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Effect of manganese ions on the incorporation of dideoxynucleotides by bacteriophage T7 DNA polymerase and *Escherichia coli* DNA polymerase I

(magnesium/fidelity/mutagenesis/DNA sequence/termination)

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ABSTRACT **Incorporation of dideoxynucleotides by T7 DNA polymerase and *Escherichia coli* DNA polymerase I is more efficient when Mn^{2+} rather than Mg^{2+} is used for catalysis. Substituting Mn^{2+} for Mg^{2+} reduces the discrimination against dideoxynucleotides approximately 100-fold for DNA polymerase I and 4-fold for T7 DNA polymerase. With T7 DNA polymerase and Mn^{2+}, dideoxynucleotides and deoxynucleotides are incorporated at virtually the same rate. Mn^{2+} also reduces the discrimination against other analogs with modifications in the furanose moiety, the base, and the phosphate linkage. A metal buffer, isocitrate, expands the $MnCl_2$ concentration range effective in catalyzing DNA synthesis. The lack of discrimination against dideoxynucleoside triphosphates using T7 DNA polymerase and Mn^{2+} results in uniform terminations of DNA sequencing reactions, with the intensity of adjacent bands on polyacrylamide gels varying in most instances by less than 10%.**

DNA synthesis catalyzed by DNA polymerases requires a 3′ hydroxyl group on the primer for nucleophilic attack of the incoming deoxynucleoside triphosphate (1). 2′,3′-Dideoxynucleoside triphosphates (ddNTPs), as well as other nucleotides lacking a 3′ hydroxyl group, act as chain terminators. ddNTPs have been useful in the characterization of DNA polymerases, in the preparation of substrates, and in DNA sequence analysis (2).

Most nucleotide analogs are incorporated less efficiently than unmodified nucleotides, the extent of discrimination depending on both the analog and the DNA polymerase. *Escherichia coli* DNA polymerase I incorporates a dideoxynucleoside monophosphate (ddNMP) nearly a thousand times slower than it does the corresponding dNMP (3). T7 DNA polymerase discriminates against a ddNTP severalfold (4). With both enzymes, the relative rates of ddNMP and dNMP incorporation are dependent on neighboring DNA sequences. This differential incorporation leads to variation in band intensities in autoradiographs of DNA sequencing gels; adjacent fragments that terminate with a ddNMP can vary in intensity by more than 50-fold with DNA polymerase I (2) and severalfold with T7 DNA polymerase (4).

In addition to polymerase activity, T7 DNA polymerase (T7 gene *5* protein) has a 3′ to 5′ exonuclease activity (5). This activity can interfere with analysis of the polymerization reaction since it hydrolyzes both 3′ terminal ddNMPs and dNMPs (4). To circumvent this complication, we use a mutant form of T7 DNA polymerase that has no detectable exonuclease activity but retains a high level of polymerase activity (5). In addition, the T7 DNA polymerase used in this study consists of a complex of T7 gene *5* protein and *E. coli* thioredoxin, the latter conferring high processivity on the polymerization reaction (6, 7). DNA polymerase I has both 3′ to 5′ and 5′ to 3′ exonuclease activities. We use a form that is missing the first 323 residues at the amino terminus; this enzyme lacks the 5′ to 3′ exonuclease activity but has normal levels of polymerase and 3′ to 5′ exonuclease activities (8). Since the 3′ to 5′ exonuclease activity of DNA polymerase I hydrolyzes ddNMP-terminated substrates at a rate 1000 times slower than those terminated with a dNMP, its effect on these studies should be minimal (3).

All known DNA polymerases require a divalent cation for catalysis (see ref. 9). Catalysis by Mg^{2+} has been most extensively studied, since in general it results in the highest activity. However, DNA polymerases are able to use other divalent cations, in particular Mn^{2+}. We show that when Mn^{2+} is substituted for Mg^{2+}, both T7 DNA polymerase and DNA polymerase I incorporate ddNMPs more efficiently. These properties offer advantages for DNA sequencing.

MATERIALS

Enzymes. The T7 DNA polymerase was a complex of *E. coli* thioredoxin and a mutant T7 gene *5* protein that has a deletion of residues 117–143 (5). This mutation inactivates the 3′ to 5′ exonuclease activity to a level $<10^{-8}$ that of native T7 DNA polymerase, which is not detectable, whereas the polymerase activity on primed M13 DNA (35,000 units/mg) is 9-fold higher. We refer to this deletion mutant T7 gene *5* protein–*E. coli* thioredoxin complex as "T7 DNA polymerase." The large fragment of *E. coli* DNA polymerase I (Klenow fragment) was purified to homogeneity from the strain CJ155 (8). We refer to this enzyme as "DNA polymerase I." *Thermus aquaticus* DNA polymerase, avian myeloblastosis virus reverse transcriptase, and T4 DNA polymerase were gifts from United States Biochemical.

DNA and Nucleotides. M13mGP1-2 DNA is a 9950-base derivative of M13mp8 (4). The M13 primer (GTTTTCCCAGTCACGAC) was prepared by A. Nussbaum (Harvard Medical School). The 5′ fluorescein-labeled primer (Fam primer) was from Applied Biosystems (10). Azidothymidine 5′-triphosphate was a gift of W. Miller (Burroughs Wellcome). Fluorescein-labeled ddATP (ddATP-512) and ddTTP (ddTTP-526) were from DuPont (11). All other nonradioactive nucleotides were from Pharmacia. [α-^{32}P]ddATP was from Amersham. All other radioactive nucleotides were from New England Nuclear.

RESULTS

High Concentrations of Mn^{2+} Reduce the Specific Activity and the Processivity of T7 DNA Polymerase, an Effect Overcome by the Addition of Isocitrate. T7 DNA polymerase activity in the presence of increasing concentrations of Mg^{2+} and Mn^{2+} is shown in Fig. 1. The optimal concentrations

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Abbreviation: dd (prefix), 2′,3′-dideoxy.

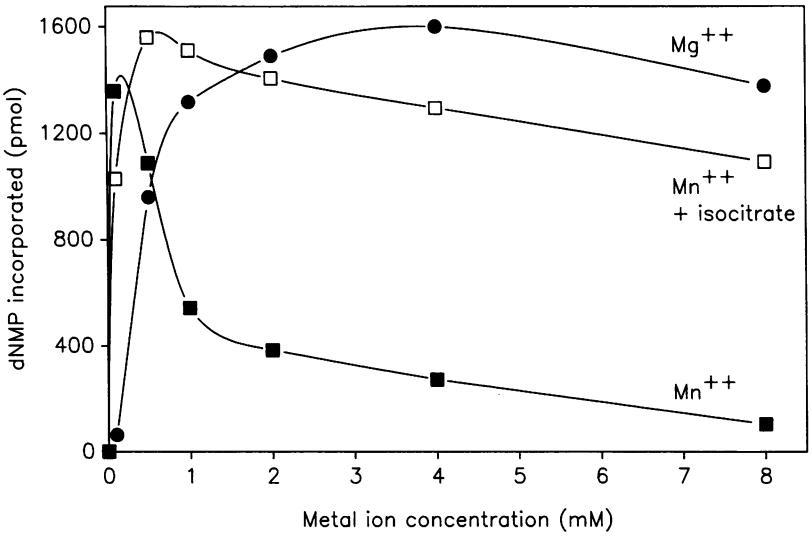

FIG. 1. T7 DNA polymerase activity in the presence of Mg^{2+}, Mn^{2+}, or Mn^{2+} plus isocitrate. Activity was measured using primed M13 DNA (5). Reaction mixtures (90 μl) contained 1 μg of primed mGP1-2 DNA, 20 mM Tris·HCl (pH 7.5), 5 mM dithiothreitol, 100 μM dGTP, 100 μM dATP, 100 μM dCTP, 100 μM [^{3}H]dTTP (20 cpm/pmol), and the indicated concentrations of either $MgCl_2$ or $MnCl_2$. Where indicated, 15 mM sodium DL-isocitrate was added. Mixtures were incubated at 37°C for 60 sec, and the reactions were initiated by the addition of 0.6 pmol of T7 DNA polymerase (a 2-fold excess over the template) in 10 μl containing 20 mM Tris·HCl (pH 7.5), 5 mM dithiothreitol, and 0.05% bovine serum albumin. Incubations were for 60 sec at 37°C. Reactions were stopped by the addition of 3 ml of 1 M HCl at 0°C, and the acid-insoluble radioactivity was determined (6). Total nucleotide incorporated is shown. ●, Mg^{2+}; ■, Mn^{2+}; □, Mn^{2+} plus isocitrate.

were 4 mM Mg^{2+} and 0.1 mM Mn^{2+}. These values are similar to those observed for DNA polymerase I (12, 13). At 0.1 mM, most of the Mn^{2+} will be complexed to the dNTPs (400 μM) and the DNA (30 μM), leaving a free Mn^{2+} concentration of 3 μM (14).

Since the optimal Mn^{2+} concentration was less than 1 mM and higher concentrations were strongly inhibitory, we tested the ability of various chelators of Mn^{2+} ($K_{app} = 10^{-3}$ to 10^{-7} M) to act as metal buffers to maintain the free Mn^{2+} concentration at a constant, submillimolar level over a wide range of added $MnCl_2$. Two such buffers, isocitrate and citrate, expanded the $MnCl_2$ concentration range effective in catalyzing DNA synthesis by T7 DNA polymerase; the stimulation resulting from the addition of 15 mM isocitrate, a concentration that will maintain the free Mn^{2+} concentration at 30–300 μM over a 1–10 mM range of $MnCl_2$ ($K_{app} \approx 10^{-3.7}$ M), is shown in Fig. 1.

T7 DNA polymerase is highly processive with Mg^{2+}, a single polymerase molecule incorporating thousands of nucleotides on the same template without dissociating (4–7). When the processivity was measured at a Mn^{2+} concentration that gave the highest specific activity in the absence of isocitrate (0.1 mM), it was likewise very high, comparable to that with Mg^{2+} (data not shown). At increasing Mn^{2+} concentrations (>0.5 mM), there was a large reduction in the processivity of the enzyme, an effect that was reversed by the addition of 15 mM isocitrate.

T7 DNA Polymerase and DNA Polymerase I Discriminate Against ddNTPs to a Lesser Extent with Mn^{2+} than with Mg^{2+}. The rates at which T7 DNA polymerase and DNA polymerase I use ddCTP relative to dCTP with either Mg^{2+} or Mn^{2+} are compared in Fig. 2. DNA synthesis was carried out using a 5′-^{32}P-labeled primer annealed to an M13 DNA template and various ratios of ddCTP to dCTP. In these reactions, the primers will be extended until a ddCMP has been incorporated, resulting in a series of fragments whose average length is a function of the ratio of ddCTP to dCTP and of their relative rate of use. T7 DNA polymerase synthesized fragments that were of significantly greater length with Mg^{2+} than with Mn^{2+}; the ratio of ddCTP to dCTP had to be increased 4- to 6-fold with Mg^{2+} to produce fragments of average length comparable to those with Mn^{2+} (Fig. 2 *A* and *B*).

DNA polymerase I and Mg^{2+} extended most of the primers several hundred nucleotides without incorporating a ddCMP, even in the presence of a 3:1 molar ratio of ddCTP to dCTP (Fig. 2*C*); this ratio resulted in the termination of virtually all of the primers within the first 30 nucleotides with T7 DNA polymerase and Mg^{2+} (Fig. 2*A*). In the presence of Mn^{2+}, ddCMP was incorporated much more efficiently; the average lengths of the fragments synthesized by DNA polymerase I and Mn^{2+} were equal to those synthesized by T7 DNA polymerase and Mn^{2+} when the former contained a 4- to 6-fold higher ratio of ddCTP to dCTP (compare Fig. 2 *D* and *B*). The effects on ddGTP, ddATP, and ddTTP were similar; when Mn^{2+} was substituted for Mg^{2+}, the ratio of each ddNTP to the corresponding dNTP had to be decreased by a factor of 4 to 6 with T7 DNA polymerase and 80 to 160 with DNA polymerase I to obtain fragments of comparable average length.

To determine quantitatively the use of a dNTP relative to the corresponding ddNTP, we measured the incorporation of [^{3}H]dAMP and [α-^{32}P]ddAMP, where the reactions contained a 10:1 molar ratio of dATP to ddATP (Table 1). With T7 DNA polymerase and Mg^{2+}, dAMP was incorporated 3.7 times more efficiently than ddAMP. This ratio varied with Mg^{2+} concentration from 7.5 at 1 mM to 3.1 at 50 mM; it varied with pH from 6.4 at pH 7.0 to 2.9 at pH 8.5. The relative rates with Mn^{2+} were strongly dependent on its concentration. At the concentration that gave the maximum polymerase activity (0.1 mM), there was no significant change in the ratio of rates compared to that observed with Mg^{2+}. As the concentration was increased, the discrimination between dATP and ddATP decreased; at 2 mM Mn^{2+}, they were incorporated at rates within 5% of one another. The addition of isocitrate to the reactions containing 2 mM Mn^{2+} increased only slightly the discrimination against ddATP. When a mixture of the two metal ions was present, the effect of Mn^{2+} was dominant—i.e., with 2 mM Mn^{2+} and 5 mM Mg^{2+}, the extent of discrimination against ddATP was identical to that with Mn^{2+} alone.

With DNA polymerase I, the effect of Mn^{2+} on the incorporation of dAMP to ddAMP was similar to that observed with T7 DNA polymerase; however, in each case ddATP was discriminated against to a greater extent. With Mg^{2+}, the ratio of dAMP to ddAMP incorporated was 150 times higher with DNA polymerase I; with Mn^{2+}, this ratio was 4 times higher. When both metals were present, the effect of Mn^{2+} was again dominant. The addition of isocitrate to 2 mM Mn^{2+} increased by 5-fold the discrimination against ddATP.

We determined the effect of Mn^{2+} on the relative incorporation of dAMP to ddAMP by other DNA polymerases, using the assays described in Table 1 and Fig. 2. *T. aquaticus* DNA polymerase discriminated against ddATP by factors of 600 and 60 in the presence of 5 mM Mg^{2+} and 2 mM Mn^{2+}, respectively. Avian myeloblastosis virus reverse transcriptase discriminated against ddATP by factors of 50 and 8 in the presence of 5 mM Mg^{2+} and 2 mM Mn^{2+}, respectively. We could not detect any incorporation of ddAMP by T4 DNA polymerase with either ion.

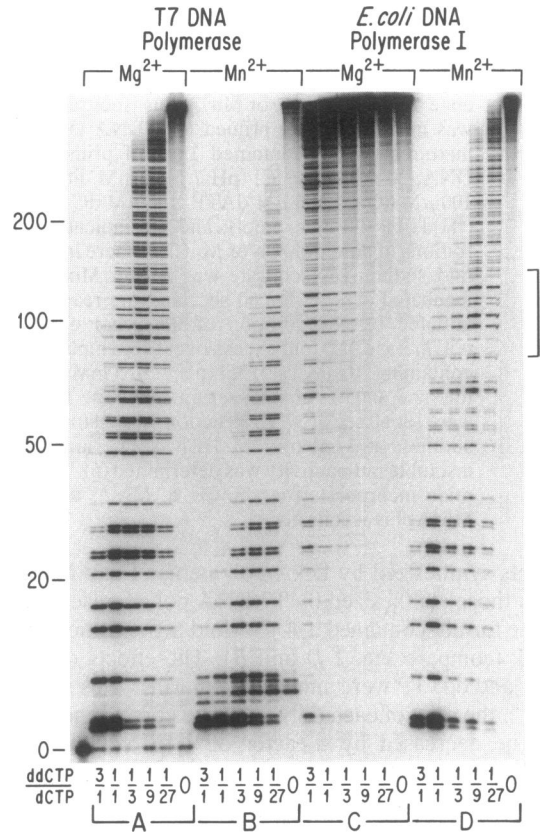

FIG. 2. Incorporation of ddCMP by T7 DNA polymerase and DNA polymerase I in the presence of Mg^{2+} or Mn^{2+}. 5'-^{32}P-labeled primer was annealed to single-stranded M13 mGP1-2 DNA (6). Reaction mixtures (8 μl) contained 10 ng of primed mGP1-2 DNA, 20 mM Tris·HCl (pH 7.5), 5 mM dithiothreitol, 50 μM of all four dNTPs, and either 5 mM $MgCl_2$ (*A* and *C*) or 2 mM $MnCl_2$ plus 5 mM sodium isocitrate (*B* and *D*). The mixtures contained 150 μM, 50 μM, 17 μM, 5.6 μM, 1.9 μM, or no ddCTP (lanes from left to right; molar ratios of ddCTP to dCTP are shown below the lanes). Mixtures were incubated at 37°C for 1 min, and the reactions were initiated by the addition of 2 μl containing 0.3 pmol (100-fold excess over template) of either T7 DNA polymerase (*A* and *B*) or DNA polymerase I (*C* and *D*) diluted in 20 mM Tris·HCl (pH 7.5), 5 mM dithiothreitol, and 0.05% bovine serum albumin. Incubations were for 10 min at 37°C, and the reactions were stopped by the addition of 10 μl of 90% (vol/vol) formamide, 50 mM EDTA, and 0.05% bromphenol blue. Samples were heated at 80°C for 2 min prior to loading on a 8% acrylamide/0.4% *N,N'*-methylenebisacrylamide gel containing 7 M urea in a buffer of 100 mM Tris borate (pH 8.9) and 1 mM EDTA. After electrophoresis the gel was dried and autoradiographed. The far left lane contains a sample without enzyme. Markers refer to the number of nucleotides incorporated. The bracket at the right corresponds to the region analyzed in Fig. 3.

Mn^{2+} also Increases the Relative Incorporation of Other Analogs. The assay described in Fig. 2 can be used to compare the relative rate of incorporation of any nucleotide analog by determining the concentration required to synthesize fragments of a given size range in the presence of a constant amount of the corresponding dNTP, if the analog is a chain terminator, or the corresponding ddNTP, if it is not. We used this assay and the double-labeling technique (Table 1) to compare the relative rates of incorporation of additional analogs by T7 DNA polymerase with either Mg^{2+} or Mn^{2+} (Table 2). The reactions with Mn^{2+} were carried out at a concentration (2 mM) that results in dAMP and ddAMP being incorporated at comparable rates.

Analogs that contained modifications in the furanose moiety were all incorporated more efficiently when Mn^{2+} was substituted for Mg^{2+}. When the 3' position contained an azido group (AzTTP), it was used 3 times more efficiently with Mn^{2+} than with Mg^{2+}; however, AzTTP was used 60 times less efficiently than ddTTP with either ion. When the 2' position contained a hydroxyl group (rATP), it was used 18 times more efficiently with Mn^{2+} than with Mg^{2+}; however, even with Mn^{2+}, rATP was discriminated against by a factor of 2000. rGMP, rCMP, and rUMP were also incorporated at rates slower than that of dGMP, dCMP, and dTTP by a factor of >10,000 with Mg^{2+} and 1500–3000 with Mn^{2+}. When both the 2' and 3' positions were modified [3'-deoxyadenosine 5'-triphosphate (3'-dATP)], the analog was used 40 times more efficiently with Mn^{2+}; however, even with Mn^{2+}, 3'-dAMP was incorporated less efficiently than dAMP by a factor of 4000.

Table 1. Relative incorporation rates of dAMP to ddAMP by T7 DNA polymerase and DNA polymerase I in the presence of Mg^{2+} and Mn^{2+}

Ion(s)	dAMP/ddAMP incorporation rate ratio: T7 DNA polymerase	dAMP/ddAMP incorporation rate ratio: DNA polymerase I
Mg^{2+} (5 mM)	3.7	550
Mn^{2+} (0.1 mM)	4.0	15
Mn^{2+} (2 mM)	1.0	3.9
Mn^{2+} (2 mM) + isocitrate (15 mM)	1.1	20
Mn^{2+} (2 mM) + Mg^{2+} (5 mM)	1.0	4.4

Polymerase activity was measured using primed M13 DNA (5). Reaction mixtures (140 μl) contained 10 μg of primed mGP1-2 DNA, 20 mM Tris·HCl (pH 7.5), 5 mM dithiothreitol, 100 μM dGTP, 100 μM dTTP, 100 μM dCTP, and 100 μM [^{3}H]dATP (320 cpm/pmol), and 10 μM [α-^{32}P]ddATP (3200 cpm/pmol), and the indicated final concentrations of $MgCl_2$, $MnCl_2$, and sodium isocitrate. Mixtures were preincubated at 37°C for 1 min, and reactions were initiated by the addition of 10 pmol of either T7 DNA polymerase or DNA polymerase I (a 3-fold excess over template) diluted in 10 μl of Tris·HCl, pH 7.5/5 mM dithiothreitol/0.05% bovine serum albumin. Reactions were for 10 min at 37°C and were stopped by the addition of 50 mM EDTA, and the acid-insoluble radioactivity was determined (5). The ratio of dAMP incorporated to 10 times the ddAMP incorporated is shown, to correct for the 10-fold higher concentration of dATP to ddATP.

The effect of Mn^{2+} on the incorporation of analogs in the base or the phosphate linkage varied with the modification. 5-Bromodeoxyuridine 5'-triphosphate was discriminated against by a factor of 4.5 with either ion. dITP, on the other hand, was used 3 times more efficiently when Mn^{2+} was substituted for Mg^{2+}. Whereas Mn^{2+} reduced the discrimination against the analog that contained either the dideoxyribose or the inosine modification by a factor of 3–4, it reduced the discrimination against the analog containing both modifications (ddITP) by a factor of 40. ddATP-512 and ddTTP-526, which also have both a dideoxyribose and modifications in the base, were used more efficiently by a factor of 9 and 25, respectively, when Mn^{2+} was substituted for Mg^{2+}. Mn^{2+} reduced the discrimination against deoxyadenosine 5'-[α-thio]triphosphate by a factor of 3, while deoxythymidine 5'-[β,γ-methylene]triphosphate was not used with either ion.

Mn^{2+} Increases the Uniformity of ddNMP Incorporation. We compared the effect of Mn^{2+} and Mg^{2+} on the variability of ddNMP incorporation at different sequences. A fluorescently labeled primer was extended in the presence of all four dNTPs and either ddCTP or all four ddNTPs at equal concentrations, and, after denaturing gel electrophoresis, the fluorescence corresponding to each termination site was determined (Fig. 3). With DNA polymerase I and Mg^{2+}, adjacent bands differed in intensity by up to 50-fold (Fig. 3 *A* and *B*, scans 1). When Mn^{2+} was substituted for Mg^{2+}, the intensities varied at the same sequences; however, the magnitude of the variability was less (Fig. 3 *A* and *B*, scans 2).

Table 2. Relative rates of the use of various nucleotide analogs by T7 DNA polymerase in the presence of Mg^{2+} and Mn^{2+}

Nucleotides compared	Nucleotide incorporation rate ratio	
	Mg^{2+} (5 mM)	Mn^{2+} (2 mM)
dATP/ddATP*	3.8	1.0
dATP/rATP*	35,000	2000
dATP/3'-dATP†	160,000	4000
dATP/ddATP-512†	70	8.0
dATP/dATP[α-thio]‡	4.5	1.5
dGTP/dITP‡	7.0	2.5
dGTP/ddITP†	120	3.0
dGTP/dc^{7}GTP‡	1.5	1.2
dTTP/AzTTP†	180	60
dTTP/ddTTP-526†	1,000	40
dTTP/5-Br-dUTP‡	4.5	4.5
dTTP/p[CH_2]ppdT‡	>5,000	>5000

ddATP-512, 2',3'-dideoxy-7-deazaadenosine 5'-triphosphate with a fluorescein derivative linked to N-7 of adenine (11); dATP[α-thio], 2'-deoxyadenosine 5'-[α-thio]triphosphate; dc^{7}GTP, 2'-deoxy-7-deazaguanosine 5'-triphosphate; AzTTP, 3'-azido-3'-deoxythymidine 5'-triphosphate; ddTTP-526, 2',3'-dideoxy-7-deazathymidine 5'-triphosphate with a fluorescein derivative linked to C-5 of thymine (11); 5-Br-dUTP, 5-bromodeoxyuridine 5'-triphosphate; p[CH_2]ppdT, deoxythymidine 5'-[β,γ-methylene]triphosphate; 3'-dATP, 3'-deoxyadenosine 5'-triphosphate.

*Ratios were determined using 100 μM [^{3}H]dATP and either 10 μM [α-^{32}P]ddATP or 100 μM [α-^{32}P]rATP, as described in Table 1. The ratios for the other rNTPs and ddNTPs were determined as described below.

†The ratios were determined using the gel assay described in Fig. 2. Each ratio with Mn^{2+} was determined by carrying out reactions using various amounts of the analog with a constant amount of the corresponding dNTP and comparing the maximum extension lengths to those using various amounts of the corresponding ddNTP with the same amount of the dNTP. The value shown is the ratio of the concentration of the analog to that of the unmodified ddNTP required to give extensions of comparable maximum lengths. Since dNMPs and ddNMPs are incorporated at identical rates with 2 mM Mn^{2+}, this ratio is also the relative rate of incorporation of the analog to the unmodified dNMP. Each ratio with Mg^{2+} was determined by carrying out reactions using various amounts of the analog with a constant amount of the corresponding dNTP, and comparing the maximum extension lengths to those with Mn^{2+}: the ratio of the concentration of the analog required to give extensions of the same maximum length with Mg^{2+} compared to Mn^{2+} was multiplied by the ratio obtained with Mn^{2+}.

‡The ratios were determined using the gel assay described in Fig. 2. Reactions were carried out using various concentrations of the dNTP with a fixed concentration of the corresponding ddNTP, and the maximum extension lengths were compared to those obtained using various concentrations of the analog in place of the dNTP with the same concentration of the ddNTP. The value shown is the ratio of the analog to that of the dNTP required to obtain extensions of comparable maximum lengths.

With T7 DNA polymerase and Mg^{2+}, ddNMPs were incorporated more uniformly; the intensities of most bands differed by less than a factor of 3, and there was a maximum variability of 10-fold (Fig. 3 *A* and *B*, scans 3). When Mn^{2+} was substituted for Mg^{2+}, the uniformity was dependent on its concentration. At 0.1 mM Mn^{2+}, the pattern of relative intensities was identical to that obtained with Mg^{2+} (data not shown). However, at 2 mM Mn^{2+}, ddNMPs were incorporated with very high uniformity; the intensities of most bands were within 10% of adjacent ones, and there was a maximum variation of 25% (Fig. 3 *A* and *B*, scans 4).

DISCUSSION

We show that the metal ion used for catalysis plays a major role in the ability of both T7 DNA polymerase and *E. coli*

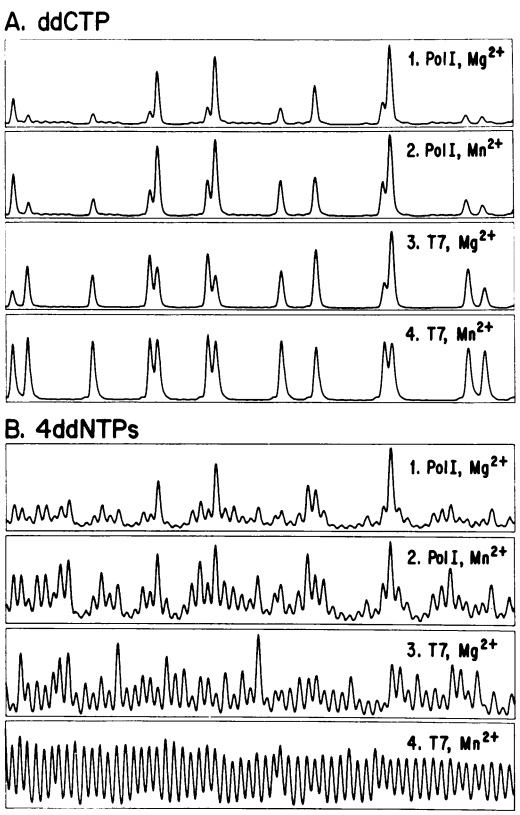

FIG. 3. Sequence-dependent variation in ddNMP incorporation by T7 DNA polymerase and DNA polymerase I. Fluorescein-labeled primer was annealed to mGP1-2 DNA in a mixture (10 μl) containing 2 μg of mGP1-2 DNA, 5 ng of primer (equal molar ratio to the template), 20 mM Tris·HCl (pH 7.5), 50 mM NaCl, and either 5 mM $MgCl_2$ (*A* and *B*, scans 1 and 3) or 2 mM $MnCl_2$ (*A* and *B*, scans 2 and 4); the mixture was heated to 65°C for 2 min and then cooled to 20°C over 30 min. Reaction mixtures (18 μl) contained 2 μg of primed mGP1-2 DNA, 20 mM Tris·HCl (pH 7.5), 5 mM dithiothreitol, 50 mM NaCl, either 5 mM $MgCl_2$ (*A* and *B*, scans 1 and 3) or 2 mM $MnCl_2$ (*A* and *B*, scans 2 and 4), each of the four dNTPs either at 50 μM (*A* and *B*, scans 1) or at 300 μM (*A* and *B*, scans 2–4), and the ddNTPs as follows. Scans in *A*: 1, 500 μM ddCTP; 2 and 3, 10 μM ddCTP; 4, 2 μM ddCTP. Scans in *B*: 1, each of the four ddNTPs at 500 μM; 2, each of the four ddNTPs at 10 μM; 3, each of the four ddNTPs at 5 μM; 4, each of the four ddNTPs at 1 μM. Mixtures were incubated at 37°C for 1 min, and the reactions were initiated by the addition of 2 μl containing 3 pmol (5-fold excess over template) of DNA polymerase I (*A* and *B*, scans 1 and 2) or T7 DNA polymerase (*A* and *B*, scans 3 and 4) diluted in 20 mM Tris·HCl (pH 7.5), 5 mM dithiothreitol, and 0.05% bovine serum albumin. Reactions were for 10 min at 37°C and were stopped by the addition of 8 μl of 25 mM EDTA/1 M potassium acetate (pH 5.0), and 56 μl of ethanol. After centrifugation, the DNA was resuspended in 6 μl of 80% (vol/vol) formamide/10 mM Tris·HCl, pH 8.0/1 mM EDTA. The samples were heated at 80°C for 2 min prior to loading on the Applied Biosystems model 370A automated sequencing system using an 8% acrylamide/0.4% *N,N'*-methylenebisacrylamide gel containing 7 M urea in a buffer of 100 mM Tris borate, pH 8.9/1 mM EDTA. The raw data of the output from the single channel optimized to detect fluorescein is shown. In each panel, the leftmost nucleotide is T7 base 5790, and the rightmost is 5729 (4); the corresponding region is indicated in Fig. 2 by a bracket.

DNA polymerase I to incorporate a nucleotide lacking a 3' hydroxyl group: ddNTPs are used more efficiently when Mn^{2+} is substituted for Mg^{2+}. What is the mechanism by which the metal ion influences the recognition of ddNTPs? Both ddNTPs and dNTPs bind to DNA polymerase I with the same affinity in the presence of Mg^{2+}, so the discrimination is not simply a difference in the respective K_d values (15). Polymerization is thought to proceed via a pentacovalent trigonal-bipyramid intermediate at the α-phosphate of the

incoming dNTP with the metal ion chelating the β- and γ-phosphates at the apical position, facilitating their release (3, 13, 16). It seems likely that in the absence of a 3′ hydroxyl group, the Mg^{2+}–nucleotide complex has a configuration that is sterically hindered either in the formation of this intermediate or in the release of the pyrophosphate group. The furanose ring has a puckered configuration, the orientation of which is dependent on its 2′ and 3′ substituents (see ref. 17). Perhaps the furanose moiety assumes different configurations depending on both the metal ion and the presence of 2′ and 3′ hydroxyl groups, and it is these structural differences in the metal–substrate complex, rather than the presence or absence of a specific hydroxyl group, that limit the rate of incorporation.

Many DNA polymerases have been shown to be more mutagenic with Mn^{2+} (see refs. 9 and 14). Most studies have focused on the incorporation of incorrect bases or base analogs, with a decrease in fidelity by a factor of 2–10 generally observed (12). In the case of DNA polymerase I, the fidelity varies with the metal ion only at occasional, specific sequences (18). Of those sites affected, most show a greater frequency of misincorporation with Mn^{2+} than with Mg^{2+}, although some have a higher fidelity with Mn^{2+}. Previous studies have also shown that DNA polymerase I incorporates ribonucleoside monophosphates (rNMPs) in the presence of Mn^{2+}, but not Mg^{2+} (19, 20); rGMP and rCMP were incorporated at rates similar to that of dGMP and dCMP, rAMP was incorporated only slowly, and rUMP was not incorporated at all. Extensive synthesis did not occur, even with rGTP or rCTP. We find that both T7 DNA polymerase and DNA polymerase I incorporate rNMPs more efficiently with Mn^{2+} than with Mg^{2+}; however, in contrast to these earlier studies, under our assay conditions each rNMP was still incorporated less efficiently than the corresponding dNMP by a factor of more than a thousand.

The optimal free Mn^{2+} concentration for T7 DNA polymerase activity is ≈3 μM, while higher levels are strongly inhibitory. In contrast, high levels of Mn^{2+} (>50 μM) are necessary for ddNTPs to be used most efficiently relative to dNTPs. One mechanism that could account for this would be if high Mn^{2+} concentrations altered the kinetics of the polymerization reaction, whereby the rate-limiting step was changed to one less dependent on the furanose structure. In a related study, Beckman *et al.* (14) showed that the decrease in fidelity by DNA polymerase I only occurs at high (>100 μM) Mn^{2+} concentrations. They compared the dissociation constants of Mn^{2+} with the enzyme, template, and dNTPs and concluded that although the stimulation of polymerase activity at low Mn^{2+} levels was due to its interactions with the enzyme, the inhibition at higher Mn^{2+} levels was likely due to its interactions with the template.

We do not understand the mechanism for the variability in incorporation of ddNMPs at specific sequences. Any model must take into account the following observations: (*i*) The sequence specificity is different for T7 DNA polymerase and DNA polymerase I, despite their high degree of homology (21). (*ii*) With DNA polymerase I, the substitution of Mn^{2+} for Mg^{2+} reduces the variability of ddNMP incorporation but does not change the specificity. (*iii*) With T7 DNA polymerase, substituting Mn^{2+} at low concentrations for Mg^{2+} has no effect on either the specificity or variability of ddNMP incorporation. Only at high Mn^{2+} concentrations, at which dNMPs and ddNMPs are incorporated at comparable rates, is the relative incorporation of ddNMPs at adjacent sequences highly uniform. These results imply that the role of Mn^{2+} in reducing this variability is an indirect consequence of its ability to reduce the discrimination between dNTPs and ddNTPs.

DNA polymerase I has a low processivity, dissociating after the incorporation of less than 10 nucleotides (4, 6). There is a strong correlation between the frequency at which the enzyme dissociates from a site during DNA synthesis in the absence of ddNTPs, and the extent of discrimination against the incorporation of a ddNMP at that site (unpublished results). This suggests that DNA polymerase I incorporates dNMPs and ddNMPs at similar rates during processive synthesis; however, when synthesis is nonprocessive, dNMPs are incorporated preferentially over ddNMPs. This model could account for the greater variability in ddNMP incorporation by DNA polymerase I compared to T7 DNA polymerase, since the latter has a processivity two orders of magnitude greater than the former (4, 6).

Regardless of the mechanism whereby ddNMPs are incorporated uniformly with T7 DNA polymerase and Mn^{2+}, this property should be of use for DNA sequencing, particularly by automated procedures (10, 11). In addition, the higher efficiency of incorporation of ddNMP analogs such as fluorescein-labeled ddNMPs enables the use of lower concentrations of these relatively expensive compounds.

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