Molecular mechanisms of manganese mutagenesis

(DNA polymerase I/error discrimination)

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ABSTRACT The mechanism by which DNA polymerase discriminates between complementary and noncomplementary nucleotides for insertion into a primer terminus has been investigated. Apparent kinetic constants for the insertion of dGTP and dATP into the hook polymer (C)_{10-12} with Escherichia coli DNA polymerase I (large fragment) were determined. The results suggest that the high specificity of base selection by DNA polymerase I is achieved by utilization of both $K_m$ and $V_{max}$ differences between complementary and noncomplementary nucleotides. The molecular basis for the increased error frequency observed with DNA polymerase I in the presence of Mn$^{2+}$ has also been investigated. Our studies demonstrate that when Mn$^{2+}$ is substituted for Mg$^{2+}$, there is a higher ratio of insertion of incorrect to correct dNTP by the polymerase activity, accompanied by a decreased hydrolysis of a mismatched dNMP relative to a matched dNMP at the primer terminus by the 3',5' exonuclease activity. Kinetic analysis revealed that in the presence of Mn$^{2+}$, the $k_{cat}$ for insertion of a complementary dNTP is reduced, whereas the catalytic rate for the insertion of a mismatched nucleotide is increased. The apparent $K_m$ values for either complementary or noncomplementary nucleotide substrates are not significantly altered when Mg$^{2+}$ is replaced by Mn$^{2+}$. The rate of hydrolysis of a mismatched dNMP at the primer terminus is greater in the presence of Mg$^{2+}$ than Mn$^{2+}$, whereas the rate of hydrolysis of a properly base-paired terminal nucleotide is greater in Mn$^{2+}$ than Mg$^{2+}$. These studies demonstrate that both the accuracy of base selection by the polymerase activity and the specificity of hydrolysis by the 3',5' exonuclease activity are altered by the substitution of Mn$^{2+}$ for Mg$^{2+}$.

All DNA polymerases require a divalent cation for activity. Mg$^{2+}$ is considered to be the physiological activator; however, other divalent cations such as Mn$^{2+}$, Co$^{2+}$, Be$^{2+}$, and Ni$^{2+}$ can substitute for Mg$^{2+}$. Of these metal ions, Mn$^{2+}$ has been the most extensively studied (for review, see ref. 1). Mn$^{2+}$ is a known mutagen and carcinogen (2, 3), and substitution of Mn$^{2+}$ for Mg$^{2+}$ results in an increased frequency of misincorporation in vitro. Increased error frequency with Mn$^{2+}$ has been observed with Escherichia coli DNA polymerase I (4–6), T4 DNA polymerase (7–10), DNA polymerase α (11, 12), and avian myeloblastosis virus reverse transcriptase (13).

The mechanism by which Mn$^{2+}$ decreases the fidelity of DNA synthesis is not well understood. For DNA polymerases with a proofreading 3',5' exonuclease activity, Mn$^{2+}$ could exert its effects on either or both of the two base-pairing selection steps involved in discrimination between complementary and mismatched nucleotides: (i) insertion of a properly base-paired nucleotide into the primer terminus by the polymerase activity (base selection) and/or (ii) removal of a non-base-paired nucleotide at the primer terminus by the 3',5' exonuclease activity prior to chain extension (proofreading).

Recently the specificity of base selection by the polymerase activity and the effects of Mn$^{2+}$ on this base-pairing step have been investigated in two laboratories. Goodman et al. (10) have examined the ability of T4 DNA polymerase to discriminate between dATP and 2-aminopurine deoxynucleoside triphosphate in the presence of either Mg$^{2+}$ or Mn$^{2+}$ by determining the apparent $K_m$ and $V_{max}$ values for the insertion of these nucleotides into a gapped, natural DNA template/primer. In these studies it was found that substitution of Mn$^{2+}$ for Mg$^{2+}$ resulted in a reduction by a factor of 10 in the $K_m$ for the “mismatched” nucleotide 2-aminopurine deoxynucleoside triphosphate, a relatively smaller reduction (by a factor of 2–3) in the $K_m$ for the complementary nucleotide dATP, and very large and equivalent reductions (by a factor of 40) in the $V_{max}$ values for both matched and mismatched nucleotides. It was further found, in agreement with the “passive polymerase” model of base selection (10, 14), that the $V_{max}$ values for insertion of dATP and 2-aminopurine deoxynucleoside triphosphate were identical in either Mg$^{2+}$ or Mn$^{2+}$. On the other hand, Fersht et al. (6) examined the effects of substitution of Mn$^{2+}$ for Mg$^{2+}$ on the ability of E. coli DNA polymerase I to discriminate between complementary and noncomplementary nucleotide substrates by determining the rate constant $k_{cat}/K_m$ for insertion of either complementary nucleotides (dATP and dTTP) or noncomplementary nucleotides (dCTP and dGTP) into the synthetic alternating copolymer poly(dA-dT). In these studies it was shown that the substitution of Mn$^{2+}$ for Mg$^{2+}$ resulted in a marked decrease in the rate constant $k_{cat}/K_m$ for insertion of the complementary dNTPs while the values of this rate constant for noncomplementary dNTPs were relatively unaffected. Similar to the results of Goodman et al. (10), the apparent $K_m$ value for the noncomplementary nucleotide dGTP was reduced in the presence of Mn$^{2+}$. However, in contrast to the results with T4 DNA polymerase, the apparent $K_m$ values for the complementary nucleotides were markedly increased at high Mn$^{2+}$ concentrations (1 mM) with DNA polymerase I. In addition, the $V_{max}$ values for insertion of complementary nucleotides were higher than those for noncomplementary nucleotides in either Mn$^{2+}$ or Mg$^{2+}$.

In the present studies we have examined the effects of substitution of Mn$^{2+}$ for Mg$^{2+}$ on the specificity of both base selection and proofreading by the large fragment of DNA polymerase I. In our studies the hook polymer poly(dC)-oligo(dG) was used as template-primer to determine the specificity of the polymerase for insertion of nucleotides both complementary (dGTP) and noncomplementary (dATP or dTTP) to the template. The same hook polymer containing either a complementary or a mismatched nucleotide at the primer terminus was also used to examine the effects of Mg$^{2+}$ and Mn$^{2+}$ on the specificity of hydrolysis by the 3',5' exonuclease activity of DNA polymerase I.

MATERIALS AND METHODS

Unlabeled deoxynucleoside triphosphates (dNTP), deoxynucleoside monophosphates (dNMP), and poly(dC) were purchased from P-L Biochemicals. $^3$H- and $^{32}$P-labeled
dNTPs were purchased from ICN. The purity of dNTPs was determined by polyethyleneimine-cellulose thin-layer chromatography using solvent systems as described (15). In assays that measured nucleotide turnover, the labeled dNTPs were purified by chromatography on hydroxylapatite to remove deoxynucleoside mono- and diphosphates essentially as described by Bernardi (16). The large fragment of DNA polymerase I of E. coli was obtained from New England Nuclear, and terminal deoxynucleotidyl transferase was purchased from either New England Nuclear or Collaborative Research (Waltham, MA).

Preparation of Hook Polymers. The hook polymer (dC)194-d(G)12 was synthesized with terminal deoxynucleotidyl transferase using (dC)194-d(G)12 as primer and dGTP as substrate essentially as described by Fisher and Korn (17). The average chain length of poly(dC) was determined from its sedimentation coefficient according to Studier (18). The average number of dG monomers added per dC194 was determined in a parallel reaction containing labeled dGTP.

Poly(dC)-oligo(dG) hook polymers end-labeled with a mismatched nucleotide at the primer terminus (either dAMP or dTMP) were prepared as described (19) with 2.5 μM (dC)194-d(G)12 (3' ends) and either 10.8 μM [3H]dATP (10,892 cpm/pmol) or 2.3 μM [3H]dTTP (35,494 cpm/pmol). Approximately 20% of the available ends were labeled with [3H]dAMP and 4% with [3H]dTMP.

Nucleotide Insertion Assays. The rate of insertion of a complementary nucleotide was determined by measuring the rate of incorporation of dGMP into the hook polymer (dC)194-d(G)12. Reaction mixtures contained in a final volume of 0.05 ml the following: 50 mM Hepes buffer (pH 7.8), either 5 mM MgCl2 or various concentrations of MnCl2, 1.3 μM (dC)194-d(G)12, [3H]dGTP, and the large fragment of DNA polymerase I diluted in 0.1 M Hepes buffer, pH 7.8/0.2 M KCl/10 mM 2-mercaptoethanol/1 mg of bovine serum albumin per ml. The reaction was stopped after 10 min at 37°C by the addition of 2 ml of 5% trichloroacetic acid containing 0.02 M sodium pyrophosphate. The radioactive precipitate was collected, washed, and counted as described (19).

The rate of insertion of a mismatched nucleotide was determined by measuring its rate of turnover (the template-dependent conversion of dNTP to dNMP). Reaction mixtures were as described above except that either [32P]dATP or [32P]dTTP was used as substrate. The reaction was stopped by the addition of 5 μl of 100 mM EDTA after 45 min at 37°C. Aliquots were applied to the origin of a presoaked polyethyleneimine-cellulose thin-layer plate to which unlabeled 5' dNMP markers had previously been applied. The plates were developed with 1 M sodium formate (pH 3.4) for dTMP and dGMP and 0.5 M sodium formate (pH 3.4) for dAMP. Markers were visualized by UV absorption and [3H]dNMP was quantitated as described (20). Control reactions included identical reaction mixtures that were not incubated and reaction mixtures that were incubated but lacked divalent cation, enzyme, or template-primer. Reactions were carried out under conditions in which the reaction being measured was linear with time and enzyme concentration.

The rate of insertion of dGTP was determined from the rate of incorporation into polynucleotide since in the presence of either Mg2+ or Mn2+ or both, the rate of template-dependent conversion of dGTP to dGMP was only 0.5–1% of the rate of incorporation. The rate of misinsertion of dATP was determined from the rate of turnover after incorporation at primer ends had reached a plateau and did not contribute to the rate of incorporation.

The rates of template-dependent conversion of dNTP to dNMP as well as the rates of incorporation of dNMP into polymer were expressed in a common unit (μmol/30 min per mg of enzyme at 37°C). Apparent Km and Vmax values were determined by the computer program WOENZYME, which is a modification of the program HYPERB of Hanson et al. (21), based on the method of Bliss and James (22).

3',5' Exonuclease Assay. The standard reaction mixture contained in a final volume of 0.15 ml: 50 mM Hepes buffer (pH 7.8), 5 mM MgCl2 or 0.5 mM MnCl2, 200–400 μM (nucleotide equivalents) (dC)194-[3H]dG12 or (dC)194-dG12-[3H]dAMP (A0.2, 0.01–0.4 unit of the large fragment of DNA polymerase I. The reaction mixture was incubated at 37°C, and 20-μl aliquots were applied to 2.4-cm DE-81 paper discs after 0, 3, 6, 9, 12, and 15 min of incubation. The circles were washed and radioactivity was counted as described (23).

RESULTS

Effects of Mg2+ and Mn2+ Concentrations on the Rates of Insertion of Complementary and Noncomplementary Nucleotides. The effects of Mn2+ and Mg2+ concentrations on the rate of insertion of either the complementary nucleotide (dGTP) or the noncomplementary nucleotide (dATP) into the hook polymer poly(dC)-oligo(dG) are shown in Fig. 1. With the complementary nucleotide as substrate (Fig. 1A), the optimal Mn2+ concentration was 0.1–0.2 mM and very sharp, whereas the optimal Mg2+ concentration was 10- to 50-fold higher and very broad. The rate of dGTP insertion at the Mn2+ optimum was approximately half that at the optimal Mg2+ concentration. In contrast, with a noncomplementary nucleotide as substrate (Fig. 1B), the rate of insertion at optimal Mn2+ concentrations was approximately 15- to 20-times that at optimal Mg2+ concentrations. The Mn2+ optimum for dATP misinsertion was considerably broader than the corresponding optimum for the insertion of dGTP.

In the presence of 5 mM Mg2+, the effects of increasing Mn2+ concentrations on the rates of insertion of either dGTP or dATP are shown in Fig. 2. Mn2+ inhibited the rate of insertion of dGTP and stimulated the rate of insertion of dATP at all concentrations shown.

Effect of Mn2+ on Base Selection by the Polymerase Activity. In order to evaluate the ability of DNA polymerase I to discriminate between nucleotide substrates that are either complementary or noncomplementary to the template, the rate of nucleotide insertion was measured as a function of dNTP concentration with the hook polymer poly(dC)-oligo(dG) as template-primer. We chose to use this template-primer for several reasons. A hook polymer in which the primer is covalently linked to the template limits the enzyme to extension of a single type of primer terminus and, consequently, there is no uncertainty as to which strand is serving as template and which as primer. Furthermore, the stability of the G-C base pair allows very little fraying of the primer terminus and, as a result, little “peelback” hydrolysis of the primer strand. Finally, since we are interested in comparing the rates of phosphodiester bond formation with complementary and noncomplementary nucleotide substrates, the use of a primed homopolymer as template-primer would ensure that the nature of the primer terminus, the non-variable substrate in phosphodiester bond formation, was held constant for each phosphodiester bond formed—i.e., for the complementary substrate dGTP, each phosphodiester bond formed would generate an identical primer terminus, whereas for the noncomplementary substrate dATP, the rapid hydrolysis of the misinserted dAMP would regenerate the original dGMP primer terminus. In this regard, when the rate of hydrolysis of a mismatched primer terminus—i.e., poly(dC)-oligo(dG)-[3H]dAMP—by the 3',5' exonuclease activity of the enzyme was measured at saturating substrate concentrations, it was found to be 20- to 30-fold greater than the rate of turnover of dATP to dAMP (data not shown), indicating that the rate of nucleotide turnover is not limited by the rate of hydrolysis of a mismatched primer terminus.

Table 1 shows the apparent Km and Vmax values for inser-
tion of the complementary nucleotide dGTP into poly(dC)oligo(dG) in the presence of either 5 mM Mg\(^{2+}\) or various concentrations of Mn\(^{2+}\), either alone or in combination with 5 mM Mg\(^{2+}\). Also given in Table 1 are the calculated rate constants \(k_{cat}/K_m\). Substitution of Mn\(^{2+}\) for Mg\(^{2+}\) or addition of Mn\(^{2+}\) to Mg\(^{2+}\) did not significantly affect the \(K_m\) for dGTP, whereas the turnover number \(k_{cat}\) decreased. At optimal Mn\(^{2+}\), \(k_{cat}\) was approximately 50\% that at optimal Mg\(^{2+}\) and at inhibitory Mn\(^{2+}\) concentrations \(k_{cat}\) was approximately 10\% that at optimal Mg\(^{2+}\). In the presence of both Mg\(^{2+}\) and Mn\(^{2+}\), the kinetic constants were as in Mn\(^{2+}\) alone.

Table 1 also shows the effect of Mn\(^{2+}\) on the insertion of mismatched nucleotides. Substitution of Mn\(^{2+}\) for Mg\(^{2+}\) resulted in a 20- to 30-fold increase in the turnover number for insertion of the noncomplementary nucleotide dATP opposite a template C and in no significant change in the apparent \(K_m\) for dATP. In the presence of both Mg\(^{2+}\) and Mn\(^{2+}\), the reaction parameters behaved as in Mn\(^{2+}\) alone. Insertion of dTTP opposite a template C was not detectable with Mg\(^{2+}\) as divalent cation. In Mn\(^{2+}\), however, insertion of dTTP was measurable. Thus, when Mn\(^{2+}\) is substituted for Mg\(^{2+}\), the enzyme is less able to discriminate between complementary and noncomplementary nucleotides, primarily due to a decreased \(V_{max}\) for insertion of complementary dTTP and an increased \(V_{max}\) for insertion of a noncomplementary dNTP, with no significant effects on apparent \(K_m\) values for either complementary or noncomplementary nucleotides. However, \(K_m\) differences are utilized by the polymerase to discriminate between complementary and noncomplementary base pairs in either Mn\(^{2+}\) or Mg\(^{2+}\).

**Effect of Mn\(^{2+}\) on the Specificity of Proofreading by the 3',5' Exonucleolytic Activity**. To determine whether the decreased specificity of base selection by the polymerase activity is the only mechanism responsible for the increased error frequency seen in the presence of Mn\(^{2+}\) or whether Mn\(^{2+}\) also affects the proofreading of primer termini by the 3',5' exonuclease activity, we also investigated the effects of substitution of Mn\(^{2+}\) for Mg\(^{2+}\) on the rates of hydrolysis of poly(dC)-oligo(dG) containing either a complementary (dGMP) or noncomplementary (dAMP) nucleotide at the 3' terminus. Fig. 3 shows that the rate of hydrolysis of the mismatched dAMP primer terminus is 2 to 3 times greater in 5 mM Mg\(^{2+}\) than in 0.5 mM Mn\(^{2+}\). Similar results were obtained when the mismatched primer terminus was dTMP (data not shown). In contrast, the rate of hydrolysis of the dGMP primer terminus is at least 2-fold higher in Mn\(^{2+}\) than in Mg\(^{2+}\). Thus, substitution of Mn\(^{2+}\) for Mg\(^{2+}\) alters the specificity of the 3',5' exonuclease activity for a mismatched primer terminus.

Studies in which the rate of d(C)\(_{104}\)\(^{3}H\)d(G)\(_{12}\) hydrolysis was measured as a function of polynucleotide concentration in either 5 mM Mg\(^{2+}\), 0.5 mM Mn\(^{2+}\), or both showed that the \(K_m\) for polynucleotide (45 nM) was not significantly altered in the presence of Mn\(^{2+}\), whereas the \(V_{max}\) in 5 mM Mg\(^{2+}\) was lower by a factor of approximately 2 than that either in 0.5 mM Mn\(^{2+}\) or in 0.5 mM Mn\(^{2+}\) and 5 mM Mg\(^{2+}\) (data not shown). Thus, the presence of Mn\(^{2+}\) results in an increased rate of hydrolysis of primer termini containing complementing nucleotides.

**DISCUSSION**

The high fidelity with which a DNA polymerase faithfully replicates a DNA template is the result of two error discrimination steps: (i) base selection, or the insertion of a nucleotide at the primer terminus that is complementary to the corresponding nucleotide in the template strand, and (ii) proofreading, or the 3',5' exonucleolytic hydrolysis of a mismatched or unpaired nucleotide at the primer terminus prior to further chain extension. Base selection is thought to utilize the difference in free energy between correct and incorrect base pairings. It has been demonstrated that the apparent \(K_m\) values for mismatched nucleotide substrates are at least one to two orders of magnitude higher than the \(K_m\) values for complementary nucleotides (6, 24). However,
whether the polymerase actively discriminates against mismatched nucleotide substrates at the insertion step by lowering the rate of phosphodiester bond formation with noncomplementary nucleotide substrates has not been resolved (6, 10, 25).

The illiterate polymerase model for nucleotide insertion (10, 14) suggests that DNA polymerases play only a passive role in error discrimination and insert any dNTP resident at their active sites with equal probability. This hypothesis predicts that error frequency is determined primarily by $K_m$ differences between complementary and noncomplementary nucleotide substrates and is supported by the findings that the 6-fold preference of T4 DNA polymerase for incorporation of adenine over 2-aminopurine nucleotides can be entirely accounted for by the free energy difference between A-T and 2-aminopurine·T base pairs. Similarly, the 22-fold preference of DNA polymerase α for insertion of dTMP rather than dCMP across from a template 2-aminopurine can be accounted for by the 25-fold difference in the $K_m$ values for dCMP and dTMP determined with 2-aminopurine-containing templates. Further support for the passive polymerase model comes from the observations that the $V_{\text{max}}$ values for insertion of dTTP or dCTP across from a template 2-aminopurine are identical, as are the $V_{\text{max}}$ values for insertion of either 2-aminopurine deoxyxynucleoside triphosphate or dATP across from a template T (10, 14, 26).

The results of our studies demonstrate that the high specificity of base selection by DNA polymerase I is achieved by utilization of both $K_m$ and $V_{\text{max}}$ differences between complementary and noncomplementary nucleotide substrates. The apparent $K_m$ for the complementary nucleotide dGTP is lower by a factor of approximately 20 than that for the noncomplementary nucleotide dATP, whereas the $V_{\text{max}}$ for dGTP insertion is approximately 5000-fold higher than that for dATP. Thus, DNA polymerase I actively discriminates between complementary and noncomplementary nucleotides resident at the active site, possibly by assuming a different conformation on binding of a nucleotide substrate that is complementary to the corresponding nucleotide in the template. These results are in contrast to those of Goodman et al. (10), who found identical $V_{\text{max}}$ values for insertion of dATP and 2-aminopurine deoxyxynucleoside triphosphate with T4 DNA polymerase. Whether these two DNA polymerases differ in the mechanisms by which they discriminate between complementary and noncomplementary nucleotide substrates or whether 2-aminopurine deoxyxynucleoside triphosphate is not sufficiently “noncomplementary” to test the discriminatory ability of the enzyme is not clear at present.

The present studies suggest that the increased error frequency is observed with *E. coli* DNA polymerase I when Mn$^{2+}$ is substituted for Mg$^{2+}$ is due to a decrease both in the specificity of base selection by the polymerase activity and in the specificity of proofreading by the $3',5'$ exonuclease activity. Furthermore, the reduced specificity observed

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**Table 1. Effects of Mn$^{2+}$ on the specificity of insertion of either complementary or mismatched nucleotides**

<table>
<thead>
<tr>
<th>Divalent cation</th>
<th>$K_m$, µM</th>
<th>$V_{\text{max}}$, µmol/30 min per mg</th>
<th>$k_{\text{cat}}$, sec$^{-1}$</th>
<th>$k_{\text{cat}}/K_m$, liter/mol per sec</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insertion of dGTP (G-C pair)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5.0 mM Mg$^{2+}$</td>
<td>3.7 ± 0.5</td>
<td>1120 ± 53</td>
<td>42.4</td>
<td>1.2 x 10$^7$</td>
</tr>
<tr>
<td>0.1 mM Mn$^{2+}$</td>
<td>3.5 ± 1.2</td>
<td>515 ± 66</td>
<td>19.5</td>
<td>5.6 x 10$^6$</td>
</tr>
<tr>
<td>0.2 mM Mn$^{2+}$</td>
<td>2.6 ± 0.7</td>
<td>524 ± 37</td>
<td>19.8</td>
<td>7.6 x 10$^6$</td>
</tr>
<tr>
<td>0.3 mM Mn$^{2+}$</td>
<td>2.5 ± 0.7</td>
<td>315 ± 32</td>
<td>11.9</td>
<td>4.8 x 10$^6$</td>
</tr>
<tr>
<td>1.0 mM Mn$^{2+}$</td>
<td>1.7 ± 0.6</td>
<td>123 ± 12</td>
<td>4.65</td>
<td>2.7 x 10$^6$</td>
</tr>
<tr>
<td>2.0 mM Mn$^{2+}$</td>
<td>2.4 ± 0.5</td>
<td>140 ± 8</td>
<td>5.29</td>
<td>2.2 x 10$^6$</td>
</tr>
<tr>
<td>5.0 mM Mg$^{2+}$ + 0.1 mM Mn$^{2+}$</td>
<td>2.1 ± 0.4</td>
<td>581 ± 36</td>
<td>22.0</td>
<td>1.0 x 10$^7$</td>
</tr>
<tr>
<td>5.0 mM Mg$^{2+}$ + 1.0 mM Mn$^{2+}$</td>
<td>1.9 ± 0.5</td>
<td>222 ± 14</td>
<td>8.39</td>
<td>4.5 x 10$^6$</td>
</tr>
<tr>
<td>Insertion of dATP (A-C mispair)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5.0 mM Mg$^{2+}$</td>
<td>63.7 ± 11</td>
<td>0.17 ± 0.03</td>
<td>0.0064</td>
<td>1.0 x 10$^6$</td>
</tr>
<tr>
<td>0.3 mM Mn$^{2+}$</td>
<td>78.9 ± 32</td>
<td>8.02 ± 1.8</td>
<td>0.303</td>
<td>3.8 x 10$^5$</td>
</tr>
<tr>
<td>1.0 mM Mn$^{2+}$</td>
<td>52.9 ± 16</td>
<td>5.2 ± 0.5</td>
<td>0.197</td>
<td>3.7 x 10$^4$</td>
</tr>
<tr>
<td>2.0 mM Mn$^{2+}$</td>
<td>32.5 ± 2</td>
<td>7.45 ± 0.15</td>
<td>0.282</td>
<td>8.7 x 10$^3$</td>
</tr>
<tr>
<td>5.0 mM Mg$^{2+}$ + 0.5 mM Mn$^{2+}$</td>
<td>58.0 ± 10</td>
<td>6.62 ± 0.36</td>
<td>0.250</td>
<td>4.3 x 10$^3$</td>
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<tr>
<td>Insertion of dTTP (T-C mispair)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5 mM Mn$^{2+}$</td>
<td>119 ± 30</td>
<td>7.34 ± 0.8</td>
<td>0.278</td>
<td>2.3 x 10$^3$</td>
</tr>
</tbody>
</table>

The concentration of dGTP was varied over a 150-fold concentration range (0.72 µM–107 µM). dATP and dTTP concentrations were varied over a 200-fold range (10 µM–2 mM). Apparent $K_m$ and $V_{\text{max}}$ values are reported as mean ± 1 SEM.

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**Fig. 3.** Effect of Mg$^{2+}$ (○) and Mn$^{2+}$ (●) on the rate of hydrolysis of either a matched or a mismatched nucleotide at the primer terminus. One hundred percent was 20,500 cpm for poly(dC)$_{194}$-[3H](dT)$_{12}$ and 33,000 cpm for poly(dC)$_{194}$-(dG)$_{12}$-[3H](dA)$_{12}$. For hydrolysis of the matched primer terminus (A) the concentration of the enzyme was 10 times that for hydrolysis of mismatched primer termini (B).
in Mn⁴⁺ is not influenced by the addition of 5 mM Mg⁴⁺. The decreased specificity of base selection by the polymerase, at least for purine-pyrimidine mispairing, is due to both a reduction in kcat for insertion of the complementary nucleotide substrate dGTP and an increase in the catalytic rate for insertion of the noncomplementary nucleotide substrate dATP. The apparent Km values for dGTP and dATP were not significantly affected by substitution of Mn⁴⁺ for Mg⁴⁺. Whether or not altered Km values or kcat values or both are responsible for the increased misinsertion of dTTP seen in Mn⁴⁺ is unresolved, since we were not able to detect insertion of dTTP opposite a template C in the presence of Mn⁴⁺.

Previous studies on the effects of substitution of Mn⁴⁺ for Mg⁴⁺ on the kinetic parameters for insertion of 2-aminopurine deoxynucleoside triphosphate as a mismatched nucleotide with T4 DNA polymerase I (10) suggested that the major effect was a differential decrease in the Km for insertion of 2-aminopurine deoxynucleoside triphosphate relative to dATP. The Vmax for insertion of 2-aminopurine deoxynucleoside triphosphate was identical to that for dATP in either Mg⁴⁺ or Mn⁴⁺, and when Mn⁴⁺ was substituted for Mg⁴⁺, both reactions were inhibited to an equivalent extent—i.e., 40-fold. Whether the differences in the effects of Mn⁴⁺ in the two studies are the result of true differences in the mechanism of base selection by the two polymerases requires further investigation.

The results of Goodman et al. (10) and those of the present study differ from those reported by Fersht et al. (6) with E. coli DNA polymerase I. These investigators (6) reported that the primary effect of substituting Mn⁴⁺ for Mg⁴⁺ is a large increase in apparent Km for the catalytic rate for insertion of complementary nucleotides, accompanied by relatively smaller changes in kcat/Km values for the insertion of noncomplementary nucleotides. These discrepancies may be the result of differences in experimental conditions. For example, in the present study Hepes buffer (50 mM, pH 7.8) was used for determination of apparent kinetic constants for insertion of nucleotides either in the presence of Mg⁴⁺ or Mn⁴⁺, whereas in the studies of Fersht et al. (6), potassium phosphate (65 mM, pH 7.4) was used as buffer when MgCl₂ was divalent cation and Tris-HCl (100 mM, pH 7.6) was the buffer when MnCl₂ was used. We have found that the Km values for nucleotide insertion are significantly affected by the nature of the buffer used—e.g., with Mg⁴⁺ as divalent cation, the apparent Km value for insertion of the mismatched nucleotide dATP into poly(dC)-oligo(dG) was approximately 15- to 20-fold higher in 50 mM phosphate buffer (pH 7.4) than in 50 mM Hepes buffer (pH 7.8) (unpublished observation). Alternatively, the discrepancy could be due to a difference in the template-primed used.

Previous studies on the effects of Mn⁴⁺ on the 3'-5' exonuclease activity of DNA polymerase I suggested that inhibition of proofreading does not play a role in Mn⁴⁺ mutagenesis. Sirover et al. (5) demonstrated no change in the extent of hydrolysis of a mismatched primer terminus when Mg⁴⁺ was replaced by Mn⁴⁺, and Miyaki et al. (27) showed that the rate of hydrolysis of a properly base-paired primer terminus was stimulated by Mn⁴⁺. The results of our studies with the hook polymer poly(dC)-oligo(dG) show that, whereas the rate of hydrolysis of a properly base-paired primer terminus is stimulated in Mn⁴⁺, the rate of hydrolysis of a mismatched primer terminus is inhibited. Thus, the decreased ability of the enzyme to hydrolyze a mismatched nucleotide at the primer terminus when Mg⁴⁺ is replaced by Mn⁴⁺ could allow extension of mismatched primer termini, resulting in increased error frequency.

Our finding that the apparent Km values for insertion of either dATP or dGTP are not significantly affected by the presence of Mn⁴⁺ suggests that the mutagenicity of Mn⁴⁺ is not due to an increase in the binding affinity of a mismatched dNTP for the enzyme-template complex. The fact that Mn⁴⁺ alters both the specificity of base selection by the polymerase activity as well as the specificity of proofreading by the 3'-5' exonuclease activity suggests that the effects of Mn⁴⁺ are not mediated solely through its interaction with dNTP. Our results suggest that the mutagenicity of Mn⁴⁺ is likely to be due to its interaction with the enzyme-template complex, possibly altering the conformation at the active sites of the polymerase and exonuclease activities.

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