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Selective Inhibition of the Polymerase Activity of DNA Polymerase I. Further evidence for Separate Active Sites for Polymerase and 3′ to 5′ Exonuclease Activities†

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ABSTRACT: The chelating agent 1,10-phenanthroline, in the presence of a reducing agent, selectively inhibits the polymerase activity, but not the 3′ to 5′ exonuclease activity, of DNA polymerase I. The inhibition of DNA synthesis by 1,10-phenanthroline is potentiated by the addition of copper salts, as was previously reported by D'Aurora et al. [D'Aurora, V., Stern, A. M., & Sigman, D. S. (1977) *Biochem. Biophys. Res. Commun. 78*, 170]. Kinetic analysis demonstrates that a 1,10-phenanthroline–metal complex is a noncompetitive inhibitor of polymerase activity with deoxynucleoside triphosphate as the variable substrate and a competitive inhibitor with primer/template as the variable substrate. These results suggest that a 1,10-phenanthroline–metal complex competes with the primer terminus for the active site of the polymerase activity, but not the exonuclease activity, of DNA polymerase I. They also support our hypothesis that the polymerase and 3′ to 5′ exonuclease activities are catalyzed by separate active sites and that the primer terminus of the primer/template, which serves as a substrate for both activities, can bind to either of the catalytic sites. Some important implications follow from the delineation of separate active sites for the polymerase and 3′ to 5′ exonuclease activities with respect to the mechanism of DNA synthesis and the role of the 3′ to 5′ exonuclease activity in maintaining the fidelity of DNA synthesis.

Escherichia coli DNA polymerase I is a single polypeptide chain of 109 000 daltons which contains 1 g-atom of zinc/mol of enzyme (Springgate et al., 1973). The protein catalyzes several reactions including (1) extension of DNA chains in the 5′ to 3′ direction, (2) exonucleolytic degradation of DNA in the 5′ to 3′ direction, and (3) exonucleolytic degradation of DNA in the 3′ to 5′ direction. It is possible to separate the 5′ to 3′ exonuclease activity from the other activities of the enzyme by limited proteolysis (Brutlag et al., 1969; Klenow & Henningsen, 1970; Jacobsen et al., 1974). This treatment results in the splitting of the polypeptide chain into two fragments: a large fragment of 75 000 daltons which retains the polymerase and 3′ to 5′ exonuclease activities and a smaller fragment of 35 000 daltons which retains only the 5′ to 3′ exonuclease activity.

We have recently demonstrated that the catalytic sites for the polymerase activity and the 3′ to 5′ exonuclease activity of DNA polymerase I can be functionally dissociated by specific inhibitors of exonuclease activity (Que et al., 1978). We have shown that the 3′ to 5′ exonuclease activity can be selectively inhibited by nucleoside 5′-monophosphates, whereas the polymerase activity is not inhibited. In this report, we will present data showing that 1,10-phenanthroline selectively inhibits the polymerase activity of DNA polymerase I while the 3′ to 5′ exonuclease activity is not inhibited.

The chelating agent 1,10-phenanthroline inhibits a variety of DNA and RNA polymerases, many of which have been shown to contain stoichiometric amounts of zinc (Slater et al., 1971; Scrutton et al., 1971; Springgate et al., 1973; Valenzuela et al., 1973; Auld et al., 1975; Lattke & Wesser, 1977; Wandzulak & Benson, 1978). Dialysis of DNA polymerase I against excess 1,10-phenanthroline results in the gradual removal of zinc from the protein, accompanied by a corresponding loss of DNA polymerase activity (Springgate et al., 1973). However, the addition of 1,10-phenanthroline to a DNA polymerase assay results in instantaneous inhibition of polymerase activity, suggesting that inhibition of DNA polymerase activity can result either from the removal of zinc from the protein or from the formation of an enzyme–phenanthroline complex.

Inhibition of polymerase activity by 1,10-phenanthroline has been found to be relieved at high DNA concentrations (Slater et al., 1971), and it has been proposed that the enzyme-bound zinc interacts with DNA. It has further been proposed that zinc has a mechanistic role in nucleotidyl transfer reactions, coordinating and promoting the nucleophilicity of the 3′-hydroxyl group of deoxyribose of the primer terminus, thus facilitating the attack of the 3′-hydroxyl group at the α-

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phosphoryl group of the incoming deoxynucleoside triphosphate (Slater et al., 1972; Springgate et al., 1973; Mildvan, 1974). However, D'Aurora et al. (1977) have recently shown that the instantaneous inhibition of DNA polymerase activity by 1,10-phenanthroline occurs only in the presence of a reducing agent such as 2-mercaptoethanol and that the inhibitory effect of 1,10-phenanthroline is potentiated by the addition of copper salts. They have suggested that the requirement for a reducing agent is for reduction of Cu^{2+}, present as a contaminant in assay reagents, to Cu^{+} and that the inhibitory species is a phenanthroline–Cu^{+} (2:1) complex. Thus, the instantaneous inhibition of DNA polymerase activity by 1,10-phenanthroline may occur by a mechanism other than interaction with protein-bound zinc.

Experimental Procedures

Materials. Unlabeled deoxynucleoside triphosphates were purchased from either P-L Biochemicals or Sigma Chemical Co. [^{3}H]dTTP (50 Ci/mmol) was purchased from Amersham Corp. Poly(dA-dT) was purchased from either Grand Island Biological or Miles Laboratories and was dialyzed before use against 10 mM Tris-HCl, pH 7.4, and 60 mM KCl. Phenanthroline analogues were obtained from K and K Chemicals. *E. coli* B (3/4 log) was purchased from Grain Processing Corp. Terminal deoxynucleotidyl transferase was purchased from Boehringer-Mannheim Corp. Chelex (50–100 mesh) was obtained from Bio-Rad Laboratories. Solutions used to assay polymerase or 3′ to 5′ exonuclease activities of DNA polymerase I were made "metal free" by batchwise treatment with Chelex-100 according to Willard et al. (1969).

Preparation of Enzymes and Polynucleotides. DNA polymerase I was prepared from *E. coli* B according to Jovin et al. (1969) through step VII and further purified by phosphocellulose chromatography as described by Slater et al. (1972). When assayed separately under the conditions described below, the ratio of polymerase activity to 3′ to 5′ exonuclease activity of DNA polymerase I was approximately 15:1. [^{3}H]poly(dT)$_{50}$ was synthesized with terminal deoxynucleotidyl transferase by using oligo(dT)$_4$ and [^{3}H]dTTP (220 cpm/pmol) in a molar ratio of 1:50 according to Setlow & Kornberg (1972). [^{3}H]poly(dA-dT) was synthesized with DNA polymerase I in a primed reaction according to Schachman et al. (1960) by using [^{3}H]dTTP (10 cpm/pmol). Polynucleotide concentrations are expressed as nucleotide equivalents.

DNA Polymerase Assay. The reaction mixture contained in a final volume of 0.3 mL: 67 mM potassium phosphate buffer, pH 7.5; 6.7 mM $MgCl_2$; 0.66 mM 2-mercaptoethanol; 3.3 mM ammonium sulfate; 33 μg/mL of bovine serum albumin; 33 μM [^{3}H]dTTP, 88 cpm/pmol; 5–10 μM poly(dA-dT); and 0.15 unit of DNA polymerase. After incubation at 37 °C for 10 min, the reaction was stopped by the addition of 2 mL of cold 5% trichloroacetic acid containing 10 mM sodium pyrophosphate. The precipitate was collected, washed, and counted as previously described (Byrnes et al., 1976). One unit of DNA polymerase catalyzes the incorporation of 10 nmol of total nucleotide in 30 min at 37 °C.

3′ to 5′ Exonuclease Assay. The reaction mixture contained in a final volume of 0.2 mL the following: 50 mM Hepes buffer, pH 7.4; 5 mM $MgCl_2$; 0.5 mM 2-mercaptoethanol; 10 mM KCl; 2 μM [^{3}H]poly(dT)$_{50}$, 220 cpm/pmol; and 0.15 unit of 3′ to 5′ exonuclease. The reaction mixture was incubated at 37 °C, and 20-μL aliquots were applied to 2.4-cm circles of Whatman DE-81 paper after 0, 4, 8, 12, 16, and 20 min of incubation. The circles were washed, dried, and counted as described by Brutlag & Kornberg (1972). Initial velocities were determined from a least-squares fit of nucleotide hydrolyzed plotted vs. time. One unit of 3′ to 5′ exonuclease catalyzes the hydrolysis of 10 nmol of nucleotide in 30 min at 37 °C.

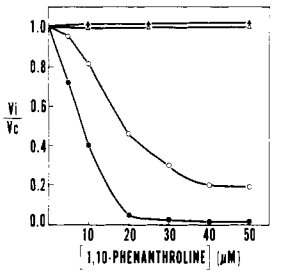

FIGURE 1: Effects of 1,10-phenanthroline on the polymerase and 3′ to 5′ exonuclease activities of DNA polymerase I in the absence (○, △) and presence (●, ▲) of 10 μM $CuSO_4$. Polymerase (○, ●) and exonuclease (△, ▲) activities were assayed separately as described under Experimental Procedures except (1) in the polymerase assay, the concentration of poly(dA-dT) was 10 μM and (2) in the exonuclease assay, the concentration of [^{3}H]poly(dT)$_{50}$ was 2 μM. V_c and V_i represent the initial rate of the uninhibited and inhibited reactions, respectively, for polymerase and exonuclease activities. Initial velocities are expressed as picomoles of nucleotide incorporated per minute in the polymerase assay and as picomoles of nucleotide released per minute in the exonuclease assay. One hundred percent activity represents 45 ± 2 pmol of [^{3}H]dNMP incorporated per min for polymerase activity and 18 ± 2 pmol of [^{3}H]dTMP released per min for exonuclease activity.

Binding Assay. The reaction mixture contained in a final volume of 0.2 mL the following: 67 mM Hepes buffer, pH 7.4; 1 mM 2-mercaptoethanol; 18.6 μM [^{3}H]poly(dA-dT), 10 cpm/pmol; and 0.3–7 pmol of DNA polymerase I. After incubation at 20 °C for 10 min, the reaction was stopped by chilling in ice and the addition of 2 mL of cold buffer A (10 mM Hepes buffer, pH 7.4; 5 mM 2-mercaptoethanol; and 50 mM NaCl). The reaction mixture was filtered on a Millipore filter (Type HA, 2.4-cm diameter), previously soaked in buffer A, and washed with 60 mL of buffer A. The filter was dried and counted in toluene–Omnifluor scintillant in a liquid scintillation counter.

Kinetic data were analyzed by use of the PROPHET Computer System, sponsored by the Chemical Biological Information Handling Program, Division of Research Resources, National Institutes of Health.

Results

Selective Inhibition of the Polymerase Activity of DNA Polymerase I. The effects of 1,10-phenanthroline on the polymerase activity and the 3′ to 5′ exonuclease activity of DNA polymerase I are shown in Figure 1. In the presence of 2-mercaptoethanol, the addition of increasing concentrations of 1,10-phenanthroline resulted in a progressive inhibition of polymerase activity, as measured by the incorporation of [^{3}H]dTMP into poly(dA-dT). Little or no inhibition of polymerase activity was observed in the absence of 2-mercaptoethanol (data not shown), in agreement with the results of D'Aurora et al. (1977), whereas in the presence of 2-mercaptoethanol, poly(dA-dT) synthesis was inhibited approximately 50% at 18 μM 1,10-phenanthroline. Copper salts, in the presence of thiols, potentiated the inhibitory effect of 1,10-phenanthroline, as was also previously reported by D'Aurora et al. (1977). In the presence of 10 μM $CuSO_4$, the concentration of 1,10-phenanthroline required for 50% inhibition was reduced to 8 μM. $CuSO_4$ alone, either in the presence or in the absence of 2-mercaptoethanol, had no effect on polymerase activity. Other analogues of phenanthroline which are not metal chelators, such as 1,5-phenanthroline,

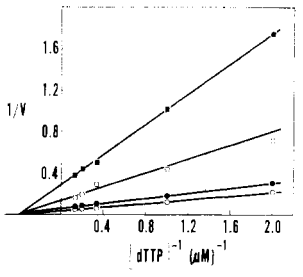

FIGURE 2: Double-reciprocal plot of the initial rate of nucleotide incorporation (V) vs. dTTP concentration at various fixed concentrations of 1,10-phenanthroline. The assay conditions are as under Experimental Procedures except that poly(dA-dT) concentration was 5 μM. The concentrations of 1,10-phenanthroline were 0 (○), 10 (●), 20 (□), and 30 μM (■). Initial velocities are expressed as picomoles of nucleotide incorporated per minute.

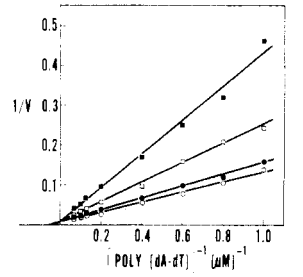

FIGURE 3: Double-reciprocal plot of the initial rate of nucleotide incorporation (V) as a function of poly(dA-dT) concentration at various fixed concentrations of 1,10-phenanthroline. Initial velocities are expressed as picomoles of nucleotide incorporated per minute. The concentrations of 1,10-phenanthroline were 0 (○), 10 (●), 20 (□), and 30 μM (■).

4,7-phenanthroline, and 1,10-phenanthroline-5,6-dione, also had no effect on polymerase activity.

No inhibition of 3′ to 5′ exonuclease activity, as measured by the release of [^{3}H]dTMP from [^{3}H]poly(dT)$_{50}$, was observed over the same concentration range of 1,10-phenanthroline, either in the presence or in the absence of 2-mercaptoethanol and/or $CuSO_4$. In these experiments, poly(dA-dT) and [^{3}H]polyd(T)$_{50}$ were present at half-saturating concentrations.

We have also observed that the optimal Mg^{2+} concentration for both polymerase and exonuclease activities remained constant in the presence and absence of 1,10-phenanthroline. The degree of inhibition of polymerase activity was unchanged at all concentrations of Mg^{2+} tested, indicating that the inhibition of DNA polymerase activity by 1,10-phenanthroline is not due to the chelation of Mg^{2+} (data not shown).

These results demonstrate that 1,10-phenanthroline, probably as a phenanthroline–Cu^+ complex, selectively inhibits the polymerase but not the 3′ to 5′ exonuclease activity of DNA polymerase I.

Kinetic Studies. To elucidate the mechanism of inhibition of polymerase activity by 1,10-phenanthroline, we have determined the pattern of inhibition with either deoxynucleoside triphosphate or primer/template as variable substrate. The plot of the reciprocal of the rate of poly(dA-dT) synthesis as a function of the reciprocal of the dTTP concentration at 0, 10, 20, and 30 μM 1,10-phenanthroline is shown in Figure 2. Similar results were obtained in the presence of 10 μM $CuSO_4$ except that lower concentrations of 1,10-phenanthroline were required for inhibition. The inhibition pattern indicates that a 1,10-phenanthroline–Cu^+ complex is noncompetitive with the substrate dTTP.

Figure 3 shows the reciprocal of the initial rate of nucleotide incorporation plotted as a function of the reciprocal of the

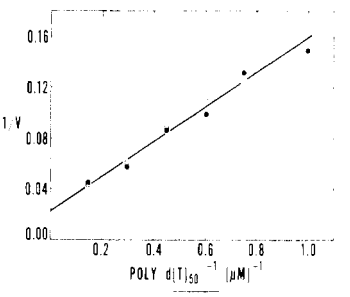

FIGURE 4: Double-reciprocal plot of the initial rate of nucleotide released (V) as a function of the poly(dT)$_{50}$ concentration in the absence (○) and presence (●) of 75 μM 1,10-phenanthroline. Initial velocities are expressed as picomoles of nucleotide released per minute.

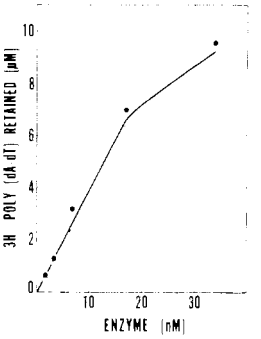

FIGURE 5: Binding of [^{3}H]poly(dA-dT) to DNA polymerase I in the absence (○) and presence (●) of 500 μM 1,10-phenanthroline.

poly(dA-dT) concentration at 0, 10, 20, and 30 μM 1,10-phenanthroline. The data indicate that the type of inhibition is competitive and suggest that the inhibitor is competing with the primer terminus for binding at the active site of the polymerase activity. A competitive pattern of inhibition was also observed in the presence of 10 μM $CuSO_4$, although the concentrations of 1,10-phenanthroline required for inhibition were lower.

Since the primer terminus of the primer/template is also a substrate for the 3′ to 5′ exonuclease activity of DNA polymerase I, we have investigated the effect of 1,10-phenanthroline on the rate of hydrolysis of [^{3}H]poly(dT)$_{50}$ as a function of poly(dT)$_{50}$ concentration. Single-stranded DNA was used to measure the 3′ to 5′ exonuclease activity of DNA polymerase I to prevent interference by the 5′ to 3′ exonuclease activity of this enzyme, which preferentially hydrolyzes double-stranded DNA. The reciprocal of the rate of hydrolysis of poly(dT)$_{50}$ as a function of the reciprocal of the poly(dT)$_{50}$ concentration, in the presence and absence of 75 μM, 1,10-phenanthroline, is shown in Figure 4. The polymerase inhibitor has no effect on the 3′ to 5′ exonuclease activity, indicating that a 1,10-phenanthroline–Cu^+ complex does not compete with the primer terminus for binding at the exonuclease active site.

Binding Studies. Our kinetic data would suggest that, although a 1,10-phenanthroline–Cu^+ complex inhibits the binding of the primer terminus at the active site of the polymerase activity, it does not inhibit binding of the primer terminus at the active site of the exonuclease activity. We have therefore investigated the effect of 1,10-phenanthroline on the binding of [^{3}H]poly(dA-dT) to DNA polymerase I by the method of filtration on cellulose nitrate membrane filters. In these experiments [^{3}H]poly(dA-dT) and DNA polymerase I were incubated in the absence of Mg^{2+} to prevent degradation of the [^{3}H]poly(dA-dT) by either the 3′ to 5′ exonuclease or the 5′ to 3′ exonuclease activities of DNA polymerase I. The

binding of DNA to DNA polymerase I has previously been shown not to require exogenous divalent cation (Englund et al., 1969; Slater et al., 1972). As shown in Figure 5, 1,10-phenanthroline, at a concentration which completely inhibits DNA polymerase activity, has no effect on the binding of poly(dA-dT) to the enzyme.

Discussion

We have recently put forth a proposal that the catalytic site for the 3′ to 5′ exonuclease activity is distinct from the catalytic site for the polymerase activity on *E. coli* DNA polymerase I and that the primer terminus of the primer/template, which serves as a substrate for both activities, can bind to either of these catalytic sites (Que et al., 1978). This hypothesis was based on the observations that the 3′ to 5′ exonuclease activity can be selectively inhibited by nucleoside 5′-monophosphates, whereas the DNA polymerase activity is not inhibited, and that nucleoside 5′-monophosphates are competitive inhibitors of exonuclease activity but not of polymerase activity when measured as a function of primer/template concentration. The present study further demonstrates the functional dissociation of these two catalytic sites.

We have observed that the polymerase activity of DNA polymerase I is selectively inhibited by 1,10-phenanthroline while the exonuclease activity is not inhibited. The presence of 2-mercaptoethanol is required for inhibition, and copper salts potentiate the inhibitory effect of 1,10-phenanthroline on polymerase activity. These results are similar to those reported by D'Aurora et al. (1977) and support their proposal that the inhibitory species is a 1,10-phenanthroline–Cu^+ complex. Whether other metal–phenanthroline complexes can also inhibit polymerase activity has not been investigated. The removal of enzyme-bound zinc by 1,10-phenanthroline has also been shown to result in the loss of polymerase activity (Springgate et al., 1973). Thus the role of enzyme-bound zinc in the catalysis of polymerization is still unknown. It is also not known whether zinc is involved in the catalysis of hydrolysis by the 3′ to 5′ exonuclease activity of the enzyme.

Kinetic analysis demonstrates that a 1,10-phenanthroline–Cu^+ complex competitively inhibits the polymerase activity but not the exonuclease activity with primer/template as variable substrate, suggesting that 1,10-phenanthroline-Cu^+ competes with the primer terminus for binding at the polymerase active site but not at the exonuclease active site of DNA polymerase I.

The observations that inhibition of DNA polymerase activity by a 1,10-phenanthroline–Cu^+ complex is competitive with primer terminus and noncompetitive with deoxynucleoside triphosphate are similar to the observations with terminal deoxynucleotidyl transferase (Chang & Bollum, 1970) and are also consistent with the observation of Slater et al. (1971) that at saturating levels of DNA, DNA polymerase I is insensitive to 1,10-phenanthroline. However, in contrast to the previous finding that 1,10-phenanthroline prevents the binding of a polynucleotide to terminal deoxynucleotidyl transferase (Chang & Bollum, 1970), we have found that 1,10-phenanthroline has no effect on the binding of DNA polymerase I to poly(dA-dT). This result is consistent with our finding that hydrolysis of DNA by the 3′ to 5′ exonuclease activity of DNA polymerase I is not inhibited by 1,10-phenanthroline, since one would expect both polymerase and exonuclease activities to be inhibited by an agent that prevented binding of DNA to the enzyme.

The present study, together with our previous observation that the 3′ to 5′ exonuclease activity of DNA polymerase I can be selectively inhibited by nucleoside 5′-monophosphates while the polymerase activity is not inhibited (Que et al., 1978), provides strong evidence that the active sites for the polymerase and 3′ to 5′ exonuclease activities of DNA polymerase I are separate and distinct and that the primer terminus of the primer/template, a substrate for both activities, can bind to either of these active sites. When the primer terminus is bound at the polymerase active site, it is a substrate for chain extension, and when it is bound at the exonuclease site, it is a substrate for hydrolysis. This is depicted schematically in Figure 6, which shows the primer terminus either bound at the polymerase site (Figure 6A) or bound at the exonuclease active site (Figure 6B).

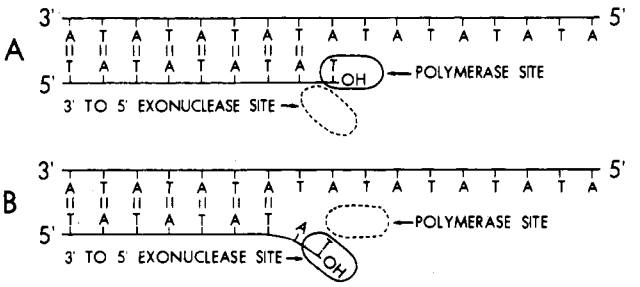

FIGURE 6: Schematic diagram of polymerase and 3′ to 5′ exonuclease sites of DNA polymerase I.

In the proposed model the binding of a primer terminus at the polymerase site is restricted to termini that are able to base-pair with the template strand of the primer/template and is dictated by the base sequence of the template. In contrast, binding of a primer terminus at the exonuclease site requires that the 3′-hydroxyl terminus be unpaired and is, therefore, favored for termini that are not able to base-pair with the template. Thus, when a primer contains a base-paired terminus, chain extension would be favored over hydrolysis, whereas when a non-base-paired nucleotide is incorporated at the primer terminus, hydrolysis would be favored over chain extension. This is consistent with the observations of Brutlag & Kornberg (1972) that a non-base-paired primer terminus is quantitatively removed before chain extension occurs, whereas a base-paired primer terminus is quantitatively retained.

The proposed model provides an efficient mechanism for error correction during DNA synthesis and extends the proposal of Kornberg (1974) that the high fidelity of DNA synthesis is the result of two base-pairing selection steps, one of which is the determination that the primer terminus is properly base-paired to the template before addition of the next nucleotide and the other is the selection of the correct nucleotide for polymerization.

That the function of the 3′ to 5′ exonuclease activity of DNA polymerase I is to correct errors that may occur during DNA synthesis was first suggested by Goulian et al. (1968) and Kornberg (1969) and was subsequently demonstrated by Brutlag & Kornberg (1972) in studies which showed that 3′ to 5′ exonuclease activity preferentially hydrolyzed primer termini which are not base-paired with the template. This led Brutlag & Kornberg (1972) to propose that the 3′ to 5′ exonuclease may serve a proofreading function by removing mismatched nucleotides incorporated at the primer terminus prior to chain extension. More recently, the function of the 3′ to 5′ exonuclease activity has been extrapolated to include proofreading of the primer terminus after the addition of each nucleotide (Topal & Fresco, 1976; Cox, 1976). However, our recent studies demonstrate that the active sites for polymerase and 3′ to 5′ exonuclease activities are separate and distinct and suggest that the fate of the primer terminus, extension

or excision, depends on whether it binds at the polymerase or the exonuclease active site. These studies argue against the suggestion that the 3′ to 5′ exonuclease proofreads the growing DNA chain at each step of DNA synthesis, since binding of the primer terminus at the exonuclease active site would necessarily result in its hydrolysis.

Several other implications follow from the delineation of separate active sites on DNA polymerase I for the polymerase and 3′ to 5′ exonuclease activities, with respect to the mechanism of DNA synthesis and the role of the 3′ to 5′ exonuclease activity in maintaining the fidelity of DNA synthesis. It would suggest that the two base-pairing selection steps which together result in the high fidelity of DNA synthesis (Kornberg, 1974) are both functions of the polymerase activity of DNA polymerase I. For polymerization to occur, both the incoming deoxyribonucleoside triphosphate and the nucleotide at the primer terminus must be properly base-paired with the corresponding nucleotides in the template strand. Thus, every nucleotide is checked twice by the polymerase activity, once before and once after polymerization; both steps involve specificity imposed by Watson–Crick base pairing. It would also suggest that the 3′ to 5′ exonuclease activity is not an obligatory component of DNA synthesis unless a mismatched nucleotide has been incorporated. When the exonuclease activity is deficient or inhibited, although the polymerase activity is able to proofread the primer terminus, the enzyme lacks the ability to excise the mismatched nucleotide that has been incorporated. This would be analogous to DNA synthesis with only a single base-pairing selection step. Thus, the editing function of DNA polymerase is not a function solely of the exonuclease activity but is shared jointly by the polymerase and the 3′ to 5′ exonuclease activities.

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