Mechanisms of Error Discrimination by *Escherichia coli* DNA Polymerase I†

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ABSTRACT: The mechanism of base selection by DNA polymerase I of *Escherichia coli* has been investigated by kinetic analysis. The apparent K_M for the insertion of the complementary nucleotide dATP into the hook polymer poly(dT)–oligo(dA) was found to be 6-fold lower than that for the noncomplementary nucleotide dGTP, whereas the V_{max} for insertion of dATP was 1600-fold higher than that for dGTP. The ratio of K_{cat}/K_M values for complementary and mismatched nucleotides of 10^4 demonstrates the extremely high specificity of base selection by DNA polymerase I and is in agreement with results obtained with a different template-primer, poly(dC)–oligo(dG) [El-Deiry, W. S., Downey, K. M., & So, A. G. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 7378]. Studies on the effects of phosphate ion on the polymerase and 3′- to 5′-exonuclease activities of DNA polymerase I showed that, whereas the polymerase activity was somewhat stimulated by phosphate, the exonuclease activity was markedly inhibited, being 50% inhibited at 25 mM phosphate and greater than 90% inhibited at 80 mM phosphate. Selective inhibition of the exonuclease activity by phosphate also resulted in inhibition of template-dependent conversion of a noncomplementary dNTP to dNMP and, consequently, markedly affected the kinetic constants for insertion of noncomplementary nucleotides. The mutagenic metal ion Mn^{2+} was found to affect error discrimination by both the polymerase and 3′- and 5′-exonuclease activities of DNA polymerase I. Substitution of Mn^{2+} for Mg^{2+} resulted in both decreased specificity of base selection by the polymerase activity and decreased specificity of proofreading by the 3′- to 5′-exonuclease activity. Furthermore, in the presence of Mn^{2+} but not Mg^{2+}, mismatched primer termini could be extended by the polymerase, resulting in an increased error frequency. In addition to lowering the proofreading specificity of the 3′- to 5′-exonuclease, Mn^{2+} also increased the sensitivity of the 3′- to 5′-exonuclease to selective inhibition by nucleoside 5′-monophosphates (5′NMPs). Thus, Mn^{2+}-induced mutagenesis may be due not only to the relaxed specificity of the polymerase in base selection and the relaxed specificity of the 3′- to 5′-exonuclease in proofreading but also to increased sensitivity of proofreading to inhibition by 5′NMP. We have demonstrated that template-dependent turnover of noncomplementary dNTP to dNMP requires a 3′-hydroxyl group at the primer terminus and can be abolished by inhibition of the 3′- to 5′-exonuclease activity of the enzyme. These data suggest that "kinetic proofreading" does not play a major role in maintaining the accuracy of DNA synthesis with DNA polymerase I.

The accuracy with which genetic information is transmitted during DNA replication is largely determined by the fidelity of DNA polymerases. With prokaryotic DNA polymerase, fidelity is thought to be the result of two error discrimination steps: the selection of a correct nucleotide for incorporation into the 3′-terminus of a growing DNA chain by the polymerase activity; and the hydrolysis of an incorrect nucleotide at the primer terminus by the proofreading 3′- to 5′-exonuclease activity of the DNA polymerase. Elucidation of the mechanisms by which the polymerase and 3′- to 5′-exonuclease activities of DNA polymerases carry out these error discrimination steps, and the factors which influence their effectiveness, has been approached in a variety of ways in different laboratories [for a review, see Loeb and Kunkel (1982)]. We have recently determined the specificity of base selection by the large fragment of DNA polymerase I of *Escherichia coli* by using a hook homopolymer, poly(dC)–oligo(dG), as template-primer to measure the kinetic constants for phosphodiester bond formation with either complementary or noncomplementary nucleotide substrates (El-Deiry et al., 1984). We found 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 substrates. We further found that the mutagenic metal ion Mn^{2+} decreases the specificity of base selection primarily by increasing the V_{max} for insertion of a noncomplementary nucleotide.

To determine whether the mechanism of base selection by DNA polymerase I is general and applicable to other template-primers, we have determined the kinetic constants for insertion of either a complementary or a noncomplementary nucleotide into the primer terminus of the hook polymer poly(dT)–oligo(dA). In addition, the role of the proofreading 3′- to 5′-exonuclease in error discrimination was investigated. We have also investigated the mechanism of Mn^{2+}-induced mutagenesis using the hook polymer poly(dT)–oligo(dA) as template-primer. The mutagenic metal ion Mn^{2+} was found to reduce the specificity of both base selection and proofreading by DNA polymerase I and to increase the sensitivity of the proofreading exonuclease to inhibition by nucleoside 5′-monophosphates. The contribution of "kinetic proofreading" to the template-dependent conversion of dNTP to dNMP has also been investigated.

Materials and Methods

The large fragment of *E. coli* DNA polymerase I was obtained from New England Nuclear at a specific activity of 15 600 units/mg of protein, 1 unit being equivalent to the incorporation of 20 nmol of dNMP in 30 min at 37 °C. The enzyme was routinely diluted for assay in freshly prepared 100

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mM *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (Hepes) buffer, adjusted to pH 7.8 with KOH, 0.2 M KCl, 10 mM 2-mercaptoethanol, and 1 mg/mL bovine serum albumin (BSA). KCl was omitted from the dilution buffer in experiments where the effect of salt concentration on exonuclease activity was being studied.

The sodium salts of deoxynucleoside 5′-triphosphates (dNTPs), dideoxynucleoside 5′-triphosphates (ddNTPs), and ribo- and deoxyribonucleoside 5′-monophosphates (5′NMPs) were purchased from P-L Biochemicals. [^{3}H]dNTPs were purchased from ICN, and their purity was determined by thin-layer chromatography on poly(ethylenimine)–cellulose (obtained from Brinkmann Instruments) using solvent systems as described by Randerath and Randerath (1964). In turnover experiments, [^{3}H]dNTPs were used only if contaminating [^{3}H]dNMP was less than 0.5% of the total radioactivity, and in most experiments, only 0.1–0.2% of the total radioactivity was present as [^{3}H]dNMP after incubation in the absence of either DNA template-primer, DNA polymerase I, or divalent cation. [α-^{32}P]DideoxyATP was obtained from Amersham. Terminal deoxynucleotidyl transferase was purchased from Collaborative Research and contained 185 295 units/mg of protein. Poly(dT), of average chain length 350 nucleotides, was obtained from Sigma, and $(dT)_{12-18}$ was obtained from P-L Biochemicals.

Preparation of Polynucleotides. The hook polymer $(dT)_{350}$–$(dA)_{14}$, either unlabeled or with the dAMP primer uniformly labeled with ^{3}H, was prepared in a reaction containing 200 mM potassium cacodylate, pH 7.4, 0.5 mM $CoCl_2$, 2.85 μM $(dT)_{350}$, 867 μM dATP, 1 mg/mL BSA, 1 mM 2-mercaptoethanol, and 1940 units/mL terminal deoxynucleotidyl transferase. Reactions were incubated for 120 min at 37 °C and treated as described previously (El-Deiry et al., 1984). The $(dT)_{350}$–$(dA)_{14}$–[^{3}H][^{3}H]$(dG)_{0.07}$ hook polymer (8600 cpm/pmol) was prepared in a reaction mixture containing 50 mM Hepes, pH 7.8, 0.5 mM $MnCl_2$, 2.12 μM poly(dT)–oligo(dA) hook polymer, 11.1 μM [^{3}H]dGTP, 1 mM 5′-AMP, and 5 units/mL of the large fragment of *E. coli* DNA polymerase I. The reaction was incubated for 90 min at 37 °C. [^{3}H]$(dT)_{121}$ (3.65 μM, 314 cpm/pmol of dTMP) was prepared in a 2.0-mL reaction containing 200 mM potassium cacodylate, pH 7.4, 1 mM $CoCl_2$, 3.65 μM $(dT)_{12-18}$, 410 μM [^{3}H]dTTP, 1 mg/mL BSA, 1 mM 2-mercaptoethanol, and 492 units/mL terminal deoxynucleotidyl transferase. The reaction was incubated for 240 min at 37 °C.

Kinetics of Nucleotide Insertion. Rates of dNTP incorporation were determined essentially as previously described (El-Deiry et al., 1984). Typical reaction mixtures contained the following in a final volume of 0.05 mL: 50 mM Hepes, pH 7.8, 20 mM KCl, 100 μg/mL bovine serum albumin, 285 nM $(dT)_{350}$–$(dA)_{14}$, 0.185 unit/mL (0.17 nM) large fragment of *E. coli* DNA polymerase I, either $MgCl_2$ or $MnCl_2$, and [^{3}H]dNTP. Other details are given in the figure legends. Reactions were incubated for 5 min at 37 °C and stopped with 2.0 mL of cold 5% trichloroacetic acid and 20 mM sodium pyrophosphate. Precipitates were collected on GF/C filters, washed, dried, and counted as previously described (Byrnes et al., 1976).

Rates of template-dependent turnover of [^{3}H]dNTP to [^{3}H]dNMP were also measured as previously described (El-Deiry et al., 1984). Reactions contained the same reagents as the incorporation assays above, except that either [^{3}H]dGTP or [^{3}H]dCTP was used as substrate, 7.4 units/mL or 6.8 nM large fragment of *E. coli* DNA polymerase I was used, and incubations at 37 °C varied from 20 min with Mn^{2+} to 60 min with Mg^{2+} as divalent cation. Reactions were stopped with ethylenediaminetetraacetic acid (EDTA) at a final concentration of 10 mM; 1 M sodium formate, pH 3.4, was used to separate 5′dGMP from deoxynucleoside di- and triphosphates and DNA precursors, and 0.5 M sodium formate, pH 3.4, was used to separate 5′dCMP from precursors by thin-layer chromatography on poly(ethylenimine)–cellulose. Samples were treated as previously described (El-Deiry et al., 1984).

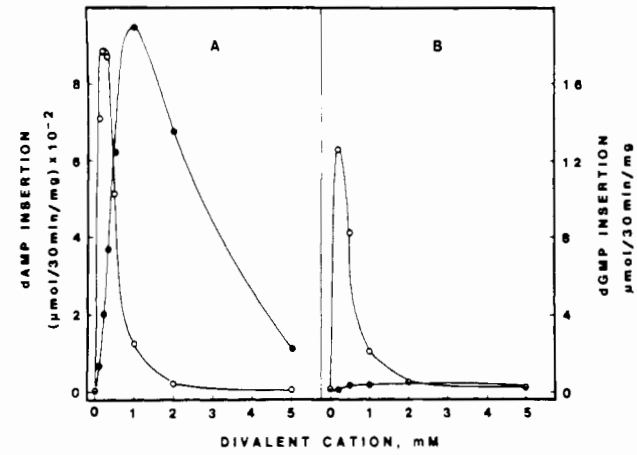

FIGURE 1: Effect of Mg^{2+} (●) and Mn^{2+} (○) concentrations on the rate of insertion into $(dT)_{350}$–$(dA)_{14}$ of either the complementary nucleotide dATP (panel A) or the noncomplementary nucleotide dGTP (panel B). Rates were determined by incorporation for dATP and by turnover for dGTP as described under Materials and Methods.

Rates of insertion of either complementary or noncomplementary nucleotides were determined by summing the rates of incorporation and turnover and are expressed as micromoles of nucleotide per 30 min per milligram of enzyme at 37 °C. Under standard assay conditions, the rate of turnover of a complementary nucleotide was less than 1% of the rate of incorporation and could be ignored. Similarly, the rate of incorporation of a mismatched nucleotide was less than 2% of the rate of turnover. Reactions were carried out under conditions where the reaction being measured was linear with time and enzyme concentration. Apparent K_M and V_{max} values were determined as previously described (El-Deiry et al., 1984).

3′- to 5′-Exonuclease Assays. Typical reaction mixtures contained in a final volume of 0.10 mL the following: 50 mM Hepes buffer, pH 7.8, either 1.71 μM $(dT)_{350}$–[^{3}H]$(dA)_{14}$, 0.53 μM $(dT)_{350}$–$(dA)_{14}$–[^{3}H]$(dG)_{0.07}$, or 0.12 μM [^{3}H]$(dT)_{121}$, 0.5–7 nM large fragment of *E. coli* DNA polymerase I, and either $MgCl_2$ or $MnCl_2$ at concentrations given in the figure legends. Reactions were incubated at 37 °C, and 20-μL aliquots were removed and applied to 2.4-cm DE81 filter paper circles at 0 min and three other time points up to 30 min. The circles were washed, and exonuclease activity was quantitated as previously described (Que et al., 1979).

RESULTS

Effect of Mg^{2+} and Mn^{2+} Concentration on the Rates of Insertion of Complementary and Noncomplementary Nucleotides. The divalent cation profiles for insertion of either the complementary nucleotide (dATP) or the noncomplementary nucleotide (dGTP) into the hook polymer $(dT)_{350}$–$(dA)_{14}$ are shown in Figure 1. With the complementary nucleotide dATP as substrate (panel A), the Mn^{2+} optimum concentration was 0.2–0.3 mM and very sharp, whereas the optimal Mg^{2+} concentration was 1.0 mM and considerably broader. With the mismatched nucleotide dGTP as substrate (panel B), the Mn^{2+} optimum concentration was also found to be 0.3 mM and very sharp, and the Mg^{2+} op-

Table I: Apparent Kinetic Constants for Insertion of Complementary and Noncomplementary Nucleotides into Poly(dT)–Oligo(dA)[a]

divalent cation	K_M (μM)	V_{max} [μmol (30 min)$^{-1}$ mg^{-1}]	k_{cat} (s^{-1})	k_{cat}/K_M (L mol^{-1} s^{-1})
Insertion of dATP (A·T Pair)				
1.0 mM Mg^{2+}	8.74 ± 1.2	1413 ± 42	53.4	6.1×10^6
5.0 mM Mg^{2+}	3.27 ± 0.42	139 ± 3.2	5.26	1.6×10^6
0.2 mM Mn^{2+}	3.46 ± 0.48	1075 ± 29	40.6	1.2×10^7
1.0 mM Mn^{2+}	13.6 ± 2.0	151 ± 5.3	5.71	4.2×10^5
Insertion of dGTP (G·T Pair)				
1.0 mM Mg^{2+}	51.5 ± 10	0.86 ± 0.05	0.033	6.4×10^2
5.0 mM Mg^{2+}	71.0 ± 21	0.81 ± 0.08	0.031	4.4×10^2
0.3 mM Mn^{2+}	62.9 ± 3	34.7 ± 0.5	1.31	2.1×10^4
1.0 mM Mn^{2+}	44.6 ± 21	3.86 ± 0.42	0.146	3.3×10^3
Insertion of dCTP (C·T Pair)				
0.3 mM Mn^{2+}	213 ± 16	18.2 ± 0.4	0.69	3.2×10^4

[a] Substrate dNTP concentrations were varied as follows: dATP, 1.7–202 μM; dGTP, 5.5 μM–1 mM; dCTP, 2.1 μM–2 mM. Apparent K_M and V_{max} values were determined as described under Materials and Methods and are reported as mean values ± SEM. In Mn^{2+}-activated reactions, dNTP was added as a 1:1 complex with cation.

timum concentration was very broad. However, in the presence of Mg^{2+}, the rate of insertion of dGTP into poly(dT)–oligo(dA) was very low, only 2–5% of that seen in Mn^{2+}.

Mechanism of Base Selection by the Large Fragment of E. coli DNA Polymerase I. Table I shows the apparent K_M and V_{max} values as well as k_{cat}/K_M values for the insertion of the complementary nucleotide dATP and the mismatched nucleotide dGTP into the hook polymer poly(dT)–oligo(dA). At optimal Mg^{2+} concentration, 1 mM, the apparent K_M for the complementary substrate dATP was found to be 6-fold lower than that for the noncomplementary substrate dGTP, while the V_{max} for insertion of the complementary substrate was found to be 1600-fold higher than that for insertion of the noncomplementary substrate. Thus, the enzyme prefers to insert a dAMP rather than a dGMP across from a template T by a factor of 10^4. The large difference in k_{cat}/K_M value between complementary and mismatched nucleotides demonstrates the extremely high specificity of base selection by *E. coli* DNA polymerase I. Furthermore, these results suggest that DNA polymerase I actively discriminates between complementary and noncomplementary nucleotides by lowering the rate of phosphodiester bond formation between a primer terminus and a noncomplementary dNTP.

Effect of Substitution of Mn^{2+} for Mg^{2+} on the Specificity of Base Selection. The effects of substitution of Mn^{2+} for Mg^{2+} on the apparent Michaelis constants for insertion of complementary dATP and mismatched dGTP into the poly(dT)–oligo(dA) hook polymer are also shown in Table I. Substitution of 0.2 mM Mn^{2+} for 1 mM Mg^{2+} had no significant effect on either the K_M or the V_{max} values for insertion of dATP. At higher concentrations of either Mg^{2+} or Mn^{2+}, the V_{max} value for insertion of dATP was 9–10-fold lower than that at optimal metal ion concentrations, whereas the apparent K_M for dATP was not significantly changed.

When the noncomplementary nucleotide dGTP was the substrate, substitution of 0.3 mM Mn^{2+} for 1 mM Mg^{2+} had no effect on the apparent K_M value for insertion but increased the V_{max} value approximately 40-fold. At higher Mn^{2+} concentrations, i.e., 1 mM, the V_{max} for insertion of dGTP across from a template T was approximately 10-fold lower than at 0.3 mM Mn^{2+}, although still 4-fold higher than in the presence of Mg^{2+}. Higher Mg^{2+} concentrations, i.e., 5 mM, did not significantly affect the apparent K_M values for either dATP or dGTP; however, the V_{max} for insertion of dATP was markedly inhibited while that for dGTP misinsertion was unaffected. This is in agreement with other studies in which inhibitory Mg^{2+} concentrations were found to be mutagenic (Kunkel & Loeb, 1979; Hillebrand & Beattie, 1984).

The insertion of dCTP with poly(dT)–oligo(dA) as template-primer was not detectable in the presence of Mg^{2+}; however, it becomes easily measurable at 0.3 mM Mn^{2+}, suggesting that the substitution of Mn^{2+} for Mg^{2+} increased the misinsertion of dCTP across from a template T. However, it is not clear whether Mn^{2+} affected the misinsertion of dCTP by affecting the K_M for dCTP, or the V_{max}, since the unfavored pyrimidine/pyrimidine mispair was only detectable in Mn^{2+}.

Similar to the results obtained with the poly(dC)–oligo(dG) hook polymer (El-Deiry et al., 1984), the increased ratio of utilization of noncomplementary to complementary nucleotides in the presence of low concentrations of Mn^{2+} appears to be due primarily to an increased V_{max} for insertion of the mismatched nucleotides, with no significant effects on the K_M values for insertion of either matched or mismatched nucleotides. These results are in contrast to those of Fersht et al. (1983), in which the substitution of Mn^{2+} for Mg^{2+} was found to result in markedly lower apparent K_M values for insertion of noncomplementary nucleotides. This discrepancy may be explained by the different buffers that were used in the two studies.

Inhibition of the 3′- to 5′-Exonuclease Activity by Phosphate Buffer Increases the Apparent K_M for Insertion of Mismatched Nucleotides. In previous studies in our laboratory, we found that the apparent K_M value for insertion of mismatched dATP into the poly(dC)–oligo(dG) hook polymer in the presence of Mg^{2+} was 15–20-fold higher in 50 mM phosphate buffer (pH 7.4) than in Hepes buffer (pH 7.8) (El-Deiry et al., 1984). Similar results were found with the poly(dT)–oligo(dA) hook polymer; i.e., the apparent K_M for dGTP was markedly increased in phosphate buffer relative to Hepes buffer while the apparent K_M for dATP was essentially the same in both buffers (data now shown).

In order to elucidate the effects of phosphate buffer on the insertion of noncomplementary nucleotides, the effects of increasing concentrations of phosphate buffer on the template-dependent turnover of dGTP to dGMP (insertion) with poly(dA-dT) as template-primer were determined (Figure 2). The rate of turnover of dGTP to dGMP was found to be extremely sensitive to inhibition by phosphate buffer, 50% inhibition occurring at 25 mM phosphate and greater than 90% inhibition at 80 mM phosphate.

Since template-dependent generation of dGMP represents alternate incorporation and hydrolysis of dGMP at the 3′-hydroxy termini of DNA by polymerase and 3′- to 5′-exonuclease activities of DNA polymerase I, and since the apparent K_M for insertion of complementary nucleotides, which is determined from incoporation rates, is not affected by phosphate buffer, it would appear that the major effect of phosphate buffer is on the exonuclease activity. That this is the case is seen in Figure 3, which shows the effect of increasing concentrations of either potassium phosphate or KCl on the rate of hydrolysis of $[^3H](dT)_{121}$. The sensitivity of the 3′- to 5′-exonuclease to inhibition by phosphate is very similar to that of the turnover of dGTP to dGMP. The activity of the exonuclease is less sensitive to inhibition by KCl, being only 50% inhibited at 100 mM. These data suggest that the increase in the apparent K_M for mismatched nucleotide insertion in phosphate buffer is due to inhibition of the 3′- to 5′-exonuclease activity of DNA polymerase I. Phosphate ion

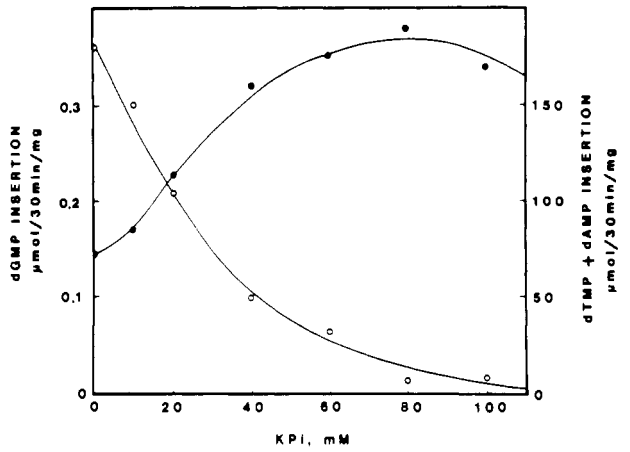

FIGURE 2: Effect of increasing concentration of potassium phosphate, pH 7.8, on the rate of insertion of either the mismatched nucleotide [^{3}H]dGTP (○) or the complementary nucleotides [^{3}H]dATP and dTTP (●) into poly(dA-dT). All reactions contained 50 mM Hepes buffer, pH 7.8, 20 mM KCl, and 50 μM dTTP. The concentrations of [^{3}H]dATP or [^{3}H]dGTP were 10 μM. Rates of insertion were determined by summing rates of incorporation and turnover as described under Materials and Methods.

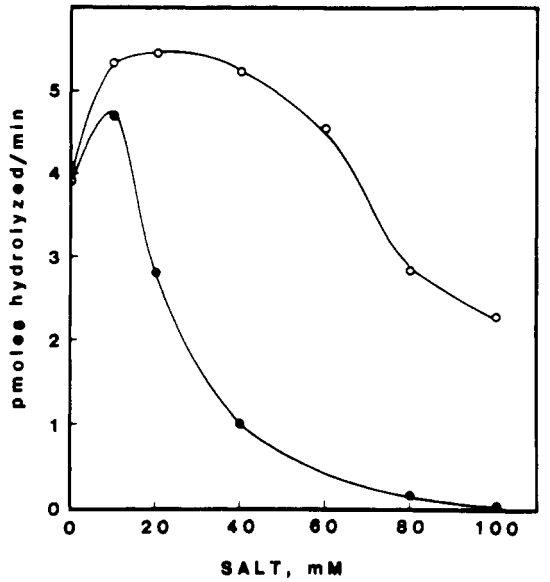

FIGURE 3: Effect of increasing concentration of either KCl (○) or KP_i (●) on the rate of hydrolysis of [^{3}H]$(dT)_{121}$. Reaction mixtures contained 5 mM $MgCl_2$ and 50 mM Hepes buffer, pH 7.8. Rates of hydrolysis were determined by a least-squares analysis of [^{3}H]dTMP released from [^{3}H]poly(dT) over time.

does not inhibit polymerase activity (Figure 2); in fact, the rate of incorporation of [^{3}H]dATP and dTTP into poly(dA-dT) was stimulated about 3-fold at 80 mM phosphate buffer. Thus, phosphate selectively inhibits the turnover of dNTP to dNMP by DNA polymerase I, thereby affecting the kinetic constants for insertion of noncomplementary nucleotides but not complementary nucleotides. The sensitivity of the 3′- to 5′-exonuclease to phosphate ion may explain the markedly higher K_M values for insertion of noncomplementary nucleotides determined in phosphate buffer (Fersht et al., 1983) as compared to Hepes buffer (El-Deiry et al., 1984; present results). The sensitivity of the 3′- to 5′-exonuclease activity to inhibition by KCl and phosphate buffer may also explain the lack of inhibition of the proofreading activity of DNA polymerase I by nucleoside monophosphates with synthetic polynucleotide substrates reported by Loeb et al. (1981). In those studies, phosphate buffer, KCl, or both were present in the reaction mixtures.

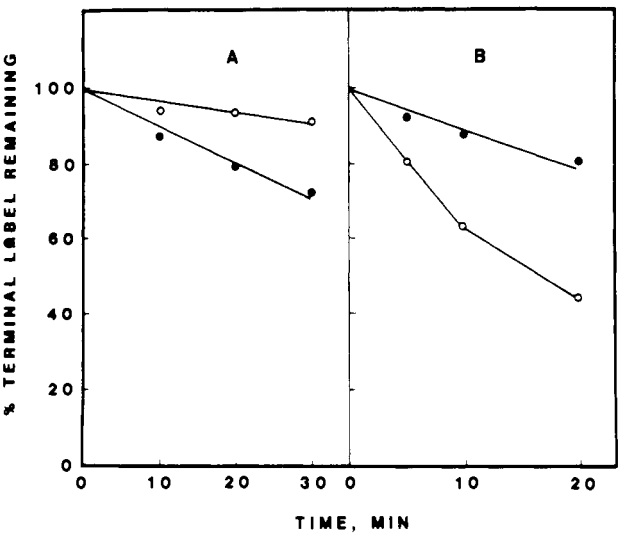

FIGURE 4: Hydrolysis of $(dT)_{350}$–[^{3}H]$(dA)_{14}$ (panel A) or $(dT)_{350}$–$(dA)_{14}$–[^{3}H]$(dG)_{0.07}$ (panel B) in the presence of either 2 mM Mg^{2+} (●) or 0.5 mM Mn^{2+} (○). For hydrolysis of the complementary primer terminus, the concentration of the enzyme was 10 times that for hydrolysis of the noncomplementary primer terminus. Reactions were carried out as described under Materials and Methods.

Effects of Substitution of Mn^{2+} for Mg^{2+} on Error Discrimination by the 3′- to 5′-Exonuclease Activity. In order to determine whether Mn^{2+} affects the specificity of proofreading, the rate of hydrolysis of matched and mismatched primer termini by the proofreading 3′- to 5′-exonuclease activity of DNA polymerase I was measured in the presence of either Mg^{2+} or Mn^{2+}. The results (Figure 4) show that the rate of hydrolysis of the mismatched dGMP primer terminus of poly(dT)–oligo(dA)–[^{3}H]dGMP was approximately 2-fold higher in Mg^{2+} than in Mn^{2+}, whereas the rate of hydrolysis of the complementary dAMP primer terminus of poly(dT)–oligo(dA)–[^{3}H]dAMP was 2–3-fold higher in Mn^{2+} than in Mg^{2+}. Thus, the specificity of the proofreading 3′- to 5′-exonuclease for the mismatched primer terminus is altered in the presence of Mn^{2+}. This altered specificity could be an important factor in the mutagenicity of Mn^{2+}, since there is a greater likelihood for a mismatched primer terminus to be extended when the proofreading ability of the polymerase is decreased.

The importance of altered specificity of proofreading as a mechanism of Mn^{2+}-induced mutagenesis was further tested by examining the fate, i.e., excision or extension, of a mismatched primer terminus, poly(dT)–oligo(dA)–[^{3}H]dGMP, in the presence of dideoxyATP (Figure 5). DideoxyATP was used as a substrate for chain extension by the polymerase instead of dATP because, once the mismatched terminus is elongated by incorporation of ddAMP, it is resistant to hydrolysis by the 3′- to 5′-exonuclease activity (Brutlag & Kornberg, 1972). In the absence of ddATP, the rate of hydrolysis of dGMP from $(dT)_{350}$–$(dA)_{14}$–[^{3}H]$(dG)_{0.07}$ was approximately 3-fold higher in the presence of Mg^{2+} than in Mn^{2+} (Figure 4). Addition of ddATP did not protect the dGMP primer terminus from hydrolysis by the exonuclease activity in the presence of Mg^{2+}, suggesting that the mismatched primer terminus was hydrolyzed prior to chain extension (Brutlag & Kornberg, 1972). Addition of 0.5 mM 5′AMP, an inhibitor of 3′- to 5′-exonuclease activity, resulted in 25% inhibition of exonuclease activity; however, addition of both 5′AMP and ddATP did not provide any further protection than addition of 5′AMP alone. In contrast, addition of either 5′AMP or ddATP to the Mn^{2+}-catalyzed reaction resulted in approximately 50% inhibition of hydrolysis of

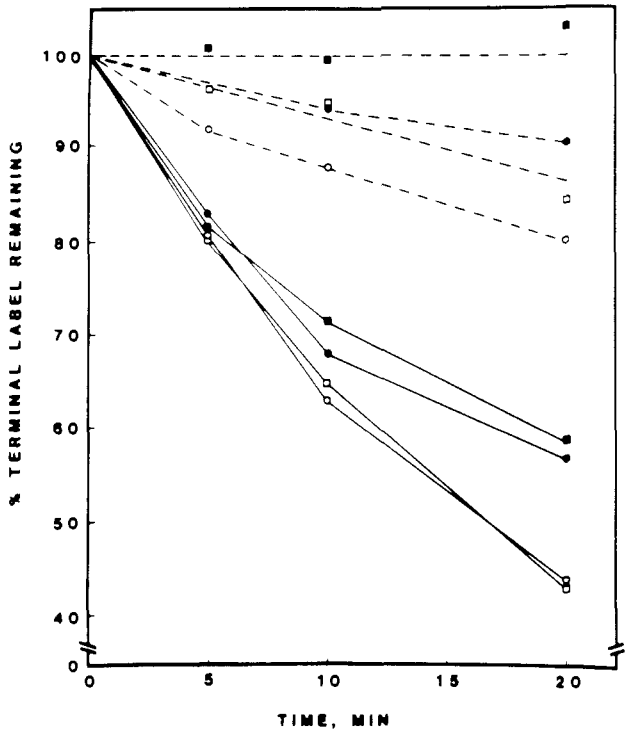

FIGURE 5: Effect of dideoxynucleoside triphosphates and nucleoside 5′-monophosphates on the rate of hydrolysis of $(dT)_{350}$–$(dA)_{14}$–[^{3}H]$(dG)_{0.07}$. Reactions contained either 2 mM Mg^{2+} (solid lines) or 0.5 mM Mn^{2+} (dashed lines). Reactions conditions were as described under Materials and Methods except for no additions (○), the addition of 0.5 mM 5′AMP (●), the addition of 10 μM ddATP (□), or the addition of 0.5 mM 5′AMP and 10 μM ddATP (■).

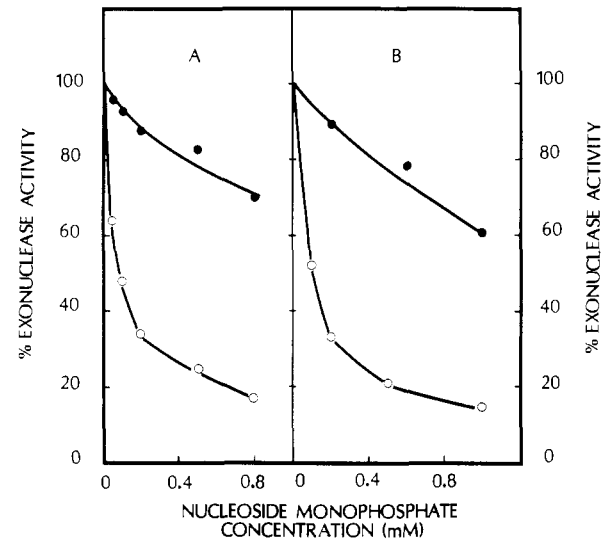

FIGURE 6: Effect of dNMP concentration on the rate of hydrolysis of [^{3}H]$(dT)_{12}$ by the 3′- to 5′-exonuclease activity in the presence of either Mg^{2+} or Mn^{2+}. The effect of either dAMP (panel A) or dGMP (panel B) on exonuclease activity is shown as a percent of the uninhibited reaction in the presence of either 5 mM Mg^{2+} (●) or 0.5 mM Mn^{2+} (○).

poly(dT)–oligo(dA)–[^{3}H]dGMP, and the presence of both 5′AMP and ddATP resulted in complete protection of the mismatched primer terminus. Noncomplementary dideoxynucleotides, e.g., ddGTP, did not protect the mismatched primer terminus in the presence of either Mg^{2+} or Mn^{2+} (data not shown), further suggesting that protection of the mismatched primer terminus was the result of its extension by incorporation of ddAMP.

Effect of Substitution of Mn^{2+} for Mg^{2+} on the Sensitivity of the 3′- to 5′-Exonuclease Activity to Inhibition by Nucleoside 5′-Monophosphates. Nucleoside 5′-monophosphates (5′NMPs) are competitive inhibitors of the 3′- to 5′-exonuclease activity but have no inhibitory effect on polymerase activity (Byrnes et al., 1977; Que et al., 1978). Figure 6 shows the effect of increasing concentration of either dAMP (panel A) or dGMP (panel B) on the rate of hydrolysis of [^{3}H]-poly(dT) in the presence of either 5 mM $MgCl_2$ or 0.5 mM $MnCl_2$. With either purine 5′NMP, the inhibition of 3′- to 5′-exonuclease activity was markedly increased in the presence of Mn^{2+} vs Mg^{2+}. Thus, the decreased specificity of the proofreading exonuclease for a mismatched primer terminus in the presence of Mn^{2+}, coupled with the increased sensitivity of the exonuclease to inhibition by 5′NMP, suggests that Mn^{2+}-induced mutagenesis may be the result of effects on proofreading as well as base selection.

Lack of Evidence for Preinsertion Proofreading. Our studies on the mechanism of base selection by DNA polymerase I are based on the premise that the template-dependent turnover of dNTPs to dNMPs is the result of incorporation of dNMP at the primer terminus by the polymerase activity followed by hydrolysis to free dNMP by the 3′- to 5′-exonuclease activity and that the rate of turnover of a noncomplementary nucleotide is limited by the rate of phosphodiester bond formation. Thus, it is important to determine whether any alternate pathway exists for turnover.

A general mechanism of error discrimination by DNA polymerases has been suggested by Hopfield (1974), in which proofreading occurs prior to phosphodiester bond formation by the hydrolysis of an unstable enzyme–dNTP intermediate to pyrophosphate and dNMP. Evidence supporting this hypothesis, known as kinetic proofreading or preinsertion proofreading, has come from studies with human KB cell DNA polymerase β, which was found to catalyze significant turnover of dNTP to dNMP in spite of an apparent absence of 3′- to 5′-exonuclease activity. It was suggested that, under conditions in which translocation of the polymerase is prevented, the dNTP turnover reaction reflects "the successful competition by the hydroxyl ion of water with the 3′-hydroxyl primer terminus for the incoming dNTP in an intermediate state that is short of completion of the phosphodiester bond" (Wang et al., 1974).

In the present study, we have taken two different approaches to determining whether or not preinsertion proofreading plays a significant role in maintaining the fidelity of DNA synthesis. We have investigated the ability of a dideoxy-terminated template-primer to support the DNA template-dependent conversion of dNTP to dNMP, i.e., whether dNTP turnover can occur in the absence of a 3′-hydroxyl group at the primer terminus. Since the mechanism of preinsertion proofreading as proposed involves competition between the hydroxyl ion of water and the 3′-hydroxyl group at the primer terminus for the conversion of dNTP to dNMP, this pathway of preinsertion hydrolysis would be favored in the absence of the 3′-hydroxyl group at the primer terminus. We further determined whether the template-dependent conversion of dNTP to dNMP can be inhibited by a selective inhibitor of the 3′- to 5′-exonuclease activity.

The rate of turnover of dGTP to dGMP with the hook polymers poly(dT)–oligo(dA) and poly(dT)–oligo(dA)–ddA as template-primers is shown in Figure 7. The rate of dGTP turnover was significantly lower when poly(dT)–oligo(dA)–ddA, which lacks a terminal 3′-hydroxyl group, was substituted for poly(dT)–oligo(dA) as template-primer; the rate of turnover with ddA-terminated template-primer was only 10% of

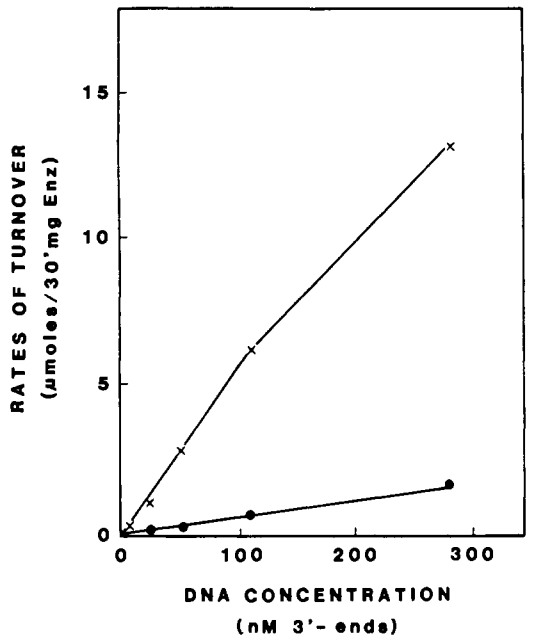

FIGURE 7: Rates of turnover of noncomplementary dNTP as a function of DNA concentration with either 3′-deoxy- or 3′-dideoxy-terminated primer. Reactions were carried out with 56 μM [^{3}H]dGTP, 0.3 mM Mn^{2+}, and either $(dT)_{350}$–$(dA)_{14}$ (×) or $(dT)_{350}$–$(dA)_{14}$–$(ddA)_1$ (●) as template-primer. The rate of template-dependent conversion of dGTP to dGMP is plotted as a function of primer terminus concentration. The ratio of primer termini to enzyme molecules varied from 4/1 to 42/1.

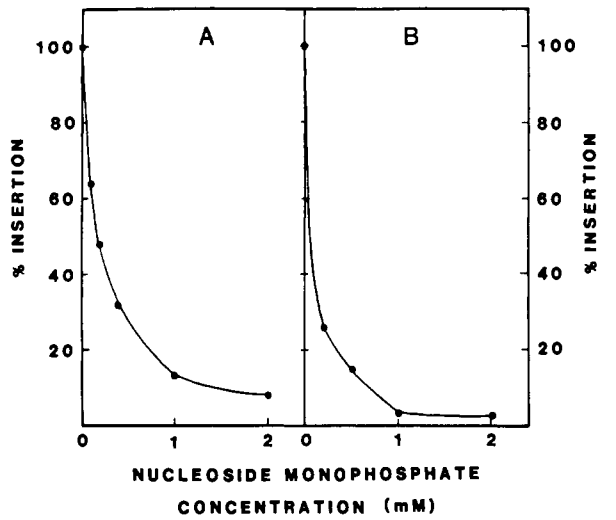

FIGURE 8: Effect of 5′NMP on the turnover of mismatched nucleotides. Reactions were carried out in the presence of 0.3 mM Mn^{2+} and either $(dT)_{350}$–$(dA)_{14}$ and 56 μM [^{3}H]dGTP (panel A) or $(dC)_{194}$–$(dG)_{12}$ and 53 μM [^{3}H]dATP (panel B). The turnover rate is plotted as a percent of the uninhibited reaction as a function of the concentration of either dGMP (panel A) or 5′AMP (panel B).

that with poly(dT)–oligo(dA). This suggests that prior formation of a phosphodiester bond at the 3′-hydroxyl group of the primer terminus is required for turnover of dNTP to dNMP and that preinsertion proofreading does not occur to any significant extent. The small amount of turnover of dGTP to dGMP observed with poly(dT)–oligo(dA)–ddA as template-primer is likely due to the fact that not all of the primer termini are blocked by ddAMP, possibly the result of the hydrolysis of ddAMP by the 3′- to 5′-exonuclease activity. We have observed that a ddAMP primer terminus is hydrolyzed by the 3′- to 5′-exonuclease activity at approximately $^1/_{10}$th the rate of a dAMP primer terminus with Mn^{2+} as divalent cation and at $^1/_{40}$th the rate with Mg^{2+} as divalent cation (data not shown).

Another approach to determining the contribution of preinsertion proofreading to the template-dependent generation of dNMP is to examine the effect of inhibiting the 3′- to 5′-exonuclease activity on the turnover reaction. These studies are based on the findings that the active site for the 3′- to 5′-exonuclease activity is functionally and spatially distinct from the polymerase active site and that the 3′- to 5′-exonuclease activity can be selectively inhibited by 5′NMP (Que et al., 1978; Ollis et al., 1985). Since there is only a single binding site for dNMP, inhibition of turnover of dNTP to dNMP by 5′NMP would be attributable to inhibition of proofreading by the 3′- to 5′-exonuclease activity of DNA polymerase and not to inhibition of the hydrolysis of enzyme–dNTP intermediates. Figure 8 demonstrates the sensitivity of the turnover reaction to inhibition by 5′NMPs. Panel A shows that with $(dT)_{350}$–$(dA)_{14}$ as template-primer and [^{3}H]dGTP as noncomplementary nucleotide, turnover is inhibited 90% at a concentration of 1 mM 5′dGMP, whereas with $(dC)_{194}$–$(dG)_{14}$ (panel B) and [^{3}H]dATP, greater than 95% inhibition of turnover is achieved at 1 mM 5′AMP. These experiments demonstrate that most, if not all, of the template-dependent turnover of mismatched dNTP to dNMP requires a 3′-hydroxyl group at the primer terminus and an active 3′- to 5′-exonuclease, suggesting that the turnover of dNTP to dNMP requires prior phosphodiester bond formation.

Discussion

In the present studies, we have extended our investigation of the mechanism by which DNA polymerase I discriminates between dNTP substrates that are complementary or noncomplementary to the corresponding nucleotide in the template by determining the kinetic constants for insertion of either dATP or dGTP into the primer terminus of the hook polymer poly(dT)–oligo(dA). The data suggest that DNA polymerase I utilizes both K_M and V_{max} differences to discriminate between dNTP substrates that are complementary or noncomplementary to the template base; i.e., the apparent K_M for the noncomplementary substrate dGTP is approximately 6-fold higher than that for the complementary substrate dATP whereas the V_{max} for insertion of dGTP is approximately 1600-fold lower than that for insertion of dATP. Thus, the specificity of the enzyme for inserting an A rather than a G opposite a template T is of the order of 10^4. These results are qualitatively similar to those obtained with a different template-primer, poly(dC)–oligo(dG), in which the 10^5-fold preference of the enzyme for inserting a G rather than an A opposite a template C resulted from a 17-fold difference in the apparent K_M between complementary and noncomplementary dNTPs and a 5000-fold difference in V_{max} (El-Deiry et al., 1984). Thus, the data with two different templates support a mechanism of base selection in which the DNA polymerase plays an active role in discriminating between complementary and noncomplementary dNTP substrates, possibly by undergoing a conformational change when a correct base pair is presented to the active site. The data do not support a passive polymerase model (Goodman et al., 1983) in which the enzyme inserts any dNTP present at the active site at the same rate and only discriminates between complementary and noncomplementary substrates on the basis of base-pairing free energies, modified by stereochemical constraints.

Other studies have also led to the suggestion that DNA polymerase I undergoes a conformational change upon binding of a dNTP substrate that is complementary to the template base. The studies of Bryant et al. (1983) and Mizrahi et al.

(1985), using isotope partitioning and rapid quench kinetic methods, also support an active polymerase model. These authors have suggested that the rate-determining step in DNA synthesis may be a first-order isomerization of the enzyme–polynucleotide–dNTP complex which precedes phosphodiester bond formation.

The present studies on the effects of substitution of Mn^{2+} for Mg^{2+} on the fidelity of DNA synthesis with poly(dT)–oligo(dA) agree well with our previous studies using poly(dC)–oligo(dG) as template-primer (El-Deiry et al., 1984). The decrease in fidelity of DNA polymerase I in the presence of Mn^{2+} was again demonstrated to be due to both a decrease in specificity of base selection by the polymerase activity and also a lowered specificity of proofreading by the 3′- to 5′-exonuclease activity of DNA polymerase I.

The lowered specificity of base selection in the presence of Mn^{2+} was found to be due primarily to a marked increase in the k_{cat} value for insertion of a mismatched dNTP, whereas substitution of Mn^{2+} for Mg^{2+} had little effect on the k_{cat} value for insertion of a complementary nucleotide (at optimal cation concentration). The apparent K_M values for insertion of either matched or mismatched nucleotides were not significantly different when Mn^{2+} was substituted for Mg^{2+}. These results are in contrast to those reported by Fersht et al. (1983), who reported significantly lower K_M values for noncomplementary dNTPs in Mn^{2+} vs Mg^{2+}. We believe that the discrepancy between our results and those of Fersht et al. can be explained by the fact that, in the study of Fersht et al., kinetic constants for nucleotides were determined in phosphate buffer when Mg^{2+} was the divalent cation and in tris(hydroxymethyl)-aminomethane (Tris) buffer when Mn^{2+} was the divalent cation. In the present studies as well as our previous studies on manganese mutagenesis (El-Deiry et al., 1984), Hepes buffer was used for determining the kinetic constants of the enzyme with both Mg^{2+} and Mn^{2+} as divalent cation. In light of our results on the inhibitory effect of phosphate ion on the 3′- to 5′-exonuclease activity of DNA polymerase I and, as a consequence, on the rate of turnover of noncomplementary dNTPs, it is likely that buffer effects can account for the discrepancy in results between the two laboratories.

Similar to the results of our previous studies with the poly(dC)–oligo(dG) hook polymer, Mn^{2+} was also found to decrease the specificity of proofreading with poly(dT)–oligo(dA) as substrate for the exonuclease. The rate of hydrolysis of the properly base-paired primer terminus was higher in Mn^{2+} than in Mg^{2+}, whereas the rate of hydrolysis of a mismatched primer terminus was higher in Mg^{2+} than in Mn^{2+}. The decreased ability of the proofreading exonuclease to hydrolyze a mismatched nucleotide at the primer terminus in the presence of Mn^{2+} would be expected to result in increased extension of mismatched primer termini by the polymerase, and, thus, in increased error frequency. The present results support this hypothesis. In the presence of Mg^{2+}, there was very little protection of a mismatched primer terminus from hydrolysis by the exonuclease activity either in the presence of a complementary dideoxynucleoside triphosphate or in the presence of a 5′NMP exonuclease inhibitor, or both. However, when Mn^{2+} was substituted for Mg^{2+}, there was complete protection of the mismatched primer terminus from exonuclease action when both complementary ddNTP and 5′NMP were present. Only partial protection was seen in the presence of either ddNTP or 5′NMP alone. Furthermore, noncomplementary ddNTP could not substitute for complementary ddNTP in protecting the mismatched primer terminus with Mn^{2+} as divalent cation.

The effects of Mn^{2+} on the specificity of base selection and proofreading by DNA polymerase I with both G·C-containing templates and A·T-containing templates are at least qualitatively the same, suggesting that base-specific effects of Mn^{2+} are unlikely to account for its mutagenic effect. On the other hand, Hillebrand and Beattie (1985) have found that with natural DNA templates, the fidelity of DNA polymerase I in the presence of Mn^{2+} versus Mg^{2+} was sequence dependent. In these studies, Mn^{2+}-induced misincorporation was found to correlate with the occurrence of G·C base pairs 5′ to the misincorporation. Since a G·C base pair at the primer terminus stabilizes the primer terminus to excision by the exonuclease activity (Pless et al., 1981; Patten et al., 1984), it is possible that the effects of Mn^{2+} on the specificity of proofreading may account for the sequence-dependent effects seen with natural DNA templates. The results of the present studies are consistent with our previous suggestion that the mutagenicity of Mn^{2+} is likely due to its interaction with the enzyme–template complex, possibly altering the conformation at the active sites of the polymerase and 3′–5′-exonuclease activities. However, whether the effects of Mn^{2+} are primarily on the enzyme conformation or secondary to changes in the DNA structure awaits further studies.

Kinetic proofreading has been proposed as a mechanism for maintaining the fidelity of DNA synthesis whereby a DNA–enzyme–noncomplementary dNTP intermediate is hydrolyzed to free dNMP prior to phosphodiester bond formation (Hopfield, 1974). In this error discrimination mechanism, the hydroxyl group of H_2O competes with the hydroxyl group of the primer terminus in a nucleophilic attack on the incoming dNTP. The present studies demonstrate that the template-dependent conversion of noncomplementary dNTP to dNMP is dependent on the presence of a 3′-hydroxyl group at the primer terminus and requires an active 3′- to 5′-exonuclease. Thus, these findings do not support kinetic proofreading as an important contributor to the high fidelity of DNA synthesis with DNA polymerase I.

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β Subunit of Rat Liver Mitochondrial ATP Synthase: cDNA Cloning, Amino Acid Sequence, Expression in *Escherichia coli*, and Structural Relationship to Adenylate Kinase†

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ABSTRACT: The amino acid sequence of all but a few N-terminal residues of the β subunit of rat liver ATP synthase has been determined from cDNA clones. Rat liver F_1-β is shown to contain 17 amino acid differences from that reported for F_1-β of bovine heart, 2 differences of which involve differences in charge. This may account in part for the observation that bovine heart F_1 binds nucleotides with much greater affinity than the rat liver enzyme. Rat liver F_1-β also contains homologous regions with another nucleotide binding protein, adenylate kinase, for which high-resolution structural studies are available. Adjacent to one of these homologous regions is an eight amino acid stretch which bears striking homology to the phosphorylation region of the (Na^+,K^+)-ATPase. The combination of these two homology regions may constitute at least part of a nucleotide binding domain in F_1-β. Significantly, both rat liver and bovine heart β contain these regions of homology, whereas the 17 amino acid differences between the two enzymes lie outside this region. The possibility of a second nucleotide binding domain which differs between the two enzymes is discussed. A cDNA clone containing all the regions of homology as well as 11 of the 17 amino acid differences between the bovine heart and rat liver β subunits has been ligated into the bacterial expression vector pKK223-3. After transformation of a protease-deficient strain of *Escherichia coli*, this cDNA clone is expressed as a 36-kilodalton protein. Finally, further cDNA library screening and primer extension analysis using several oligonucleotide probes generated cDNAs which always terminated prior to that region of the β gene coding for the N-terminal region. A strong secondary structure of regulatory significance may, therefore, be a unique characteristic of the rat liver mRNA coding for the F_1-β subunit in this region.

The ATP synthase of rat liver mitochondria is responsible for the production of ATP from the respiration-derived electrochemical gradient across the mitochondrial inner membrane. The enzyme complex is composed of two parts, F_1 and F_0, each being a multisubunit complex. The hydrophilic and membrane-extrinsic F_1 complex contains the catalytic regions of the molecule and has been shown to bind and hydrolyze nucleotides [for recent reviews, see Cross (1981), Senior and Wise (1983), Wang (1983), Amzel and Pedersen (1983), and Hatefi (1985)]. The enzyme subunit structure is highly conserved throughout evolution and, from bacteria to mammals, is generally agreed to be composed of five different subunit types in the ratio of $\alpha_3\beta_3\gamma\delta\epsilon$ (Senior & Brooks, 1971; Catterall & Pedersen, 1971; Catterall et al., 1973; Esch & Allison, 1979). The primary sequences of the three largest subunits, α, β, and γ, are also highly conserved (Walker et al., 1982, 1985), and, intriguingly, significant homology exists between the β subunit and numerous other nucleotide binding proteins (Walker et al., 1982; Fry et al., 1985). On the basis of these homologies and on chemical modification studies of β using nucleotide analogues, the suggestion has been raised that the mechanism of nucleotide binding may have similar themes in differing proteins.

Experiments described here were undertaken with a 3-fold purpose in mind. First, obtaining the predicted amino acid sequence of the β subunit will aid in understanding the characteristic nucleotide binding of the rat liver F_1-ATPase. Reports from this and other laboratories indicate that the F_1-ATPase from rat liver binds nucleotides with much less affinity than does the bovine heart enzyme (Catterall & Pedersen, 1972; Cross & Nalin, 1982). Moreover, a maximum of only four nucleotide binding sites are readily detected in the rat liver enzyme (Williams et al., 1987), whereas six sites are readily detected in similar binding assays in the bovine heart enzyme (Cross & Nalin, 1982). These differences between closely related enzymes may arise from primary sequence differences. Second, the acquisition of the F_1-β amino acid sequence will aid and complement the X-ray crystallographic structure determination currently in progress. A 9-Å map of rat liver F_1 has been obtained (Amzel et al., 1982) and is currently being extended with data up to a resolution of 3.5 Å. Third, obtaining a cDNA clone and the development of

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