THE ROLE OF METAL IONS IN THE MECHANISMS OF DNA AND RNA POLYMERASES

Authors: Albert S. Mildvan
Fox Chase Cancer Center
Philadelphia, Pennsylvania

Lawrence A. Loeb
Joseph Gottstein Memorial Laboratories
University of Washington School of Medicine
Seattle, Washington

Referee: Cheng-Wen Wu
Department of Biophysics
Albert Einstein College of Medicine
Bronx, New York

INTRODUCTION

DNA and RNA polymerases are of interest not only to molecular biologists, as the catalysts of the replication and transcription of DNA, but also to coordination chemists because they are Zn-containing enzymes which require Mg^{2+} as well, and to enzymologists because they derive their unusually high specificity, in part, from a nonprotein macromolecule, the DNA template.

The functioning of various DNA polymerases in the replication of DNA,[1-4] and of RNA polymerases in the transcription of DNA templates[5-7] have been thoroughly considered elsewhere. The purpose of this review is to examine in detail the mechanistic roles of the "built-in" and added metal ions, the structural basis of the unexpectedly high specificity of polymerase enzymes, and the possible mechanisms of lowering of this specificity by certain metal ions. In addition, a problem unique to RNA polymerases will be considered, the unique ability of this class of enzymes to transform a thermodynamically stable enzyme-bound intermediate, a DNA — RNA hybrid, to the thermodynamically less stable, but biologically necessary products, single stranded RNA and the double-helical DNA template.

Finally, the implications of these findings in the design of chemotherapeutic agents for malignant disease will be considered.

MECHANISTIC STUDIES OF DNA POLYMERASE

DNA polymerases catalyze the central reaction in the biosynthesis of DNA, the ac-

curate copying of a polynucleotide template. As indicated by Figure 1, the reaction is a nucleophilic attack of the 3′-OH primer terminus upon the α-phosphorus of a deoxynucleoside triphosphate substrate with the displacement of pyrophosphate. The reaction requires a template-primer complex, four deoxynucleotide substrates, and a divalent cation activator such as Mg^{2+} or Mn^{2+}[1].

The earliest studies of the interaction of templates and substrates with DNA polymerase I were carried out by Kornberg and co-workers.[1,8,9] In the presence of phosphate buffer, they found one binding site on Pol I for the template,[8] one site for deoxynucleoside triphosphate substrates, and a separate binding site for deoxynucleoside monophosphates.[9] In phosphate buffer, the nucleoside monophosphate is believed to bind at the primer terminus site, substituting for the growing point of the DNA. From the paramagnetic effects of a spin-labeled analog of ATP (tempo-ATP) on the longitudinal relaxation rates of the C_2 proton of AMP in the presence of Pol I, Krugh determined the distance between these sites (7.1 ± 0.6 Å) to be appropriate for direct interaction.[10] A reasonable mechanism consistent with these findings was proposed by Kornberg,[1] which, however, did not address itself to the role of the various metal ions required for catalysis.

The Presence and Role of Bound Zn^{2+} in DNA Polymerases

The long known requirement of Zn^{2+} for cell division,[11] and the provocative finding by Chang and Bollum that the enzyme terminal deoxynucleotidyl transferase was inhibited by *o*-phenanthroline,[12] led us to examine a variety of DNA polymerases for their content of Zn^{2+} and of other metals.[13] DNA polymerases from animal,[13] bacterial,[13,14] and viral sources[14] have been found to contain stoichiometric quantities of tightly bound Zn^{2+} (Table 1). In addition, Zn^{2+} was found in the DNA polymerase from avian myeloblastosis virus, or "reverse transcriptase,"[15-17] and in the DNA polymerases of other RNA tumor viruses.[18]

Of the various polymerase enzymes listed in Table 1, in only two cases, *E. coli* DNA-polymerase I[14] and AMV DNA polymerase,[16] have the essentiality of Zn^{2+} for catalytic activity been established by demonstrating enzyme inactivation upon removal of Zn^{2+} and enzyme reactivation upon the replacement of $Zn.^{2+}$ An essential role for Zn in all polymerases has been suggested.[13-18] This is based on their inhibition by the chelating agent, *o*-phenanthroline, and little or no inhibition by its nonchelating analog, *m*-phenanthroline. The inhibition by *o*-phenanthroline is not due to chelation of the added divalent cation, $Mg.^{2+}$ As seen in Table 1, 50% inhibition by *o*-phenanthroline is achieved at concentration of 0.01 to 1.0 m*M*, clearly lower than the Mg^{2+} concentration of 2 to 10 m*M* in the reaction mixture. However, the validity of this criterion for the essential role of Zn^{2+} has recently been questioned by Sigman and co-workers,[19] who found that the initial inhibition of DNA-polymerase I by *o*-phenanthroline was due to the formation of an inhibitory phenanthroline-Cu (I) chelate which formed in the presence of micromolar levels of contaminating Cu^{2+} and of thiol compounds which are often present in polymerase assay mixtures. Hence, at present it seems appropriate only to refer to *E. coli* DNA-polymerase I and AMV DNA-polymerase as Zn metalloenzymes. It will be important to determine if other chelators in the absence of contaminating Cu^{2+} and reducing agents inhibit the various DNA polymerases.

Two indirect lines of evidence suggest that the bound Zn^{2+} in DNA polymerase interacts with the DNA template-primer complex. First, the bound Zn^{2+} on *E. coli* DNA-polymerase I has a large effect on the nuclear quadrupolar relaxation rate of $^{79}Br^-$.[14] This effect is diminished by 70% with a sharp end point when one molecule of a polynucleotide binds per enzyme molecule. However, it is unaltered by the substrate dTTP, suggesting that the polynucleotide, but not the substrate, displaces 70% of the Br^- from the bound $Zn.^{2+}$[14] Second, in most cases in which a kinetic analyses of the inhi-

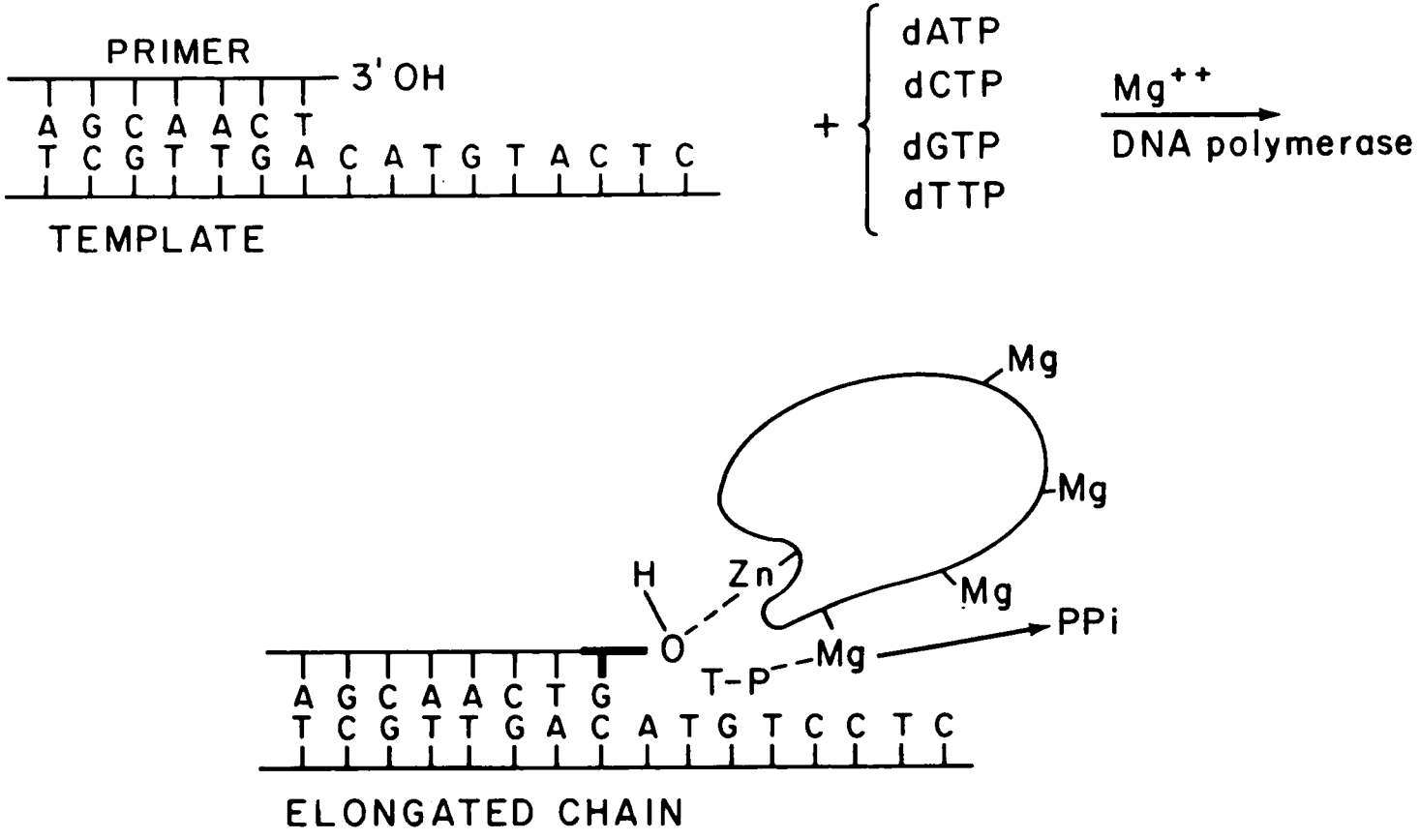

FIGURE 1. DNA polymerase reaction.

TABLE I

Zinc in DNA and RNA Polymerases

Source	Zinc (g-atom/mol)	o-Phenanthroline for 50% inhibition (mM)	Ref.
DNA Polymerases			
Bacteria			
E. coli DNA Pol I	1.8	0.04	13
E. coli DNA Pol I	1.0 ± 0.15	—	14
Bacteriophage			
T_4 phage	1.0	—	14
Eucaryotes			
Sea urchin	4.2	0.4	13
Human lymphocytes	ND*	1.0	13
Human placenta-α	ND	0.5	111
Human placenta-β	ND	0.5	111
RNA tumor viruses			
Avian myeloblastosis	1.3	0.4	15,16
Avian myeloblastosis	1.8—2.0	0.1	17
Murine leukemia	1.4	0.01	18
Woolly monkey	1.0	0.01	18
RNA polymerases			
E. coli RNA polymerase	2	0.04	49
B. subtilis	2.0	1.2	53
E. gracilis (I)	2.2	0.01	112
E. gracilis (II)	2.2	0.3	113
Yeast (1)	2.4	0.3	51
Yeast (B)	1.0 ± 0.4	0.6	52
Rat liver RNA polymerase	ND	0.025	114
T_7 RNA polymerase	2—4	—	50

* ND, not determined.

bition by *o*-phenanthroline has been made, a competitive component to this inhibition (i.e., a slope effect in a double-reciprocal plot) with respect to DNA, but not with respect to substrates, has been detected. These findings have been made with DNA-polymerase I from *E. coli*[13] and from sea urchin nuclei.[13] However, with AMV DNA-polymerase, inhibition by *o*-phenanthroline is noncompetitive,[16] which could be due either to inactivation of the enzyme by rapid removal of the Zn^{2+} or to a structural role of the Zn^{2+} in catalysis. The potent inhibition of Pol I by the *o*-phenanthroline-Cu (I) complex[19] may well require a re-examination of all of these effects.

Because of their indirect competitive nature, at best, these studies must be considered inconclusive; but they do suggest a possible catalytic role of Zn^{2+}, namely coordination of the 3′-OH primer terminus (Figure 2), facilitating its deprotonation, and thereby preparing it to attack the α-phosphorus atom of the substrate.[14] This role for Zn^{2+} is analogous to that proposed for terminal nucleotidyl transferase,[12] and long considered for carbonic anhydrase in the so-called Zn-hydroxide mechanism.[20]

The deprotonation of the 3′-OH group of the primer terminus would be expected to be accomplished by a nearby general base (Figure 3A). Evidence for such a general base has recently been obtained by the suicidal inactivation of Pol I by 2′-3′ epoxy ATP (Figure 3B).[21]

The Requirement for Added Divalent Metal Cations

In addition to the tightly bound zinc in DNA polymerases, these enzymes also re-

FIGURE 2. Mechanism of displacement of bromide ion from DNA-polymerase-bound Zn^{2+} by the DNA analog $(dT)_{6-9}$[14] (From Springgate, C. F., Mildvan, A. S., Abramson, R, Engle, J. L., and Loeb, L . A., *J. Biol. Chem.*, 249, 5987, 1973. With permission.)

quire an added divalent metal cation for activity, presumably Mg^{2+} in vivo. It has long been known that Mn^{2+} can substitute for $Mg,^{2+}$ [1,22] and it has recently been shown that Ni^{2+}, Co^{2+}, and perhaps even Zn^{2+} can serve as metal activators.[23] This is not surprising in view of the ability of these divalent cations to substitute for Mg^{2+} with a variety of phosphotransferase enzymes.[24] The rate of polymerization achieved with these alternative cations is significant. For example, with *E. coli* DNA-polymerase I (Pol I) and activated DNA, the maximal rate of nucleotide incorporation with Mn^{2+}, $Co,^{2+}$ and Zn^{2+} is 153, 57, and 4% of that achieved with $Mg.^{2+}$ [23] As all activities associated with DNA polymerases require a metal activator, the use of Mn^{2+} or Co^{2+} as substitutes for Mg^{2+} in these reactions may provide a means to probe the mechanism of catalysis by these enzymes.

This problem has been investigated with Pol I in Tris Cl^- buffer by replacing the diamagnetic Mg^{2+} with paramagnetic $Mn.^{2+}$ [22] Binding studies using EPR reveal one tight-binding active site for Mn^{2+} on the enzyme with a dissociation constant ($\sim 1\ \mu M$) in agreement with its kinetically determined activator constant (1.2 μM), four intermediate binding sites ($K_D = 29\ \mu M$), and approximately 20 weak-binding, inhibitory Mn^{2+} sites with dissociation constants (0.8 mM) in agreement with the inhibitor constant of Mn^{2+} (0.6 mM).[22] Comparison of the binding and kinetic data indicates that occupancy of the tight site by Mn^{2+} activates the enzyme, and occupancy of the weak sites inhibits activity. With Mg^{2+} as activator, a comparison of kinetic and binding properties indicates that the tight and intermediate sites must be occupied for enzyme activity, suggesting an essential role for the intermediate sites as well.[22] In accord with this view, nuclear relaxation studies of water indicate that deoxynucleotide substrates such as dTTP interact with Mn^{2+} at both the tight and intermediate sites to decrease the number of coordinated water ligands by ~ 1.[22] Multiple deoxynucleoside triphosphate binding sites have also been detected kinetically by their complex inhibitory effect.[25] However, the precise role of the intermediate metal-binding sites in catalysis is, at present, unclear.

To examine in greater detail the structure and conformation of the ternary Pol I — Mn^{2+}-dTTP complex with Mn^{2+} at the active site, the longitudinal and transverse nuclear-relaxation rates of the phosphorus atoms and protons of the bound dTTP substrate were studied.[26] The ^{31}P relaxation rates were measured at 40.5 MHz, and those of the protons were measured at two frequencies, 100 and 220 MHz, to permit evaluation of the correlation time. Analysis of the longitudinal relaxation rates permitted calculation of the distances from Mn^{2+} to five protons and 3 phosphorus atoms of dTTP in both the binary Mn-dTTP complex and the ternary DNA polymerase-Mn-dTTP complex.[26] These distances were used to construct molecular stick models of both complexes (Figure 4A).[26] The uniqueness of these models was tested by constructing space-filling models and by a computer search among 47,000 conformations, rejecting those structures which produced a total van der Waals overlap greater than 0.4 Å and which required distances which exceed the error limits of 5.5 and 7.5% for the binary and ternary complexes, respectively. By these tests the structures of Figure 4A provide a highly unique fit to the data.[26]

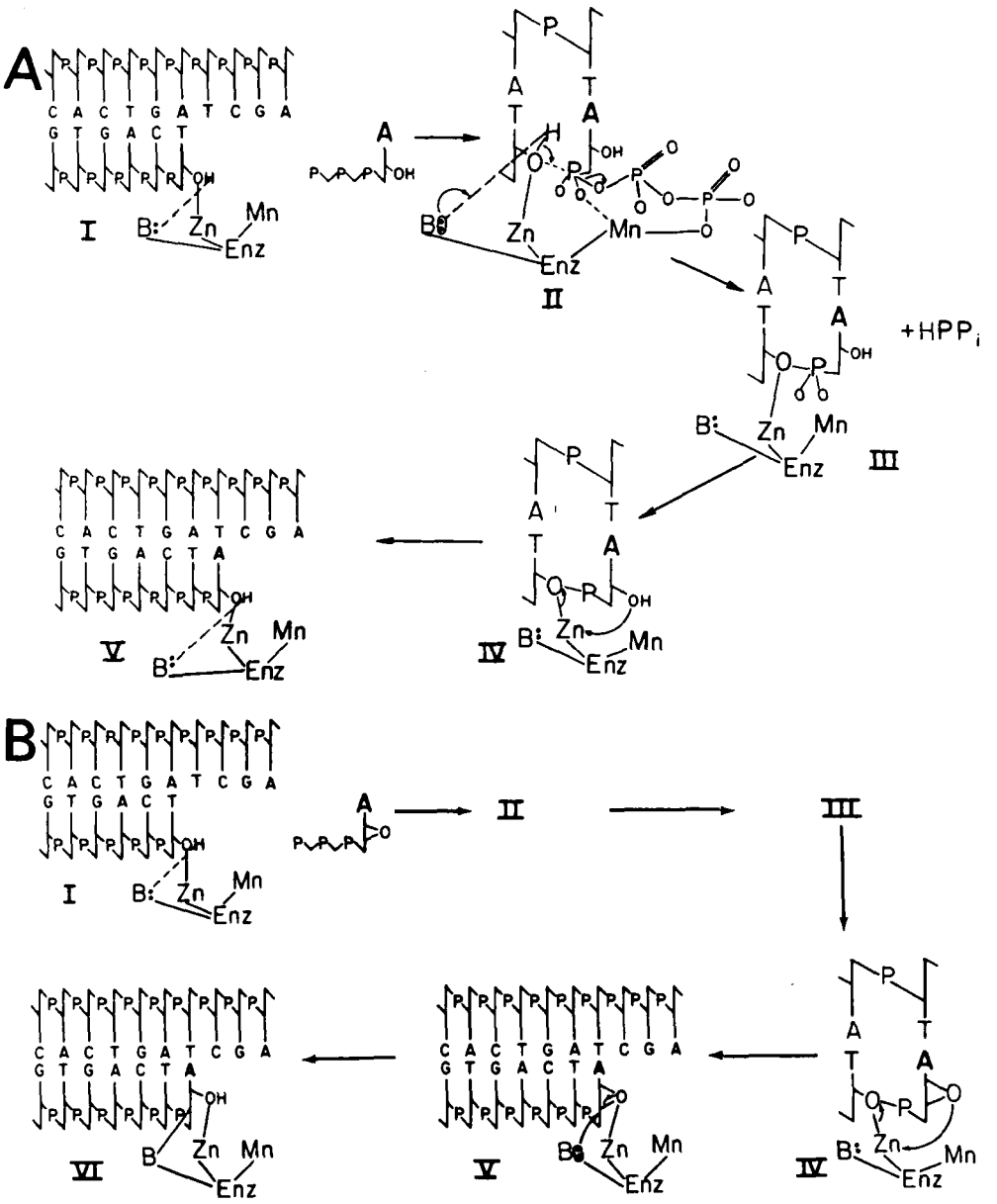

FIGURE 3. Mechanism of (A) the DNA polymerase reaction and (B) the suicidal inactivation of DNA polymerase by 2′-3′-epoxy ATP[21] (From Abboud, M. M., Sim, W. J., Loeb, L. A., and Mildvan, A. S., *J. Biol. Chem.*, 253, 3415, 1978. With permission.)

Two major differences are noted between the binary and ternary complexes. First, in the binary complex all three of the phosphoryl groups of dTTP are directly coordinated to Mn^{2+}. On DNA polymerase only the γ-phosphoryl group remains coordinated by the enzyme-bound Mn^{2+}. The distance from Mn^{2+} to the β phosphorus atom (4.9 Å) is greater than that expected for direct coordination (2.9 ± 0.2 Å). The intermediate distance to the reaction-center α-phosphorus atom (4.2 Å) is most simply explained by the rapid averaging of <15% inner-sphere coordination with >85% second-sphere coordination, possibly with an intervening water ligand. The resulting polyphosphate conformation is puckered and somewhat strained. Hence, an important role of the divalent cation activator in catalysis is to link the enzyme to the γ-phosphoryl group

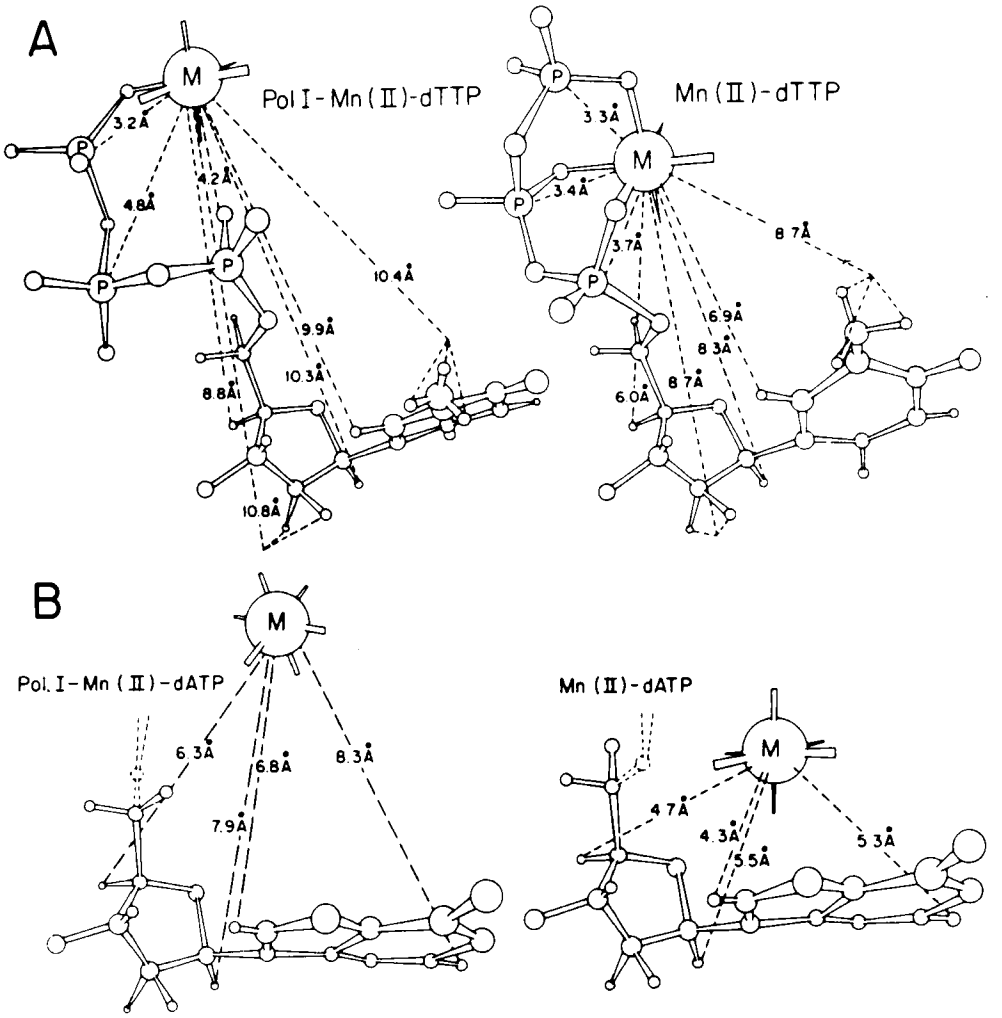

FIGURE 4. Conformations in solution of binary Mn^{2+} complexes and ternary Pol I-Mn^{2+} complexes of (A) dTTP and (B) dATP[26] (From Sloan, D.L., Loeb, L.A., Mildvan, A.S., and Feldmann, R. J., *J. Biol. Chem.*, 250, 8913, 1975. With permission.)

of the substrate to assist the departure of the leaving pyrophosphate group and, possibly also, to facilitate nucleophilic attack on the α-phosphorus atom by strain and by hydrogen bonding through a coordinated water ligand.[26] Consistent with an active Pol I-metal-substrate bridge of this structure, the stable bidentate metal complex β,γ $Co^{3+}(NH_3)_4$ dATP is not a substrate for Pol I even in the presence of added Mg^{2+}.[27] In addition to an incorrect phosphoryl coordination, the stable metal ion cannot dissociate its amine ligands to coordinate to the protein.

A second important difference between the binary and ternary complexes is in the conformation about the thymine-deoxyribose bond of dTTP. Such glycosidic conformations are quantitatively described by the torsion angle χ. In the present case, this is the dihedral angle between N_1-C_6 of the thymine and C_1'-O_1' of deoxyribose when viewed along the glycosidic N_1-C_1' bond. The χ value of 40 ± 5° in the binary complex increases to 90 ± 5° in the ternary complex (Figure 4A). Interestingly, the latter torsion angle of 90° is indistinguishable from that found for deoxynucleotidyl units in double-helical DNA.[28,29] Hence, the binding of the subtrate Mn-dTTP to the enzyme DNA polymerase, in absence of template, has changed the substrate conformation to that

of a nucleotidyl unit in the product — double-helical DNA. Similarly, a 90° torsion angle is also found for the purine-nucleotide substrate Mn-dATP when bound to DNA polymerase (Fig. 4B).[26]

As discussed below, this enzyme-induced change in substrate conformation may well represent an error-preventing mechanism. Analogous effects are observed with a purine-substrate dATP (Figure 4B). The folded structure of the binary Mn-dATP complex causes the nucleotide to assume a χ angle of 90°. In the ternary complex, this conformational angle of 90° is preserved, although the substrate conformation has changed to an open one. These effects are summarized diagramatically in Figure 5, which postulates that the substrate changes its conformation upon binding to the enzyme.[30] An enzyme induced conformational change, rather than the selection of a minor 90° conformational species of the free substrate by the enzyme, is indicated by the lower limits to the second-order rate constant for the binding of all forms of dTTP to the Pol I — Mn^{2+} complex as determined from the NMR data ($k_{on} \geq 1.1 \times 10^9 M^{-1}$ sec^{-1}).[26,30] Thus the total dTTP binds to the enzyme-Mn^{2+} complex at diffusion controlled rate. The NMR data, as well as theoretical calculations of the conformations of thymidine, TMP, uridine, UMP, and crystal structures of nucleosides and nucleotides summarized elsewhere,[30] indicate that the subpopulation of dTTP with $\chi = 90°$ represents a thermodynamically unfavored minor fraction of the total dTTP. Hence, the selection by Pol I of the pyrimidine substrate with the correct conformational angle of 90° would require an impossibly large second-order rate constant ($\geq 10^{11} M^{-1}$sec^{-1}). A kinetic and thermodynamic scheme for the binding and conformation change of dTTP on DNA polymerase has been proposed[30] that is consistent with the measured equilibrium constants[22] and lower limit rate constants.[26] It indicates that the enzyme shifts the conformational equilibrium of dTTP toward the 90° form, primarily by inhibiting the rate of the reverse conformation change from 90° to 40°.[30]

When the structure of enzyme-bound Mn-dTTP (Fig. 4A) is superimposed by computer onto the double-helical structure of DNA-B (Fig. 6), the resulting location of the α-phosphorus atom and the leaving pyrophosphate group of the bound substrate relative to the attacking 3′-OH group of the preceding nucleotide unit is consistent

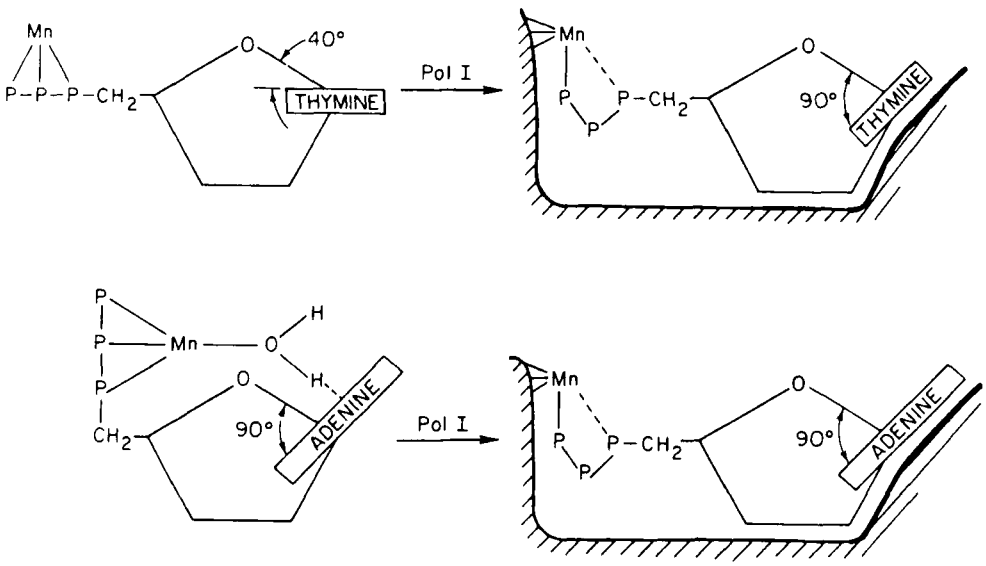

FIGURE 5. Diagram of conformational changes occuring in the Mn-dTTP and Mn-dATP complexes upon binding to Pol I.[30]

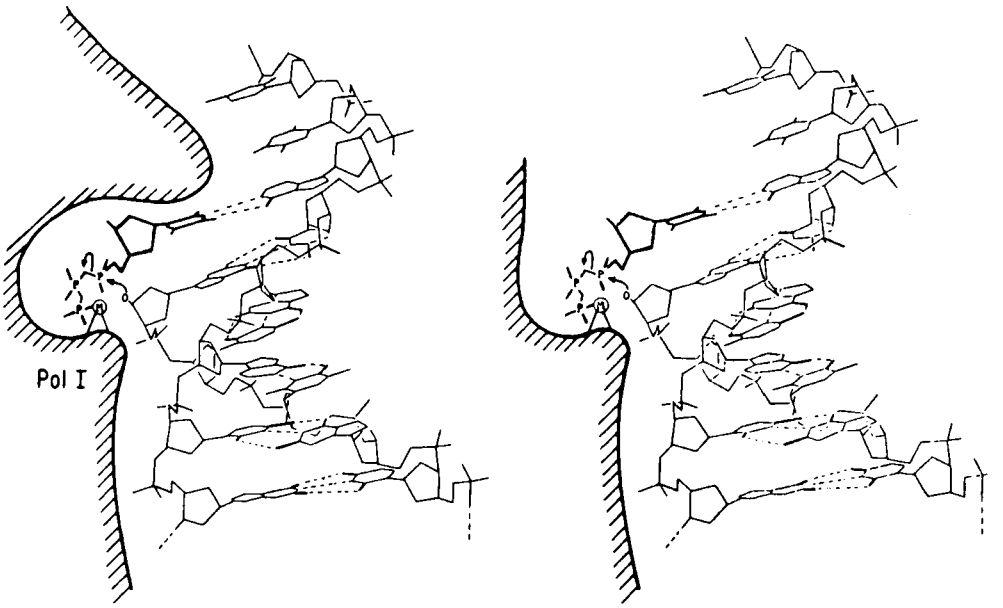

FIGURE 6. Superposition of conformation of Pol I-bound Mn-dTTP into the double helical B-DNA structure, showing the difference between an enzyme of high fidelity (left) and an enzyme of low fidelity (right).[26,30]

with an in-line nucleophilic displacement on the α-phosphorus.[26] Hence, the DNA-polymerase reaction, like those catalyzed by ribonuclease[31] and phosphoribosylpyrophosphate synthetase,[32] appears to proceed by an in-line mechanism with inversion at phosphorus. In-line displacements have recently been found in the nucleotidyl transfer reactions catalyzed by UDP-glucose pyrophosphorylase,[32a] acetyl CoA synthetase,[32b] and RNA polymerase.[32c] The role of the added divalent cation in DNA polymerase is to form a metal bridge between the enzyme and the terminal phosphoryl group of the substrate, facilitating the departure of the pyrophosphate leaving group.[26]

With human DNA polymerase-β, a change in the divalent-cation activator from Mg^{2+} to Mn^{2+} produces an order of magnitude lowering of the K_M values of the substrates and template in accord with a mechanism of this type.[33]

Effects of Monovalent Cations

Many DNA polymerases are stimulated as much as 3- to 5-fold by monovalent cations, particularly K^+ and NH_4^+ at concentrations up to 50 m*M*.[4,34] At higher concentrations of monovalent cations, most DNA polymerases are inhibited. For example, Bollum[35] has reported that calf-thymus DNA polymerase-α is inhibited 90% by 100 m*M* LiCl, NaCl, KCl, or NH_4Cl. Inhibition by Na^+ or K^+ of DNA polymerases from human KB cells[36] HeLa cells,[37] rabbit[38] and mouse testis,[39] has been studied. Inhibition by monovalent cations has been used to distinguish between DNA polymerase-α and -β from eucaryotic cells since the latter enzyme is not inhibited by concentrations as great as 300 m*M*.[35] Also, a DNA polymerase coded for by Herpes virus is uniquely stimulated by both Na^+ and K^+.[40] Little is known about the mechanism of both the stimulatory and inhibitory effects of monovalent cations on DNA polymerases.

MECHANISTIC STUDIES OF RNA POLYMERASE

RNA polymerases are generally more complex in structure and function than are

the DNA polymerases.[5-7] RNA polymerase from *E. coli* is known to consist of at least 5 subunits $\alpha_2\beta\beta'\sigma$[5-7] with a globular arrangement.[41] The total molecular weight (500,000) is 3- to 5-fold greater than those of DNA polymerases. The enzyme from *E. coli* can transcribe either a single- or double-stranded DNA template to form single-stranded RNA. Building on a purine-nucleotide initiator, the enzyme can initiate transcription at any point of the DNA template. The presence of the σ subunit enables the enzyme to dissociate more slowly (i.e., to bind more tightly) by a factor of 10^5 at a particular DNA sequence called the promoter region, as compared with its rate of dissociation from nonspecific sites on DNA, and, thereby, to preferentially initiate RNA synthesis at such a sequence.[42,43]

Kinetic analyses have been interpreted to indicate at least four complex substeps in the RNA-polymerase reactions,[5,5a,7] namely template binding, RNA chain initiation, RNA chain elongation, and RNA chain termination, and release.

The two covalent substeps are given by Equations 1 and 2:

$$\text{Pur Nuc} + \text{N}_{ppp} \xrightarrow[M^{2+}]{\text{DNA}} \text{Pur Nuc–pN} + \text{PP}_i \qquad (1)$$

$$\text{Pur Nuc–pN} + n\text{N}_{ppp} \xrightarrow[M^{2+}]{\text{DNA}} \text{Pur Nuc–(pN)}_{n+1} + n\text{PP}_i \qquad (2)$$

The first step is termed chain initiation, and all subsequent ones are termed chain elongation. In the chain initiation step, the enzyme catalyzes the transfer of the nucleotidyl group of the substrate, bound at the elongation site, to the initiator, bound at the initiation site, with the displacement of pyrophosphate. All subsequent nucleotidyl transfer (elongation) reactions occur at the elongation site, extending the RNA chain.

The initiator is a purine-containing mononucleotide or oligonucleotide with an available 3′-OH group, such as AMP, ADP, ATP, CrATP, ApA, or ApU.[5-7,44,45] The nuceloside-triphosphate substrates are defined by the DNA template with an error rate of 10^{-4}.[46] The template may be either double- or single-stranded DNA in vitro, the former being the biologically functional template. When double-stranded DNA functions as the template, the two DNA strands separate on the enzyme[47] to permit Watson-Crick base pairing between the incoming substrates and the transcribed DNA strand. As RNA chain elongation proceeds, a DNA-RNA hybrid structure might form as an intermediate. Although DNA-RNA hybrid helices are generally more stable than DNA-DNA double helices,[48] the ultimate and biologically necessary products of the reaction are single-stranded RNA and the DNA-DNA double-helical template. Hence, the enzyme must also catalyze the separation of the stable DNA-RNA intermediate complex to permit the reformation of the less stable double-stranded DNA template.

The Role of Bound Zinc in RNA Polymerases

In 1971, the detection of Zn^{2+} in various DNA polymerases,[13] and its presence in RNA polymerase from *E. coli* were reported.[49] Subsequently, Zn^{2+} has been found in RNA polymerases from a virus,[50] yeast,[51,52] and from *Bacillus subtilis*[53] (Table 1). While the presence of Zn^{2+} in RNA polymerases is widespread,[49-53] the essentiality of Zn^{2+} for enzyme activity has not been rigorously established by removal and replacement experiments, with the possible exception of phage-T_7 RNA polymerase[50] (see below). Apparently, many RNA polymerases are irreversibly inactivated upon the removal of Zn.[50-54] The widely used criterion of inhibition by *o*-phenanthroline, but not by its nonchelating analog, 1,7-phenanthroline, while suggestive, is insufficient to establish the essentiality of Zn,[2+] especially in view of Sigman's recent finding of the potent inhibition of RNA polymerase by the phenanthroline-Cu (I) complex.[55] With

phage-T_7 RNA polymerase, Coleman has observed a rough correlation of the specific activity of various purified fractions with their Zn^{2+} content. The enzyme was inhibited in a time-dependent manner by a variety of metal complexing agents such as EDTA, Chelex, CN^-, azide, sulfide, and *o*-phenanthroline.[50] The inactivation of T_7 RNA polymerase by *o*-phenanthroline correlated well with the loss of Zn^{2+} from the enzyme, and certain enzyme preparations were specifically activated by added Zn^{2+} in a time-dependent manner[50] characteristic of the replacement of Zn^{2+} lost by certain metalloenzymes.[14,56,57]

Assuming Zn^{2+} to be an essential component of RNA polymerase as based on its presence in enzymes from a variety of sources (Table 1) and on the presumptive evidence summarized above, its possible role will be considered. First, unlike DNA polymerases which appear to contain only one Zn^{2+}/mol, many RNA polymerases contain multiple Zn^{2+} ions, despite single initiation and elongation sites (Table 1). This suggests multiple roles for Zn^{2+}, including purely structural ones. Indeed, the two Zn^{2+} ions in the enzyme from *B. subtilis* appear to have different affinities for the enzyme.[53]

A catalytic role of Zn^{2+} has also been suggested[49,53,54] as interacting with the template and/or the initiator. Interaction with the template might facilitate promoter site selection.[54] Interaction with the initiator might facilitate priming in a manner analogous to the role proposed for Zn^{2+} in DNA polymerase (Figures 2 and 3).[13,14,21,22] Evidence for interaction of the bound Zn^{2+} with DNA is that the Zn^{2+} is located predominantly on the β' subunit of the enzyme from *E. coli*[58] which binds DNA,[59] and on the analogous subunit from *B. subtilis*.[53] However, a significant amount of Zn^{2+} is also located on the β subunit of the *E. coli* enzyme on which the initiation and elongation nucleotide-binding sites are located.[58-61] The biosynthetic replacement of Zn^{2+} by Co^{2+} in the enzyme from *E. coli* produced relatively few and small kinetic changes, but these changes were in template binding and in initiation.[54] Thus, the $(Co^{2+})_2$-enzyme showed a twofold lower K_M for a DNA template and twofold less efficient initiation by a number of biochemical tests. The optical spectrum of the $(Co^{2+})_2$ enzyme showed peaks at 584 and 703 nm with extinction coefficients of 200 and 335 M^{-1} cm^{-1}, respectively. This is suggestive of an approximately tetrahedral or pentacoordinate complex[54] as is generally found in Zn metalloenzymes.[62-66] Nucleoside-triphosphate substrates and initiators decreased the absorbancy at the 703 nm band while the template analog, $d(pT)_{10}$, decreased the absorbancy at both bands.[54] Of course such effects on the bound Co^{2+} could be indirect, resulting from protein conformation changes due to the more distant binding of template, initiators, and substrates.[54] Similarly, the inhibition of initiation by *o*-phenanthroline and the competition between the initiator GTP and the inhibitor *o*-phenanthroline,[49] while suggestive of a role for Zn^{2+} in initiation, need not have resulted from a direct interaction of *o*-phenanthroline with the enzyme-bound Zn^{2+}.[49] Such findings could, for example, have been due to inhibition by the Cu(I)-phenanthroline complex.[55] As with DNA polymerases, more direct studies of the role of the bound Zn^{2+} in RNA polymerases are needed.

The Role of the Added Divalent Cation in RNA Polymerase

All RNA polymerases require a divalent cation such as Mg^{2+} or Mn^{2+} for activity. A direct Mn^{2+}-binding study by EPR and by measurements of water proton-relaxation rates revealed the presence of one tight Mn^{2+} binding site per molecule of *E. coli* RNA polymerase with a dissociation constant less than 10 μM, and approximately six weaker Mn^{2+} binding sites with dissociation constants 100-fold greater.[67] Although the role of the six weaker sites is not clear, four lines of evidence[44,45,67] indicate that the one tight Mn^{2+} binding site functions as the active site for RNA chain elongation. The first is the agreement of the K_M values of the elongation substrates MnUTP and MnATP (in the ApA initiated reaction) with their respective dissociation constants from the tight

Mn^{2+} site.[67] The second is the greater interaction of elongation substrates (UTP and ATP) than of specific initiators (ApA and ApU) with the enzyme-bound Mn^{2+}, as monitored by changes in the relaxation rate of water protons coordinated to the bound Mn^{2+}.[67] Third, the specific initiators ApA and ApU do not interfere with the binding of elongation substrates near the enzyme-bound Mn^{2+}, indicating that the Mn^{2+} is not at the initiation site. On the contrary, ApA raises the affinity of the enzyme-Mn^{2+} complex for UTP,[67] and both ApA and ApU cause the elongation substrates to displace water ligands from the enzyme-bound Mn^{2+}.[44] Fourth, the specific initiators ApU and CrATP are 10 Å from the bound Mn^{2+}, while the elongation substrate ATP (at saturating ApA) is only 5 Å from the bound Mn^{2+}, as determined by NMR and EPR studies.[44,45]

The tight binding of a single Mn^{2+} at the elongation site and the selective interaction of CrATP at the initiation site have provided two paramagnetic reference points which have been used to map the conformations and arrangement of substrates and initiators on RNA polymerase from *E. coli*.[44,45,67] The stable trivalent metal-ATP complexes CrATP and $Co^{3+}(NH_3)_4$ ATP do not function as substrates for RNA polymerase even in the presence of Mg^{2+} or Mn^{2+}, presumably because the metal cannot coordinate to the protein. They bind only weakly to the elongation site as shown by the fact that they are weak competitive inhibitors. However, CrATP, which is paramagnetic, binds moderately tightly to the initiation site (K_D = 75 μM), as determined kinetically by its activation of the incorporation of UTP into poly r(AU) and by its competition with the authentic initiators ApA and γ-^{32}P-ATP.[45] Moreover, CrATP is incorporated into RNA unless blocked by the authentic initiator ApA,[45] which forms a tight 1:1 complex only at the initiation site.[45,67]

Geometry of Bound Mn^{2+}, Initiator, and Substrate on RNA Polymerase

Using the tightly bound Mn^{2+} as a paramagnetic reference point, seven distances to ApU, bound solely at the initiation site (9.0 to 10.5 Å), and three distances to ATP, bound solely at the elongation site (4.0 to 5.7 Å), in the presence of an initiator have been calculated from $1/T_1$ measurements.[44] These distances have been used to construct a molecular model of the conformation of the bound initiator, ApU (Fig. 7), and of the bound substrate. The conformational angle (χ) at the glycosidic bond of the bound-substrate ATP, based on only three distances,[44] is indistinguishable from 90°, which is appropriate for base-pairing with the β-helical form of the bound DNA-template strand. To position the bound substrate with respect to the bound initiator, initiator to substrate distances are required. These distances were obtained using CrATP at the initiator site and MgCTP at the substrate site.[45] Distances of 7.9 to 11.0 Å from the bound initiator CrATP to the H_5, H_6 and H_1' protons of the bound pyrimidine substrate CTP defined an average χ value of 90°, which is in agreement with that found for ATP. A diagram (Fig. 8) and molecular model (Figure 9) of the initiator-substrate-metal interaction consistent with all of these distances indicate that the terminal base of the initiator can stack with that of the substrate and that an associative (S_N2) displacement of the leaving pyrophosphate of the substrate by the initiator is possible.[45]

However, the bound initiator, as shown by the structure of ApU (Fig. 7), differs from the binary Mn-ApU complex which is approximately helical[44] and from free ApU which is internally stacked.[68] Enzyme-bound ApU is clearly neither helical (i.e., is neither a DNA or RNA helix) nor stacked, but rather assumes a looplike structure (Figure 7).[44] Hence, if the enzyme-bound DNA template were helical, the enzyme could play a catalytic role in separating the newly synthesized RNA strand from the DNA template as the growing RNA strand passed through the initiator site (Fig. 10).[44]

To avoid paramagnetic contamination and the introduction of additional metal com-

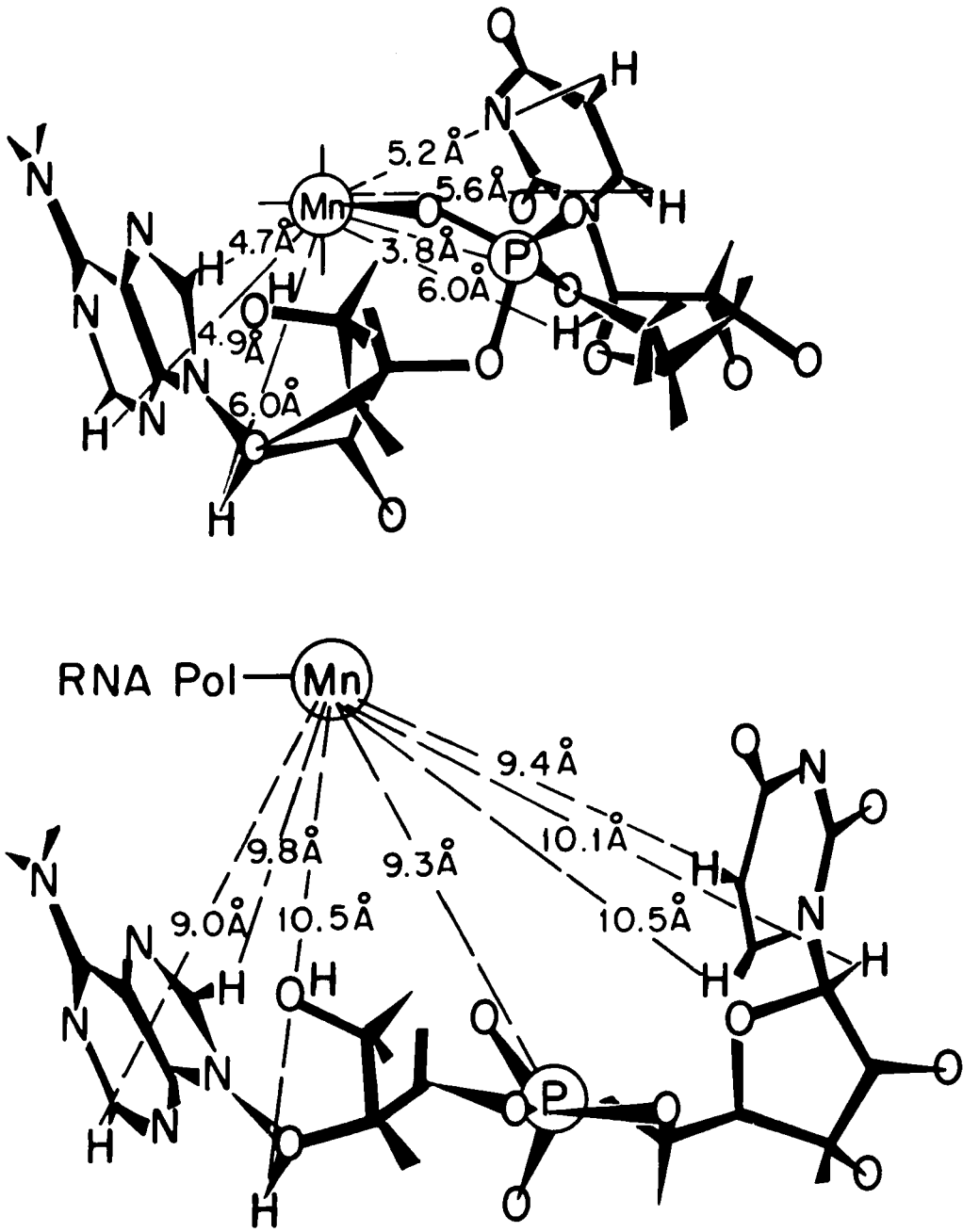

FIGURE 7. Conformations of adenylyl (3′ →5′) uridine (ApU) in its binary complex with Mn^{2+} (upper) and in its ternary complex with RNA polymerase and Mn^{2+} (lower) [44] (Reprinted with permission from Bean, B. L., Koren, R., and Mildvan, A. S., *Biochemistry*, 16, 3322, 1977. Copyright by The American Chemical Society.)

plexes, all of the distance measurements by NMR were made in the absence of the DNA template, i.e., with inactive complexes.[44,45] Hence, the structural models of Figures 7 to 9 might alter in the presence of the template.[44,45] However, such structural alterations by the template are probably small. This is because the conformations of the bound substrates in the absence of template are appropriate for base-pairing with B-DNA,[44,45] and the presence of the template does not significantly alter the affinity of the enzyme for Mn^{2+},[67] ApU,[44,67] ATP,[44,67] or CrATP.[45]

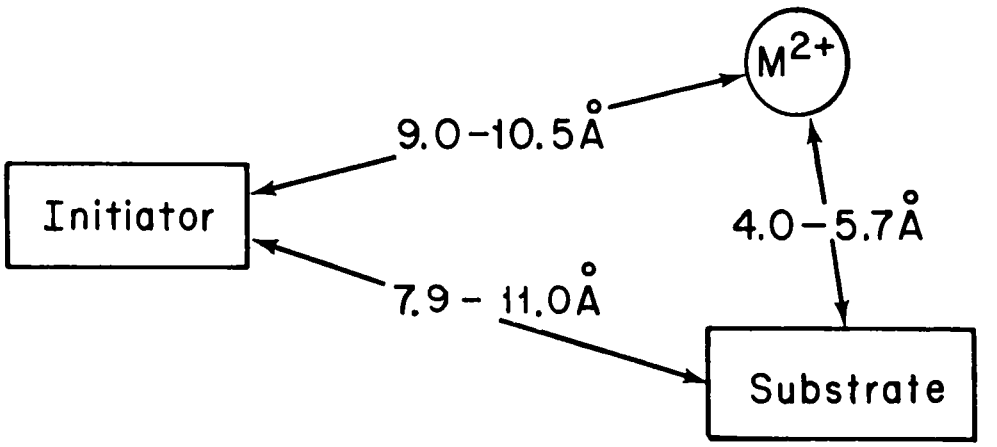

FIGURE 8. Spatial arrangement of the functional sites on RNA polymerase based on NMR measurements.[44,45] (Reprinted with permission from Stein, P. J. and Mildvan, A. S., *Biochemistry*, 17, 2675, 1978. Copyright by the American Chemical Society.)

Kinetics of Formation of RNA Polymerase Complexes

The lower limits to the second-order rate constants for the binding of the substrate ATP and the initiator ApU to the Mn^{2+} complex of RNA polymerase are $5.3 \times 10^9 M^{-1}$ sec^{-1} and $8 \times 10^9 M^{-1}$ sec^{-1}, respectively, indicating diffusion-controlled binding of these small components.[30,44] A similar conclusion has previously been reached for the combination of a high-molecular-weight double-helical DNA template with RNA polymerase based on an estimated rate constant of $10^8 M^{-1}$ sec^{-1}.[69] Although the substrate MnATP[44] and the template[47] appear to be predominantly in the "correct" conformation prior to binding, the initiator ApU is clearly not (Fig. 7).[44] Hence, the diffusion-controlled formation of an enzyme-ApU complex with a looplike conformation of ApU (Figure 7) implies a rapid change of conformation of the initiator after binding, as is the case for the substrates of DNA polymerase[26,30] (Figure 5).

FIDELITY OF DNA AND RNA POLYMERASES

It is generally assumed that errors in DNA replication are rare events occurring only frequently enough to permit the divergence of species through mutations, yet rare enough not to significantly alter essential genetic information.[70] In contrast, errors in RNA synthesis could be more easily tolerated due to the multiple copies and rapid turnover of RNA. The need for conserving genetic information has stimulated considerable study of possible mechanisms for generating the exceptional fidelity of DNA replication.

On the basis of spontaneous mutation rates in procaryotic and eucaryotic cells, it is usually estimated that the stable misincorporation of a base during DNA replication occurs with a frequency of only 10^{-7} to 10^{-10} mutations per base pair synthesized.[71,72] This accuracy appears to be achieved by a multistep process (Figure 11). Watson-Crick base pairing alone provides little more than an additional hydrogen bond (2 to 3 kcal) for the correct base-pairing interaction over an incorrect interaction, which would yield an error rate of only 10^{-2}.[73,74] Secondly, polymerases may amplify Watson-Crick base pairing by appropriate structural constraints on the bound substrate and template.[26,30] Such "error prevention" could decrease the frequency of misincorporation by three orders of magnitude to the minimal values of 10^{-5} observed in vitro[30,75,76] (Table 2). Alternatively, prokaryotic DNA polymerases contain a 3′ → 5′-exonuclease activity which by kinetic proof-reading could, in principle, have the same quantitative

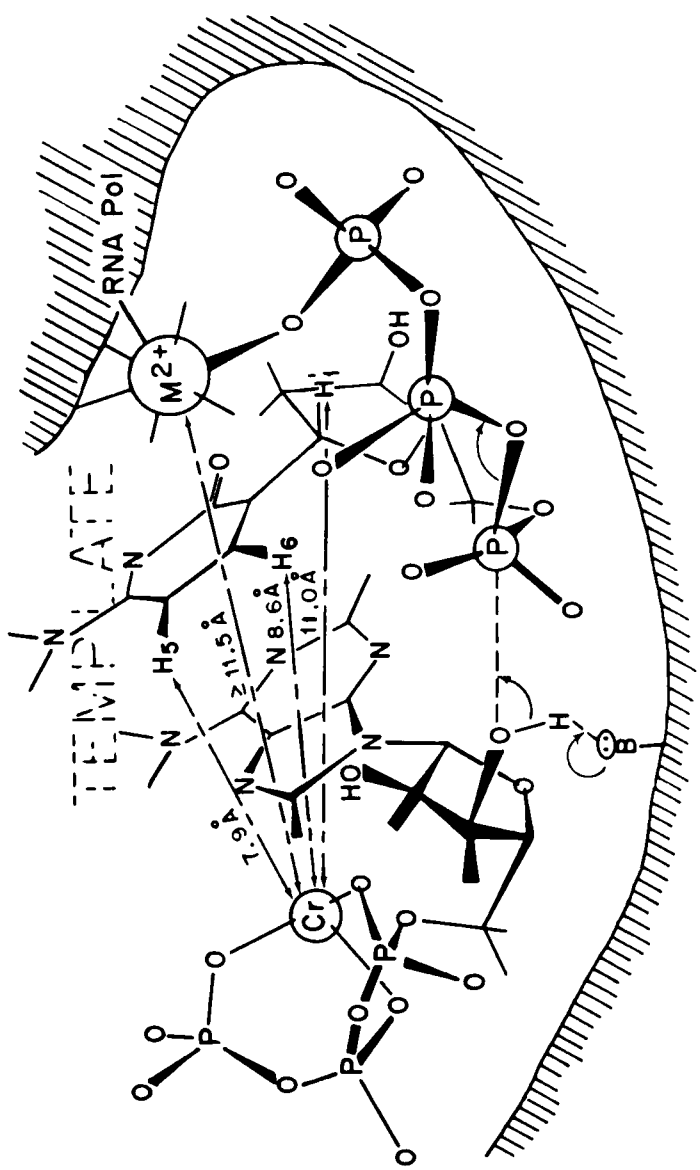

FIGURE 9. Arrangement of the initiator, CrATP, the elongation substrate, CTP, and the divalent activator, Mn^{2+}, on RNA polymerase consistent with the indicated distances[45] and with those from Mn^{2+} to the initiator and substrate.[44] (Reprinted with permission from Stein, P. J. and Mildvan, A. S., *Biochemistry*, 17, 2675, 1978. Copyright by the American Chemical Society.)

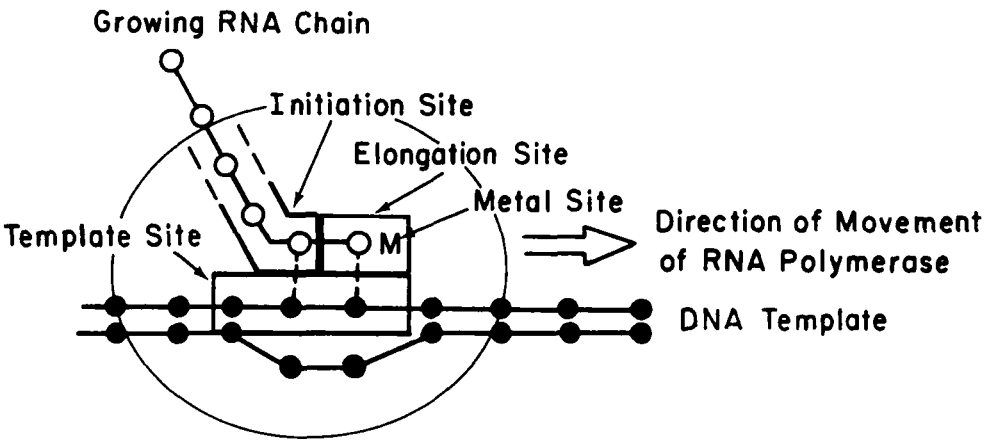

FIGURE 10. Arrangement of sites on RNA polymerase showing how passage of the growing RNA chain through the initiation site could separate the RNA from the DNA template.[44] (Reprinted with permission from Bean, B. L., Koren, R., and Mildvan, A. S., *Biochemistry*, 16, 3322, 1977. Copyright by the American Chemical Society.)

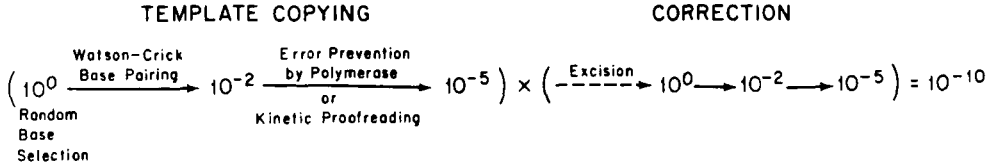

FIGURE 11. Proposed mechanisms for achieving low error rates in DNA synthesis in vitro (10^{-5}) and in vivo (10^{-10}). The in vivo error rate assumes that the excision of the noncomplementary nucleotide is accompanied by the removal of complementary nucleotides in amounts which are very small compared to the reciprocal of the error rate in vitro.

effect[74,77] (Figure 11). However, a proof-reading mechanism cannot be operative in eukaryotic DNA polymerases since these enzymes copy polynucleotide templates with high fidelity, yet lack a 3′ → 5′-exonuclease activity.[78,79] The excision of a mismatched nucleotide by an endonuclease, followed by another polymerase-catalyzed reaction with a similar error rate of 10^{-5}, would yield the low initiation rates observed in vivo (Fig. 11).[3] Indeed, recent evidence indicates the presence of endonucleases that excise mismatched nucleotides in newly synthesized daughter strands of DNA.[80]

Certain metal ions have been shown to alter the fidelity by which purified DNA polymerases copy polynucleotide templates in vitro and to act as mutagens and carcinogens in vivo. Thus, an analysis of the effects of these cations on purified polymerases is pertinent to understanding the basis for fidelity, and may provide clues to understanding their mutagenicity.[81]

Error Rates of DNA and RNA Polymerases

Measurements of the fidelity of DNA or RNA synthesis are usually carried out with synthetic polynucleotide templates containing only one or two nucleotide species by determining the frequency at which noncomplementary nucleotides present at equal concentrations are incorporated into an acid-insoluble product. Control experiments are required to rigorously document the purity of substrates and polynucleotides and to establish that the radioactive nucleotide precursor is incorporated in phosphodiester linkage into the newly synthesized product as single noncomplementary substitutions.[82] Assays are carried out using complementary and noncomplementary nucleotides la-

TABLE 2

Fidelity in Copying Polynucleotide Templates

Source of DNA polymerase	Template	Noncomplementary nucleotide incorporated	Error rate	Ref.
Procaryotic DNA polymerases				
E. coli polymerase I	poly[d(A-T)]	dGTP	1/70,000	75,115
Bacteriophage T_4	poly[d(A-T)]	dCTP	1/12,000	101,116
Eucaryotic DNA polymerases				
Sea urchin nuclei	poly[d(A-T)]	dCTP	1/12,000	116
Calf thymus (3S)	poly (A)·oligo(dT)	dCTP	1/180,000	78
Human lymphocyte (6S-8S)	poly[d(A-T)]	dCTP	1/12,000	115
Human placenta-α	poly[d(A-T)]	dGTP	1/10,000	111
Human placenta-β	poly[d(A-T)]	dGTP	1/40,000	111
HeLa-α	poly(dC)·oligo(dG)	dATP	1/3,400	117
HeLa-β	poly(dC)·oligo(dG)	dATP	1/8,800	117
RNA polymerase				
E. coli	poly[d(A-T)]	dCTP	1/2,400	46
E. coli	poly[d(A-T)]	dGTP	1/42,000	46
Reverse transcriptases				
Avian myeloblastosis virus	poly(A)·oligo(dT)	dCTP	1/300—800	82
Avian myeloblastosis virus	poly[d(A-T)]	dGTP	1/3,000	82
Rous sarcoma virus	poly(C)·oligo d(G)	dATP	1/900	118
Rauscher leukemia virus	poly(A)·oligo d(T)	dCTP	1/400	119

beled with different radioactive isotopes. The frequency of misincorporation can be determined in the same reaction by the ratios of the two incorporated isotopes.[82]

It might be argued that the error rates observed with synthetic polynucleotides are not relevant to those which would be observed with a natural DNA template. Synthetic polynucleotides may have conformations differing from that of natural DNA. Recently, an assay has been developed by Weymouth and Loeb for measuring the accuracy of copying a natural DNA template.[83] The assay is based on the frequency by which a single-base mutation in ϕX DNA changes to a wild-type copy during catalysis by purified DNA polymerases. Initial estimates of error rates for avian-myeloblastosis DNA polymerase and *E. coli* DNA polymerase I are 1:700 and >1:8000, respectively,[84] corroborating the results obtained with synthetic polynucleotides.

A compilation of the fidelity of DNA synthesis by DNA and RNA polymerases is given in Table 2. The frequency of misincorporation by DNA polymerases ("reverse transcriptase") from RNA tumor viruses varies from $\sim$1:300 with homopolymer templates to $\sim$1:7000 with alternating polynucleotides.[82] One of the reverse transcriptases, avian-myeloblastisis DNA polymerase, fails to excise noncomplementary deoxynucleotides at the 3′-primer terminus even when the molar ratio of enzyme to termini is 100:1.[85] *E. coli* RNA polymerase is more accurate in template copying;[46] it also does not appear to contain an associated exonuclease, but detailed studies are lacking. Eucaryotic DNA polymerases lack any associated exonucleases[86] and have error rates approaching 10^{-5}.[78,79] Procaryotic DNA polymerases with similarly low error rates contain an associated 3′ → 5′-exonuclease which has been postulated to preferentially excise noncomplementary nucleotides during or immediately after phosphodiester bond formation.[77] This concept has been formalized mathematically by Hopfield[74] and Ninio,[87] and generalized to all template-directed biological processes of high specificity.[74,88]

Mechanism of Base Selection by Polymerases

As indicated in Figure 11, two classes of mechanisms have been proposed to explain the 10^3-fold greater fidelity in template copying by polymerases than is obtainable from Watson-Crick base-pairing:

1. "Error prevention", amplification of the Watson-Crick base-pairing scheme prior to the covalent reactions.
2. "Proof-reading", the hydrolysis of mispaired nucleotides during or immediately after the covalent reactions.

The absence of 3′ → 5′-exonuclease activities in eucaryotic DNA polymerases,[86,89,90] RNA polymerase, and reverse transcriptases[85] rules out proof reading by these enzymes. Even in the case of *E. coli* Pol I, which contains a 3′ → 5′-exonuclease activity, this activity is not kinetically competent to generate the low error rates of 10^{-5}.[91] With bacteriophage-T_4 DNA polymerase, proof reading remains a viable hypothesis and is supported by the high rates of deoxynucleoside monophosphate generation during polymerization and by genetic studies.[92] However, even in this system, experiments suggesting "error prevention" have been reported.[93,94] These observations are, on balance, incompatable with the idea that proof reading is a significant mechanism for the high fidelity observed with DNA and RNA polymerases in vitro.

The alternative hypothesis of "error prevention" is supported by studies of the conformations of enzyme-bound substrates on DNA[26] and RNA polymerases[44,45] from *E. coli*. On both enzymes, the conformational angles χ at the glycosidic bonds of bound purine and pyrimidine substrates are all 90° (Figures 4, 5, and 9). In at least one case (Pol I-Mn-dTTP), this results from a 50° conformation change of the substrate (dTTP)

upon binding to the Pol I-Mn^{2+} complex[26] (Fig. 4, and 5). The conformation angle of 90° is that found in the double helical DNA B.[28,29] Hence, the binding of substrates to Pol I and to RNA polymerase, even in the absence of template, changes the conformation of the substrates to that of a nucleotidyl unit in the DNA double helix (Figures 4, 5, and 9). Such a conformation change of the substrate would position it in a manner appropriate for base pairing with the template (Figure 6). The bound substrate in its 90° conformation, when aligned by the enzyme with the rigid-template base, will, if incorrect, be rejected by steric overlap of the hydrogen-bond donors of the template and substrate (Figure 12).[30,95] If the substrate is correct, its interaction with the template would be entropically facilitated by the enzyme (Figures 6 and 12). The tightening of binding of the template to the enzyme in the presence of the correct nucleotide[94] is consistent with our observation of substrate orientation by the enzyme.[26] This selective amplification of the Watson-Crick base-pairing scheme could result in the observed error rate of 10^{-5} mistakes per correct nucleotide incorporated. For such an orientational effect of an enzyme on a substrate to be fully operative, the substrate and template should be held rigidly by the enzyme. This point was examined by studying the fluorescence polarization of enzyme-bound substrate analogs, a sensitive method of measuring the local rotational freedom of these analogs.[30,96] The analogs used were εATP and εdATP which have appropriately long fluorescence lifetimes for polarization studies.[97] Preliminary results in the presence of Mn^{2+} and in the absence of templates[30] indicated a correlation between fidelity, as measured by error rate, and the rigidity of the purine ring of the bound-substrate analog, as measured by its fluorescence polarization. Thus, Pol I, an enzyme of high fidelity, *E. coli* RNA polymerase, an enzyme of intermediate fidelity, and AMV reverse transcriptase, an enzyme of rather low fidelity, polarize the fluorescence of their bound substrate analogs by ≥18, 6.4 and 3.3%, respectively.[30] Independent evidence for some residual local mobility of the bound substrate CTP on RNA polymerase has been obtained by NMR.[45] The diamagnetic effects of this enzyme on the longitudinal and transverse relaxation rates of the protons of bound CTP yield a rotational correlation time for CTP (1 to 13 nsec) which is significantly shorter than the rotation time of the entire enzyme molecule (130 nsec). Hence, the larger error rates observed with RNA polymerase and reverse transcriptase may result from greater freedom of motion of the bound substrate, as suggested by the structure on the right of Figure 6.

In the past, other structural mechanisms of error prevention have been suggested which remain to be tested experimentally. These include template-induced changes in the conformation of the enzymes such that only the correct substrate[98] or the correct base pair is accommodated.[99]

The Maximum Fidelity of DNA and RNA Polymerases

When an enzyme has evolved to the point where it can optimally position and immobilize both the substrate and the template, the error rate should then be limited by the finite amount of the incorrect tautomer of the template base or of the substrate base present in the complex. As originally suggested by Watson and Crick,[95] enolization of a nucleic-acid base interconverts hydrogen-bond donors and acceptors, which could result in a mismatch. It is of interest that the tautomeric equilibrium ratio [imino] to [amino] for cytidine in water (from 10^{-5} to 10^{-4})[100] approaches the lowest error rates reported for DNA and RNA polymerases (10^{-5} to 10^{-4})[46,75,76,101] (Table 2). Such equilibrium considerations are applicable to Pol I, which appears to equilibrate rapidly with its substrates prior to each rate-limiting nucelotidyl transfer reaction.[22,25] Clearly, polymerases cannot shift the tautomeric equilibria by directly interacting with the bases of the substrate or the template since this would interfere with the substrate-template base pairing. Media of lower dielectric constant than water are known to shift the

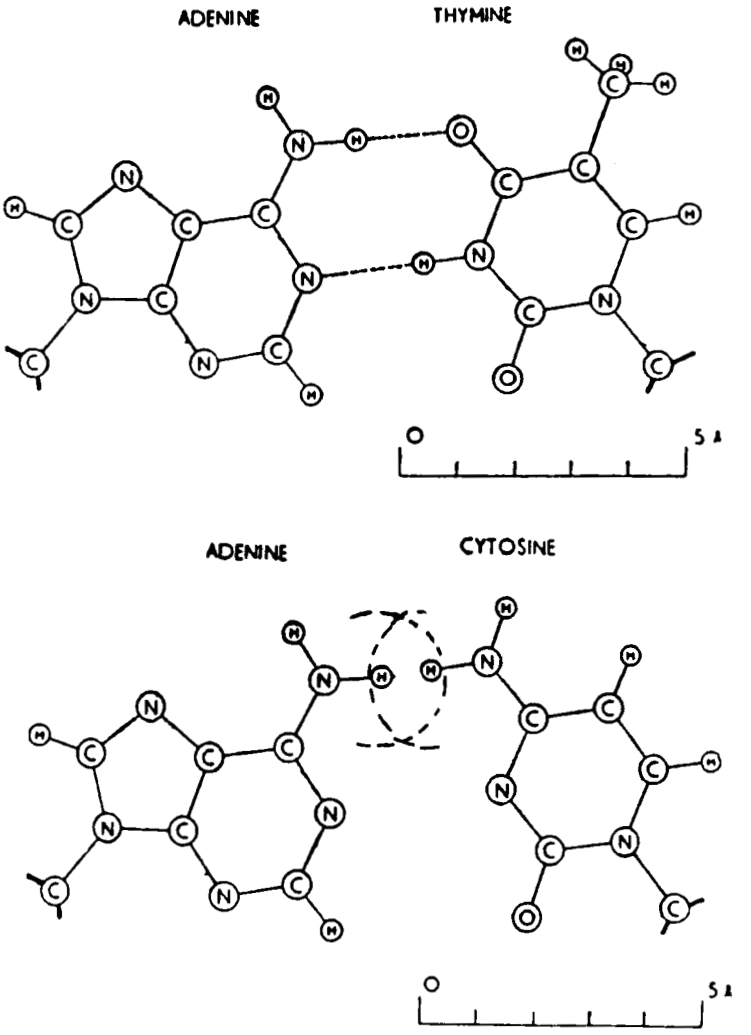

FIGURE 12. (Upper) Correct Watson-Crick A-T interaction.[95] (Lower) Incorrect A-C interaction hypothetically forced into the correct orientation, showing the highly unfavorable (1.4 Å) van der Waals overlap of the H-bond donors.[30] (From Mildvan, A. S., Stein, P. J., Abboud, M. M., Koren, R., and Bean, B. L., *Protons and Ions involved in Fast Dynamic Phenomena*, Laszlo, P., Ed., Elsevier, Amsterdam, 1978, 413, With permission.)

tautomeric equilibria toward the "wrong" forms.[100,102] Hence, polymerase enzymes could shift the tautomeric equilibria toward the correct forms by increasing the local dielectric constant at the binding sites of the substrate and template to a value greater than that of water. The fluorescence enhancement we have observed when εdATP binds to Pol I suggests a decrease rather than an increase in the local dielectric constant.[30] Also, decreasing the dielectric constant of the reaction mixture by the addition of up to 30% glycerol produced a threefold decrease in the fidelity by which AMV DNA polymerase copies poly [d(A-T)].[103] Hence, an error rate of 10^{-5} may reflect the inability of polymerase enzymes to shift the tautomeric [enol] to [keto] equilibrium ratio to a value lower than 10^{-5}.

Effect of Divalent Cations on Fidelity

The cations, Mn^{2+}, Co^{2+}, and Ni^{2+} can substitute for Mg^{2+} in activating DNA poly-

merases from diverse organisms, with two- to threefold diminutions in the fidelity of DNA synthesis at concentrations which produce maximal enzyme activity.[23] Interestingly, substitution of Mn^{2+} for Mg^{2+} increases the accuracy of RNA synthesis with *E. coli* RNA polymerase.[46] With DNA polymerases, the effects of substitution of Mn^{2+} for Mg^{2+} have been most extensively studied.

The effects of varying concentrations of free Mn^{2+} on the fidelity of copying poly [d(A-T)] by Pol I[104] have been compared with the dissociation constants of Mn^{2+} from the various components of the system.[22] At levels of Mn^{2+} where only the active site is occupied ($\leqslant 10^{-5} M$) a threefold increase in error rate is observed.[104] This is due, possibly, to a small change in the conformation of the enzyme or in the conformation or mobility of the enzyme-bound substrate. Such changes could also explain the high incorporation of ribonucleotides under these conditions,[26,105] since ribonucleotides would tend to be in the 3′-endo conformation while deoxyribonucleotides would tend to be 2′-endo.[28,29] We have previously pointed out that 2′-endo sugar conformations could assist in the further immobilization of the base.[26] At higher than activating concentrations of free Mn^{2+}, a preferential inhibition of the incorporation of the complementary nucleotide occurs, resulting in a large enhancement of the error rate to values approaching 10^{-2}.[104] Possible mechanisms for this enhanced misincorporation are (1) a reduction in the ratio of exonuclease to polymerase activity, (2) a secondary interaction of Mn^{2+} with the complementary substrate, either dATP or dTTP so as to change its base-pairing specificity, (3) Mn^{2+} binding to ancillary sites on the enzyme, and (4) Mn^{2+} binding to nucleotides on the template, causing changes in base-pairing specificity.[106]

The selective inhibition by Mn^{2+} of the 3′ → 5′-exonuclease is not found.[104,107] On the contrary, the formation of deoxynucleoside monophosphates is increased at high levels of Mn^{2+}.[91] Moreover, Mn^{2+} also enhances misincorporation with AMV DNA polymerase, an enzyme lacking any 3′ → 5′-exonuclease activity.[85,105,106]

The specific interaction of Mn^{2+} with the bases of certain substrates cannot account for these effects since similar enhancements of infidelity are observed with many combinations of polynucleotide templates and substrates.[106] The large increases in misincorporation with Pol I occur in the range of free Mn^{2+} concentrations (100 to 140 μM) at which the binding of Mn^{2+} to the template is increasing most rapidly.[22,104] This suggests modification of the template to be the mechanism of mutagenesis. However, since further binding of Mn^{2+} to the enzyme occurs at weak sites ($K_D \sim 0.8$ mM),[22] further experiments are required to firmly establish the sites of metal binding which greatly reduce fidelity.

As shown by preincubation studies, Be^{2+}, a nonactivating, slowly exchanging cation, forms a stable complex with AMV DNA polymerase. This results in a 20-fold enhancement of the rate of misincorporation.[108]

Those metal ions which cause increased misincorporation by AMV DNA polymerase in such in vitro assays are also known to be mutagenic and/or carcinogenic.[81,109] This correlation has recently been confirmed with *E. coli* Pol I.[107] The infidelity of DNA synthesis observed in vitro provides a reasonable explanation for mutagenesis and carcinogenesis by metal ions.[81,110]

CHEMOTHERAPEUTIC APPROACHES TO MALIGNANT DISEASES SUGGESTED BY MECHANISTIC STUDIES

As we have pointed out elsewhere,[13,121] regardless of the precise role of Zn^{2+}, its presence and apparent essentiality in various DNA and RNA polymerases renders this metal a potential target for chemotherapeutic attack.[121-123] Short-lived, highly radioactive isotopes of Zn^{2+} might be biosynthetically incorporated or exchanged into DNA and RNA polymerases to provide local irradiation of DNA in rapidly dividing cells.[13]

While the universal presence of Zn^{2+} in polymerases suggests difficulties in selectively interfering with DNA replication in animal tumor viruses or in malignant cells, certain Zn^{2+}-selective chelators might be concentrated in particular types of malignant cells. Alternatively, DNA replication in malignant cells with diminished concentrations of Zn^{2+} may be more easily inhibited by chelating agents. Also, RNA tumor viruses have been found to be inactivated more rapidly by *o*-phenanthroline than are cellular DNA polymerases,[16] permitting the selective inactivation of the former enzymes.[121]

The apparent suicidal inactivation of various DNA polymerases by 2′-3′-epoxy ATP provides a highly specific reagent for this class of enzymes.[21] As we have noted previously,[121] the selectivity of such reagents for error-prone DNA polymerases, which might be causally related to the progression of malignant disease,[76] could be increased as follows. As suggested in Figure 6 (right), error-prone polymerase enzymes may be unable to change the torsion angle of their substrates to the 90° form upon binding them. Rather, they may accept those substrate conformations which pre-exist free in solution, such as the 40° conformation for Mn-dTTP (Figure 3A). Thus, nucleotide inhibitors of DNA polymerase, the torsion angles of which are locked at 40°, might show a preference for those error-prone polymerases associated with malignant disease.[121]

ACKNOWLEDGMENTS

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