Poliovirus RNA-dependent RNA Polymerase (3Dpol)

DIVALENT CATION MODULATION OF PRIMER, TEMPLATE, AND NUCLEOTIDE SELECTION

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We have analyzed the divalent cation specificity of poliovirus RNA-dependent RNA polymerase, 3Dpol. The following preference was observed: Mn$^{2+}$ > Co$^{2+}$ > Ni$^{2+}$ > Fe$^{2+}$ > Mg$^{2+}$ > Cu$^{2+}$ > Ca$^{2+}$, and Zn$^{2+}$ was incapable of supporting 3Dpol-catalyzed nucleotide incorporation. In the presence of Mn$^{2+}$, 3Dpol activity was increased by greater than 10-fold relative to that in the presence of Mg$^{2+}$. Steady-state kinetic analysis revealed that the increased activity observed in the presence of Mn$^{2+}$ was due, primarily, to a reduction in the $K_M$ value for 3Dpol binding to primer/template, without any significant effect on the $K_M$ value for nucleotide. The ability of 3Dpol to catalyze RNA synthesis de novo was also stimulated approximately 10-fold by using Mn$^{2+}$, and the enzyme was now capable of also utilizing a DNA template for primer-independent RNA synthesis. Interestingly, the use of Mn$^{2+}$ as divalent cation permitted 3Dpol activity to be monitored by following extension of 5'-32P-end-labeled, heteropolymeric RNA primer/template. The kinetics of primer extension were biphasic because of the enzyme binding to primer/template in both possible orientations. When bound in the incorrect orientation, 3Dpol was capable of efficient addition of nucleotides to the blunt-ended duplex; this activity was also apparent in the presence of Mn$^{2+}$, 3Dpol efficiently utilized dNTPs, ddNTPs, and incorrect NTPs. On average, three incorrect nucleotides could be incorporated by 3Dpol. The ability of 3Dpol to incorporate the correct dNTP, but not the correct ddNTP, was also observed in the presence of Mg$^{2+}$. Taken together, these results provide the first glimpse into the nucleotide specificity and fidelity of the poliovirus polymerase and suggest novel alternatives for the design of primer/templates to study the mechanism of 3Dpol-catalyzed nucleotide incorporation.

Positive-strand RNA viruses represent an existing and emerging threat to the United States public health. For example, as many as 4 million Americans are currently infected by hepatitis C virus. Hepatitis C virus is capable of establishing a persistent infection, which leads to cirrhosis of the liver and, in some cases, liver cancer (1). Fortunately, highly effective therapies to treat chronic RNA virus infection do not exist. Replication of the genomes of all RNA viruses requires the virus-encoded RNA-dependent RNA polymerase (RdRP).1

RdRP activity is unique to virus-infected, human cells; therefore, the RdRP is a suitable target for the development of antiviral agents. Although RdRPs from several viruses have been purified and characterized to some extent, a large gap remains in terms of our understanding of the biochemical mechanism of this class of nucleic acid polymerase relative to those classes of polymerase involved in cellular processes such as replication and transcription (2–8). A detailed kinetic and thermodynamic description of RdRP-catalyzed nucleotide incorporation should permit this enzyme to be distinguished from cellular polymerases, thus facilitating the development of RdRP-specific inhibitors useful for the treatment of RNA virus infection.

Biological and biochemical studies of poliovirus genome replication have been ongoing for decades (9). These studies have shown that RNA structures at the 3'-end of the genome (10), and possibly at the 5'-end of the genome (11), specify the site of assembly of the replication complex. The exact composition and order of assembly of this complex remains to be determined. However, both viral and host factors have been implicated in replica assembly and/or function (12–15). After complex assembly, poliovirus RNA-dependent RNA polymerase, 3Dpol, initiates RNA synthesis by using the protein primer, 3B (VPg). 3Dpol has been studied intensively for many years because of its key role in poliovirus genome replication. Therefore, this enzyme is an ideal model system to use in the study of RdRP mechanism and for the elucidation of RdRP structure-function relationships.

In vitro studies employing pure, active 3Dpol have identified many of the biochemical properties and enzymatic activities associated with this enzyme. In addition to oligo(dT)- and oligo(rU)-dependent poly(rU) polymerase activity (3, 4), 3Dpol is capable of uridylylating VPg and utilizing the resulting VPg-pU product as a primer for poly(rU) synthesis (16). 3Dpol has terminal transferase activity (17) and strand displacement activity (18). Also, 3Dpol has the ability to multimerize (19), and Kirkegaard and colleagues (20) have suggested that multimerization may be required for nucleic acid binding, polymerase activity (21), and virus viability (22). Recently, we demonstrated that 3Dpol is sufficient for template switching, and this enzyme is capable of catalyzing primer-independent RNA synthesis (23). Finally, a high resolution crystal structure is available for 3Dpol (19). The overall topology of 3Dpol is quite similar to that of the other classes of nucleic acid polymerase in that the enzyme resembles a right hand with “fingers,” “palm,” and “thumb” subdomains. The palm subdomain contains four structurase; PAGE, polyacrylamide gel electrophoresis; TLC, thin-layer chromatography; NTP, nucleoside-5'-triphosphate; dNTP, 2'-deoxy nucleoside-5'-triphosphate; dTMP, 2',3'-dideoxynucleoside-5'-triphosphate; dNMP, nucleoside-5'-monophosphate; dNMP, 2',3'-dideoxynucleoside-5'-monophosphate; ddNMP, 2',3'-dideoxynucleoside-5'-monophosphate; PPi, pyrophosphate.
5'-32P Labeling of Oligonucleotides

DNA and RNA oligonucleotides were end-labeled by using [γ-32P]ATP and T4 polynucleotide kinase essentially as specified by the manufacturer. Reactions typically contained 11 μM [γ-32P]ATP, 10 μM DNA, or RNA oligonucleotide, and 0.4 unit/μl T4 polynucleotide kinase. Unincorporated nucleotide was removed by passing the sample over two consecutive 1-ml Sephadex G-25 (Sigma) spin columns.

5'-32P Labeling of DNA Ladder

Labeling of the DNA ladder was performed by using [γ-32P]ATP and T4 polynucleotide kinase as specified by Life Technologies, Inc.

3Dpol Assays

Reactions contained 50 mM HEPES, pH 7.5, 10 mM 2-mercaptoethanol, 5 mM MgCl2 or MnCl2, 60 μM ZnCl2, 500 μM NTP, primer/template and 3Dpol. Reactions were quenched by the addition of EDTA to a final concentration of 50 mM. Specific concentrations of primer/template and 3Dpol, along with any deviations from the above, are indicated below or in the appropriate figure legend.

Divalent Cation Modulation of 3Dpol Poly(r(U) and Poly(r(G) Polymerase Activity

Reactions contained 0.5 μM 3Dpol, 500 μM nucleotide (UTP or GTP), 0.2 μM radiolabeled nucleotide ([α-32P]UTP or [α-32P]GTP), either MgCl2 or MnCl2 (5 mM) and either dT15 (1.88 μM) or poly(rA) (93.4 μM AMP), or dG15 (1.88 μM) and poly(rC) (93.4 μM CMP), or dT15/rA30 (1 μM) or dG15/rC30 (1 μM). Reactions were initiated by the addition of 3Dpol and incubated at 30 °C for 5 min. Reaction volumes were 25 μl.

Optimal Divalent Cation Concentration for Maximal Activity

Reactions contained 3Dpol (0.1 μM), dG6 (4.7 μM), poly(rC) (93.4 μM CMP), [α-32P]GTP (500 μM), [α-32P]UTP (0.2 μCi/μl, 0.07 μM) and either MgCl2 or MnCl2. Reactions were initiated by addition of 3Dpol and incubated at 30 °C for 5 min at which time the reactions were quenched by addition of EDTA to a final concentration of 50 mM. Reaction volumes were 25 μl. Products were analyzed by DE81 filter binding.

RNA Synthesis de Novo: Template Specificity

Reactions contained 3Dpol (0.5 μM), GTP (500 μM), [α-32P]GTP (0.2 μCi/μl, 0.07 μM), either MgCl2 (5 mM) or MnCl2 (5 mM) and either rC10 (10 μM), rG10 (10 μM), or poly(rC) (300 μM) CMP). Reactions were initiated by addition of 3Dpol and incubated at 30 °C for 10 min. Reaction volumes were 50 μl. Products were analyzed by DE81 filter binding.

Purity of [α-32P]NTPs

[α-32P]NTPs were diluted to 0.1 μCi/μl in ddH2O, and 1 μl was spotted in triplicate onto TLC plates. TLC plates were developed in 0.3 M potassium phosphate, pH 7.0, dry, and exposed to a PhosphorImager screen. Imaging and quantitation were performed by using the ImageQuant software from Molecular Dynamics. The purity was used to correct the specific activity of NTPs in reactions to calculate accurate concentrations of product. Purity was checked before or after each experiment and ranged from 50 to 90%.

Expression and Purification of 3Dpol

Expression and purification of 3Dpol was as described previously (23, 24).

Purification of Synthetic Oligonucleotides

DNA and RNA oligonucleotides were purified by denaturing PAGE. Gels consisted of: 19% acrylamide, 1% bisacrylamide, 7 M urea and 1 × TBE (89 mM Tris base, 89 mM boric acid, and 2 mM EDTA). The oligonucleotide ladder was visualized by UV shadowing. A gel slice containing only the full-length oligonucleotide was removed, and the nucleic acid was electroeluted from the gel in 1 × TBE by using an Elutrap apparatus (Schleicher & Schuell). Oligonucleotides were desalted on Sep-Pak columns (Millipore) as specified by the manufacturer. Oligonucleotides were typically suspended in TBE, 10 mM Tris, 1 mM EDTA, pH 8.0, aliquoted, and stored at −80 °C until use. Concentrations were determined by measuring the absorbance at 260 nm by using calculated extinction coefficients (25).

5'-32P Labeling of GpG

GpG was end-labeled by using [γ-32P]ATP and T4 polynucleotide kinase essentially as specified by the manufacturer. Reactions typically contained 1 μM [γ-32P]ATP, 10 μM GpG, and 0.4 unit/μl T4 polynucleotide kinase. Reactions were quenched by heating the reaction at 60 °C for 5 min.

Annealing of Heteropolymeric Primer/Template

1 μM end-labeled RNA primer was mixed with 9 μM unlabeled RNA primer and 10 μM unlabeled RNA template in T10E1, and heated to 90 °C for 1 min and slowly cooled to 10 °C at a rate of approximately 5 °C/min in a Progene thermocycler.

3Dpol Assays

Reactions contained 50 mM HEPES, pH 7.5, 10 mM 2-mercaptoethanol, 5 mM MgCl2 or MnCl2, 60 μM ZnCl2, 500 μM NTP, primer/template and 3Dpol. Reactions were quenched by the addition of EDTA to a final concentration of 50 mM. Specific concentrations of primer/template and 3Dpol, along with any deviations from the above, are indicated below or in the appropriate figure legend.

Divalent Cation Modulation of 3Dpol Poly(r(U) and Poly(r(G) Polymerase Activity

Reactions contained 0.5 μM 3Dpol, 500 μM nucleotide (UTP or GTP), 0.2 μM radiolabeled nucleotide ([α-32P]UTP or [α-32P]GTP), either MgCl2 or MnCl2 (5 mM) and either dT15 (1.88 μM) and poly(rA) (93.4 μM AMP), or dG15 (1.88 μM) and poly(rC) (93.4 μM CMP), or dT15/rA30 (1 μM) or dG15/rC30 (1 μM). Reactions were initiated by the addition of 3Dpol and incubated at 30 °C for 5 min. Reaction volumes were 25 μl.

Optimal Divalent Cation Concentration for Maximal Activity

Reactions contained 3Dpol (0.1 μM), dG6 (4.7 μM), poly(rC) (93.4 μM CMP), [α-32P]GTP (500 μM), [α-32P]UTP (0.2 μCi/μl, 0.07 μM) and either MgCl2 or MnCl2. Reactions were initiated by addition of 3Dpol and incubated at 30 °C for 5 min at which time the reactions were quenched by addition of EDTA to a final concentration of 50 mM. Reaction volumes were 25 μl. Products were analyzed by DE81 filter binding.

RNA Synthesis de Novo: Template Specificity

Reactions contained 3Dpol (0.5 μM), GTP (500 μM), [α-32P]GTP (0.2 μCi/μl, 0.07 μM), either MgCl2 (5 mM) or MnCl2 (5 mM) and either rC10 (10 μM), rG10 (10 μM), or poly(rC) (300 μM) CMP). Reactions were initiated by addition of 3Dpol and incubated at 30 °C for 10 min. Reaction volumes were 50 μl. Products were analyzed by DE81 filter binding.

Steady-state Kinetic Analysis of 3Dpol

Kinetic constants, Kd and Vmax, were determined by using the assay described above. The concentration of 3Dpol employed in these experiments ranged from 0.01 to 0.5 μM depending upon the substrate and cation employed. The Vmax values reported in Table III have been normalized to 0.01 μM 3Dpol to facilitate comparison of the various substrates. Concentrations of the varied substrate, nucleic acid or nucleotide, ranged from 0.25 × Kd to 4 × Kd. The concentration of the substrate that remained constant was 5–10 × Kd. Single time points were taken that were in the linear range for product formation. Reaction rates were plotted as a function of substrate concentration, and these data were fit to a hyperbola by nonlinear regression using the program KaleidaGraph (Synergy Software, Reading, PA) to obtain the kinetic constants. In one instance, the determination of the Kd value for GTP in the presence of MgCl2, the enzyme was not saturated with dG15/rC30 primer/template, thus the Kd(app) is reported. However, the true Kd was calculated by using Equation 1.
Reactions were performed as described under "Experimental Procedures." Activity values are reported using one significant figure, and the S.E. of the data was less than 10%.

| Substrate | Activity |
|-----------|----------|
| dT15/poly(rA) | 1,000 pmol/min/μg |
| dT15/rA30 | 10 |
| dG15/poly(rC) | 200 |
| dG15/rC30 | 2 |

K_M(app)GTP = K_M,GTP (1 + K_M(app)GTP \frac{[dG15/rC30]}{[dT15/rA30]})

(1)

**Table I**

| Substrate | GMP incorporated |
|-----------|------------------|
| Mg2+ | Mn2+ |
| 0.5 mM | 55 | 172 |
| 1 mM | 386 | 2,058 |
| 2.5 mM | 691 | 2,256 |
| 5 mM | 826 | 2,384 |
| 10 mM | 759 | 2,162 |
| 50 mM | 0 | 590 |

**Table II**

| Concentration | GMP incorporated |
|---------------|------------------|
| 0.5 mM | 55 pmol |
| 1 mM | 386 pmol |
| 2.5 mM | 691 pmol |
| 5 mM | 826 pmol |
| 10 mM | 759 pmol |
| 50 mM | 0 pmol |

Phosphatase Treatment of [α-32P]-GTP- and [γ-32P]-GTP-labeled RNA Products

Phosphatase reactions were performed by using calf intestinal alkaline phosphatase and either [α-32P]-GTP- or [γ-32P]-GTP-labeled RNA essentially as described by the manufacturer. Reactions contained 0.1 unit/μl calf intestinal alkaline phosphatase and either [α-32P]-GTP-labeled RNA (300,000 cpm, 15 pmol) or [γ-32P]-GTP-labeled RNA (300,000 cpm, 100 pmol). Reaction volumes were 25 μl. Reactions were initiated by addition of 3Dpol and incubated at 37 °C for 10 min. Reaction volumes were 25 μl. In all cases, 5 mM was the optimal concentration for maximal activity. Products were analyzed by DE81 filter binding.

**Product Analysis**

**DE81 Filter Binding**—10 μl of the quenched reaction was spotted onto DE81 filter paper discs and dried completely. The discs were washed three times for 10 min in 250 μl of 5% dioxane acid sodium phosphate and rinsed in absolute ethanol. Bound radioactivity was quantitated by liquid scintillation counting in 5 ml of Ecoscint scintillation fluid (National Diagnostics).

**TLC**—1 μl of the quenched reaction was spotted onto TLC plates. TLC plates were developed in 0.3 M potassium phosphate, pH 7.0, dried, and exposed to a PhosphorImager screen.

**Denaturing PAGE**—Sample preparation and electrophoresis were as described previously (26). Briefly, 1 μl of the quenched reaction was added to 9 μl of loading buffer: 90% formamide, 50 mM Tris borate, 0.025% bromphenol blue, 0.025% xylene cyanole and where appropriate a 10-fold excess of unlabeled RNA (trap strand) relative to the end-labeled RNA under investigation was added. Samples were heated at 70 °C for 2-5 min prior to loading 5 μl on a 1 x TBE, 7 M urea polyacrylamide gel of the appropriate percentage. Highly cross-linked gels contained 2% bisacrylamide. Electrophoresis was performed in 1 x TBE at 75 watts. Gels were visualized and quantitated by using a PhosphorImager.

**RESULTS**

**Transition Metal Support 3Dpol-catalyzed Nucleotide Incorporation**—We determined the effect of Mn2+ on 3Dpol-catalyzed nucleotide incorporation with the following substrates: dT15/poly(rA), dT15/rA30, dG15/poly(rC), and dG15/rC30 (Table I). The stimulation of 3Dpol activity observed by using Mn2+ was substrate-dependent and varied from 5- to 100-fold the activity determined in the presence of Mg2+ (Table I). The 10-fold decrease in activity with dT15/poly(rA) was due, most likely, to a decrease in the solubility in the presence of Mn2+ as a whole precipitate could be observed after centrifugation of this reaction mixture. The precipitate formed in the presence or absence of enzyme. This phenomenon was not observed with other primer/template substrates.

The optimal concentration for maximal 3Dpol activity was 5 mM (Table II). Concentrations of divalent cation greater than 10 mM were inhibitory (Table II). The observed inhibition did not appear to be due to precipitation of nucleic acid and/or enzyme. The K_M value for primer/template substrates was reduced by an average of 25-fold in the presence of Mn2+ relative to the corresponding values measured in the presence of Mg2+ (Table III). A 3-fold reduction in V_max was observed for dT15/rA30 by using Mn2+ instead of Mg2+; however, a 30-fold increase in V_max was observed for dG15/rC30 by using Mn2+ instead of Mg2+. The K_M values for UTP and GTP with the corresponding primer/template substrates were similar, 62 and 116 μM, respectively, and the V_max values were as expected based on the kinetic analysis of primer/template substrates discussed above. The increase in V_max observed in the presence of Mn2+ when dG15/rC30 was employed did not result from a change in K_M value for GTP (Table III). Thus, an increase in the number of productive 3Dpol-dG15/rC30 complexes formed may occur by using Mn2+ instead of Mg2+, Mn2+, Co2+, Ni2+, and Fe2+ supported higher levels of activity than Mg2+ (Table IV). Ca2+ and Cu2+ supported lower levels of activity than Mg2+ (Table IV). Zn2+ was incapable of supporting activity (Table IV).

Mn2+ Increases the Efficiency of 3Dpol-catalyzed RNA Synthesis de Novo—Recently, we reported that 3Dpol initiates RNA synthesis when poly(rC) and GTP are employed as the sole substrates (23). Primer-independent RNA synthesis did not result from a polynucleotide-phosphorylase-like activity as template was required (data not shown). Poly(rC) and GTP appear to be the most efficient substrates for this reaction as neither poly(rA) and UTP nor poly(rU) and ATP could be used to demonstrate convincingly synthesis of RNA (data not shown). In addition, rC30, but not dC30, was also a template for primer-independent RNA synthesis, albeit at a level 30-fold lower than observed by using poly(rC) (Table II). As shown in Table II, Mn2+-stimulated primer-independent RNA synthesis by 15-fold relative to reactions performed in the presence of Mg2+ when either poly(rC) or rC30 was employed as template. In the presence of Mn2+, dC30 was also utilized as a template (Table II) and utilization of the dC30 template by 3Dpol was now only 2-fold less efficient than utilization of the rC30 template. Products of this reaction resolved by denaturing PAGE and...
TABLE III
Steady-state kinetic analysis of 3Dpol

| Substrate varied | Cofactor        | $K_{m}$  | $V_{max}$  | $V_{max}/K_{m}$ |
|------------------|----------------|----------|------------|-----------------|
| Nucleic acid     |                | $\mu M$  | $\mu M/min$| $\times 10^{-3}$|
| dT$_{15}$/rA$_{30}$ | MgCl$_2$     | 24.6 ± 2.8 | 0.182 ± 0.008 | 7.40            |
| dT$_{15}$/rC$_{30}$ | MnCl$_2$     | 0.88 ± 0.08 | 0.064 ± 0.002 | 73              |
| dG$_{15}$/rC$_{30}$ | MgCl$_2$     | 13.2 ± 3.9  | 0.005 ± 0.001 | 0.38            |
| dT$_{15}$/rC$_{30}$ | MnCl$_2$     | 0.75 ± 0.25  | 0.148 ± 0.014 | 197             |
| rC$_{30}$         | MnCl$_2$     | 0.034 ± 0.004 | 0.028 ± 0.008 | 824             |

TABLE IV
Divalent cation specificity of 3Dpol

| Divalent cation | UMP incorporated |
|----------------|------------------|
| Zn$^{2+}$       | 0                |
| Cu$^{2+}$       | 3                |
| Ca$^{2+}$       | 4                |
| Mg$^{2+}$       | 9                |
| Fe$^{2+}$       | 31               |
| Ni$^{2+}$       | 38               |
| Co$^{2+}$       | 97               |
| Mn$^{2+}$       | 275              |

$^{a}$ Taken from Arnold and Cameron (23).

$^{b}$ This value was not determined under saturating conditions of dG$_{15}$/rC$_{30}$ and is therefore an apparent $K_{m}$ value. The calculated $K_{m}$ value is 80 $\mu M$ (see “Experimental Procedures”).

visualized by phosphorimaging were greater than unit length and similar in appearance to those produced via template switching when dG$_{15}$/rC$_{30}$ and GTP are employed as substrates (23).

What is the mechanism of initiation of these long RNA products? One possibility is that the terminal transferase activity of 3Dpol adds GMP to the 3'-end of rC$_{30}$ thus creating a "snap-back" substrate that is efficiently extended by 3Dpol. However, by using a 5'-end-labeled rC$_{30}$ template, we were only able to show the incorporation of a single GMP into rC$_{30}$ (data not shown). Moreover, the kinetics of formation of this product were too slow to support the hypothesis that rC$_{30}$G RNA was the substrate used by 3Dpol to produce long products (data not shown). A second possibility is that RNA synthesis is initiated de novo. To test this possibility, we performed an experiment employing rC$_{30}$ and [γ-32P]GTP as substrates. If RNA synthesis initiates de novo, then long RNA products should incorporate the label. Product RNA was labeled by using [γ-32P]GTP, thus long products most likely result from de novo initiation (Fig. 1B). The primary product of this reaction was the dinucleotide, pppGpG. Also, tri-, tetra-, and pentanucleotide products were observed. The dinucleotide product was assigned based upon the comigration of this product with 32PpGpG on polyacrylamide gels (data not shown). To keep a complete inventory of all products formed during the course of the reaction when [γ-32P]GTP was employed, reaction mixtures were also resolved by TLC; over half of the nucleotide was utilized based on PPi accumulation (Fig. 1C). Phosphatase treatment of [γ-32P]GTP-labeled RNA showed a time-dependent loss of label by using the DE81 filter paper method (Fig. 2A) and PAGE (Fig. 2B) without any change in label associated with the control, [α-32P]GTP-labeled RNA (Fig. 2, A and B). Greater than 95% of the counts associated with [γ-32P]GTP-labeled RNA originated from [γ-32P]GTP, thus confirming that this RNA was initiated de novo.

Quantitative analysis of the kinetics of product formation in reactions employing either [γ-32P]GTP or [α-32P]GTP as substrates showed that both reactions displayed burst kinetics (Fig. 3A). In both cases, the steady-state rates (linear phases) of product formation were identical; however, the burst amplitude of PPi formation measured by TLC was 5-fold greater than that of RNA measured by using the DE81 filter binding method. This difference likely reflects the inability of DE81 filter paper to retain dinucleotide product. Although the burst of PPi formation cannot be used directly to quantitate active sites, the burst can be exploited to compare the “active” fraction of various enzyme preparations.

In contrast, quantitation of the kinetics of [γ-32P]GTP-labeled RNA formation should report directly on the concentration of active sites if a burst of product formation is observed. When this analysis was performed, an apparent burst of labeled RNA was observed (Fig. 3B). However, based on the concentration of enzyme employed, the burst amplitude was 6–7-fold greater than the enzyme concentration. Because the enzyme concentration was determined by measuring the protein absorbance at 280 nm under denaturing conditions and using a calculated extinction coefficient of 71,830 M$^{-1}$ cm$^{-1}$ (24, 27, 28), it is unlikely that the enzyme concentration was grossly underestimated. The most reasonable explanation for this observation is that multiple rounds of abortive initiation events occur producing dinucleotide product prior to synthesis of completely elongated RNA.

Steady-state kinetic analysis of this reaction in the presence of Mn$^{2+}$ showed that the catalytic efficiency of this reaction is greater than either of the primer-dependent reactions characterized (Table III). The $K_{m}$ value of 3Dpol for GTP in the de novo reaction was virtually identical to that measured for GTP in the primer-dependent reaction (Table III). The ability to saturate the enzyme with reasonably low levels of template RNA coupled with the high catalytic efficiency could be useful for the rapid characterization of the nucleic acid binding properties of 3Dpol by evaluating the ability of "competitor" nucleic acids to inhibit RNA synthesis de novo.

Mn$^{2+}$ Stimulates 3Dpol-catalyzed Extension of Heteropolymeric RNA Primer/templates—The observation that the number of productive 3Dpol-dG$_{15}$/rC$_{30}$ complexes that formed was increased by using Mn$^{2+}$ instead of Mg$^{2+}$ suggested that an increase in the utilization of heteropolymeric RNA primer/templates might also be observed by using Mn$^{2+}$. Two different primer/template substrates were employed (see Fig. 4, A and D). Both substrates consist of a 15-nucleotide primer and a 21-nucleotide template, which when annealed form a primer/
template substrate containing a 15-base pair duplex and a 6-nucleotide, single-stranded 5’-overhang. These primer/templates differ from each other in two significant ways. First, the calculated $T_M$ values are different (29). Primer/template I has a calculated $T_M$ value of $-70$ °C; primer/template II has a calculated $T_M$ value of $80$ °C. Second, by using primer/template I and UTP, multiple cycles of correct nucleotide incorporation should occur; whereas by using primer/template II and ATP, only a single cycle of correct nucleotide incorporation should occur.

With primer/template I, where multiple cycles of nucleotide incorporation should occur by using UTP as the sole nucleotide substrate, primer was extended to the end of template (Fig. 4B). Once the primer was extended to the end of template, however, additional nucleotides ($>20$) were added, most likely a result of slippage synthesis. Products consistent with template switching were not observed. With primer/template II, where a single round of nucleotide incorporation should occur by using ATP as the sole nucleotide substrate, the first nucleotide was incorporated and misincorporation was noted (Fig. 4E). However, with each round of misincorporation, subsequent cycles of misincorporation became less efficient as very few primers could be extended to the end of template.

In both cases, the kinetics of primer extension were biphasic (Fig. 4C and F). The first phase was faster than could be measured by manual quenching of the reaction. When primer/template I was employed, the amplitude of the first phase represented 65% of this substrate. Whereas 85% of primer/template I was utilized during the course of the reaction, only 70% of primer/template II was utilized. When the kinetics of primer extension in the presence of Mn$^{2+}$ from primer/template II were compared with the kinetics in the presence of Mg$^{2+}$, the primary difference observed was that more complexes formed in the presence of Mn$^{2+}$ than in the presence of Mg$^{2+}$, both productive (note difference in $y$ intercept in Fig. 5A) and nonproductive (note difference in end points in Fig. 5A). This conclusion was the same whether the first correct nucleotide (Fig. 5A) or all four nucleotides (Fig. 5B) were provided. However, it should be noted that the use of all four nucleotides supported higher levels of primer extension in the presence of both Mg$^{2+}$ and Mn$^{2+}$ than the use of a single nucleotide.

3Dpol Adds Nontemplated Nucleotides to Blunt-ended, Heteropolymeric RNA Primer/Template—We reasoned that the biphasic nature of the kinetics was a reflection of enzyme binding in the “correct” orientation in some cases (fast incorporation) and in the “incorrect” orientation in others (slower incorporation). We performed experiments with primer/templates I and II in which the template strand was end-labeled instead of the primer strand (Fig. 6, A and C). Nontemplated addition of nucleotides was observed with both primer/templates in the presence of either Mn$^{2+}$ or Mg$^{2+}$ (Fig. 6, B and D). The reaction was more efficient in the presence of Mn$^{2+}$ than in the presence of Mg$^{2+}$. Consistent with the amplitudes observed when labeled primers were employed, the template strand of primer/template I was utilized by 3Dpol with a lower efficiency than the template strand of primer/template II.
The ability of 3Dpol to add non templated nucleotides to the blunt end of an RNA primer/template was somewhat surprising. However, it has been reported previously that the reverse transcriptase from human immunodeficiency virus has a similar activity (30). Terminal transferase activity of 3Dpol would yield similar results if single-stranded RNA were present in the reactions described above. We performed an experiment in which either the end-labeled primer (Fig. 7A) or template (Fig. 7C) strand of primer/template II was incubated with 3Dpol, ATP, and either Mg$^{2+}$ or Mn$^{2+}$ as the divalent cation. In all cases, the kinetics and/or products of the terminal transferase reaction were substantially different from those observed by using a template-labeled primer/template (Fig. 7, B and D). The ability of 3Dpol to partition in both possible orientations on heteropolymeric RNA primer/templates must be considered in any quantitative analysis of 3Dpol-catalyzed RNA synthesis.

**3Dpol Is an RdRP and a Reverse Transcriptase**—The ability to monitor 3Dpol activity by primer extension permitted us to evaluate the specificity and fidelity of 3Dpol-catalyzed nucleotide incorporation. In the presence of Mg$^{2+}$, both the correct rNMP and dNMP were incorporated to the greatest extent (Fig. 8B). With the correct rNTP, 40% of primers were extended (Fig. 8B, lane 4). In most cases, primers were extended to the end of template and additional nucleotides were added. The addition of extra nucleotides was most likely the result of slippage synthesis. However, it is also plausible that the extra nucleotides were added in a nontemplated fashion as discussed above. With the correct dNTP, 30% of primers were extended (Fig. 8B, lane 8). Whereas some primers were extended to the end of template, products with only a single dNMP incorporated accumulated to the greatest extent. Products greater than unit length were not observed. In the presence of Mn$^{2+}$, the correct dNMP was not incorporated at all. The n + 1 product observed in lane 12 of Fig. 8B must arise from rNTP contamination of the ddNTP stock. This conclusion is based on the migration of this product through the polyacrylamide gel; the n + 1 product present in lane 12 of Fig. 8B is migrating slower than expected for a ddNMP-incorporated product (cf. Fig. 8B, lane 12, and Fig. 8C, lane 26).

In the presence of Mn$^{2+}$, a 2-fold increase in primer utilization was observed when either the correct rNTP (Fig. 8C, lane 18) or the correct dNTP (Fig. 8C, lane 22) was employed. 84% of primers were extended when the correct rNTP was utilized; and 72% of primers were extended when the correct dNTP was utilized. Interestingly, by using Mn$^{2+}$ as the divalent cation, the correct ddNTP was utilized, and 34% of primers were extended (Fig. 8C, lane 26). The efficiency of correct rNMP and dNMP incorporation was also stimulated by using Mn$^{2+}$. In both cases, 90% of extended primers reached the end of template and addition of extra nucleotides was enhanced significantly (cf. Fig. 8B, lanes 4 and 8, and Fig. 8C, lanes 18 and 22). Mn$^{2+}$ Decreases the Fidelity of 3Dpol-catalyzed Nucleotide Incorporation—In the presence of Mg$^{2+}$, the efficiency of utilization of incorrect rNTPs (Fig. 8B, lanes 1–3), dNTPs (Fig. 8B, lanes 5–7), and ddNTPs (Fig. 8B, lanes 9–11) was less than 20%
Whereas utilization of dGTP was increased in the presence of Mn2⁺, significant accumulation of products was in the presence of Mn2⁺ and incubated at 30 °C; reactions were quenched at the indicated times by addition of EDTA to a final concentration of 50 mM. Products were resolved by electrophoresis on a denaturing 20% polyacrylamide gel. C, kinetics of primer extension in the reaction described in B were determined by quantitating product by using the ImageQuant software. D, 15/21-mer primer/template II employed in the experiment described in E. E, reactions contained 3Dpol (5 μM), end-labeled primer/template II (1 μM), ATP (500 μM), and MnCl₂ (5 mM). Reactions were initiated by addition of 3Dpol and incubated at 30 °C; reactions were quenched at the indicated times by addition of EDTA to a final concentration of 50 mM. Products were resolved by electrophoresis on a denaturing 20% polyacrylamide gel. F, kinetics of primer extension in the reaction described in E were determined by quantitating product by using the ImageQuant software.

of the value measured for incorporation of the correct rNTP. Incorrect rNTPs were utilized better than incorrect dNTPs, and ddNTPs were not utilized at all. Again, as discussed above, the n + 1 products observed in lanes 9–11 of Fig. 8B must result from rNTP contamination of the ddNTP stocks. In the presence of Mn2⁺, incorrect rNTPs were utilized efficiently. 53, 59, or 68% of primers were extended by using ATP (Fig. 8C, lane 15), CTP (Fig. 8C, lane 16), or GTP (Fig. 8C, lane 17), respectively, as the incorrect rNTP. In most instances, primers could not be extended to the end of template. The most significant accumulation of products was in the n + 1 to n + 3 range. Whereas utilization of dGTP was increased in the presence of Mn2⁺ (21% of primers extended), utilization of the other two incorrect dNTPs was similar to that observed in the presence of Mg2⁺. The use of Mn2⁺ had very little effect on utilization of incorrect ddNTPs.

Finally, in some instances, an apparent activation of primer cleavage was observed. This increase in primer cleavage was apparent in the presence of Mg2⁺ (lanes 6, 9, and 10 of Fig. 8B) and was stimulated by using Mn2⁺ (lanes 15, 19, 20, 21, 23, 24, and 25 of Fig. 8C). Primer cleavage was not due to ribonuclease contamination of our 3Dpol preparations as cleavage was not observed in all reactions. Moreover, in most cases, ribonuclease activity does not require a divalent cation, thus an activity difference in the presence of Mg2⁺ relative to Mn2⁺ would not be expected (31). Interestingly, primer cleavage occurred primarily after misincorporation of nucleotides. Primer cleavage was not evident in reactions incorporating correct ribonucleotides (lane 18, Fig. 8C), deoxynucleotides (lane 22, Fig. 8C), or dideoxynucleotides (lane 26, Fig. 8C). Additional studies will be necessary to determine the molecular basis for this observation.

**DISCUSSION**

We have performed a comprehensive, quantitative evaluation of the dNTP binding specificity of poliovirus RNA-dependent RNA polymerase, 3Dpol. The primary, universal effect of Mn2⁺ on 3Dpol was a substantial (20–30-fold) reduction in the KM value of the enzyme for primer/template (Table III). 3Dpol activity was stimulated by an additional 20-fold over that expected based solely on the reduction in KM value for primer/template when dG15/rC30 was analyzed in the presence of Mn2⁺ (Table III). This additional increase in activity was not due to changes in the KM value of 3Dpol for GTP as this value was not affected significantly by using Mn2⁺ instead of Mg2⁺ (Table III). Therefore, we concluded that by using Mn2⁺ the number of productive 3Dpol-dG15/rC30 complexes formed was increased by 20-fold relative to the number formed by using Mg2⁺.

The ability of nucleic acid polymerases to utilize transition metals, especially Mn2⁺, as the divalent cation cofactor instead of Mg2⁺ is well established (33–37). The primary effect of Mn2⁺ relative to Mg2⁺ is that nucleotide specificity is relaxed, that is nucleotides with the inappropriate sugar or base can be incorporated more efficiently (35, 36). The classic explanation for the observed relaxation in nucleotide specificity in the presence of Mn2⁺ is that fewer geometrical constraints exist with this divalent cation for coordination of the nucleotide phosphates and active site ligands, which is a prerequisite to phosphoryl transfer (36). In fact, even for 3Dpol, it has been shown by Morrow and colleagues (38) that the use of transition metals as divalent cation cofactor for this enzyme can overcome, to some extent, effects of mutations at positions of the enzyme that alter the position or identity of the active site residues that are involved in metal coordination. Also, it has been noted that Mn2⁺ is capable of relaxing template specificity (39). In this regard, it worth noting that template specificity of 3Dpol was also relaxed by using Mn2⁺; a DNA template supported RNA synthesis in the presence of this cofactor (Table II).

In this study, we observed a dramatic reduction in the KM value for primer/template by employing Mn2⁺ as the divalent cation cofactor instead of Mg2⁺. To date, similar observations have not been made for any other nucleic acid polymerase. However, the ability of Mg2⁺ to increase formation of productive polymerase-nucleic acid complexes has been noted previously by Modrich and colleagues (40). It has been shown that...
transition metals, such as Mn$^{2+}$, bind much more tightly to the phosphodiester backbone of nucleic acid than Mg$^{2+}$ (41). Therefore, it is plausible that stability and/or concentration of primer/template duplex is increased due to the enhanced charge neutralization of the phosphodiester backbone in the presence of Mn$^{2+}$. If more primer/template duplex exists at lower concentrations in the presence of Mn$^{2+}$ than in the presence of Mg$^{2+}$, then an apparent reduction in the $K_M$ value for primer/template would be observed as this is the competent form of the substrate.

Alternatively, it is possible that 3Dpol has not evolved to bind to a charged template, that is the enzyme is incapable of effectively neutralizing the phosphodiester backbone. The strong binding of Mn$^{2+}$ to the phosphodiester backbone would overcome this problem thereby increasing the affinity of 3Dpol for primer/template, in addition to possibly increasing the number of complexes that form. If this hypothesis is correct, then a mechanism for neutralization of the biological templates might exist. The virus-encoded 3AB protein may fulfill such a role because it has nonspecific RNA binding activity (42) and can increase the use of homo- and heteropolymeric primer/templates (43, 44). Similar scenarios have been well established for negative-strand RNA viruses such as Sendai virus (45).

We reported previously that 3Dpol is capable of primer-independent RNA synthesis when poly(rC) is employed as template (23). In this study, we demonstrated that primer-independent RNA synthesis resulted from initiation de novo and was also stimulated by using Mn$^{2+}$ as the divalent cation cofactor. Overall, the reaction sequence employed by 3Dpol in catalyzing RNA synthesis de novo is quite similar to that observed for the same type of reaction catalyzed by replicases for RNA viruses such as Qβ (46) and brome mosaic virus (47). It is currently unclear whether RNA synthesis de novo catalyzed by 3Dpol has any biological significance. However, the ability of a polymerase that clearly uses a protein primer in vivo to support RNA synthesis de novo has significant implications on the conclusions that should be drawn when similar observations are made with polymerases from RNA virus systems which lack significant biological characterization. For example, a recent report on the RdRP from bovine viral diarrhea virus showed that this enzyme is capable of initiating RNA synthesis de novo (48). However, in the absence of data characterizing the 5'-end of viral RNA, it may be premature to completely rule out the possibility of primed synthesis in the mechanism of initiation of pesti- and hepacivirus genome replication.

The kinetics of primer extension were biphasic with both heteropolymeric primer/template substrates employed (Fig. 4, C and F). We anticipated that the reaction would be monophasic with the kinetics of formation of extended primers being described best by a single exponential. This assumption was based on the fact that the $K_M$ values measured for dT<sub>15</sub>/rA<sub>30</sub>...
and dG15/rC30 in the presence of Mn2⁺ were in the 1 μM range, and a 3Dpol concentration of 5 μM was employed in this reaction, thus approximately 90% of the primer/template should be bound to enzyme.

One possible explanation for biphasic kinetics given the aforementioned assumptions was that two different 3Dpol primer/template complexes formed. Whereas one complex would be competent for primer extension (first, fast phase), the other would be unproductive requiring some type of rearrangement of the initial enzyme-primer/template complex or enzyme dissociation from primer/template prior to formation of a complex that was competent for primer extension (second, slow phase). By employing a template-labeled primer/template, it was apparent that the enzyme was capable of binding to primer/template in both orientations and adding nucleotides to the blunt-end of the duplex (Fig. 6). That the addition was not terminal transferase activity was ruled out by qualitative and quantitative comparison of the single-stranded RNA primer or template (Fig. 7). Therefore, the “lost” fraction was found, and the slow phase, most likely, reflected dissociation of the enzyme from the unproductive conformation to bind to primer/template in the productive conformation.

Partitioning of the enzyme between the productive and unproductive conformations was not equal and differed for the two primer/template substrates employed in this study. Both substrates are identical in length of primer, template, and substrates are identical in length of primer, template, and/or integrity of the duplex region of nascent chain is important for processive synthesis. Therefore, the “lost” fraction was found, and the slow phase, most likely, reflected dissociation of the enzyme from the unproductive conformation to bind to primer/template in the productive conformation.

Finally, we noted that cleavage of the primer occurs in reac-
tions incorporating incorrect rNMPs and dNMPs. Primer cleavage occurred in the presence of Mg\(^{2+}\) but was most striking in the presence of Mn\(^{2+}\), because of the increased levels of misincorporation observed by using this cofactor. Primer cleavage may result from pyrophosphorolysis. If an appropriately base-paired duplex is a prerequisite to efficient translocation and PPi release requires translocation (49), then it is conceivable that after misincorporation PPi may have sufficient time to attack the misaligned duplex. However, our results would also suggest that PPi is capable of attacking phosphodiester bonds other than the ultimate bond. Of course, this reaction would also suggest that PPi is capable of attacking phosphodiester bonds other than the ultimate bond. Of course, this reaction could provide a mechanism for error correction. Studies are in progress to characterize this reaction more completely.

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Poliovirus RNA-dependent RNA Polymerase (3D<sup>pol</sup>): DIVALENT CATION MODULATION OF PRIMER, TEMPLATE, AND NUCLEOTIDE SELECTION
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