Metal-induced Infidelity of DNA Synthesis

R. A. Zakour¹, L. K. Tkeshelashvili², C. W. Shearman¹,
R. M. Koplitz¹, and L. A. Loeb¹

¹ The Joseph Gottstein Memorial Cancer Research Laboratory Department of Pathology SM-30,
University of Washington, Seattle, WA 98195, USA
² Institute of Physics, Academy of Sciences of the Georgian S.S.R. Tbilisi, 380077 USSR

Summary. A number of metals have been demonstrated to be mutagens in pro-
caryotic and eucaryotic organisms as well as carcinogens in experimental ani-
mals. Epidemiologic studies have indicated that Ni, Cr, and As are involved in
human carcinogenesis. We have hypothesized that the active molecular species is
the cation and that metal induced mutations result from incorrect base-substi-
tutions during DNA replication. This is supported by the observations that
metal ions diminish the fidelity of DNA synthesis in vitro using a variety of
DNA polymerases. There is a significant correlation between the metals that
decrease fidelity and those that have been reported to be mutagenic and car-
cinogenic. Thus, metal carcinogens are no exception to the general postulate
that carcinogens can be identified by their effects on DNA.

Key words: Metal ions – DNA polymerase – Miscoading

A number of metals have been shown to be mutagens and carcinogens (Sunderman
1978) and to affect the accuracy of DNA replication (Sirover and Loeb 1976). In
vivo systems are too complicated to begin to unravel the mechanisms by which
metals induce mutations and the effects of metal ions on the fidelity of DNA rep-
lication. Our approach to this problem has been to examine DNA synthesis in vi-
tro, to determine the effects of different metal ions on the fidelity of this process,
and then to ask whether alterations in the fidelity of DNA synthesis are related to
the mutagenic and carcinogenic properties of these metals.

Until very recently, all assays of the fidelity of DNA synthesis in vitro measured
the ability of DNA polymerases to copy homopolymer or alternating copolymer
templates. These templates contained only one or two nucleotides and the
mismatched nucleotide was identified simply as one not complementary to the tem-
plate nucleotides. Using this assay, the effects of both activating and nonactivating
metals on the fidelity of DNA synthesis have been studied. The template that we
have chosen for critical measurements of fidelity is poly (d(A – T)), a synthetic
polynucleoside consisting of deoxythymidine and deoxyadenosine monophosphates. Poly (d(A-T)) can be synthesized to contain less than 1 in $2 \times 10^6$ mistakes, using de novo reaction with E. coli DNA polymerase I (Agarwal et al. 1979). Copied correctly, only dAMP and dTMP should be incorporated into the newly synthesized product. By using ($\alpha$-32P)-dTTP, unlabeled dATP and (3H)-dGTP or (3H)-dCTP, one can simultaneously measure the incorporation of complementary and non-complementary nucleotides (Battula and Loeb 1975). The incorporation of either dCTP or dGTP would represent errors. The frequency of mis-incorporation is obtained from the ratio of (3H) to (32P) in the acid-insoluble product. Control experiments have been done to show that the (3H) label in the reaction product is in the non-complementary nucleotides and not in any radioactive contaminants.

Measurements of the frequency of mis-incorporation by DNA polymerases when copying polynucleotide templates have been summarized in a recent review (Loeb et al. 1979). In general, they vary from $10^{-3}$ for DNA polymerases from RNA tumor viruses to $10^{-5}$ for prokaryotic DNA polymerases. In this paper we review the effects of metals on the fidelity of DNA using poly (d(A-T)) as a template to report initial results using a biologically active natural DNA template, DNA from the bacteriophage $\phi$ X 174.

**Results**

A. Metal Activators and Fidelity

The activating metal for DNA polymerase in vivo is Mg$^{2+}$. In vitro, DNA polymerases from animal (Wang et al. 1974), viral (Seal et al. 1979) and bacterial sources can also use Mn$^{2+}$, Co$^{2+}$, or Ni$^{2+}$ as the activating metal. For example, with avian myeloblastosis virus DNA polymerase and a DNA template, the maximal rates of nucleotide incorporation with Mn$^{2+}$, Co$^{2+}$, and Ni$^{2+}$ were 65%, 25%, and 7%, respectively, of that achieved with Mg$^{2+}$ (Sirover and Loeb 1977). Minimal activity has also been reported with Zn$^{2+}$. The effects of Mg$^{2+}$, Mn$^{2+}$, and Co$^{2+}$ concentrations on the incorporation of complementary and noncomplementary nucleotides with poly (d(A-T)) as a template has been measured. At activating concentrations of Mg$^{2+}$ (2 mM), human placenta DNA polymerase-β incorporates one molecule of dGTP for every 40,000 molecules of dTTP and dATP polymerized (Seal et al. 1979). This error rate is invariant with respect to Mg$^{2+}$ concentration. At the optimal activating concentration of Mn$^{2+}$ (0.1 mM), the error rate with the same enzyme is 1:15,000. At greater than activating concentrations of Mn$^{2+}$ there is a progressive decrease in the incorporation of the complementary nucleotide but not of the non-complementary nucleotide, thus yielding a further increase in the frequency of misincorporation. At concentrations as great as 2 mM, the error rate approached 1:3,600. A similar enhancement in the error rate is observed with both activating and inhibiting concentrations of Co$^{2+}$. The decreased fidelity with increased Mn$^{2+}$ concentration has been observed with all templates and noncomplementary nucleotides tested.

The enhancement of misincorporation with an alternate metal activator appears to be a characteristic finding with DNA polymerases (Table 1). For example, substitution of Mn$^{2+}$ for Mg$^{2+}$ results in an increase in misincorporation by *E. coli* DNA polymerase I (Sirover et al. 1979), T4 DNA polymerase (Hall and Lehman...
Metal-induced Infidelity of DNA Synthesis

Table 1. Effect of metal activator on fidelity

| DNA polymerase     | Mg<sup>2+</sup> (5 mM) | Mn<sup>2+</sup> (0.1 mM) | Mn<sup>2+</sup> (2 mM) | Co<sup>2+</sup> (0.4 mM) | Co<sup>2+</sup> (5 mM) |
|--------------------|------------------------|-------------------------|-----------------------|-------------------------|------------------------|
| AMV                | 1/1,680                | 1/760                   | 1/500                 | 1/1,100                 | 1/200                  |
| E. coli I          | 1/20,000               | 1/10,000                | 1/1,000               | 1/7,500                 | 1/7,000                |
| Human placenta-α   | 1/6,000                | 1/1,900                 | 1/300                 | 1/1,300                 | 1/450                  |
| Human placenta-β   | 1/20,000               | 1/9,000                 | 1/2,000               | 1/5,000                 | 1/1,300                |

All assays were carried out with poly (d(A-T)) as a template and dATP, (α<sup>32</sup>P)-dTTP and (γ<sup>3</sup>H)-dGTP at 25 μM (Kunkel and Loeb 1979).

1968), DNA polymerases-α and -β (Linn et al. 1976; Seal et al. 1979), and avian myeloblastosis virus DNA polymerase (Battula and Loeb 1975; Dube and Loeb 1975). The fact that Mn<sup>2+</sup> and Co<sup>2+</sup> alter the fidelity of the DNA polymerase that do not have an associated exonuclease (AMV, DNA polymerase-α and -β) indicates that these metal ions do not promote misincorporation by inhibiting an error-correcting exonucleolytic activity. Ni<sup>2+</sup> can also substitute for Mg<sup>2+</sup> as a metal activator. However, the amount of synthesis achieved with Ni<sup>2+</sup> as the metal activator has not been sufficient to accurately measure the changes in the fidelity of DNA synthesis with any DNA polymerase except AMV DNA polymerase, in which case Ni<sup>2+</sup> also promotes misincorporation (Sirover and Loeb 1977).

To relate the measurements with alternate metal activators to a situation that would be expected to occur in cells, the effects of these activators on Mg<sup>2+</sup>-activated DNA synthesis have been investigated. Co<sup>2+</sup>, Mn<sup>2+</sup>, and Ni<sup>2+</sup> have been shown to enhance misincorporation by DNA polymerases in the presence of activating amounts of Mg<sup>2+</sup> (Dube and Loeb 1975; Miyaki et al. 1977; Seal et al. 1979). Thus, these metal activators could alter the fidelity of DNA polymerases in cells even in the presence of Mg<sup>2+</sup>.

B. Non-activating Metal Ions and Fidelity

Beryllium, a known animal carcinogen, has been shown to decrease the fidelity of catalysis with M. luteus DNA polymerase (Luke et al. 1975) and AMV DNA polymerase (Sirover et al. 1979). Be<sup>2+</sup> is unable to substitute for Mg<sup>2+</sup> as a metal activator. However, as a non-activating cation, Be<sup>2+</sup> alters the fidelity of DNA synthesis in the presence of Mg<sup>2+</sup>. Preincubation of the enzyme but not the template, primer or substrates with high concentrations of Be<sup>2+</sup> resulted in an increased error rate (Sirover et al. 1979). This finding suggests that Be<sup>2+</sup> can interact with some non-catalytic site on DNA polymerase and thereby alter the fidelity of DNA synthesis. Be<sup>2+</sup> has also been shown to alter the fidelity of DNA polymerase-α from human fibroblasts (Radman et al. 1977), DNA polymerases-α and -β from human placenta and E. coli DNA polymerase I (Seal et al. 1979).

C. Screening for Metals that Alter Fidelity

To date, over 40 metal compounds have been tested in graded concentrations for their effects on the fidelity of DNA synthesis. The method of analysis and the re-
FIDELITY ASSAY
SCREEN FOR MUTAGENS AND/OR CARCINOGENS

In Vitro Assay

- Template: poly [d(A-T)]
- Correct Substrates: dATP + [α-32P]dTP
- Incorrect Substrate: [3H]dGTP
- DNA Polymerase
- Mg2+

+ Exogenous Agents
  (Metal Cations)

Known Carcinogens and/or Mutagens

| Increased Misincorporation | No Change in Fidelity |
|---------------------------|-----------------------|
| Ag                        | Ag                    | Al                     |
| As                        | Be                    | As                     |
| Be                        | Cd                    | Ba                     |
| Cd                        | Co                    | Co                     |
| Co                        | Cr                    | Fe                     |
| Cr                        | Cu                    | K                      |
| Cu                        | Mn                    | Rb                     |
| Mn                        | Ni                    | Mg                     |
| Ni                        | Pb                    | Na                     |
| Pb                        |                       | Se                     |
|                           |                       | Sr                     |
|                           |                       | Zn                     |

Fig. 1. Screening for metal ions that alter fidelity. The data for this table is compiled from references (Sirover and Loeb 1976; Tkeshelashvili et al. 1979; Tkeshelashvili et al. 1980)

Results are summarized in Fig. 1. In the initial study, 22 of these metal salts were tested using a triple-blind protocol in which the assays, computations, and designation of each unknown compound with respect to fidelity were carried out independently (Sirover and Loeb 1976). Compounds which increased infidelity by more than 30% at two or more concentrations were scored as positive. Metals were designated as carcinogens or mutagens by an evaluation of the literature prior to assessment of their effects on fidelity. An enhancement in the infidelity of DNA synthesis was observed with all of the known mutagens and/or carcinogens tested (Ag, Be, Cd, Co, Cr, Mn, Ni, Pb). The evidence in the literature on the mutagenicity or carcinogenicity of three of the metal cations was considered equivocal. Of these, Cu2+ increased misincorporation; Fe2+ and Zn2+ did not alter fidelity. All other metal salts that were tested were considered to be neither carcinogenic nor mutagenic, and they did not increase misincorporation. Only a few of the metal salts that did not alter fidelity are listed in Fig. 1.

With only a few exceptions, these results have been confirmed by Miyaki et al. (1977) and Sirover et al. (1979), using E. coli DNA polymerase I, and by Seal et
al. (1979), using DNA polymerases-α and -β from human placenta. Most recently, we have observed that neither arsenic (AsO₄, As₂O₃) nor selenium (SeO₂) diminish fidelity with *E. coli* DNA polymerase I (Tkeshelashvili et al. 1979). Furthermore, selenium, which has been reported to have an anticarcinogenic effect, does not reduce the mutagenic effect of manganese in titration experiments; containing varying amounts of these two metal ions. We have also examined the effect of different ionic species of chromium and have found that both Cr(III) and Cr(VI) alter the fidelity of *E. coli* DNA polymerase I (Tkeshelashvili et al. 1979). Chain initiation by RNA polymerases can be stimulated by Pb²⁺, Cd²⁺, Co²⁺, Cu²⁺, and Mn²⁺, whereas Zn²⁺, Mg²⁺, Li⁺, Na⁺, and K⁺ are inhibitory (Hoffman and Niyogi 1977). The similarity between the effects caused by particular metal ions on fidelity with DNA polymerases and on chain initiation with RNA polymerase could suggest that metal interaction with the DNA template is the common underlying mechanism for these two phenomena.

**D. Fidelity of DNA Synthesis with Natural DNA Templates**

All of the aforementioned studies on the effects of metal ions on the fidelity of DNA synthesis depended on measuring the incorporation of non-complementary nucleotides using synthetic polynucleotide templates of limited composition. It has been assumed that the results with such a model system are similar to those that would be obtained copying natural DNA containing all four bases. It is known, however, that slippage of the primer relative to the template can occur when
Table 2. Effect of metal activator on the fidelity of copying \( \Phi X174 \)

| Metal ions       | Concentration (mM) | Nucleotides incorporated \( \Phi X174 \) template | Error rate |
|------------------|--------------------|-----------------------------------------------|------------|
| Mg\(^{2+}\)      | 5.0                | 0                                             | 1/17,000   |
|                  | 5.0                | 1,690                                         | 1/17,000   |
| Mn\(^{2+}\)      | 0.25               | 780                                           | 1/2,240    |
| Co\(^{2+}\)      | 2.5                | 1,350                                         | 1/4,150    |
| Cr\(^{6+}\).Mg\(^{2+}\) (7.5 mM) | 0.05      | 1,269                                         | 1/6,850    |
|                  | 5.0                | 529                                           | 1/1,646    |

The template was \( \Phi X174 \) am3 DNA primed with a restriction fragment located 83 nucleotides away from the am locus. Some of the data in this table was taken from Kunkel and Loeb (1979) and Tkeshelashvili et al. (in prep.)

Primed templates of a repeating nucleotide sequence are copied. Thus, metal-mediated changes in the fidelity of DNA synthesis could result from such slippage of the primer on the template, an event that presumably does not occur during copying of natural DNA templates. Also unique to homopolymers or repeating heteropolymers is the fact that a single non-complementary nucleotide can occupy a looped-out structure without changing the reading frame of subsequent codons. Thus, metals could enhance misincorporation by increasing the frequency of such looped-out structures. To circumvent these limitations, a system has been recently developed (Weymouth and Loeb 1978) in our laboratory to monitor the fidelity of in vitro DNA synthesis using a natural DNA template, DNA from the bacteriophage \( \Phi X174 \) carrying a suppressible nonsense mutation, amber 3 (am3) (Fig. 1). Certain nucleotide substitutions within the am3 locus that occur during in vitro replication of this DNA will cause a reversion to the wild type phenotype. Transfection with the in vitro replicated \( \Phi X174 \) DNA of \( E. coli \) spheroplasts under non-suppressive conditions permits one to assay for revertants at the am3 locus. Thus, measurement of the reversion frequency of the progeny phage indicates the accuracy with which the DNA in the region of this mutation was copied. So far with the assay, two metal activators, Mn\(^{2+}\) and Co\(^{2+}\), and one non-activating cation, Cr\(^{3+}\), have been demonstrated to enhance misincorporation by \( E. coli \) DNA polymerase I (Table 2). Thus, it is likely that metal mutagens and carcinogens also increase infidelity with natural DNA templates.

**Discussion**

The exact mechanism by which certain divalent metal ions decrease the fidelity of DNA synthesis in vitro is not known. On the basis of the available data, three alternatives can be unambiguously eliminated, while three others may still be considered viable mechanisms and will require further investigation.
The following three possibilities by which metal ions decrease the fidelity of in vitro DNA synthesis are no longer tenable mechanisms:

1. Precipitation of Noncomplementary Nucleotides. It can be argued that the observed increase in error frequency at high metal concentration represents the selective acid precipitation of metal ion complexes containing unincorporated noncomplementary nucleotides. However, physical and enzymatic studies of the products synthesized with AMV DNA polymerase (Battula and Loeb 1975), E. coli DNA polymerase (Sirover et al. 1979), and DNA polymerases-α and -β (Seal et al. 1979), indicate that the noncomplementary nucleotides are incorporated into a polynucleotide chain, predominantly as single-base substitutions.

2. Metal-Substrate Interactions. Metal-induced infidelity does not appear to result from selective interactions between particular metals and particular nucleotides. For example, it could be argued that Co²⁺ selectively interacts with the noncomplementary nucleotide and reduces its effective concentration in the reaction mixture. However, at a high concentration of Co²⁺ (5 mM), the incorporation of dGTP as the complementary nucleotide with a poly (C) template is markedly inhibited, whereas the incorporation of dGTP as the noncomplementary nucleotide with poly (d(A-T)) as the template is undiminished (Loeb et al. 1979).

3. Inhibition of “Proof-Reading” Exonuclease by Metal Ions. The possibility that decreases in fidelity with divalent metal ions are mediated by inhibition of 3'→5' exonucleolytic activity is also unlikely. Eukaryotic DNA polymerases and DNA polymerases from RNA tumor viruses are devoid of such an activity (Luke et al. 1975), yet mutagenic metal ions decrease the fidelity of these enzymes.

The decrease in fidelity of metal ions during in vitro DNA synthesis can be explained most directly by any one or more of the following types of interactions (Fig. 3).

1. Altered Substrate Conformation. The ability of Mn²⁺, Co²⁺, Ni²⁺, and possibly Zn²⁺ to substitute for Mg²⁺ as a metal activator focuses on the possibility that the mechanism of change in fidelity by these metals occurs by a substitution at the substrate binding site on the polymerase. Using a variety of DNA polymerases, the frequency of misincorporation at activating concentrations of Mn²⁺ and Co²⁺ is two- or three-fold greater than that observed with Mg²⁺. Magnetic resonance studies indicate that the interaction of the metal activator involves an enzyme-metal substrate bridge complex involving the γ-phosphoryl group of the substrate (Slater et al. 1972). Studies with E. coli DNA polymerase I in the absence of template indicate that the bound metal changes the conformation of the substrate to that of the nucleotidyl unit in double-helical DNA. Sloan et al. (1975), noted that this conformation could reduce the frequency of misincorporation. Thus, it could be argued that differences in conformation of the bound substrate with different metal activators might account for differences in the fidelity of DNA synthesis. However, this mechanism would be restricted to those metals that can serve as activators for DNA polymerases.
Possible Mechanisms for Metal-induced Infidelity

I. Altered Substrate Conformation

II. Altered Enzyme Conformation

III. Altered Template Base Specificity

2. Altered Enzyme Conformation. The decrease in fidelity observed at inhibiting concentrations of metal activators suggests binding of metals at sites in addition to the active site. Ancillary binding sites for Mn$^{2+}$ were detected on E. coli DNA polymerase I by nuclear magnetic resonance studies (Slater et al. 1972). The demonstrations that nonactivating metal cations alter the fidelity of other DNA polymerases is compatible with this concept. Also, evidence has been presented that Be$^{2+}$, a nonactivating cation, binds to AMV DNA polymerase directly and diminishes the fidelity of DNA synthesis in vitro (Sirover and Loeb 1976). Thus, interactions of metals or metal-nucleotide complexes at distant sites could change the conformation of the polymerase so as to promote misincorporation.

3. Altered Template-Base Specificity. The direct interaction of metal ions with phosphates and bases on polynucleotides have been measured by a number of physical techniques (Eichhorn and Shin 1968; Slater et al. 1972). Eichhorn and Shin (1968) initially observed that metal cations can cause enhanced mispairing upon renaturation of polynucleotides. Conceivably, the metal ions can directly in-
terfere with complementary base-pairing or cause a shift in the keto-enol equilibria of the nucleotide. Recent studies by Murray and Flessel (1976) indicate that Mn$^{2+}$ and Cd$^{2+}$ promote mispairing during hybridization of the synthetic templates. Moreover, the mispairing with Mn$^{2+}$ can be demonstrated to occur at millimolar concentrations.

Our results indicate that mutagenic metal ions decrease the fidelity of DNA synthesis. This has been demonstrated with purified DNA polymerases using both synthetic and natural DNA templates. We argue that in studying fidelity of DNA synthesis by DNA polymerases, one is studying mutagenesis in vitro. Correlations observed between alterations in fidelity in vitro and mutagenicity or carcinogenicity in vivo are in accord with the hypothesis that infidelity during DNA synthesis may cause mutations. However, it should be recognized that metal ions have many other effects in vivo. Considerable evidence will be required to document whether or not alterations in the fidelity of DNA synthesis are causally associated with mutations and malignancy. Irrespective of a defined mechanism, the correlation between alterations in fidelity and mutagenicity and/or carcinogenicity indicates the practicality of using fidelity assays as a screen for evaluating possible mutagens and carcinogens. Since these assays are carried out in vitro in defined homogeneous systems, it is possible to design experiments to understand how metals alter the fidelity of DNA synthesis.

With respect to metals, diminished fidelity, and somatic mutations, the following hypothesis can be generated. Metal-induced mutations may occur by the interaction of metal ions with the DNA template or with the DNA polymerase. In the latter case, a normal polymerase could be exposed to an abnormal concentration of physiologically required metals, or to exogenous metals that are usually not present during cellular metabolism. Alternatively, metal ions that are normally not used for DNA replication could serve as activators for DNA polymerases that have been previously altered. In either case, an abnormal polymerase-metal combination might decrease the fidelity with which the DNA is replicated, and thus lead to the synthesis of DNA containing mutations. This newly synthesized DNA may contain certain critical errors (e.g., genes which code for altered polymerases). Furthermore, continued replication of the DNA by an altered polymerase or in the presence of mutagenic metals could also lead to an accumulation of additional errors during subsequent rounds of replication. Such critical errors and/or cascading errors caused by an accumulation of mutations may account for the progressive change in cellular properties during tumor progression.

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