MANGANESE AS A MUTAGENIC AGENT DURING IN VITRO DNA SYNTHESIS

Dipak K. Dube and Lawrence A. Loeb

The Institute for Cancer Research

The Fox Chase Cancer Center

7701 Burholme Avenue

Philadelphia, Pa. 19111

Received October 1,1975

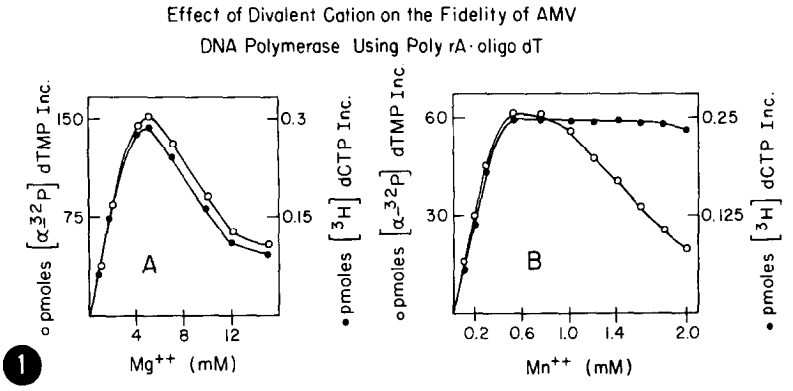
SUMMARY: The effect of Mn^{2+}, a known mutagen, on the fidelity of DNA synthesis in vitro by avian myeloblastosis DNA polymerase has been determined. Substitution of Mn^{2+} for Mg^{2+} leads to an enhanced incorporation of non-complementary deoxynucleotides as well as complementary ribonucleotides with either poly (A) or poly (C) as templates. Since this polymerase lacks any detectable deoxyribonuclease activity, the in vitro mutagenic effect of Mn^{2+} in promoting errors in base-pairing does not result from any diminished proof-reading function.

Manganese has been shown to be mutagenic in vivo (1) and to reduce the fidelity of DNA synthesis in vitro(2). The in vitro studies have been mainly limited to procaryotic DNA polymerases, which have an associated error-correcting 3' → 5' exonuclease (3, 4). Thus it is not known whether the manganese-mediated increase in incorrect nucleotide incorporation with these polymerases is due to the suppression of their associated nucleases, or to an alteration in their base-selective function. To gain insight into the mechanism by which Mn^{2+} alters base-selection, we studied the effect of Mn^{2+} on the fidelity of DNA synthesis using the DNA polymerase from avian myeloblastosis virus (AMV). The high frequency of non-complementary nucleotide incorporation by this enzyme (5) has facilitated studies on the function of polymerases in base-selection. This enzyme is devoid of any detectible exodeoxynuclease activity; it fails to excise mismatched nucleotides present at the 3'-terminus (6). Thus any observed changes in the frequency of non-complementary nucleotide incorporation in the presence of Mn^{2+} would reflect changes in the base-selection process of the enzyme.

We have purified AMV DNA polymerase to homogeneity from isolated virions by the method of Kacian and Spiegelman (7). The ratio of complementary to non-complementary nucleotide incorporation upon fractionation of the holoenzyme (α and β subunits) by a variety of techniques was invariant (8). The effect of the concentration of Mg^{2+} on the incorporation of complementary and non-complementary nucleotides with poly rA·oligo dT and poly rC·oligo dG as template-primers is seen in Fig. 1A and Fig. 2A, respectively. In these experiments, we have measured the simultaneous incorporation of the complementary and non-complementary deoxyribonucleotides for each template using nucleoside triphosphates labelled with different radioactive isotopes (5). With each template-primer the error frequency does not vary as a function of Mg^{2+} concentration.

Mn^{2+} at low concentrations can be substituted for Mg^{2+} in catalysis by all the DNA polymerases which have been studied in detail (9,10). Nuclear magnetic resonance studies on E. coli DNA polymerase I indicate that Mn^{2+} serves to coordinate the enzyme with the α and γ phosphoryl groups of the deoxynucleotide triphosphate substrate (11). We find with AMV DNA polymerase that the error frequency is greater with Mn^{2+} than with Mg^{2+}. With poly rA·oligo dT the frequency of dCMP incorporation in 0.5 mM Mn^{2+} is 1 in 250, while in 6 mM Mg^{2+} it is 1 in 500. Similarly, with poly rC·oligo dG the error rate for dAMP in 0.5 mM Mn^{2+} is 1 in 800, while in 6 mM Mg^{2+} it is 1 in 1400. With increasing concentrations of Mn^{2+} the incorporation of the complementary nucleotide is preferentially reduced. This dichotomy in inhibition by Mn^{2+} is in accord with interpretation of kinetic experiments suggesting multiple substrate binding sites on AMV DNA polymerase (8).

The products of the reaction were analyzed on alkaline sucrose gradients. The size of the products is unaffected by the concentration of non-complementary nucleotide in the reaction mixture (Fig. 3A, B). With poly rA.oligo dT the frequency of dCMP incorporation at 0.5 mM Mn^{2+}



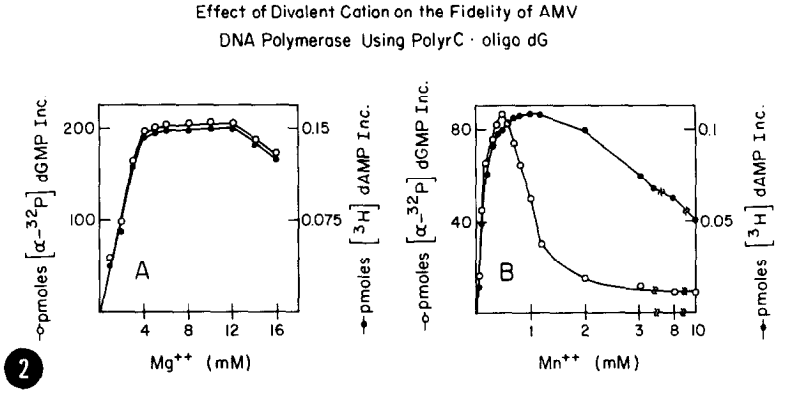


FIGURE 1: Effect of Mg^{2+} and Mn^{2+} on the fidelity of AMV DNA polymerase during catalysis using poly rA·oligo dT as a template-primer. The reaction mixture in a total volume of 25 µl contained the following: 50 mM Tris-HCl, pH 8.0; 5 mM dithiothreitol; 1 µg bovine serum albumin; 20 mM KCl; 20 µM [α-^{32}P]dTTP (5-10 cpm/pmole); 20 µM [^{3}H]dCTP (10,000 cpm/pmole); 1 µg poly rA_{500}·oligo dT_{12-18}[1:1]; 0.2-0.25 µg AMV DNA polymerase and the indicated concentration of Mg^{2+} or Mn^{2+}. The assays were incubated for 30 min at 37°C. Incorporation of the radioactive nucleotides into an acid-insoluble precipitate was determined after repeatedly precipitating the polynucleotide product with 1.0 N perchloric acid and dissolving with 0.2 M NaOH (4). All assays were carried out in triplicate and the average incorporation is listed after subtraction of the amount of incorporation without incubation, 0.005 pmoles for the non-complementary nucleotide.

FIGURE 2: Effect of Mg^{2+} and Mn^{2+} on the fidelity of AMV DNA polymerase during catalysis using poly rC·oligo dG as template-primer. The reaction conditions are the same as in Fig. 1 except 1 µg of poly rC·oligo dG was the template-primer and 20 µM [α-^{32}P]dGTP (5-10 cpm/pmole) and 20 µM [^{3}H]dATP (6000 cpm/pmole) were the substrates. In reaction carried with 6 mM Mg^{2+}, 0.5 mM Mn^{2+} or 20 mM Mn^{2+} the nucleotide content was analyzed by thin layer chromatography after incubation was completed. In all cases, less than 3% of [^{3}H]dGTP was hydrolyzed to dGMP or dG.

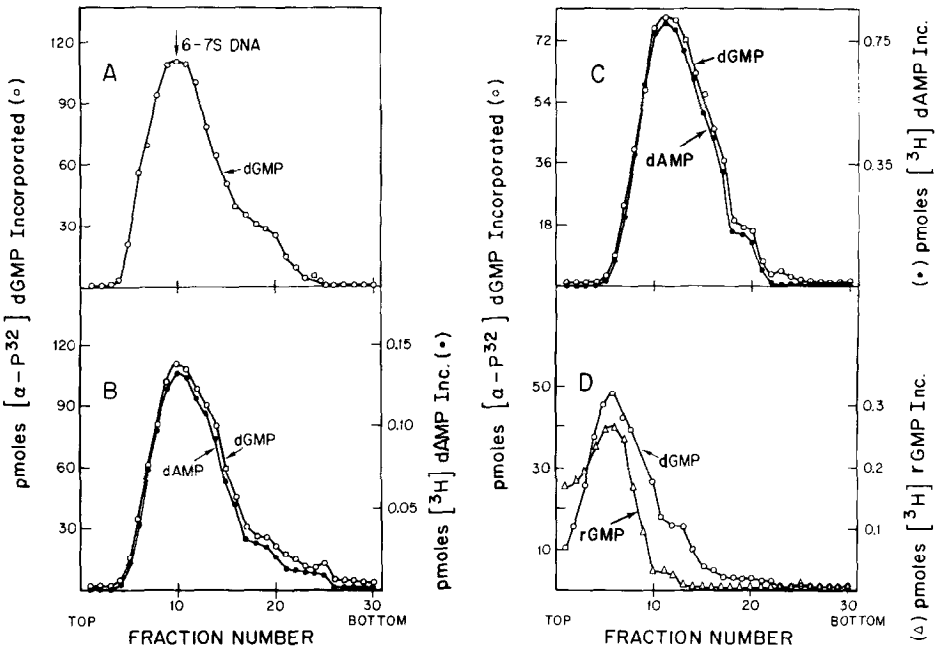


FIGURE 3: Alkaline sucrose sedimentation analysis of poly rC·oligo dG directed product. A) The assay mixture of 0.15 ml consisted of: 50 mM Tris-HCl, pH 8.0; 5 mM dithiothreitol; 0.5 mM $MnCl_2$; 20 mM KCl; 15 µg 10S poly rC·oligo dG [1:1]; 20 µM [α-^{32}P]dGTP (10 cpm/pmole); 1.25 µg AMV DNA polymerase. Incubation was carried out at 37°C for 60 min. The products were extracted with phenol and hydrolyzed with 0.3 M NaOH for 10 min at 90°C. The hydrolysate was layered over a linear 5 to 20% alkaline sucrose gradients (5 ml) containing 0.2% sarkosyl. The gradients were centrifuged in a Spinco SW 50.1 rotor at 40,000 rpm for 12 hr at 20°C. Fractions were collected from the top of the gradients and radioactivity was determined after adding 100 g of calf thymus DNA precipitating with 1.0 N perchloric acid, and collecting onto glass-fiber filters. Marker was ^{3}H-labelled 6-7S DNA. B) The assay mixture was the same except for the presence of 20 µM [^{3}H]dATP (6000 cpm/pmole). C) The reaction mixture was the same as in frame B except that $MnCl_2$ was present at a concentration of 3.5 mM. D) The assay mixture is as in frame A except for the presence of 20 µM [^{3}H]rGTP (4000 cpm/pmole). The increased ratio of ^{32}P to ^{3}H in the faster sedimenting product suggests a biased incorporation of ribonucleotides into larger molecules. The size of the product in alkali (2-4S) is in agreement with the frequency of ribonucleotide incorporation, 1 in 150.

---

is 1 in 800 (Fig. 3B), while at an inhibitory Mn^{2+} concentration of 3.5 mM it was 1 in 90 (Fig. 3C). Most importantly, the ratio of correct to incorrect nucleotide incorporation is constant across the peak, suggesting that the errors are evenly distributed throughout the polynucleotide product.

Mn^{2+} also facilitates the incorporation of complementary ribonucleotides during polymerization of deoxynucleotides. With poly rA as a template and Mn^{2+} as the divalent cation, one rUMP is incorporated for every 2375 dTMP polymerized (Table 1). The non-complementary ribonucleotide rCMP is incorporated, but at a less frequent rate. Under these conditions ribonucleotide incorporation was hardly detected with Mg^{2+}. With poly rC·oligo dG as a template the incorporation of rGMP is greatly enhanced (Table 1). The alkaline lability of the product of the reaction indicates that the ribonucleotides are indeed incorporated into the polynucleotide (Fig. 3D). E. coli Pol I has also been shown to incorporate ribonucleotides; this has been of importance in nucleotide sequencing (12, 13). Since AMV DNA polymerase has no deoxynuclease activity, and can also copy RNA, it offers certain advantages for sequence determination.

Our results indicate that the substitution of Mn^{++} for Mg^{++} results

TABLE I

Effect of Mg^{++} and Mn^{++} on the Incorporation of Ribonucleotide Monophosphates

System	Template	Divalent Metal Ion	pmole Inc. dTMP	pmole Inc. rNMP	Ratio
Control	Poly rA·oligo dT	Mn^{++} (0.5mM)	90	-	-
Plus rUTP	"	"	95	0.04	1/2375
Plus rCTP	"	"	90	0.01	1/9000
Control	"	Mg^{++} (5 mM)	150	-	-
Plus rUTP	"	"	153	0.005	1/31,000
Plus rCTP	"	"	160	0.006	1/27,000
			dGMP	rNMP	
Control	Poly rC·oligo dG	Mn^{++} (0.5mM)	65	-	-
Plus rGTP	"	"	60	0.45	1/135
Control	"	Mg(5 mM)	92	-	-
Plus rGTP	"	"	97	0.01	1/9750

The conditions for reaction are the same as those given in Figs. 1 and 2. In these experiments, the indicated ribonucleoside triphosphate and the complementary deoxyribonucleoside triphosphate were present in the same reaction mixture, each at a concentration of 20 M. The specific activity of [^{3}H]GTP, [^{3}H]CTP and [^{3}H]UTP were 10-50 x 10^3, 10^4 and 8 x 10^3 dpm/pmole, respectively. All reactions were in triplicate and incorporation without incubation was less than 0.005 pmoles.

in more frequent mistakes during catalysis using AMV DNA polymerase. Mn^{++} facilitates the incorporation of non-complementary deoxyribonucleotides as well as complementary ribonucleotides. Since this polymerase lacks any detectable exonuclease, the mutagenic effect of Mn^{2+} involves errors in base-pairing during polymerization and does not result from a diminished "proof-reading" function of this polymerase.

ACKNOWLEDGEMENTS: This study was supported by grants from the National Institutes of Health (CA-11524, CA-12818), and National Science Foundation (BMS-73-06751), by grants to this Institute from the National Institutes of Health (CA-06927, RR-05539), and from an appropriation from the Commonwealth of Pennsylvania.

REFERENCES:

1. Orgel, A. and Orgel, L.E: (1965) J. Mol. Biol., 14: 453.
2. Hall, Z.W. and Lehman, I.R: (1968) J. Mol. Biol., 36: 321.
3. Brutlag, D. and Kornberg, A: (1972) J. Biol. Chem., 247: 241.
4. Englund, P.T: (1971) J. Biol. Chem., 246: 5687.
5. Battula, N. and Loeb, L.A: (1974) J. Biol. Chem., 249: 4086.
6. Battula, N. and Loeb, L.A: (unpublished results).
7. Kacian, D.L., Watson, K.F., Burny, A. and Spiegelman, S:(1971) Biochim. Biophys. Acta., 246: 365.
8. Battula, N., Dube, D.K. and Loeb, L.A: J. Biol. Chem., in press.
9. Loeb, L.A: (1974) in The Enzymes, Ed., P. Boyer, New York, N.Y. Academic Press, Vol. X: 173.
10. Kornberg, T. and Kornberg, A: (1974) in The Enzymes, Ed. P. Boyer, New York, N.Y., Academic Press, Vol. X: 119.
11. Sloan, D.L., Loeb, L.A., Mildvan, A.S. and Feldmann, R.J., J. Biol. Chem. (in press).
12. Sanger, F., Donelson, J.E., Coulson, A.R., Kossel, H. and Fischer, D: (1973) Proc. Nat. Acad. Sci., U.S.A., 70: 1209.
13. Loewen, P.C. and Khorana, H.G: (1973) J. Biol. Chem., 248: 3489.