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Metal-induced infidelity during DNA synthesis

(beryllium ions/divalent cations/DNA nucleotidyltransferase fidelity/mutagenicity of cations)

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ABSTRACT The effect of several divalent cations on the accuracy of DNA replication *in vitro* has been examined. Only Be^{2+} altered the accuracy of DNA synthesis using purified DNA polymerase (DNA nucleotidyltransferase; deoxynucleosidetriphosphate:DNA deoxynucleotidyltransferase; EC 2.7.7.7) from avian myeloblastosis virus. The Be^{2+}-induced base substitutions occurred with all templates and with all nucleotides tested. Analysis of the product by equilibrium density centrifugation and processive hydrolysis with snake venom phosphodiesterase suggested that the noncomplementary nucleotides were present in phosphodiester linkage. Nearest neighbor studies indicated that many of the Be^{2+}-induced errors were present as single base substitutions. The enhancement of error frequency could be duplicated by the pretreatment of the enzyme, but not the template, with Be^{2+}. Glycerol gradient centrifugation dissociated the Be^{2+}–DNA polymerase complex and restored the initial error frequency of the polymerase. Thus, the weak binding of a metal cation to a DNA polymerase could alter the accuracy with which that polymerase copied DNA. Beryllium is a known carcinogen. The potential use of this system as a screening technique to detect chemical mutagens and carcinogens is considered.

Recent studies have investigated the accuracy of DNA replication *in vitro* by the use of highly purified DNA polymerases and synthetic polynucleotide templates of defined composition (1–6). Using the DNA polymerase (DNA nucleotidyltransferase; deoxynucleosidetriphosphate:DNA deoxynucleotidyltransferase; EC 2.7.7.7) from avian myeloblastosis virus (AMV), it is possible to quantitate accurately the incorporation of noncomplementary nucleotides, and to determine the reaction parameters that influence base-substitution *in vitro*. AMV DNA polymerase lacks detectable 3′–5′ proof-reading exonuclease activity (7) and thus may not excise a mismatched nucleotide (8–10).

The error frequency of AMV DNA polymerase is not influenced by most alterations in the reaction parameters (4, 5, 7). Accordingly, we have tested exogenous agents to determine their influence on the error frequency. We now report evidence that the treatment of AMV DNA polymerase by beryllium, a carcinogenic metal, increases base substitutions during polynucleotide replication. Modification of AMV DNA polymerase by beryllium is completely reversible, resulting in the restoration of the initial error frequency of the polymerase.

MATERIALS AND METHODS

Materials. Unlabeled deoxynucleotides were purchased from Calbiochem. Tritium-labeled nucleotides were purchased from New England Nuclear or from Amersham-Searle; ^{32}P-labeled nucleotides were obtained from New England Nuclear.

Poly[d(A-T)] was prepared by a *de novo* catalyzed reaction using *Escherichia coli* DNA polymerase I (11). All other synthetic polynucleotide templates were purchased from P. L. Biochemicals or Miles Laboratories. Beryllium chloride powder (99.9% chemical purity) was purchased from Alpha Products, and beryllium chloride that emits gamma rays ([γ]-$^7BeCl_2$) (99% radiometric purity) was purchased from New England Nuclear. Snake venom phosphodiesterase, micrococcal nuclease, and spleen phosphodiesterase were products of Worthington.

Purification of AMV DNA Polymerase. AMV was separated from the plasma of infected chickens by velocity and equilibrium centrifugations. The virions were disrupted (12) and the polymerase was purified by chromatography on DEAE-cellulose and on phosphocellulose (13).

DNA Polymerase Assay. Reaction mixtures (total volume 0.05 ml) in which complementary nucleotide incorporation was measured, contained 50 mM Tris·HCl (pH 8.0), 20 mM KCl, 5 mM dithiothreitol, 5 mM $MgCl_2$, 2 μg of bovine serum albumin, 25 μM [α-^{32}P]dTTP (60 dpm/pmol), 1 μg of poly(A)·oligo(dT) [1:1.5 mass ratio, $(rA)_{2000}\cdot(dT)_{12-18}$], and 0.45 μg of AMV DNA polymerase. For simultaneous quantitation of complementary and noncomplementary nucleotide incorporation, each reaction was modified to contain 26 μM [α-^{32}P]-dTTP (12 dpm/pmol) and 23 μM [^{3}H]dCTP (50,000 dpm/pmol). With 1 μg of poly(C)·oligo(dG) [$(rC)_{1500}\cdot(dG)_{12-18}$] as a template, the reaction mixture contained 25 mM Tris·HCl (pH 8.0), 10 mM KCl, 3 mM $MgCl_2$, 3 mM dithiothreitol, 20 μM [α-^{32}P]dGTP (4 dpm/pmol), 19.2 μM [^{3}H]dATP (20,000 dpm/pmol), and 0.45 μg of AMV DNA polymerase. Metals were added directly to the reaction mixture as aqueous solutions of chloride salts. All reaction mixtures were incubated for 60 min at 37°. Incorporation of radioactive nucleotides into an acid-insoluble and alkali-resistant product was determined by using the precipitation-solubilization procedure (4). All experiments were performed in triplicate and the averages were determined. All experiments were repeated several times, and incorporation of the noncomplementary deoxynucleotide was at least twice the incorporation at zero time in each experiment.

RESULTS

Metal requirements for catalysis by AMV DNA polymerase

All known DNA polymerases require either Mg^{2+} or Mn^{2+} as an added divalent cation for activity (14). As is shown in Fig. 1, AMV DNA polymerase used Mg^{2+} as an added divalent cation, and the maximum rate of polymerization with poly(A)·oligo(dT) was attained at 5 mM Mg^{2+}. In the absence of Mg^{2+}, the addition of Be^{2+} in concentrations as great as 15 mM did not result in detectable poly(dT) synthesis. Thymidine monophosphate incorporation with Be^{2+} was less than 0.3% of that obtained with 5 mM Mg^{2+}. Similarly, the divalent cations Ca^{2+} and Ba^{2+} were unable to substitute for Mg^{2+}. [However, preliminary results suggest that Co^{2+} and Ni^{2+} serve as metal activators with several DNA polymerases (results not shown).]

Abbreviations: AMV, avian myeloblastosis virus; poly(C)·oligo(dG), $(rC)_{1500}\cdot(dG)_{12-18}$; poly(A)·oligo(dT), $(rA)_{2000}\cdot(dT)_{12-18}$.

Table 1. Effects of divalent cations on complementary and noncomplementary nucleotide incorporation

Divalent cations	Complementary nucleotide (pmol of dTMP)	Noncomplementary nucleotide (pmol of dCMP)	Error frequency
Mg^{2+} (2 mM)	320	0.56	1/571
(5 mM)	600	1.16	1/517
(8 mM)	520	1.00	1/520
(15 mM)	540	0.90	1/600
Mg^{2+} (5 mM)	990	0.878	1/1128
+ Be^{2+} (2 mM)	291	0.372	1/782
+ Be^{2+} (5 mM)	133	0.294	1/453
+ Be^{2+} (10 mM)	8.6	0.114	1/75
Mg^{2+} (5 mM)	839	0.990	1/848
+ Ca^{2+} (1mM)	704	0.700	1/1005
+ Ca^{2+} (2 mM)	129	0.190	1/681
+ Ca^{2+} (5 mM)	55	0.083	1/665
Mg^{2+} (5 mM)	839	0.99	1/848
+ Sr^{2+} (4 mM)	798	0.86	1/927
+ Sr^{2+} (6 mM)	936	0.96	1/975
+ Sr^{2+} (10 mM)	845	0.86	1/982
Mg^{2+} (5 mM)	232	0.38	1/610
+ Ba^{2+} (2 mM)	240	0.42	1/578
+ Ba^{2+} (6mM)	243	0.46	1/528
+ Ba^{2+} (10 mM)	317	0.61	1/520

The frequency of noncomplementary nucleotide incorporation was determined as described in *Materials and Methods.*

Influence of metals on fidelity

In order to determine the effect of different metals on the accuracy of DNA replication, metal cations were added as chloride salts directly to the polymerase reaction. In these experiments, poly(A)·oligo(dT) was the template-primer and the simultaneous incorporation of complementary nucleotide, [α-^{32}P]dTMP, and the noncomplementary nucleotide, [^{3}H]dCMP, was determined. The error frequency, i.e., the ratio of noncomplementary to complementary nucleotide incorporated, was invariant with respect to Mg^{2+} concentration (Table 1). Only upon the addition of Be^{2+} was the error frequency increased. In this experiment, AMV DNA polymerase incorporated one noncomplementary nucleotide for every 1128 complementary nucleotides polymerized with 5 mM Mg^{2+}. The addition of graded amounts of Be^{2+} resulted in a nearly proportional increase in the frequency of dCMP incorporation. With 10 mM Be^{2+}, the error frequency was increased 15-fold to 1/75. The addition of Ca^{2+} inhibited in parallel the incorporation of the complementary and noncomplementary nucleotide and thus did not alter the error frequency. The addition of Ba^{2+} or Sr^{2+} did not affect the incorporation of either the complementary or the noncomplementary nucleotides. Similarly, various chemicals lacking known carcinogenic or mutagenic activity did not alter the fidelity of DNA synthesis in this system. These include spermidine (0.1–5 mM), KCl (5–300 mM), alteration in pH (6.0–8.5), caffeine (0.1–20 mM), and retinol (0.2–4 μg). The increased error frequency induced by Be^{2+} was not limited to poly(A)·oligo(dT) as the template nor to dCTP as the noncomplementary nucleotide (Table 2), suggesting that the interaction of Be^{2+} is with the polymerase itself.

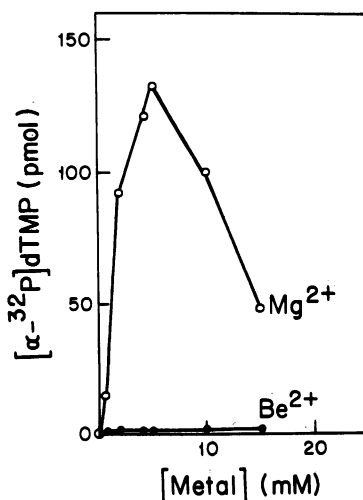

FIG. 1. Divalent cation requirement for catalysis. The reaction components are given in *Materials and Methods,* with the indicated amounts of $MgCl_2$ or $BeCl_2$. The reaction mixtures were incubated at 37° for 60 min, and acid-insoluble radioactivity was determined. Poly(A)·oligo(dT) was used as the template.

Product analysis

The products of reactions with and without Be^{2+} were analyzed by cesium sulfate equilibrium density gradient centrifugation (Fig. 2). Polynucleotide products from reactions that did not contain Be^{2+} banded at a density of 1.45, which corresponds to a 1:1 hybrid of poly(C)·poly(dG) (Fig. 2a). Polynucleotide products from reactions that contained Be^{2+} also banded at the same density (Fig. 2b). With both products all of the acid-precipitated ^{3}H radioactivity was found at this density and the error frequency was the same before and after gradient centrifugation. The error frequency of the isolated product synthesized in the presence of Be^{2+} was 3- to 4-fold higher than that synthesized in the absence of Be^{2+}.

When any of the reaction components required for polymerization (enzyme, template, Mg^{2+}, or the complementary nucleotide) were deleted, the incorporation of the noncomplementary nucleotide was less than 30% of that obtained

Table 2. Effect of Be^{2+} on catalysis with different template-primers

Template-primer	Noncomplementary nucleotide	$BeCl_2$ (mM)	Nucleotide incorporation (pmol) Complementary	Noncomplementary	Error frequency
Poly(C)·oligo (dG)	dATP	0	520	0.270	1/1925
		1	181	0.110	1/1649
		2	81	0.079	1/1025
		4	57	0.062	1/919
		5	64	0.077	1/831
Poly[d(A-T)]	dCTP	0	337	0.240	1/1402
		1	209	0.160	1/1306
		3	137	0.140	1/979
		5	97	0.140	1/689
Poly(A)·oligo (dT)	rGTP	0	610	0.074	1/8245
		1	568	0.093	1/6104
		2	503	0.186	1/2704
		5	128	0.263	1/487
		10	52	0.349	1/149

Reaction conditions for poly(A)·oligo(dT) and poly(C)·oligo(dG) are described in *Materials and Methods*. Error analysis using poly-[d(A-T)] was determined in a reaction mixture (total volume 0.05 ml) that contained 50 mM Tris·HCl (pH 8.0), 20 mM KCl, 4 mM dithiothreitol, 5 mM $MgCl_2$, 1 μg of bovine serum albumin, 100 μM dATP, 100 μM [α-^{32}P]dTTP (4 dpm/pmol), 100 μM [^{3}H]dCTP (25,000 dpm/pmol), 1 μg of poly[d(A-T)], and 0.45 μg of AMV DNA polymerase. Reaction mixtures were incubated at 37° for 60 min.

during polymerization. In addition, processive exonuclease hydrolysis of the reaction product synthesized with Be^{2+} released noncomplementary and complementary nucleotides in constant proportions (data not shown). These combined results are identical to those previously reported for control experiments (4, 15). A more exacting analysis of the incorporation of noncomplementary nucleotides during polymerization was provided by nearest neighbor analysis (Table 3). In the product synthesized without Be^{2+}, 80% of the radioactivity incorporated as [α-^{32}P]dCMP was transferred to dTMP and thus represented single base substitutions. In the product synthesized with Be^{2+} only 27% of the ^{32}P is transferred, suggesting that there was an increase in the clustering of base substitutions. More importantly, since ^{32}P is derived from the noncomplementary nucleotide (dCTP) and ^{3}H is derived from the complementary nucleotide (dTTP), the ratio of ^{32}P to ^{3}H in the recovered dTMP

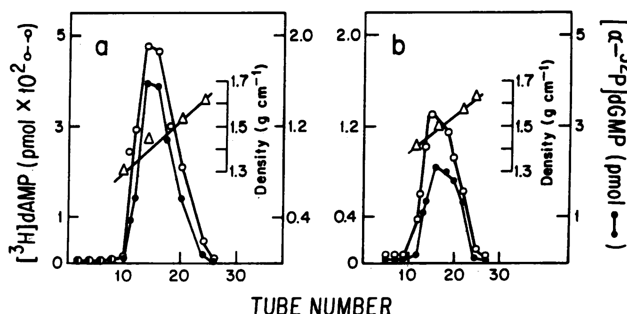

FIG. 2. Cesium sulfate sedimentation analysis of the reaction product. The reaction mixture (total volume 0.5 ml) consisted of 25 mM Tris·HCl (pH 8.0), 10 mM KCl, 3 mM $MgCl_2$, 3 mM dithiothreitol, 20 μM [α-^{32}P]dGTP (5 dpm/pmol), 20 μM [^{3}H]dATP (30,000 dpm/pmol), 10 μg of poly(C)·oligo(dG), and 4.5 μg of AMV DNA polymerase. The mixture was incubated for 60 min at 37°, and the product was isolated as described in *Materials and Methods*. The product was suspended in a $CsSO_4$ solution (final density 1.5 g cm^{-1}). The final volume was 5.0 ml and contained 0.2% Sarkosyl. The product was centrifuged in a Spinco SW 50.1 rotor for 72 hr at 20°. Fractions containing 8 drops each were collected from the top of the gradient. One milliliter of 10% trichloroacetic acid and 100 μg of denatured calf thymus DNA were added to each fraction and the precipitate was collected onto glass fiber papers. (a) Standard reactions; (b) with Be^{2+}.

indicated the frequency of single base substitutions. Thus, with Be^{2+} there was a 15-fold increase in the frequency by which dCMP occupied the position adjacent to the 3′-end of dTMP throughout the polynucleotide product.

Site of beryllium action

In order to determine whether Be^{2+} forms a stable complex with either the enzyme or the template-primer, preincubation experiments were performed (Table 4). AMV DNA polymerase or poly(A)·oligo(dT) was preincubated with 3.3 mM Be^{2+}. The remaining reaction components were then added, which reduced the Be^{2+} concentration to 0.66 mM. This concentration of Be^{2+} was below that required for increased error incorporation. As shown in Table 4, pretreatment of the template-primer at 3.3 mM Be^{2+} did not increase noncomplementary nucleotide incorporation when assays were performed at 0.66 mM Be^{2+}. The error frequency of (1/716) was within experimental error identical to that of a concurrent control (1/736). However, pretreatment of the enzyme in this experiment resulted in an increased error frequency of 1/227. Similar increases were observed upon repetition of the experiment. These results suggested that Be^{2+}-facilitated error incorporation occurred through a polymerase–Be^{2+} complex, and these results

Table 3. Nearest neighbor analysis

$BeCl_2$ (mM)	Transfer of ^{32}P after hydrolysis %C → T	%C → C	Ratio of ^{32}P to ^{3}H in dTMP
0	80	20	0.06
2	27	73	0.88

Polynucleotide products were made in the assay mixture as described in *Materials and Methods* except that 25 μM [^{3}H]dTTP (1700 dpm/pmol) and 100 μM [α-^{32}P]dCTP (9300 cpm/pmol) were used. The product was isolated and hydrolyzed with micrococcal nuclease and spleen phosphodiesterase as described (16). The extent of hydrolysis was greater than 95%. The deoxynucleoside monophosphates were separated by one-dimensional thin-layer chromatography in isopropanol:HCl:water (65:16.7:18.3), as described (17).

Table 4. Effect of pretreatment of reaction constituents with beryllium on error incorporation

Initial $[Be^{2+}]$ (mM)	Pretreated constituent	Final $[Be^{2+}]$ (mM)	Nucleotide incorporation (pmol)		Error frequency
			Complementary	Noncomplementary	
0		0	751	1.02	1/736
3.3	Poly(A)·oligo(dT)	0.66	315	0.44	1/716
3.3	AMV DNA polymerase	0.66	41	0.18	1/227

Beryllium chloride was added to AMV DNA polymerase, to poly(A)·oligo(dT), and to the remaining reaction components at a concentration of 3.3 mM. These solutions were preincubated at 37° for 10 min. The remaining reaction components were then added, and the total reaction mixtures were incubated at 37° for 60 min for error analysis. The addition of the remaining components resulted in a final beryllium concentration below that required to increase error incorporation. Error analysis was performed in a reaction mixture (total volume 0.1 ml) that contained 50 mM Tris·HCl (pH 8.0), 20 mM KCl, 5 mM $MgCl_2$, 5 mM dithiothreitol, 4 μg of bovine serum albumin, 26 μM [α-^{32}P]dTTP (12 dpm/pmol), 23 μM [^{3}H]dCTP (50,000 dpm/pmol), 2 μg of poly(A)·oligo(dT) (1:1.5 ratio), and 0.9 μg of AMV DNA polymerase.

could reflect the slow dissociation rate characteristic of Be^{2+} (36).

Dissociation of metal-polymerase complex

Studies were initiated to determine whether this complex could be dissociated and whether the normal error frequency of AMV DNA polymerase could be restored. Since Be^{2+} readily coordinates with free hydroxyl groups (18), glycerol gradient sedimentation was used to separate the Be^{2+}–polymerase complex. Analysis using [γ]-$^7BeCl_2$ (Fig. 3A and B) indicated that during centrifugation the majority of the Be^{2+} readily dissociated from the polymerase. The initial Be^{2+} concentration of 5 mM was reduced to less than 1 μM, which corresponds to less than 5 g-atoms of Be^{2+} per mole of polymerase. In a separate experiment, AMV DNA polymerase was pretreated with and without 5 mM Be^{2+}. With poly(A)·oligo(dT) as the template, the error frequency of AMV DNA polymerase was 1/900 in the absence of Be^{2+}. With 5 mM Be^{2+}, the error frequency was increased to 1/26. The metal–polymerase complex was then centrifuged in a glycerol gradient. The treated polymerase sedimented to a position identical to that of the AMV DNA polymerase treated with [γ]-$^7BeCl_2$ (Fig. 3B). Polymerase activity and error frequency were then determined. The error frequency of the peak tubes was 1/1000, as compared to a concurrent, untreated, control of 1/900. Moreover, synthesis was equal to that of

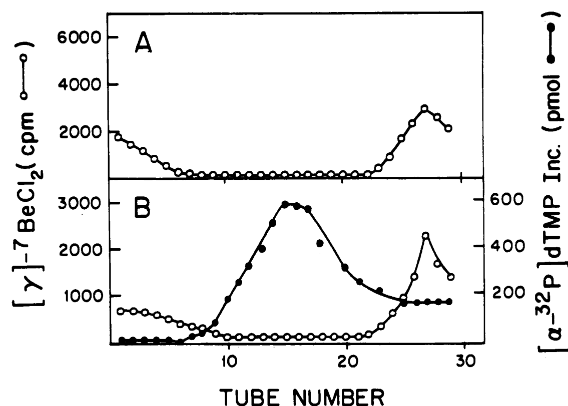

FIG. 3. Glycerol gradient analysis of beryllium-treated AMV DNA polymerase. Beryllium (5 mM) was added to AMV DNA polymerase. The mixture was incubated for 10 min at 37°. The solution was layered on a 10–35% glycerol gradient that contained 10 mM Tris·HCl (pH 7.5), 100 mM NaCl, and 1 mM K_2EDTA (pH 7.0). The solution was centrifuged in a Spinco SW 50.1 rotor at 40,000 rpm for 16 hr at 5°. Fractions containing 4 drops each were collected from the top of the gradient. In experiments using [γ]-$^7BeCl_2$, 16.5 μCi of radioactive Be^{2+} was added to the reaction mixtures. (A) [γ]-$^7BeCl_2$; (B) [γ]-$^7BeCl_2$ plus AMV DNA polymerase.

control gradients. These results suggested that the binding of Be^{2+} to AMV DNA polymerase was completely reversible, since the initial error frequency and the initial activity were restored.

DISCUSSION

DNA polymerases may have a common mechanism for catalysis. They require Mg^{2+} or Mn^{2+} for catalytic activity (14) and are known to contain stoichiometric quantities of zinc (19–23). Kinetic and nuclear magnetic resonance studies indicate that the added divalent cations, Mg^{2+} or Mn^{2+}, coordinate the nucleotide substrate with the enzyme (24) and suggest that zinc functions in the binding to the primer terminus. Be^{2+} does not appear to interact at the catalytically active Mg^{2+} sites, since Be^{2+} is unable to substitute for Mg^{2+} during catalysis and does not alter the concentration of Mg^{2+} required for maximal activity. It is unlikely that Be^{2+} substitutes for Zn^{2+}, since alteration at the primer site would not be expected to directly effect fidelity. It has been reported (25) that Be^{2+} inhibits the 3′ → 5′ proof-reading exonuclease of *Micrococcus luteus* DNA polymerase, resulting in error incorporation. AMV DNA polymerase lacks detectable 3′ → 5′ proof-reading exonuclease activity (7). Since AMV DNA polymerase cannot excise a mismatched nucleotide during polymerization (7), thus the effect of Be^{2+} on this polymerase could not be mediated by a diminution of a possible proof-reading function (16).

In this report we show that a metal cation can bind to a DNA polymerase at a noncatalytic site and alter the accuracy with which that polymerase copies DNA. Since this increase in error frequency occurs with various templates and nucleotides, it seems reasonable that similar infidelity would occur with natural DNA and RNA templates. These results suggest that the environment that surrounds the DNA replication complex may influence the accuracy of DNA replication. Thus, a momentary localization of a carcinogen may induce base substitution. Even though the effect of the metal carcinogen (26, 27) on the polymerase may be reversible, the results of this interaction would be a base-substitution in DNA.

It is proposed that the accuracy of polynucleotide replication *in vitro* could be used as a screening mechanism to detect chemical mutagens or carcinogens. To evaluate this system, it will be essential to document the correlations among mutagenicity, carcinogenicity, and alterations in fidelity. A large number of substances with diverse biological functions do not alter the accuracy of DNA replication in this *in vitro* system. Of the plethora of reaction parameters and biological compounds that have been tested, the only parameters that influence noncomplementary incorporation are (*a*) the substitution of magnesium by manganese (28), (*b*) the alteration of a specific template by a chemical carcinogen (15, 29), (*c*) extreme changes

in the ratio of nucleotide substrate concentrations (4, 5), and (*d*) Be^{2+} modification of AMV DNA polymerase. It is important to state that there may exist two possible exceptions to the proposed correlation between reduced fidelity and carcinogenesis. First, Mn^{2+} is a potent mutagen (30), but has not yet been reported to be a carcinogen. Second, preliminary results suggest that nickel chloride slightly affects noncomplementary nucleotide incorporation during Mg^{2+}-activated catalysis although nickel powder has been reported to induce tumors at the site of application (31). However, we have recently demonstrated that Ni^{2+} can substitute for Mg^{2+} with various DNA polymerases (manuscript in preparation) and it will be of interest to determine the accuracy of Ni^{2+}-activated DNA polymerization.

Many chemicals require activation by cellular enzymes (32, 33) to become carcinogenic. Ames has been able to surmount this difficulty by using a cell-free activating system coordinate with bacterial reversion analysis (34, 35). Presumably a similar activation system may be required in specific instances for this analysis *in vitro*. If infidelity tightly correlates with carcinogenesis, these studies may provide compelling evidence that DNA is the critical target of chemical carcinogens.

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