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Exonucleolytic proofreading by calf thymus DNA polymerase δ

(proofreading/mutagenesis/accuracy/3′ to 5′ exonuclease/mismatched bases)

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ABSTRACT **The fidelity of DNA synthesis by calf thymus DNA polymerase δ (pol δ) *in vitro* has been determined using an M13*lacZα* nonsense codon reversion assay. Pol δ is highly accurate, producing on average <1 single-base substitution error for each 10^6 nucleotides polymerized. This accuracy is 10- and 500-fold greater than that of DNA polymerases α and β, respectively, in the same assay. Three observations suggest that this higher fidelity results in part from proofreading of misinserted bases by the 3′ to 5′ exonuclease associated with pol δ. First, the exonuclease efficiently excises terminally mismatched bases. Second, both terminal mismatch excision and the fidelity of DNA synthesis by pol δ are reduced with increasing concentration of deoxynucleoside triphosphates in the synthesis reaction. These effects result from increasing the rate of polymerization relative to the rate of exonucleolytic excision and are hallmarks of exonuclease proofreading. Third, both terminal mismatch excision and fidelity decrease upon addition to the reaction mixture of adenosine monophosphate, a compound known to selectively inhibit the exonuclease but not the polymerase activity of pol δ. These results suggest that 3′ to 5′ exonuclease-dependent proofreading enhances the fidelity of DNA synthesis by a mammalian DNA polymerase *in vitro*.**

The high base-substitution fidelity of prokaryotic DNA polymerases results from discrimination at the base insertion step and from exonucleolytic proofreading of incorrectly inserted bases during synthesis (for review, see ref. 1). Discrimination at the insertion step also operates with eukaryotic DNA polymerases, since certain of these enzymes synthesize DNA with relatively high fidelity even in the absence of detectable associated 3′ to 5′ exonuclease activity (1–6). The role of proofreading in improving the fidelity of DNA synthesis in eukaryotes is less well defined.

Proofreading has been implied to contribute to fidelity in lower eukaryotes (7, 8). While purified DNA polymerases α and β (pol α and β) from higher eukaryotes usually have been found to lack exonuclease activity, several reports describe 3′ to 5′ exonuclease activities associated with DNA pol α (9–11) and β (12, 13). A nondissociable 3′ to 5′ exonuclease is one of the distinguishing characteristics of DNA polymerase δ (pol δ), an enzyme first purified from rabbit bone marrow (14) and more recently from calf thymus (15–17) and designated DNA polymerase δII.‡

In this report, we address two questions: (*i*) What is the fidelity of calf thymus DNA pol δ during DNA synthesis *in vitro*, and (*ii*) does the associated 3′ to 5′ exonuclease activity contribute to the observed accuracy by proofreading errors during DNA synthesis?

MATERIALS AND METHODS

Bacteriophage. Three mutant derivatives of bacteriophage M13mp2 were used in this study: M13mp2A89 (18) containing a guanine to adenine base substitution at position 89 (where +1 is the first transcribed base of the *lacZα* gene)—this change creates an opal codon, yielding a colorless phenotype; M13mp2G103 (19) containing a thymine to guanine change at position 103, creating a missense codon yielding a medium light-blue phenotype; and M13mp2A103 (20) containing a thymine to adenine change at position 103, creating a missense codon yielding a very faint-blue phenotype. All bacterial strains and other materials were as described (18, 19).

DNA Polymerases. Pol α and pol δ were purified by the methods of Wahl *et al.* (21) and Crute *et al.* (16), respectively. After the initial purification, both pol α and pol δ were further purified by phosphocellulose chromatography to remove contaminating nuclease from pol α and endonuclease from pol δ. Phosphocellulose columns (Whatman P-11; 0.4 × 5.5 cm) were equilibrated with 50 mM Tris·HCl (pH 7.5) containing 20% (vol/vol) glycerol, 100 mM NaCl, 5 mM dithiothreitol, 1 mM EDTA, 1 mM EGTA, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 μg of pepstatin per ml, and 0.5 μg of leupeptin per ml. Pol α (85 units) or pol δ (200 units, δII fraction III) were dialyzed into equilibration buffer and applied to separate phosphocellulose columns. Each polymerase was eluted at 1 ml/hr in a 20-ml linear gradient using equilibration buffer and the same buffer containing 500 mM NaCl. After the polymerase assays, each peak of activity was pooled and concentrated using an Amicon Centricon 30 concentrator. Pol α and pol δ had concentrations of 0.4 and 1 unit/μl, respectively. Conditions for polymerase assays and units of activity were defined as described (17).

Avian myeloblastosis virus (AMV) DNA polymerase and the large Klenow fragment of *Escherichia coli* DNA polymerase I [pol I (KF)] were obtained from Pharmacia. Rat Novikoff hepatoma DNA pol β was provided by Dale Mosbaugh (University of Texas, Austin).

Fidelity Assay. The base-substitution fidelity of DNA synthesis *in vitro* has been monitored using an M13mp2*lacZα* opal codon reversion assay. The experimental design is essentially that described in figure 1 of ref. 19. A gapped molecule is constructed in which the gap contains a mutant derivative of the *lacZα* coding sequence, possessing a single base change (guanine to adenine in the viral template strand at position 89). This change creates an opal termination codon, resulting in a colorless plaque phenotype under the appropriate plating conditions (19). Base-substitution errors at the opal codon during gap-filling DNA synthesis are detected as blue plaques upon transfection and the proportion of blue to total plaques (the reversion frequency) reflects the error frequency of DNA synthesis. This assay detects eight

Abbreviations: pol δ, DNA polymerase δ; pol α, DNA polymerase α; pol β, DNA polymerase β; pol I (KF), large Klenow fragment of *Escherichia coli* DNA polymerase I; AMV, avian myeloblastosis virus.

‡A polymerase with a readily dissociable nuclease was isolated from calf thymus and designated DNA polymerase δI (16, 17). This enzyme may represent an association of DNA pol α with a 3′ to 5′ exonuclease.

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different single base-substitution errors, each resulting in a blue phenotype of sufficient intensity to permit detection of a single blue revertant on a plate containing >10,000 colorless plaques. None of the plating experiments described here exceeds this detection limit.

Heteroduplex expression experiments for all eight detectable mispairs at this codon demonstrate that ≈60% of the errors produced *in vitro* can be expressed by transfection, with no significant bias for any of the eight mispairs.

Terminal Mismatch Excision Assay. In the assay for exonucleolytic removal of a mismatched base from a primer terminus, DNA from two mutant derivatives of bacteriophage M13mp2 containing single base changes at position 103 are used to construct a gapped heteroduplex molecule. This molecule contains a 3′-terminal cytosine residue in the primer (i.e., minus) strand opposite an adenine residue in the template (plus) strand. Expression of the cytosine-containing sequence results in a medium-blue plaque phenotype, whereas expression of the adenine-containing sequence results in a faint-blue plaque phenotype. Polymerization to fill the gap without excision of the cytosine will produce a double-stranded heteroduplex, which upon transfection will yield ≈60% medium-blue plaques and ≈40% faint-blue plaques that are easily distinguished. However, if the mispaired cytosine is removed prior to extension by the polymerase, subsequent correct incorporation of thymidine opposite the template adenine will yield a homoduplex molecule having exclusively a faint-blue plaque phenotype. For any given reaction condition, the proportion of medium- and faint-blue plaques obtained upon transfection of the reaction products describes the extent of terminal mismatch excision.

DNA Polymerase Reactions. Pol α and pol δ reactions contained 20 mM Tris·HCl, pH 7.5/10 mM dithiothreitol/10 mM $MgCl_2$/200 μg of bovine serum albumin per ml/20% (vol/vol) glycerol/3 mM ATP. All other DNA polymerase reactions contained 20 mM Hepes (pH 7.8), 2 mM dithiothreitol, and 10 mM $MgCl_2$. The variables, including reaction volume, time, amount of DNA polymerase, amount of gapped DNA (containing either the opal codon or the terminal mismatch), and the concentrations of the dNTPs and AMP are given in the legends to the tables and figures. After incubation at 37°C, reactions were terminated by addition of EDTA to a final concentration of 15 mM. Twenty microliters of each reaction was analyzed by agarose gel electrophoresis as described (19). All polymerase reactions reported here generated products that migrated coincident with a replicative form II fully double-stranded DNA standard (e.g., see figure 2 in ref. 19). All or a portion of the remaining DNA (as indicated in the legends) was used to infect competent *E. coli* cells to monitor plaque colors as described (19). DNA sequence analysis of (blue) revertants was performed as described (19).

RESULTS

Base-Substitution Fidelity of Pol δ. The fidelity of calf thymus pol δ was measured using the M13*lacZ*α opal codon reversion assay. As shown in Table 1, the reversion frequency of DNA copied by pol δ at equal concentrations (20 μM) of all four dNTP substrates is only slightly above the background reversion frequency of the uncopied viral DNA control. Thus, DNA synthesis by pol δ under these reaction conditions is highly accurate. In contrast, but consistent with previous results (2, 3, 6, 19, 20, 23), both pol α and pol β produce errors at much higher frequencies. After subtracting the background reversion frequency, pol δ is at least 13-fold and 580-fold more accurate than pol α and β, respectively.

Terminal Mismatch Excision by Pol δ. One possible explanation for the higher fidelity of pol δ is that its associated 3′ to 5′ exonuclease proofreads misinsertions during synthesis.

Table 1. Base-substitution fidelity of pol δ

DNA sample	Plaques scored: Total	Plaques scored: Blue	Reversion frequency, × 10^{-6}
Uncopied (viral)	2,900,000	4	1.4
Copied by			
Pol δ	2,300,000	12	5.2
Pol α	160,000	8	50
Pol β	84,000	151	1800

All copying reaction mixtures contained equimolar concentrations of dATP, dTTP, dGTP, and dCTP. The pol δ reaction mixture (150 μl) contained 20 μM each dNTP, 30 units of pol δ, and 750 ng of gapped M13mp2A89 DNA. The pol α reaction (100 μl) contained 50 μM each dNTP, 20 units of calf thymus pol α, and 500 ng of gapped DNA. The pol β reaction mixture (50 μl) contained 500 μM each dNTP, 0.32 unit of rat Novikoff hepatoma pol β, and 250 ng of gapped DNA. Incubation was at 37°C for 1 hr. Transfections were performed as described (19) using 130 μl (pol δ), 40 μl (pol α), or 15 μl (pol β) of the reaction mixture. The uncopied DNA reversion frequency was determined by transfection of cells with the same viral single-stranded template DNA preparation used to prepare the gapped substrate. The base-substitution fidelity of pol δ can be calculated from the reversion frequency by subtracting the background reversion frequency of uncopied DNA then dividing by 0.6, the probability of expressing a base-substitution error. This value of 1/160,000 is the combined error frequency for all three template positions and all eight possible mispairs. The calculated average error frequency per base is thus 1/470,000 and frequency per mispair is 1/1,300,000.

With prokaryotic DNA polymerases, a classic approach to determine potential proofreading activity is to measure exonucleolytic removal of a terminally mispaired base (24). Indeed, Lee *et al.* (25) have shown that, in manganese-activated reactions with synthetic homopolymer substrates, the 3′ to 5′ exonuclease activity of a lower molecular weight form of calf thymus pol δ hydrolyzes both noncomplementary and complementary primer termini, with a 3-fold preference for mispaired ends.

We examined the ability of pol δ to excise a terminal cytosine residue from a C (medium blue)·A (faint blue) mispair located at the 3′-hydroxyl end of a gapped M13 molecule. The results shown in Table 2 compare pol δ to AMV DNA polymerase, an enzyme having no associated 3′ to 5′ exonuclease (26), and to pol I (KF), having a well-characterized associated 3′ to 5′ exonuclease (27). Extension by AMV DNA polymerase creates a product yielding 52% medium-blue plaques, suggesting that little or no terminal mismatch excision occurs prior to synthesis by AMV DNA polymerase. In contrast, the medium-blue frequency of 14% observed with Pol I (KF) demonstrates that, under these reaction conditions (100 μM dNTP substrates), the cytosine was excised from 74% of the gapped DNA molecules by the proofreading exonuclease prior to gap-filling DNA synthesis. Under similar conditions, transfection of the products of the

Table 2. Terminal mismatch excision by pol δ

DNA copied by	Blue plaques scored: Faint	Blue plaques scored: Medium	Medium blue, %	% terminal cytosine excised
AMV pol	540	593	52	0
Pol I (KF)	1115	184	14	74
Pol δ	1055	15	1.4	98

Copying reactions (30 μl) contained 150 ng of gapped DNA substrate containing the terminal C·A mispair, 100 μM each dNTP and 20 units (as described by the supplier) of AMV DNA polymerase (AMV pol), 0.3 unit of pol I (KF), or 6 units of pol δ. Enzyme was added last to reactions prewarmed to 37°C for 3 min. Incubations at 37°C were for 60 min (AMV pol and pol δ) or 10 min [pol I (KF)]. Transfections using 2 μl of the reaction mixture were performed as described (19).

pol δ reaction resulted in only 1.4% medium-blue plaques, indicating excision of 98% of the mispaired cytosine prior to polymerization. These data demonstrate that the 3′ to 5′ exonuclease activity associated with pol δ efficiently removes a terminal mispair.

These results prompted a more extensive examination of the efficiency of terminal mismatch excision by systematic manipulation of reaction conditions to alter the exonuclease/polymerase ratio. Two variations were used. The first was to increase the dNTP substrate concentration for polymerization. The concentrations used ranged from the lowest that gave complete gap-filling up to 1 mM. As the concentration of the next correct base to be added after the mismatch (in this instance, dCTP) is increased, the rate of polymerization from this mispaired terminus should increase, permitting less time for excision of the mismatch. This "next-nucleotide" effect has been a standard method of demonstrating the contribution of proofreading to fidelity with several prokaryotic enzymes (28–30). The result in this assay should be an increase in the medium-blue (minus strand) phenotype with increasing substrate concentrations. This is exactly what is observed for both DNA polymerases [pol I (KF) and Pol δ] containing associated 3′ to 5′ exonuclease activities (Fig. 1). The medium-blue frequency (corrected by subtracting the background frequency of 0.37%) increases 52-fold for pol I (KF) (0.55% at 1 μM versus 28.5% at 1 mM) and 23-fold for pol δ (0.54% at 20 μM versus 12.6% at 1 mM). At any given dNTP concentration, pol δ was consistently more effective than pol I (KF) in excising the terminal cytosine residue for this C·A mispair. No next-nucleotide effect was observed with AMV DNA polymerase.

The second variation used to decrease the exonuclease/polymerase ratio was to add AMP to the reactions. AMP has been shown to selectively inhibit the 3′ to 5′ exonuclease, but not the polymerase activity of *E. coli* DNA polymerase I and rabbit bone marrow pol δ (31), presumably by binding to the 3′ to 5′ exonuclease active site and preventing entry of the terminally mispaired base (32). As shown in Fig. 2, AMP inhibits terminal mismatch excision by the exonucleases associated with both the Pol I (KF) and pol δ but has no effect with AMV DNA polymerase. As with the next-nucleotide effect, AMP is less effective with pol δ than with pol I (KF).

Exonuclease Proofreading During DNA Synthesis. To determine whether proofreading occurs during a polymerization excision cycle, we looked for next-nucleotide and nucleoside-5′-monophosphate effects on base-substitution fidelity using the TGA opal codon reversion assay. To obtain reliable reversion frequencies with pol δ, all reactions were performed at a constant 10-fold dNTP pool imbalance to increase misinsertions at the first position in the codon, a template thymine residue. Thus, the three incorrect substrates—dGTP, dCTP, and dTTP—were present in 10-fold molar excess over the correct substrate, dATP. In this case, the next-nucleotide effect is measured by increasing the concentration of all dNTPs while maintaining the 10:1 pool imbalance. Alternatively, AMP can be added to inhibit the exonuclease. As shown in Table 3, the reversion frequency of DNA copied by pol δ increases as either the next-nucleotide concentration is increased or AMP is added. The next-nucleotide effect is >10-fold. Furthermore, the addition of AMP increased the reversion frequency 4-fold at a next-nucleotide concentration of 200 μM. DNA sequence analysis of revertants obtained from the transfection of DNA copied at 1 mM next-nucleotide (without AMP) confirms that 19 of 21 were single base substitutions at the first position, as expected from the pool bias used.

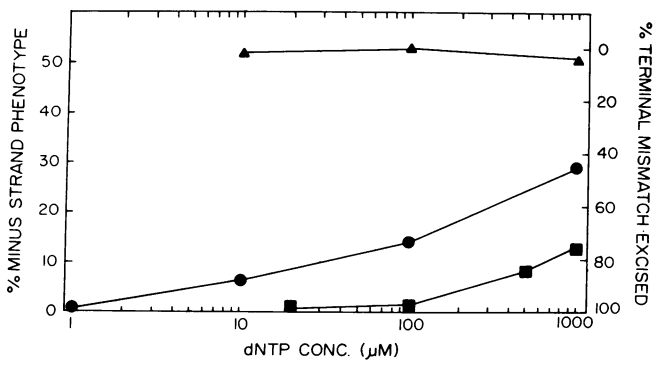

FIG. 1. Reactions were performed as described in *Materials and Methods* and in the legend to Table 2, but at the substrate concentrations shown. ▲, AMV DNA polymerase; ●, pol I (KF); ■, pol δ.

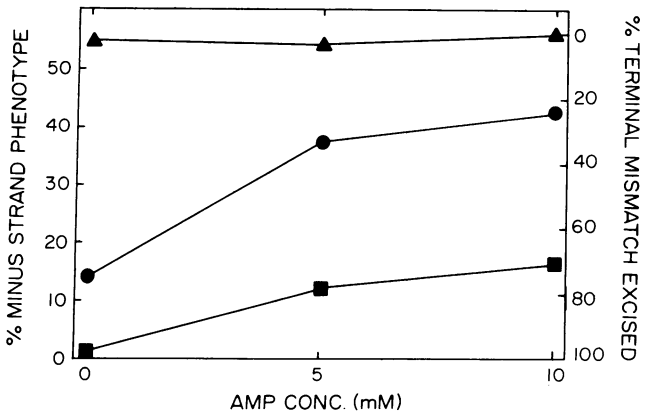

FIG. 2. Reactions were performed as described in *Materials and Methods* and in the legend to Table 2, using the indicated concentrations of AMP and 10 μM AMV DNA polymerase or 100 μM pol I (KF) and pol δ dNTPs. ▲, AMV DNA polymerase; ●, pol I (KF); ■, pol δ.

The reversion frequencies for pol δ are compared to those of AMV DNA polymerase and pol I (KF) in Fig. 3. The latter two enzymes were used as positive and negative controls, respectively, to validate the assay for proofreading and to examine the prediction, based on the data in Table 2 and Figs. 1 and 2, that pol δ should be more accurate than either AMV DNA polymerase or pol I (KF).

Consistent with previous results (30), AMV DNA polymerase is inaccurate and exhibits no next-nucleotide (Fig. 3)

Table 3. Next-nucleotide and monophosphate effects on fidelity

Incorrect dNTP, μM	Correct dNTP, μM	AMP, mM	Plaques scored		Reversion frequency (× 10^{-6})
			Total	Blue	
		Experiment 1			
100	10	0	110,000	1	9.1
1000	100	0	300,000	48	160
		Experiment 2			
100	10	0	230,000	1	4.3
200	20	0	350,000	13	37
300	30	0	340,000	21	62
1000	100	0	400,000	44	110
200	20	5	300,000	45	150

Reaction mixtures (50 μl) contained the indicated concentrations of the incorrect (dGTP, dCTP, dTTP) and correct (dATP) substrate, 10 units of pol δ, and 250 ng of gapped M13mp2A89 DNA. Transfections and plating were as described (22) using 30 μl of the reaction mixtures. The base-substitution fidelity of pol δ can be calculated from the reversion frequency (5.9 × 10^{-6}, combining experiments 1 and 2 at the lowest substrate concentration used) by first subtracting the background (1.4 × 10^{-6}; see Table 1), dividing by 0.6 (the probability of expressing an error), and then dividing by 10 to correct for the first position pool imbalance. The error frequency for the first position base at equal pools is 1/1,300,000.

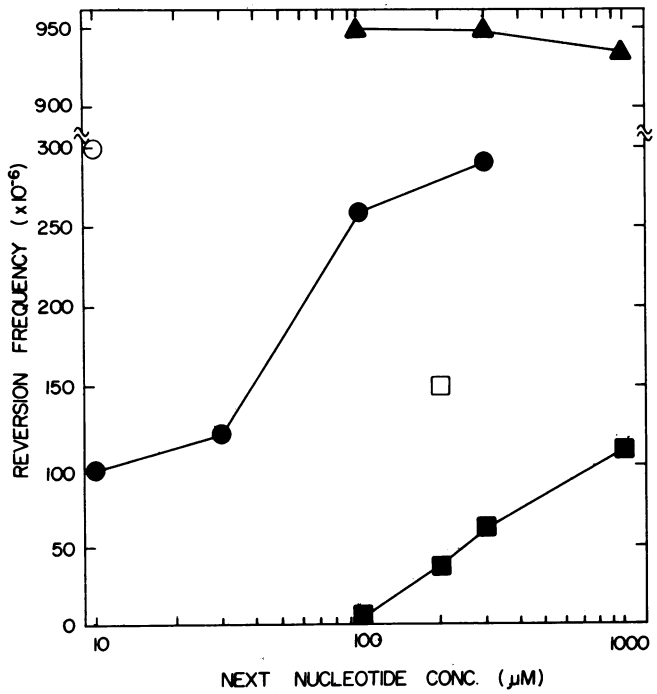

FIG. 3. Reactions (in 50 μl) were performed as described in *Materials and Methods* and in the legend to Table 3, using 20 units of AMV DNA polymerase, 0.5 unit of pol I (KF), or 10 units of pol δ. The ratios of incorrect (dGTP, dCTP, and dTTP) to correct (dATP) substrates were held constant at 10:1 as the concentration of the next correct nucleotide (dGTP) was varied from the lowest concentration consistent with complete gap-filling synthesis up to 1 mM. ▲, AMV DNA polymerase; ●, pol I (KF); ■, pol δ; ○, pol I (KF) at 10 μM next-nucleotide plus 5 mM AMP; □, pol δ at 200 μM next-nucleotide plus 5 mM AMP.

or nucleoside-5′-monophosphate proofreading effect (data not shown). Both proofreading effects are readily apparent with pol I (KF), which is 10-fold more accurate than AMV DNA polymerase. Pol δ is even more accurate than pol I (KF). The maximum accuracy difference of pol δ over pol I (KF) is 60-fold and is observed at the lowest comparable next-nucleotide concentration examined, 100 μM. The difference in fidelity between these two enzymes is, however, only 2-fold under conditions (plus AMP) that yield the highest reversion frequencies (open symbols in Fig. 3). One interpretation of these data is that pol δ and pol I (KF) have approximately the same level of discrimination at the insertion step, but pol δ can be 10- to 100-fold more accurate as a result of a more active proofreading exonuclease.

DISCUSSION

We initiated these studies to determine the base-substitution fidelity of pol δ and to determine the ability of its associated 3′ to 5′ exonuclease to proofread errors during synthesis. The data in Tables 1 and 3 can be used to calculate (see legends to tables) the base-substitution fidelity of pol δ under conditions that allow a maximum contribution of the exonuclease to fidelity—i.e., low dNTP concentration and no nucleoside-5′-monophosphate added. Under these conditions, pol δ stably misincorporates 1 base-substitution error for each 10^6 correct incorporation events. This is an approximate value since pol δ was so accurate that few revertants were observed, and since, as with other DNA polymerases (23), significant site-to-site and mispair-to-mispair differences are expected. Nevertheless, it is clear that pol δ is substantially more accurate than the forms of pol α and β examined here (Table 1) or in similar assays described in the literature (2, 3, 19, 20, 23). Pol δ is in fact considerably more accurate, in direct comparative measurements (Fig. 3), than is *E. coli* pol I (KF), which has a well-characterized proofreading exonuclease (for review, see refs. 1 and 27).

In addition to the correlation of high fidelity with the presence of a polymerase-associated 3′ to 5′ exonuclease, several other observations suggest that pol δ proofreads errors. The 3′ to 5′ exonuclease efficiently removes a terminally mispaired cytosine (Table 2). Most importantly, both terminal mismatch excision (Figs. 2 and 3) and the fidelity of DNA synthesis (Table 3) decrease under reaction conditions known to reduce the ratio of exonuclease to polymerase activity. In contrast, these same conditions have no effect on misinsertion fidelity of a non-exonuclease-containing DNA polymerase (Figs. 1–3; refs. 30 and 33).

Next-nucleotide concentration and nucleoside-5′-monophosphate-inhibition effects on proofreading observed with *E. coli* DNA pol I (30, 32, 34) and pol I (KF) suggest that such experimental variations in reaction conditions can diminish, but do not entirely eliminate, proofreading. The data in Figs. 1–3 suggest that this interpretation also applies to pol δ. Excision of a terminal cytosine from the transition mispair C·A is only diminished 30% by the reaction conditions used here. Under similar conditions (Table 3), the pol δ error frequency for first position mispairs (T·dGMP, T·dCMP, and T·dTMP) is 1/40,000 (see legend). Assuming that under these conditions 70% of the misinsertions are proofread, pol δ insertion discrimination would produce an error frequency of 1/12,000, a value similar to the estimated insertion fidelity of several non-exonuclease-containing DNA polymerases, including pol α (Table 1; refs. 1, 6, and 23). Comparing this value to the error frequency estimate of $1/10^6$ for pol δ under conditions in which the exonuclease is active, we conclude that proofreading may improve fidelity as much as 100-fold. Further analyses using additional mutant codons or the M13mp2 forward mutational assay (19) are expected to reveal substantial variations from this initial estimate, depending on the composition and position of the mispair.

The biological implications of these initial estimates of insertion and proofreading fidelity of pol δ await not only further examination of fidelity but also elucidation of the relationship (if any) of pol δ to pol α. For a number of reasons, pol α has been considered to be the major replicative DNA polymerase in animal cells (extensively reviewed in ref. 6.). Our recent work directly comparing two forms of calf thymus pol δ to pol α demonstrates numerous similarities among these enzymes. These include similar molecular weight and shape, associated DNA primase activity, identical inhibition by aphidicolin, and similar processivity and stimulation by ATP (16, 17, 21). Recent selective inhibitor studies with diploid human fibroblasts suggest the involvement of pol δ in the processes of semiconservative DNA replication and repair of damage by UV irradiation (35). The well-known difficulties in defining the subunit composition of pol α, often ascribed to proteolysis, led to the interesting speculation that pol α may be derived from pol δ or vice versa. Such a relationship could have physiological importance—e.g., in regulating the roles of these DNA polymerases and perhaps mutation rates as well. Proteolysis of pol α has, in fact, been suggested to reduce fidelity in two studies (5, 22). Two reports also describe complex high molecular weight forms of *Drosophila* pol α (4) and immunoaffinity-purified calf thymus pol α (ref. 5; M. Reyland and L. Loeb, personal communication) that have nearly 100-fold greater insertion fidelity than does pol α purified by earlier approaches that apparently has a proteolyzed catalytic subunit. Our current ability to relate these observations from different systems and laboratories is incomplete. However, one reasonable conclusion from the experiments presented here is that the 3′ to 5′

exonuclease activity associated with calf thymus pol δ contributes to the fidelity of DNA synthesis by this enzyme.

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