^6$) and BMV. Four types of RNA can be isolated

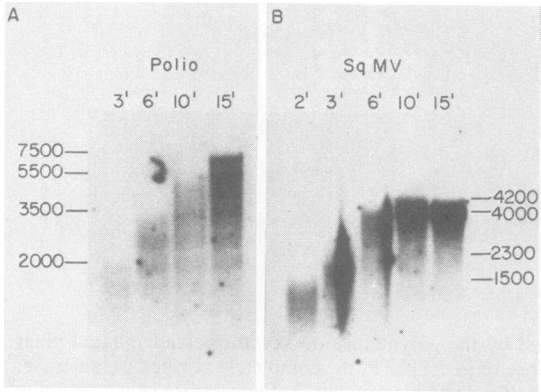

FIG. 4. Effect of reaction time on the synthesis of product RNA, using SqMV middle-component RNA and poliovirion RNA templates. At the times indicated, product RNA was isolated from reactions containing [α-^{32}P]GTP, oligo(U), and the completely purified polymerase. The size of the product RNA at each time point was then determined by CH_3HgOH-agarose gel electrophoresis. (A) Autoradiogram of the gel containing product RNA recovered from reactions with poliovirion RNA template. (B) Autoradiogram of gel containing product RNA recovered from reactions with SqMV middle-component RNA template. The approximate length in nucleotides of the largest product RNA detected at each time point is shown for each template. These values were calculated from the position of three marker RNAs (poliovirion RNA, SqMV middle-component RNA, 18S rRNA) detected by ethidium bromide staining (data not shown).

from purified BMV. In this study, only BMV RNA3 ($M_r = 0.7 \times 10^6$) was used as a template. Both BMV and TMV RNAs are known to have tRNA-like secondary structures at their 3'-ends (1, 13).

When TMV RNA was used as a template with our most purified preparations of the polymerase, no labeled product RNA was detected in our gels in the presence or absence of added oligo(U) (Fig. 5C, lanes 3 and 6). When BMV RNA3 was used in the reaction, a band of labeled product RNA was observed that had an apparent molecular weight of 0.5×10^6 (Fig. 5C, lane 2). This product RNA was significantly smaller than BMV RNA3 (Fig. 5A, lane 2) and was synthesized only in the presence of added oligo(U) (Fig. 5C, lanes 2 and 4). BMV RNA3 is known to have an internal oligo$(A)_{20}$ sequence that is 876 nucleotides from its 3'-end (1). The poliovirus polymerase apparently used the internal oligo(A) sequence as an initiation site with the oligo(U) primer to copy the 5' portion of BMV RNA3. With poliovirion RNA, the completely purified polymerase synthesized full-sized product RNA only when oligo(U) was added to the reaction (Fig. 5C, lanes 1 and 4).

When a partially purified preparation of the poliovirus polymerase was used instead of the completely purified enzyme, the results obtained with BMV and TMV RNA were different. For these experiments, the polymerase was purified through the phosphocellulose step, but the last two steps in the normal protocol were deleted (see Materials and Methods). In the presence of oligo(U), the subgenomic-sized product RNA was again synthesized on BMV RNA3 (Fig. 5B, lane 2). In the absence of oligo(U), the partially purified polymerase was also active on both BMV RNA3 and TMV RNA (Fig. 5B, lanes 5 and 6). The largest of the labeled product RNAs isolated from these reactions, however, were larger than the template RNAs. The BMV RNA3 product ($M_r = 1.5 \times 10^6$) and the TMV RNA product ($M_r = 3.2 \times 10^6$) were both about twice the size of the template RNAs. This result suggested that the product RNA from these reactions may be covalently linked to the template RNAs. Additional studies are required to investigate this possibility. With poliovirion RNA, the partially purified polymerase appeared to require oligo(U) for activity (Fig. 5B, lanes 1 and 4). In several other experiments, however, small amounts of product RNA were synthesized by the polymerase in the absence of added oligo(U). It has been possible to increase the amount of product RNA synthesized by the partially purified polymerase in the absence of oligo(U) by modifying the in vitro reaction conditions (D. C. Young and J. B. Flanegan, unpublished data). Studies are now under way to optimize the conditions for this reaction and to characterize the labeled product RNA synthesized in the absence of oligo(U).

DISCUSSION

The poliovirus RNA-dependent RNA polymerase was shown in a previous study to make predominately full-sized copies of poliovirion

TABLE 3. Elongation rates on poliovirus and SqMV RNAs

Template RNA	Mg^{2+} (mM)	Elongation rate (nucleotides per min)[a]
Polio	3	220 (210)
	7	504 (610)
$SqMV_m$	3	230
	7	635

[a] The elongation rates were determined at 30°C on poliovirion RNA and SqMV middle-component RNA. The rates at 3 mM Mg^{2+} were calculated from the data shown in Fig. 4. The rates at 7 mM Mg^{2+} were determined with data from a similar experiment (data not shown). The values in parentheses for poliovirus RNA were previously reported (26).

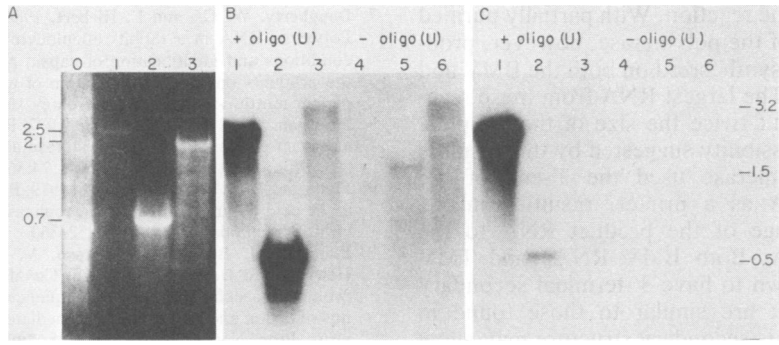

FIG. 5. Characterization of product RNA synthesized by the polymerase on two non-polyadenylated plant virus RNAs. Product RNA was synthesized in the presence of [α-^{32}P]GTP and oligo(U) where indicated. The size of the product RNA was determined by CH_3HgOH-agarose gel electrophoresis. (A) Photograph of the gel stained with ethidium bromide. Lane 0 contained HeLa cytoplasmic RNA marker. The remaining lanes contained RNA recovered from reactions containing poliovirion RNA template (lane 1), BMV RNA3 template (lane 2), and TMV RNA template (lane 3). (B) Autoradiogram of the same gel. Product RNA was synthesized by the phosphocellulose-purified polymerase in the presence (lanes 1 through 3) or absence (lanes 4 through 6) of oligo(U). (C) Same as (B), except completely purified polymerase was used in these reactions. The approximate molecular weights ($\times 10^{-6}$) for various positions in the gel are shown in (A) and (C).

RNA in vitro when oligo(U) was used as a primer (26). In this study, we found that the polymerase would copy many other RNA templates. Characterization of the product from these reactions showed that the polymerase was able to make full-sized copies of the RNAs tested.

All primed homopolymeric RNAs tested in this study were copied by the poliovirus polymerase. Although the activity observed with poly(U) · oligo(A) was about fourfold lower than that observed with the other primed homopolymers, a significant level of activity was seen in all cases. This differs from the results reported by Dasgupta et al. (6), and we have no direct explanation for this difference. It is possible, however, that we fortuitously obtained a more uniform set of homopolymers that were all copied to about the same extent by the poliovirus polymerase. In any event, our results suggest that the polymerase was not specific for poly(A) templates and was able to copy other primed homopolymers.

The polymerase was able to copy a wide variety of polyadenylated RNAs, including HeLa mRNA, rabbit globin mRNA, and virion RNAs from CpSMV, SqMV, and tobacco etch virus. With each template, the purified polymerase required oligo(U) for activity. Characterization of the product RNA by gel electrophoresis after denaturation with CH_3HgOH showed that a significant amount of full-sized product RNA was produced on each polyadenylated RNA. The poliovirus polymerase should prove useful in making full-sized labeled cRNAs for a wide variety of polyadenylated RNAs. This may be useful for RNA templates for which the synthesis of full-sized cDNA has not been possible.

In a previous study, we examined the elongation rate of the in vitro polymerase reaction on poliovirion RNA (26). The elongation rate was found to be very dependent on the in vitro reaction conditions and, in particular, on the Mg^{2+} concentration. The results presented in this study showed that the elongation rates of SqMV middle-component RNA were similar to those on poliovirion RNA. In addition, changes in the Mg^{2+} concentration changed the elongation rates in a fashion similar to that observed on poliovirion RNA. These data suggest that the absolute value of the elongation rate as well as the changes in the elongation rates observed with changes in Mg^{2+} concentration are independent of the RNA template being copied by the polymerase.

Two different types of product RNA were synthesized by the polymerase when BMV RNA3 and TMV RNA were used as templates. Both of these template RNAs are known to lack a 3'-terminal poly(A) sequence. Our highly purified poliovirus polymerase was inactive on both of these RNAs in the absence of oligo(U). When oligo(U) was added to the reaction, the polymerase was able to copy BMV RNA3 to produce a subgenomic-sized product RNA. The synthesis of this RNA appears to initiate on the internal oligo(A) sequence known to be present in BMV RNA3 (1). This oligo(A) sequence is also known to be a strong initiation site for oligodeoxythymidylate-primed DNA synthesis with retrovirus reverse transcriptase (1). Only trace amounts of product RNA were detected when oligo(U) was

deleted from the reaction. With partially purified preparations of the polymerase, however, product RNA was synthesized on both the BMV and TMV RNAs. The largest RNA from these reactions was about twice the size of the template RNA. One possibility suggested by this result is that the polymerase used the 3'-end of the template RNA as a primer, resulting in the covalent linkage of the product RNA to the template RNA. Both BMV RNA3 and TMV RNA are known to have 3'-terminal secondary structures that are similar to those found in tRNA (13). This secondary structure may allow the polymerase to use the 3'-ends of these RNAs as primers. Because the completely purified polymerase was inactive on these templates, it appears that an additional factor(s) may be required to initiate RNA synthesis on these templates. We have not yet identified this factor(s), but we have recently observed that partially purified preparations of the host factor originally described by Dasgupta et al. (6) will stimulate our most purified preparations of the polymerase to carry out the synthesis of the double-sized product on both BMV RNA3 and TMV RNA (unpublished data). These observations may have important implications concerning the mechanism of replication of these plant virus RNAs. It also raises interesting questions about the mechanism of poliovirus RNA replication, since the host factor appears to stimulate the initiation of RNA synthesis by the poliovirus polymerase on poliovirion RNA (6).

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