Increased Catalytic Activity and Altered Fidelity of Human DNA Polymerase \(\eta\) in the Presence of Manganese*

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All DNA polymerases require a divalent cation for catalytic activity. It is generally assumed that Mg\(^{2+}\) is the physiological cofactor for replicative DNA polymerases in vivo. However, recent studies suggest that certain repair polymerases, such as pol \(\lambda\), may preferentially utilize Mn\(^{2+}\) in vitro. Here we report on the effects of Mn\(^{2+}\) and Mg\(^{2+}\) on the enzymatic properties of human DNA polymerase \(\eta\) (pol \(\eta\)). pol \(\eta\) exhibited the greatest activity in the presence of low levels of Mn\(^{2+}\) (0.05–0.25 mM). Peak activity in the presence of Mg\(^{2+}\) was observed in the range of 0.1–0.5 mM and was significantly reduced at concentrations >2 mM. Steady-state kinetic analyses revealed that Mn\(^{2+}\) increases the catalytic activity of pol \(\eta\) by ~30–60,000-fold through a dramatic decrease in the \(K_m\) value for nucleotide incorporation. Interestingly, whereas pol \(\eta\) preferentially misinserts G opposite T by a factor of ~1.4–2.5-fold over the correct base A in the presence of 0.25 and 5 mM Mg\(^{2+}\), respectively, the correct insertion of A is actually favored 2-fold over the misincorporation of G in the presence of 0.075 mM Mn\(^{2+}\). Low levels of Mn\(^{2+}\) also dramatically increased the ability of pol \(\eta\) to traverse a variety of DNA lesions in vitro. Titration experiments revealed a strong preference of pol \(\eta\) for Mn\(^{2+}\) even when Mg\(^{2+}\) is present in a >10-fold excess. Our observations therefore raise the intriguing possibility that the cation utilized by pol \(\eta\) in vivo may actually be Mn\(^{2+}\) rather than Mg\(^{2+}\), as tacitly assumed.

It has been known for several decades that DNA polymerases (pol\(^2\)) require a divalent cation as an activator for phosphotidyl transfer (1–3). Two metal ions are usually coordinated by three acidic amino acids within the active site of the polymerase, so as to form a metal bridge between the enzyme and the terminal phosphoryl group of the substrate (4, 5), thereby facilitating the departure of the pyrophosphate moiety (6). Based upon its cellular abundance, it is generally believed that Mg\(^{2+}\) is the activating cofactor in vivo. However, Mn\(^{2+}\), Co\(^{2+}\), Ni\(^{2+}\), and Zn\(^{2+}\) have the capacity to substitute for Mg\(^{2+}\) under certain conditions in vitro (2, 3, 7), but usually at the consequence of reduced fidelity and in some cases decreased processivity. The effect of substituting Mg\(^{2+}\) with Mn\(^{2+}\) on the activity of A- and B-family polymerases has been widely studied (8–12). In addition to reducing fidelity, Mn\(^{2+}\) also helps facilitate translesion synthesis by certain replicative polymerases, including Escherichia coli pol I (13) and herpes simplex virus-1 UL30 protein (14) but not others, such as T4 DNA polymerase, or bovine pol \(\delta\) (12, 14).

The effect of Mn\(^{2+}\) on X-family polymerases has also been studied. In the case of pol \(\beta\), Mn\(^{2+}\) decreases the \(K_m\) value of nucleotide incorporation by ~30-fold (15), such that there is little regard for the instructions provided by the templating base (16). Recent data also suggest that the phylogenetically related pol \(\lambda\) may have actually evolved to utilize Mn\(^{2+}\) under physiological conditions, because the enzyme is active over a wide range of Mn\(^{2+}\) concentrations and is inhibited by high levels of Mg\(^{2+}\) (17, 18).

To date, there have been few studies on the effects of Mn\(^{2+}\) on the Y-family of DNA polymerases. In the single published report, Sulfolobus solfataricus Dpo4 behaved in a similar manner to the replicative polymerases, in that 5 mM Mn\(^{2+}\) increased the efficiency of lesion bypass, but with a concomitant 3–4-fold decrease in the overall fidelity of the enzyme (19). The effect of Mn\(^{2+}\) on other Y-family polymerases, including human DNA polymerases \(\gamma\), \(\iota\), and \(\kappa\), is largely unknown. We were therefore very interested in determining the effects of various metal ions on the activity of the human Y-family polymerases and in particular pol \(\iota\), because its reported biochemical properties are quite unusual. Indeed, pol \(\iota\) exhibits a remarkable template-dependent fidelity in vitro. When replicating template T in the presence of Mg\(^{2+}\), pol \(\iota\) preferentially misinserts G over the correct base A by a factor of 3–10-fold (20–24). In contrast, when replicating template A, the enzyme is relatively accurate with misincorporations occurring in the range of 10\(^{-3}\)–10\(^{-4}\) (20–22). Thus, the fidelity of pol \(\iota\) can vary by up to 100,000-fold depending upon the template sequence being replicated. Here we report on the enzymatic properties of pol \(\iota\) in vitro in the presence of various divalent cations, in particular Mg\(^{2+}\) and Mn\(^{2+}\).

**EXPERIMENTAL PROCEDURES**

**Enzymes**—N-terminal glutathione S-transferase-tagged human pol \(\iota\) was expressed and purified from baculovirus-infected insect SF9 cells as described (20). C-terminal His-tagged human pol \(\eta\) (25) was also expressed in baculovirus-infected SF21 cells and purified by nickel-agarose, Q-Sepharose, and Bio-Gel HT hydroxyapatite chromatography. N-terminal His-tagged human pol \(\kappa\) (26) was purchased from Enzymax (Lexington, KY).
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Primers and Templates—All primers, undamaged templates, and the abasic site-containing template, TXT30, were synthesized by Lofstrand Laboratories (Gaithersburg, MD) and gel-purified prior to use. The cyclobutane pyrimidine dimer containing template, TTA48, was synthesized and purified by Phoenix Biotechnologies, Inc. (Huntsville, AL). The pyrimidine-pyrimidone (6-4)-containing template, 6-4TT30, was a kind gift from Shigenori Iwai (Osaka University). The benzo[a]pyrene diol epoxide-containing template, BPDE29, was a kind gift from Don Jerina (NIDDK, National Institutes of Health). For the experiments presented in Fig. 1, the template was T50, 5′-CGT CTA GAC GAA TTC AGC GCT CAA GCT TGC TTT CGC ATG TCT TGC AGG CGG-3′, and the primer was a 22-mer that was complementary to the template nucleotides underlined. The template used for the experiments presented in Figs. 2 and 5 and Table 1, parts B and C, was T50M13, 5′-CGG TAA TGA TTC CTA CCA TGA AAA TAA AAA CGG CTT GCT TGT TCT CGA TG-3′, which corresponds to M13mp18 (nucleotides 3486–3437) (27). The primer was a 20-mer complementary to the template nucleotides underlined. The experiments described in Fig. 3 and Table 1, Part A utilized 48-mer templates UTTA and TTA48, with the sequence 5′-CTC GTC AGC ATC GTA CTA CGA TAG ATT AAG CAA TTA AGC CAG TCC GTA CCA TCG-3′. The primer was a 16-mer that was complementary to the template nucleotides underlined. The adjacent Ts were covalently linked to form a cyclobutane pyrimidine dimer (CPD) in template TTA48. The experiments described in Fig. 4 utilized three lesion-containing templates. TXT30 was a 30-mer with the sequence 5′-CTC GTC AGC ATC TTA CTC GTA CTA CGA TCA GTA TCG-3′, where the $X$ was a synthetic tetrahydrofuran moiety (abasic site). The primer was a 16-mer that was complementary to the template nucleotides underlined. The second template, BPDE29, was a 29-mer with the sequence 5′-GGT CGT CAG CAG ATT TAG AGT CGT CAG TCG-3′, where the $A$ has a benzo[a]pyrene adduct at its N6 position (28). The primer was a 16-mer that was complementary to the template nucleotides underlined. The third template, 6-4TT30, was a 30-mer with the sequence 5′-CTC GTC AGC ATC TCG ATC ATC ATA CAG TCA GTA TCG-3′, where the two $T$s indicate a 6-4 pyrimidine-pyrimidone dimer (29). The primer was a 16-mer that was complementary to the template nucleotides underlined.

Annealing Primers to Templates—100 nM of $^{32}$P-labeled primer was mixed with 150 nM of the corresponding template in 50 mM Tris-HCl (pH 8.0), 10 mM NaCl, 1.42 mM 2-mercaptoethanol, and 50 $\mu$g/ml bovine serum albumin, heated at 95 °C for 5 min, and then slowly cooled to room temperature over a period of several hours.

Primer Extension Reaction—Standard replication reactions (10 $\mu$L) contained 40 nM Tris-HCl (pH 8.0), 2.5% glycerol, 0.1 mg/ml bovine serum albumin, 10 nM dithiothreitol, 10 nM of 5′-$^{32}$P-labeled primer-template DNA, 2 nM glutathione S-transferase-pol $\iota$, 5 nM His-pol $\eta$, or His-pol $\kappa$. Reaction times, divalent metal ions, in the form of $-\text{Cl}$ salt, and dNTPs concentrations are specified in the figures and legends. After incubation at 37 °C, reactions were terminated by addition of 10 $\mu$L of 95% formamide, 10 mM EDTA, and samples were heated to 100 °C for 5 min and then immediately transferred on ice. The reaction products were separated by 15% polyacrylamide, 8 M urea gel electrophoresis and analyzed using a Fuji FLA-5100 PhosphorImager and ImageGauge software.

Steady-state Kinetics—Steady-state kinetic parameters $V_{\text{max}}$ and $K_{\text{m}}$, for incorporation opposite template T or A, were measured in standing-start reactions as described previously (20, 27, 28). Reactions contained 0.4 nM of pol $\iota$, and conditions were optimized to ensure that the reaction remains in a linear range. For reactions measuring incorporation opposite template A in the presence of 0.075 mM Mn$^{2+}$, we used 0.001–0.015 $\mu$M of dTTP and 0.0005–0.003 $\mu$M of dATP in 3-min reactions. For reactions containing 0.25 mM Mg$^{2+}$, we varied the concentration of dTTP from 0.001 to 0.015 $\mu$M and dATP from 10 to 100 $\mu$M in 2-min reactions. For reactions containing 5 mM Mg$^{2+}$, we varied the concentration of dTTP from 0.005 to 0.03 $\mu$M and dATP from 10 to 100 $\mu$M in 2.5-min reactions. In reactions where both metal ions were present (0.075 mM Mn$^{2+}$/0.25 mM Mg$^{2+}$), we used 0.001–0.02 $\mu$M of dTTP and 0.025–0.15 $\mu$M of
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FIGURE 2. Determination of the optimal concentration of Mn$^{2+}$ or Mg$^{2+}$ required for maximal activity of human pol λ and pol η in vitro. The ability of pol λ (top) and pol η (bottom) to extend a $^{32}$P-labeled 20-mer primer annealed to template "T50M13" was assayed in the presence of 50 μM of all four dNTPs and various concentrations of MnCl$_2$ or MgCl$_2$, ranging from 0.05 to 8 mM in 20-min reactions at 37 °C. The sequence of the template immediately downstream of the primer (P$_r$) is shown on the left-hand side of each gel. The arrows indicate the location of a template T that is located 6 bp from the end of the primer terminus. In the presence of Mg$^{2+}$, pol λ frequently misincorporates G opposite template T. The mispair is poorly extended compared with the correct base pair, and as a consequence a strong pause site is observed 6 bp from the primer terminus. Thus, one measurement of the overall catalytic activity of pol λ is its ability to extend beyond the normal pause site at template T. When products 7 bp and longer are plotted as a function of Mn$^{2+}$ or Mg$^{2+}$, it is clear that pol λ exhibits greatest activity at 0.075 mM Mn$^{2+}$ and 0.25–0.5 mM Mg$^{2+}$. Although pol η does not pause at template T, we used the same method to determine the optimal conditions for pol η activity. As can be seen (lower panels), pol η exhibits greatest activity in 0.05–0.1 mM Mn$^{2+}$ and 0.1–2 mM Mg$^{2+}$.

dATP in 2-min reactions. To measure incorporation opposite template T, in the presence of 0.075 mM Mn$^{2+}$, we varied dNTPs concentrations from 0.0001 to 0.0015 μM in 2.5-min reactions. Reactions in the presence of 0.25 mM Mg$^{2+}$ contained dNTPs that ranged in concentration from 0.005 to 0.03 mM in 2.5-min reactions, whereas in assays containing 5 mM Mg$^{2+}$, the dNTPs ranged from 20 to 150 μM in 2-min reactions. Reaction mixtures were subjected to 15% polyacrylamide, 8 M urea gel electrophoresis and analyzed using Fuji FLA-5100 PhosphorImager and ImageGauge software. Saturation plots of velocity as a function of dNTPs concentrations (in μM) were calculated as a percentage of the primer extension per min. $V_{max}$ and $K_m$ values were determined from a Hanes-Woolf plot by linear least squares fit as described (27) using Sigma Plot software (SPSS, Chicago).

RESULTS

Effects of Various Divalent Metal Ions on the Activity of Human Y-family Polymerases—The ability of human DNA polymerases λ, η, and κ to utilize various divalent cations as activators for polymerization was assayed in replication reactions using a radiolabeled 22-mer primer annealed to a 50-mer oligonucleotide template. Each metal ion was present as the chloride salt, at a concentration of either 0.5 or 5 mM. Quite surprisingly, of the eight divalent cations assayed, only Cu$^{2+}$ failed to support any catalysis. The remaining seven cations all promoted polymerization to some extent, with the degree of activation being acutely polymerase-specific (Fig. 1). As expected, robust synthesis was observed with pol η and pol κ in the presence of both 0.5 and 5 mM Mg$^{2+}$. In contrast, however, the activity of pol λ was far greater in 0.5 mM Mg$^{2+}$ than in 5 mM Mg$^{2+}$ (Fig. 1). All three polymerases were highly active in the presence of Mn$^{2+}$ with greater activity observed in 0.5 mM Mn$^{2+}$ compared with 5 mM Mn$^{2+}$. Co$^{2+}$ also served as an activator for all three polymerases, with pol η being the most active of the three enzymes in 0.5 mM Co$^{2+}$. Similarly, pol η was more active than either pol λ or pol κ in the presence of Ca$^{2+}$ or Zn$^{2+}$. In contrast, pol κ exhibited much greater activity in the presence of 5 mM Cd$^{2+}$ or Ni$^{2+}$, compared with either pol λ or pol η. We conclude from these studies that each human Y-family polymerase possesses its own unique ability to utilize different metals as cofactors for DNA synthesis. In the case of pol η and pol κ, the preferred activator appears to be Mg$^{2+}$. However, based upon the data presented in Fig. 1, pol λ either prefers low levels of Mg$^{2+}$ or Mn$^{2+}$ for optimal activity in vitro.

Titration of Mn$^{2+}$ or Mg$^{2+}$ in pol λ and pol η Replication Reactions—We next determined the optimum concentration of Mg$^{2+}$ or Mn$^{2+}$ required to promote peak activity of pol λ or pol η by performing primer extension assays in the presence of Mg$^{2+}$ or Mn$^{2+}$ ranging in concentrations from 0.05 to 8 mM. The template for these assays was a 50-mer oligonucleotide that has a run of five template As followed by a T, readily incorporates T opposite the five template As, but then frequently misinserts G opposite template T (29). The efficiency of G (or A) insertion opposite T is much lower than that of the incorporation of T opposite A (20). Further elongation from the mispair is also reduced compared with that of the correct A:T base pair (24), and as a consequence, there is a strong pol λ-dependent pause 5–6 bases from the starting primer terminus (Fig. 2, top, 0.1–0.5 mM Mg$^{2+}$). The percentage of replication products longer than 6 bp therefore provides
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In an attempt to further optimize the in vitro reaction conditions for pol λ synthesis, we also performed replication assays in the presence of low Mn$^{2+}$ or Mg$^{2+}$ in buffers that ranged in pH from 7.5 to 9.0 and in the presence of increasing amounts of NaCl. In general, changing the pH of the assay buffer had little effect on the activity of the enzyme. In contrast, the activity of pol λ steadily decreased as the concentration of NaCl was increased from 0 to 150 mM.

Activity of pol λ and pol η on a CPD-containing Template in the Presence of Mg$^{2+}$ and Mn$^{2+}$—We have previously reported that in the presence of 5 mM Mg$^{2+}$, pol λ is capable of inserting a base opposite the 3'T of a T-T CPD, as well as facilitating low levels of complete translesion synthesis (30, 31). We therefore wanted to reinvestigate the ability of pol λ to facilitate translesion synthesis over a range of Mg$^{2+}$ and Mn$^{2+}$ concentrations (Fig. 3). In these reactions, the 3’ end of the primer was juxtaposed to the CPD and are therefore considered "standing start" reactions. Similar to the observations reported above, maximal activity for pol λ was observed in the range of 0.075–0.25 mM Mn$^{2+}$ on the damaged template (Fig. 3A).

Under these conditions, there was significant extension of primers past the CPD. Indeed, we estimate that approximately 60% of the primer was elongated past the CPD by pol λ in the presence of 0.2 mM Mn$^{2+}$ (Fig. 3A, right-hand panel). The results with Mg$^{2+}$ were similar to our earlier published observations (30, 31), in that the 3’T of the CPD is a strong kinetic block to elongation past the 3’T was greatest in the range of 0.25–2 mM Mg$^{2+}$. Indeed, we estimate that in the presence of 0.5 mM Mg$^{2+}$, ~8% of the primers are elongated past both Ts of the CPD, compared with ~2.5% in the presence of 5 mM Mg$^{2+}$, which is consistent with our earlier observations (30, 31).

The ability of pol η to bypass a CPD in vitro is unrivalled. It does so with the same or even higher efficiency than it replicates undamaged DNA (32–34). In the presence of Mn$^{2+}$, robust bypass activity was observed from 0.05 to 0.25 mM Mn$^{2+}$, but bypass activity steadily declined as the Mn$^{2+}$ concentration increased (Fig. 3B). In contrast, pol η exhibited strong bypass activity in the presence of 0.05–8 mM Mg$^{2+}$.

We conclude from these studies that pol λ and pol η have differing affinities for metal ion activation. Translesion synthesis of a CPD by pol λ is stimulated ~3-fold by low levels of Mg$^{2+}$ and up to 25-fold in the presence of 0.2 mM Mn$^{2+}$ compared...
of the 6-4PP, it did not appreciably alter the extent of 6-4PP lesion bypass.

**pol ε Has Higher Affinity for Mn^{2+} than Mg^{2+}**—As noted earlier, in the presence of Mg^{2+} pol ε readily misinserts G opposite template T, causing the enzyme to pause. This pausing is much less evident in the presence of Mn^{2+}, with the accumulation of a significant proportion of replication products longer than 6 bp (Fig. 2, top). These two distinguishable properties gave us an opportunity to assay which cation pol ε prefers to utilize when presented with both Mn^{2+} and Mg^{2+}. In the experiments depicted in Fig. 5, pol ε was first preincubated in the presence of various concentrations of Mn^{2+} (Fig. 5A), or Mg^{2+} (Fig. 5B), for 5 min at room temperature. Reactions were started by the addition of various concentrations of either Mg^{2+} or Mn^{2+} and the radiolabeled primer/template, followed by incubation at 37 °C for 20 min. As shown in Fig. 5, it is clear that when preincubated with Mn^{2+} and then challenged with Mg^{2+}, the pol ε-dependent replication products are reminiscent of those observed in the presence of Mn^{2+} alone, with very little pausing at template T (Fig. 5A). This property occurs over a wide range of Mn^{2+}/Mg^{2+} concentrations, and is even evident when Mg^{2+} is present in a 40-fold molar excess over Mn^{2+} (see Fig. 5A, 0.05 mM Mn^{2+}/2 mM Mg^{2+}).

The preference of pol ε for Mn^{2+} is more evident in the reactions in which pol ε was preincubated in the presence of Mg^{2+} and then challenged with Mn^{2+} (Fig. 5B). In the absence of added Mn^{2+}, the Mn^{2+} reactions exhibited the classic termination pattern, with a strong pause 5–6 bp downstream of the primer. However, in all reactions containing Mn^{2+} this pausing is significantly reduced. Again, Mn^{2+} stimulation occurred over a wide range of Mn^{2+}/Mg^{2+} concentrations and was obvious even when Mg^{2+} was present in a 10–40-fold molar excess over Mn^{2+}. Based upon these observations, we conclude that pol ε preferentially utilizes Mn^{2+} as the activator for polymerization, even in reactions containing a vast molar excess of Mg^{2+}.

**Kinetics of pol ε-dependent Incorporation in the Presence of Mn^{2+} and/or Mg^{2+}**—Several groups have previously reported the kinetic parameters for pol ε-dependent nucleotide incorporation on an undamaged template. In all cases, these values were determined in the presence of 5 or 8 mM

with 5 mM Mg^{2+}. In contrast, pol ε appears to exhibit efficient bypass a CPD over a wide range of Mg^{2+} concentrations.

**General Ability of Mn^{2+} to Stimulate Translesion Synthesis by pol ε**—We next examined whether low levels of Mn^{2+} or Mg^{2+} would stimulate pol ε-dependent synthesis of a synthetic abasic site, a BPDE adduct, and a 6-4PP lesion (Fig. 4). Previous in vitro studies in the presence of 5 mM Mg^{2+} indicated that pol ε can efficiently incorporate a nucleotide opposite an abasic site, a benzo[a]pyrene lesion, and up to two bases opposite the 6-4PP; however, further extension is limited (21, 30, 35). The previous results are recapitulated here in the presence of 5–8 mM Mg^{2+} (Fig. 4). Lower concentrations of Mg^{2+} helped stimulate incorporation opposite each of the three lesions but had little effect on bypass of the lesion. In contrast, in the presence of 0.2 mM Mn^{2+}, pol ε-dependent bypass of the abasic site increased to ~18%, and bypass of the BPDE adduct increased to ~10% (Fig. 4, A and B). However, whereas Mn^{2+} appears to stimulate the ability of pol ε to incorporate opposite both bases

![Figure 4: Pol ε-dependent bypass of an abasic site, BPDE lesion, and 6-4PP lesion in the presence of Mn^{2+} or Mg^{2+}](image)

The figure shows the percentage of lesion bypass (products marked with an M of all four dNTPs and various concentrations of MnCl2 or MgCl2 ranging from 0.05 to 8 mM in 30-min reactions at 37 °C. The location of the abasic site is indicated by →AP, the BPDE lesion by →BP, and the 6-4PP indicated by →6-4. A graph depicting the percent of lesion bypass (products extended beyond the abasic site, the BPDE lesion, or the 6-4TT), in the presence of various concentrations of Mn^{2+} (M) or Mg^{2+} (□), is shown on the right-hand side of each panel.

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Mg$^{2+}$ (20–22). Under these conditions, the two most striking properties of pol $\iota$ are its ability to preferentially misinsert G opposite T and to faithfully and efficiently incorporate A opposite T. However, our present studies indicate that pol $\iota$ is much more catalytically active at much lower concentrations of Mg$^{2+}$ and Mn$^{2+}$. We have therefore determined the kinetic parameters for (mis)insertion opposite template T and A, in the presence of 0.075 mM Mn$^{2+}$, 0.25 mM Mg$^{2+}$, and 5 mM Mg$^{2+}$, either alone (Table 1, parts A and B), or in combination (Table 1, part C). These kinetic studies revealed that the increase in catalytic activity observed in our earlier experiments is largely derived from a dramatic decrease in the $K_m$ value for nucleotide incorporation. Indeed, whereas the $V_{\text{max}}$ was essentially unchanged under all assay conditions, the $K_m$ value varied by as much as 2400-fold in the presence of 0.25 mM Mg$^{2+}$ compared with 5 mM Mg$^{2+}$, and there was a further 10-fold reduction in the presence of 0.075 mM Mn$^{2+}$. The $K_m$ value for the misincorporation of G opposite T decreased by ~245-fold in the presence of 0.25 mM Mg$^{2+}$ compared with 5 mM Mg$^{2+}$, and by 1.4-fold in 0.25 mM Mg$^{2+}$, in reactions containing 0.075 mM Mn$^{2+}$. The $K_m$ value for the misincorporation of G opposite T also dropped dramatically but to a slightly lower extent. As a result, there was an overall change in the frequency of misincorporation ($f_{\text{inc}}$) under the various assay conditions. Although misincorporation of G opposite T was favored by a factor of 2.5-fold in 5 mM Mg$^{2+}$ and by 1.4-fold in 0.25 mM Mg$^{2+}$, in reactions containing 0.075 mM Mn$^{2+}$, the correct incorporation of A was actually favored ~2.3-fold more than G. Thus, in contrast to its fidelity-reducing effects on all known polymerases, Mn$^{2+}$ actually increases the fidelity of pol $\iota$ at template T. It should be

**TABLE 1**

| Cations          | Incoming Nucleotide | $V_{\text{max}}$ (% · min$^{-1}$) | $K_m$ (µM) | $V_{\text{max}}/K_m$ | $f_{\text{inc}}$ |
|------------------|---------------------|-----------------------------------|------------|-----------------------|-----------------|
| **A. Kinetics of pol $\iota$-dependent incorporation opposite template T** |                     |                                    |            |                        |                 |
| 0.075 mM Mn$^{2+}$ | A                   | $7.1 \pm 0.5$                     | $0.0011 \pm 0.0002$ | 6450                  | 1                |
|                  | G                   | $6.3 \pm 1$                       | $0.0022 \pm 0.0003$ | 2860                  | 0.44             |
| 0.25 mM Mg$^{2+}$ | A                   | $1.7 \pm 0.3$                     | $0.011 \pm 0.002$ | 150                   | 1                |
|                  | G                   | $5.9 \pm 0.9$                     | $0.012 \pm 0.002$ | 210                   | 1.4              |
| 5 mM Mg$^{2+}$   | A                   | $5.0 \pm 0.8$                     | $2.7 \pm 0.5$ | 1.85                  | 1                |
|                  | G                   | $8.3 \pm 0.9$                     | $1.8 \pm 0.3$ | 4.6                   | 2.5              |
| **B. Kinetics of pol $\iota$-dependent incorporation opposite template A** |                     |                                    |            |                        |                 |
| 0.075 mM Mn$^{2+}$ | T                   | $2.6 \pm 0.5$                     | $0.0053 \pm 0.0001$ | 4950                  | 1                |
|                  | A                   | $1.1 \pm 0.3$                     | $0.003 \pm 0.0008$ | 370                   | 0.075            |
| 0.25 mM Mg$^{2+}$ | T                   | $6.8 \pm 1.8$                     | $0.016 \pm 0.0004$ | 425                   | 0.11             |
|                  | A                   | $7.7 \pm 2$                       | $70 \pm 15$ | 0.06                  | 0.00026          |
| 5 mM Mg$^{2+}$   | T                   | $10 \pm 2$                        | $0.06 \pm 0.01$ | 167                   | 1                |
|                  | A                   | $5 \pm 1.1$                       | $90 \pm 17$ | 0.06                  | 0.00036          |
| **C. Kinetics of pol $\iota$-dependent incorporation opposite template A in the presence of both Mn$^{2+}$ and Mg$^{2+}$** |                     |                                    |            |                        |                 |
| 0.075 mM Mn$^{2+}$ | T                   | $17 \pm 2$                        | $0.016 \pm 0.003$ | 1100                  | 1                |
|                  | A                   | $17 \pm 3$                        | $0.33 \pm 0.08$ | 52                    | 0.05             |
| 0.25 mM Mg$^{2+}$ | T                   | $17 \pm 2$                        | $0.016 \pm 0.003$ | 1100                  | 1                |
|                  | A                   | $17 \pm 3$                        | $0.33 \pm 0.08$ | 52                    | 0.05             |

**FIGURE 5.** Activation of pol $\iota$ catalysis in the presence of Mn$^{2+}$ and Mg$^{2+}$. The ability of pol $\iota$ to extend a $^{32}$P-labeled 20-mer primer annealed to template "T50M13" was assayed in the presence of 0.075 mM all four dNTPs and various concentrations of MnCl$_2$ and MgCl$_2$. The experiments shown in A, pol $\iota$ was preincubated in the presence of Mn$^{2+}$ for 5 min and reactions were started by the addition of various concentrations of Mg$^{2+}$ together with the annealed primer template. Reactions were terminated after 20 min at 37 ºC. In the experiments shown in B, pol $\iota$ was preincubated in the presence of Mg$^{2+}$ for 5 min and reactions were started by the addition of various concentrations of Mn$^{2+}$ together with the annealed primer template and were terminated after 20 min at 37 ºC. The sequence of the template immediately downstream of the primer (Pr$_r$) is shown on the left hand side of each panel. The arrows indicate the location of a template T that represents a strong pause site for pol $\iota$ in the presence of Mg$^{2+}$ alone. As can be clearly seen, pausing at this site is diminished in reactions containing Mn$^{2+}$. 

Indeed, whereas the $V_{\text{max}}$ was essentially unchanged under all assay conditions, the $K_m$ value varied by as much as 2400-fold at template T and 30,000-fold at template A. For example, the $K_m$ value for the correct incorporation of A opposite template T and 30,000-fold at template A. For example, the $K_m$ value for the correct incorporation of A opposite T also dropped dramatically but to a slightly lower extent. As a result, there was an overall change in the frequency of misincorporation ($f_{\text{inc}}$) under the various assay conditions. Although misincorporation of G opposite T was favored by a factor of 2.5-fold in 5 mM Mg$^{2+}$ and by 1.4-fold in 0.25 mM Mg$^{2+}$, in reactions containing 0.075 mM Mn$^{2+}$, the correct incorporation of A was actually favored ~2.3-fold more than G. Thus, in contrast to its fidelity-reducing effects on all known polymerases, Mn$^{2+}$ actually increases the fidelity of pol $\iota$ at template T. It should be
stressed, however, that even though Mn
2+
 altered the relative ratios of (mis)incorporation opposite template T so as to favor the incorporation of the correct base, pol ϱ nevertheless remains extremely error-prone under these conditions.

At template A, 0.075 mM Mn
2+
 caused the characteristic decrease in fidelity as there was a dramatic increase in the frequency of misincorporation of A, over the correct base T (Table 1, part B). Similar to our observations above, these effects were largely driven by changes in the \( K_m \) value for nucleotide incorporation. The largest effect was on the \( K_m \) value for misinsertion of A, where it dropped from 70 to 90 \( \mu M \) in the presence of Mn
2+
 to 0.003 \( \mu M \) for Mn
2+
 (~23,000–30,000-fold difference).

Overall, the catalytic activity (\( V_{max}/K_m \)) of pol ϱ increased in the presence of low levels of Mg
2+
 or Mn
2+
. The smallest effect was observed for (mis)incorporation opposite A, where the catalytic activity only increased ~2–2.5-fold in the presence of 0.25 mM Mg
2+
 compared with 5 mM Mg
2+
. However, much larger effects were observed at template T, where the catalytic activity increased ~45–80-fold in 0.25 mM Mg
2+
 compared with 5 mM Mg
2+
. The effects of 0.075 mM Mn
2+
 were much more dramatic, with an additional ~11–3300-fold increase in catalytic activity over that observed in the presence of 0.25 mM Mg
2+
.

In the simultaneous presence of 0.075 mM Mn
2+
 and 0.25 mM Mg
2+
, the catalytic activity (\( V_{max}/K_m \)) of pol ϱ for the correct incorporation of T opposite A was intermediate to that observed in the presence of each individual cation. For example, the catalytic activity of pol ϱ was ~4.5-fold lower than in the presence of 0.075 mM Mn
2+
 alone but ~2.6-fold higher than in the presence of 0.25 mM Mg
2+
 alone. With regard to fidelity, Mn
2+
 appears to be dominant, in that pol ϱ exhibited ~200-fold lower fidelity in the presence of both Mn
2+
 and Mg
2+
 compared with that observed in the presence of 0.25 mM Mg
2+
 alone.

**DISCUSSION**

In this study, we have investigated the effects of various divalent cations on the in vitro properties of human DNA polymerases η, ϱ, and κ. The most abundant divalent cation is Mg
2+
, with an intracellular concentration of 0.21 to 0.24 mM (36, 37). Given its abundance, Mg
2+
 is therefore generally considered to be the physiologically relevant divalent metal cofactor for most DNA polymerases. In support of this notion, our analyses strongly suggest that the preferred cation for pol η and pol κ is Mg
2+
, as both enzymes are active over a wide range of Mg
2+
 concentrations. In contrast, pol ϱ exhibited greatest activity within a much more narrow concentration range of Mg
2+
 (Fig. 2). Peak activity was observed at 0.25 mM Mg
2+
, and synthesis was reduced significantly in reactions containing >2 mM Mg
2+
. As far as we are aware, all of the published reports on the biochemical properties of pol ϱ in vitro were performed in the presence of 5 or 8 mM Mg
2+
 and were therefore performed under suboptimal conditions (20–24, 28, 30, 31, 35, 38–42). Indeed, it appears that in the presence of low levels of Mg
2+
, pol ϱ may exhibit higher catalytic activity and fidelity than previously thought (Table 1).

Limited synthesis by pol η, pol ϱ, and pol κ was observed in the presence of many different divalent cations, but in most cases, the concentration required for replication occurred well outside the physiological concentration of the trace metal, and is therefore probably of little biological significance. The exception was the ability of pol ϱ to utilize low levels of Mn
2+
 as an activator for catalysis (Fig. 2). The cellular concentration of Mn
2+
 in mammalian cells is much lower than that of Mg
2+
 and is thought to be in the range of 0.1 to 40 \( \mu M \) (43–45). Our current studies indicate that in vitro, the optimum concentration of Mn
2+
 required for maximal stimulation of pol ϱ on an undamaged template occurs around 0.075 mM (75 \( \mu M \)). Although this concentration may lie outside the physiological range of Mn
2+
 available to most cells, pol ϱ nevertheless exhibited a strong preference for Mn
2+
 over Mg
2+
, even when Mg
2+
 was in a 10–20-fold excess. Our steady-state kinetic analyses revealed that Mn
2+
 stimulated the catalytic activity of pol ϱ through a dramatic decrease in the \( K_m \) value for nucleotide incorporation. Interestingly, when both Mn
2+
 and Mg
2+
 were present at optimal concentrations (0.075 and 0.25 mM, respectively), pol ϱ exhibited kinetic parameters that more closely resembled those observed in the presence of Mn
2+
 alone (Table 1, part C), which infers that Mn
2+
 is the preferred activator for polymerization.

The structural basis for the enhanced catalytic activity of pol ϱ in the presence of Mn
2+
 is presently unknown. However, it is clear that Mn
2+
 has a more relaxed coordination than Mg
2+
, thereby facilitating reactions that are suboptimal or even inhibited in the presence of Mg
2+
 (5). Indeed, the crystal structure of pol ϱ reveals that its active site is somewhat distorted and has large side chains protruding into the space normally occupied by the replicating base pair (40). Mn
2+
 may therefore simply allow greater flexibility of the active site of pol ϱ, such that it can adopt a conformation that is more favorable for catalysis.

The effects of Mn
2+
 on the fidelity of pol ϱ were unique. Although it caused a characteristic reduction in fidelity at template A, Mn
2+
 actually increased fidelity at template T, by 3–5-fold compared with the fidelity of pol ϱ in the presence of 0.25 or 5 mM Mg
2+
, respectively. Low levels of Mg
2+
 and Mn
2+
 also had dramatic effects on the ability of pol ϱ to traverse a T-T CPD. The efficiency of bypass increased from ~2.5% in the presence of 5 mM Mg
2+
 to ~8% in the presence of 0.5 mM Mg
2+
 and up to ~60% in the presence of 0.2 mM Mn
2+
. Thus, under certain conditions in vitro pol ϱ can bypass a T-T CPD relatively efficiently. Presumably conditions favorable for the pol ϱ-dependent bypass of CPDs also occur in vivo. Such an assumption is supported by the recent reports that implicate cellular roles for pol ϱ in UV-induced mutagenesis and carcinogenesis in mice and humans (46–48). The stimulatory effect of Mn
2+
 on pol ϱ-dependent lesion bypass was not limited to the T-T CPD, as bypass of an abasic site increased from ~1 to 2% in the presence of Mg
2+
 to ~18% in the presence of Mn
2+
. Similarly, bypass of a BPDE lesion also increased from ~2.5 to ~10% in the presence of Mg
2+
 and Mn
2+
, respectively, thereby raising the possibility that pol ϱ may also bypass these lesions in vivo.

In summary, pol ϱ is more active in the presence of physiological concentrations of Mg
2+
 than at concentrations previously used in vitro to study the enzymatic properties of the polymerase. We therefore suggest that future in vitro studies on pol ϱ be conducted in buffers containing low Mg
2+
 (0.25–2
Effects of Divalent Cations on the in Vitro Activity of pol λ

We also discovered that the enzyme is highly active in low concentrations of Mn²⁺. When asked to select between Mg²⁺ and Mn²⁺, pol λ clearly prefers to utilize Mn²⁺, even when Mg²⁺ is in large molar excess. Whether a similar situation occurs in vivo remains to be determined, but it nevertheless remains an intriguing possibility certainly worth considering.

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