We have analyzed the divalent cation specificity of poliovirus RNA-dependent RNA polymerase, 3Dpol. The following preference was observed: 

\[ Mn^{2+} > Co^{2+} > Ni^{2+} > Fe^{2+} > Mg^{2+} > Cu^{2+} > Zn^{2+} \]

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'-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 Mg$^{2+}$. 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). Unfortunately, highly effective therapies to treat chronic RNA virus infection do not exist. Replication of the genomes of all RNA viruses requires the enzyme resemblance a right hand with "fingers," "palm," and "thumb" subdomains. The palm subdomain contains four structural features. This paper is available online at http://www.jbc.org

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Poliovirus RNA-dependent RNA polymerase (3Dpol) DIVALENT CATATION MODULATION OF PRIMER, TEMPLATE, AND NUCLEOTIDE SELECTION

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Oligonucleotides were typically suspended in T10E1 (10 mM Tris, 1 mM salted on Sep-Pak columns (Millipore) as specified by the manufacturer. Elutrap apparatus (Schleicher & Schuell). Oligonucleotides were de-
tween 3Dpol and primer/template that permit polymerase ac-
have shown that the heteropolymeric RNA primers. One possible explanation for
tivity to be monitored by following the extension of end-labeled,
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spotted in triplicate onto TLC plates. TLC plates were developed in 0.3
or Fisher.
were from EM Science; 2.5-cm DE81 filter paper discs were from What-
motifs (A–D) found in all polymerases, in addition to a
tural motifs that permit polymerase ac-
tural significance between the RdRP and DNA polymerases and suggest novel strategies for the design of
primer/template substrates to investigate 3Dpol mechanism.

**EXPERIMENTAL PROCEDURES**

**Materials**

\[ [\alpha-^32P]GTP (>3,000 Ci/mmol), [\alpha-^32P]UTP (>6,000 Ci/mmol), and [\alpha-^32P]GTP (>6,000 Ci/mmol) were from NEN Life Science Products; \[\gamma-^32P]ATP (>7,000 Ci/mmol) was from ICN; nucleotide 5'-triphos-
phases, 2'-deoxyxycoside 5'-triphosphates, 2',3'-deoxyxycoside 5-
triphosphates (all nucleotides were ultrapure solutions), and poly(rA) were from Amersham Pharmacia Biotech, Inc.; poly(rC) and poly(U) were from Sigma; all DNA oligonucleotides were from Operon Tech-
ologies, Inc. ([Alameda, CA]; all RNA oligonucleotides were from Dharmar-
 Research, Inc. (Boulder, CO); 10-base pair DNA ladder was from Life Technologies, Inc.; T4 polynucleotide kinase and calf intestinal alkaline phosphatase were from New England Biolabs, Inc.; MgCl2, MnCl2, MnCl2, ZnCl2, and CaCl2 were from Fisher; CuCl2, FeCl2, NiCl2, CoCl2, and alkaline phosphatase were from New England Biolabs, Inc.; MgCl2, MnCl2, MnCl2, ZnCl2, and CaCl2 were from Fisher; CuCl2, FeCl2, NiCl2, CoCl2, and Gpg were from Sigma; polyethyleneimine-cellulose TLC plates were from EM Science; 2.5-cm DES1 filter paper discs were from What-
amer reagents were of the highest grade available from Sigma or Fisher.

**Expression and Purification of 3Dpol**

Expression and purification of 3Dpol was performed 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% urea and 1 \times 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 \times TBE by using an Elutrap apparatus (Schleicher & Schuell). Oligonucleotides were de-
salted on Sep-Pak columns (Millipore) as specified by the manufacturer.

Oligonucleotides were typically suspended in T10E1 (10 mM Tris, 1 mM EDTA, pH 8.0), aliquoted, and stored at \(-80 \degree C\) until use. Concentra-
tions were determined by measuring the absorbance at 260 nm by using calculated extinction coefficients (25).

**Purity of [\alpha-^32P]NTPs**

[\alpha-^32P]NTPs were diluted to 0.1 \mu Ci/\mu l in ddH2O, and 1 \mu l was spotted in triplicate onto TLC plates. TLC plates were developed in 0.3 
particle radiolabeled nucleotide ([\alpha-^32P]UTP or [\alpha-^32P]GTP), either MgCl2 or MnCl2 (5 mM) and either dT15/rA30 (10 
M) and poly(rA) (93.4 
M); \[\gamma-^32P]ATP and T4 polynucleotide kinase essentially as specified by the manufacturer. Reactions typically contained 1 \mu M [\gamma-^32P]ATP, 10 \mu M Gpg, and 0.4 unit/\mu l T4 polynucle-
tide kinase. Reactions were quenched by heating the reaction at 60 \degree C for 5 min.

**Annealing of Heteropolymeric Primer/Template**

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

**3Dpol Assays**

Reactions contained 50 mM HEPES, pH 7.5, 10 mM 2-mercapto-
ethanol, 5 mM MgCl2 or MnCl2, 60 \mu M ZnCl2, 500 \mu M 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(rU) and Poly(rG) Polymerase Activity**

Reactions contained 0.5 \mu M 3Dpol, 500 \mu M nucleotide (UTP or GTP), 0.2 \mu M radiolabeled nucleotide ([\alpha-^32P]UTP or [\alpha-^32P]GTP), either MgCl2 or MnCl2 (5 mM) and either MgCl2 or MnCl2 (5 mM) and either dC30 (10 
M), FeCl2, and CaCl2 were from Fisher; CuCl2, FeCl2, NiCl2, CoCl2, and Gpg were from Sigma; polyethyleneimine-cellulose TLC plates were from EM Science; 2.5-cm DES1 filter paper discs were from What-

**RNA Synthesis de Novo: Template Specificity**

Reactions contained 3Dpol (0.1 \mu M), dG6 (4.7 \mu M), poly(rC) (93.4 
M); \[\gamma-32P]GTP (0.2 
M) and either MgCl2 or MnCl2. Reactions were initiated by addition of 3Dpol and incubated at 30 \degree 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 \mu l. Products were analyzed by DES1 filter binding.

**Steady-state Kinetic Analysis of 3Dpol**

Kinetic constants, \(K_d\) and \(V_{max}\), were determined by using the assay described above. The concentration of 3Dpol employed in these experi-
ments ranged from 0.01 to 0.5 \mu M depending upon the substrate and cation employed. The \(V_{max}\) values reported in Table III have been normalized to 0.01 \mu M 3Dpol to facilitate comparison of the various substrates. Concentrations of the varied substrate, nucleic acid or nucle-
itrate, ranged from 0.25 \times \text{K}_d \times 4 \times \text{K}_d. The concentration of the substrate that remained constant was 5–10 \times \text{K}_d. Single time points were taken that were in the linear range for product formation. Reac-
tion 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 \(K_d\) value for a primer/template substrates that permit polymerase activity to be monitored by following the extension of end-labeled, heteropolymeric RNA primers. One possible explanation for
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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 | 10 |
| dG15/poly(rC) | 200 | 1,000 |
| dG15/rC30 | 2 | 200 |

\[ K_{M(appGTP)} = K_{M(GTP)} + \frac{1}{K_{M(appGTP)}} \] (Eq. 1)

**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 20 μl. Reactions were initiated by addition of calf intestinal alkaline phosphatase and incubated at 37 °C. Reactions were quenched by addition of EDTA to a final concentration of 50 mM. Each Phosphatase reaction was passed over two consecutive 1-ml Sephadex G-25 columns to remove any unincorporated nucleotide.

**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 ml of 5% boric acid, rinsed and 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, pH 7.0, 0.025% bromphenol blue, 0.025% xylene cyanol and where appropriate 5 mM of MgCl2, CaCl2, MgCl2, FeCl2, NiCl2, CuCl2, and MnCl2. Reactions were initiated by addition of 3Dpol and incubated at 37 °C for 5 min at which time reactions were quenched by addition of EDTA to a final concentration of 50 mM. Each quenched reaction was passed over two consecutive 1-ml Sephadex G-25 spin columns to remove any unincorporated nucleotide.

**RESULTS**

**Transition Metals 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 white 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 II). 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...
rate the label. Product RNA was labeled by using 95% of the counts associated with g (Fig. 1 on PPi accumulation (Fig. 1 resolved by TLC; over half of the nucleotide was utilized based by using the DE81 filter paper method (Fig. 2 A).

3Dpol adds GMP to the 3' end of rC30, thus creating a "snap back" substrate that is efficiently extended by 3Dpol. However, when a 5' end-labeled rC30 template, we were only able to show the incorporation of a single GMP into rC30 (data not shown). Moreover, the kinetics of formation of this product were too slow to support the hypothesis that rC30G 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 rC30 and [γ-32P]GTP as substrates. If RNA synthesis initiates de novo, then long RNA products should incorporate -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 Mn²⁺ showed that the catalytic efficiency of this reaction is greater than either of the primer-dependent reactions characterized (Table III). The Km 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.

Mn²⁺ Stimulates 3Dpol-catalyzed Extension of Heteropolymeric RNA Primer/Templates—The observation that the number of productive 3Dpol-dG15/rC30 complexes that formed was increased by using Mn²⁺ instead of Mg²⁺ suggested that an increase in the utilization of heteropolymeric RNA primer/templates might also be observed by using Mn²⁺. 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 3D\textsuperscript{pol} to add nontemplated 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 3D\textsuperscript{pol} 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 3D\textsuperscript{pol}, ATP, and either Mg\textsuperscript{2+} or Mn\textsuperscript{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 3D\textsuperscript{pol} to partition in both possible orientations on heteropolymeric RNA primer/templates must be considered in any quantitative analysis of 3D\textsuperscript{pol}-catalyzed RNA synthesis.

3D\textsuperscript{pol} Is an RdRP and a Reverse Transcriptase—The ability to monitor 3D\textsuperscript{pol} activity by primer extension permitted us to evaluate the specificity and fidelity of 3D\textsuperscript{pol}-catalyzed nucleotide incorporation. In the presence of Mg\textsuperscript{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\textsuperscript{2+}, the correct ddNMP 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\textsuperscript{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 dNTP was utilized; and 72% of primers were extended when the correct ddNTP was utilized. Interestingly, by using Mn\textsuperscript{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\textsuperscript{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\textsuperscript{2+} Increases the Fidelity of 3D\textsuperscript{pol}-catalyzed Nucleotide Incorporation—In the presence of Mg\textsuperscript{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 incorrect accumulation of products was in the lane 26 of the experiment described in B. E, reactions contained 3Dpoly (5 μM), end-labeled primer/template I (1 μM), UTP (500 μM), and MnCl₂ (5 mM). Reactions were initiated by addition of 3Dpoly 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 3Dpoly (5 μM), end-labeled primer/template II (1 μM), ATP (500 μM), and MnCl₂ (5 mM). Reactions were initiated by addition of 3Dpoly 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.

We have performed a comprehensive, quantitative evaluation of the divalent cation 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 nucleotide 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 is 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 Mn²⁺ 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 dT15/rA30
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 nucleotide selection by 3Dpol. An additional advantage of the two 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 duplex region and only differ in three readily apparent ways. First, the sequence around the primer/template junction and template overhang are different. Second, there is a subtle (10 °C) difference in the calculated Tm values for the two primer/templates. Third, by using primer/template I and UTP, the enzyme can extend to the end of template, whereas by using primer/template II and ATP, extension to the end of template is not efficient as it requires misincorporation. This third possibility was ruled out as being a significant factor by showing that partitioning of primer/template II was not affected when reactions were performed in the presence of all four NTPs (Fig. 5). Given the two remaining possibilities, a sequence dependence for binding seems most likely. Additional experiments will be required to clarify this issue.

However, to gain insight into the nucleotide specificity and fidelity of 3Dpol, we performed a series of primer-extension experiments in which the utilization of correct and incorrect NTPs, dNTPs, and ddNTPs was evaluated. Incorporation of dTMP was more efficient than incorporation of any of the incorrect NTPs or analogs (500 μM). Reactions were initiated by addition of 3Dpol and incubated at 30 °C for 10 min. Products were resolved by electrophoresis on a denaturing 20% polyacrylamide gel. C, reactions contained 3Dpol (5 μM), primer/template I (1 μM), MnCl2 (5 mM), and the indicated NTP or analog (500 μM). Reactions were initiated by addition of 3Dpol and incubated at 30 °C for 10 min. Products were resolved by electrophoresis on a denaturing 20% polyacrylamide gel. C, reactions contained 3Dpol (5 μM), primer/template I (1 μM), MnCl2 (5 mM), and the indicated NTP or analog (500 μM). Reactions were initiated by addition of 3Dpol and incubated at 30 °C for 10 min. Products were resolved by electrophoresis on a denaturing 20% polyacrylamide gel.

FIG. 7. Terminal transferase activity by 3Dpol. A, RNA oligonucleotide employed in the experiment described in B was 5′-end-labeled. B, reactions contained 3Dpol (5 μM), RNA (1 μM), ATP (500 μM), and either MnCl2 or MgCl2 (5 mM). Reactions were initiated by addition of 3Dpol and incubated at 30 °C for 10 min. Products were resolved by electrophoresis on a denaturing 20% polyacrylamide gel. C, reactions contained 3Dpol (5 μM), primer/template I (1 μM), MnCl2 (5 mM), and the indicated NTP or analog (500 μM). Reactions were initiated by addition of 3Dpol and incubated at 30 °C for 10 min. Products were resolved by electrophoresis on a denaturing 20% polyacrylamide gel.

FIG. 8. Nucleotide selection by 3Dpol in MnCl2 and MgCl2. A, 15/21-mer primer/template I employed in this experiment. B, reactions contained 3Dpol (5 μM), primer/template I (1 μM), MnCl2 (5 mM), and the indicated NTP or analog (500 μM). Reactions were initiated by addition of 3Dpol and incubated at 30 °C for 10 min. Products were resolved by electrophoresis on a denaturing 20% polyacrylamide gel. C, reactions contained 3Dpol (5 μM), primer/template I (1 μM), MnCl2 (5 mM), and the indicated NTP or analog (500 μM). Reactions were initiated by addition of 3Dpol and incubated at 30 °C for 10 min. Products were resolved by electrophoresis on a denaturing 20% polyacrylamide gel.

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$^{2+}$ and Mn$^{2+}$ 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 pyrophosphorylation. 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 could provide a mechanism for error correction. Studies are in progress to characterize this reaction more completely.

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