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Deoxynucleoside [1-thio]triphosphates prevent proofreading during *in vitro* DNA synthesis

(phosphorothioate nucleotides/fidelity of DNA synthesis/DNA polymerases)

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ABSTRACT The contribution of proofreading to the fidelity of catalysis by DNA polymerases has been determined with deoxyribonucleoside [1-thio]triphosphate substrates. These analogues, which contain a sulfur in place of an oxygen on the α phosphorus, are incorporated into DNA by DNA polymerases at rates similar to those of the corresponding unmodified deoxynucleoside triphosphates. The fidelity of DNA synthesis was measured with ϕX174 *am3* DNA; reversion to wild type occurs most frequently by a single base substitution, a C for a T at position 587. By using avian myeloblastosis virus DNA polymerase and DNA polymerase β (enzymes without a proofreading 3′→5′ exonucleolytic activity), substitution of deoxycytidine thiotriphosphate in the reaction mixture did not alter fidelity. In contrast, with DNA polymerases from *E. coli* (DNA polymerase I) and bacteriophage T4 (enzymes containing a proofreading activity), fidelity was markedly reduced with deoxycytidine [1-thio]triphosphate. DNA containing phosphorothioate nucleotides is insensitive to hydrolysis by the exonuclease associated with these prokaryotic DNA polymerases. These combined results indicate that the deoxynucleoside [1-thio]triphosphates have normal base-pairing properties; however, once misinserted by a polymerase, they are not excised by proofreading. Proofreading of a C:A mismatch at position 587 is thereby found to contribute 20-fold to the fidelity of *E. coli* DNA polymerase I and a greater amount to the fidelity of bacteriophage T4 DNA polymerase.

The ability to correct errors during DNA replication has long been recognized as one important mechanism by which an organism can potentially achieve the highly accurate replication of its genetic information. This concept stems from the observation that prokaryotic DNA polymerases contain an integrally associated 3′→5′ exonuclease activity, which can selectively remove mistakes as they occur during polymerization (1). Biochemical support for this concept was obtained by Brutlag and Kornberg (2), who demonstrated that the 3′→5′ exonuclease of *Escherichia coli* DNA polymerase I (Pol I) preferentially removes mismatched bases at primer termini before initiation of polymerization. Support for proofreading *in vivo* comes from studies with certain mutator (3) and antimutator (4) bacteriophage T4 DNA polymerases. These studies (5–8) correlated spontaneous mutation rates of bacteriophage T4 with the ratio between the polymerization reaction and the excision of a noncomplementary nucleotide at the primer terminus. Also, differences in discrimination between adenosine and its base analogue 2-aminopurine by mutant T4 DNA polymerase (9) can be accounted for by proofreading. Based on the kinetic data with substrate analogues, a number of mathematic models for proofreading have been proposed (10–13).

Our continuing interest in determining the relative importance of the several mechanisms available to the cell to achieve high fidelity (14) has led us to assess the contribution of proofreading to accuracy by direct measurements of misincorporation *in vitro*. The excision of noncomplementary bases does not occur with purified eukaryotic DNA polymerases (15), avian myeloblastosis virus (AMV) DNA polymerase (16), or possibly RNA polymerase, indicating that proofreading does not occur with these enzymes. It remains to be determined whether separate exonucleases work in concert with those polymerases during replication or transcription. We have shown that proofreading has a minimal contribution to the accuracy with which purified Pol I copies poly[d(A-T)] (17) but has a much greater contribution with natural DNA (18). Also, proofreading may be significant in multienzyme systems that function with natural DNA, as recently suggested by studies with *E. coli* DNA polymerase III holoenzyme (19). In this report, we make use of the ϕX174 fidelity assay (20, 21) to measure the fidelity of several purified DNA polymerases, using a substrate analogue that contains a sulfur atom in place of an oxygen on the α phosphorus of the deoxynucleoside triphosphate. This analogue is incorporated normally (22, 23), but the phosphorothioate diester bond is not hydrolyzed by the 3′→5′ exonuclease of Pol I. By comparing fidelity with a normal substrate versus this deoxynucleoside [1-thio]triphosphate analogue, we assess the contribution of proofreading to the fidelity of different DNA polymerases.

MATERIALS AND METHODS

Materials. Unlabeled 2′-deoxynucleoside 5′-*O*-([1-thio]triphosphate) derivatives (dNTPαS) were prepared as described (22). In all experiments, the A isomer of dATPαS was used, whereas dGTPαS and dCTPαS consisted of both the A and B isomers. Because the B isomer is not incorporated by Pol I (23), it was not considered in quantitating substrate concentrations. All other reagents including [α-^{32}P]dTTP were obtained from sources as described (24). The ϕX174 single-stranded viral DNA (template) and restriction endonuclease *Hae* III fragment Z-5 (primer) were prepared as described (24), as was homogeneous Pol I. AMV DNA polymerase was a gift of J. W. Beard (Life Sciences Research Laboratories), and homogeneous DNA polymerase β from rat (Novikoff) hepatoma was a gift of R. Meyer (University of Cincinnati). M. F. Good-

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Abbreviations: dNTPαS, 2′-deoxynucleoside 5′-*O*-([1-thio]triphosphate) derivatives; AMV, avian myeloblastosis virus; Pol I, *E. coli* DNA polymerase I.

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man (University of Southern California), and P. Englund (Johns Hopkins University) provided samples of highly purified wild-type T4 DNA polymerase.

DNA Polymerase Assays. Reaction mixtures (50 μl) contained 20 mM Tris·HCl (pH 8.0); 2 mM dithiothreitol; 10 mM $MgCl_2$; 0.2 μg of ϕX174 *am*3 viral DNA primed at a 5:1 molar ratio with Z-5 primer; Pol I (7 units, 25:1 molar ratio of enzyme to template) (1), AMV DNA polymerase (10 units) (16), phage T4 DNA polymerase (0.5 unit) (10), or DNA polymerase β (0.8 unit) (25); and 5 μM [α-^{32}P]dTTP (200–1000 cpm/pmol). The concentration of dATP, dCTP, dGTP, or the corresponding phosphorothioate derivative (A isomer) was 5 μM unless otherwise indicated. Incubation was at 37°C for 5 min (Pol I and T4 polymerase), 10 min (AMV polymerase), or 30 min (polymerase β), by which time synthesis had proceeded past the amber mutation (83 nucleotides) for all conditions reported here. Reactions were terminated by addition of EDTA to 15 mM, and duplicate aliquots were processed to determine acid-insoluble radioactivity.

Exonuclease Assays. The ϕX174 DNA substrates used for exonuclease digestion were prepared with Pol I in reactions scaled up to copy 10 μg of Z-8 primed ϕX174 DNA. Two synthetic reactions were performed with 5 μM [α-^{32}P]dTTP (5000 cpm/pmol) and either 5 μM dATP, dCTP, and dGTP or 5 μM dATPαS, dCTPαS, and dGTPαS. The reactions were incubated for 15 min at 37°C and then stopped by adding EDTA to 15 mM. The DNA was separated from nonincorporated deoxyribonucleotides on a Sephadex G-100 column (0.5 × 60 cm), pre-equilibrated and eluted with 0.5 M KCl/0.05 M Tris·HCl, pH 7.4. Approximately 0.05 μg of each DNA was used as a substrate for hydrolysis by the exonuclease activities associated with Pol I or T4 DNA polymerase. Reaction mixtures (50 μl) contained, in addition to the DNA, 30 mM Tris·HCl (pH 7.4), 2 mM dithiothreitol, 6 mM $MgCl_2$, and either 5 units of Pol I or 0.70 unit of phage T4 DNA polymerase. Reactions were incubated at 37°C for the times indicated in Fig. 1 and stopped by addition of 200 μl of 10% (wt/vol) trichloroacetic acid, followed by addition of 50 μl of calf thymus DNA (1.0 mg/ml) as a carrier. Acid-insoluble DNA was removed by centrifugation, and the acid-soluble radioactivity in the supernatant was quantitated by counting in a liquid scintillation counter.

Transfection Assay for Determination of Error Rate. The reversion frequency of the amber mutation in the copied DNA was determined by transfecting the copied DNA into *E. coli* spheroplasts and measuring the titer of the resultant progeny phage on bacterial indicators either permissive or nonpermissive for the amber mutation. A detailed account of the methodology for this assay has been published (24). All reversion frequency values are the average of duplicate determinations after subtracting the background reversion frequency of uncopied DNA from reactions not incubated at 37°C (typically 2.0–2.5 × 10^{-6}). The procedure for measuring phage titer gives results that fluctuate 2- to 3-fold from day to day; however, within an experiment, the average variation of duplicate samples is about 20%. For error rate determinations, only reversion frequencies at least 2 SD above background were considered significant.

RESULTS

Incorporation and Excision of dNTPαS. The ability of three purified DNA polymerases to incorporate dNTPαS approached that obtained with normal nucleotide substrates (Table 1). This was true for each of the three different dNTPαS used and confirmed previous observations with Pol I (22, 23). In all cases, incorporation was more than sufficient to copy past the amber mutation for determination of error rates. The simultaneous use of these same three dNTPαS, together with [α-^{32}P]dTTP, also yielded relatively normal incorporation (not shown). When this phosphorothioate-substituted DNA was used as a substrate for hydrolysis by the exonucleases associated with Pol I or wild-type phage T4 DNA polymerase, no release of acid-soluble nucleotides was observed (Fig. 1, closed symbols) in a 4-hr incubation. In this experiment, the amount of DNA polymerase was ≈10-fold greater than that of the DNA substrate. When compared to the substantial digestion observed under these same conditions with DNA synthesized with unmodified deoxynucleotide substrates (Fig. 1, open symbols), the results suggest that dNTPαS, once incorporated, cannot be excised by the 3′→5′ exonuclease activity. This conclusion is further substantiated by the recent study of Brody and Frey (26), who observed that the phosphorothioate diester bond in poly [d(A-T)] is completely resistant to the action of the exonuclease activities associated with Pol I.

Table 1. Incorporation of dNTPαS into ϕX174 DNA by purified DNA polymerases

DNA polymerase	% of control incorporation		
	dATPαS	dCTPαS	dGTPαS
AMV	69	95	100
Pol I	80	70	80
T4	43	68	67

Controls represent incorporation in the presence of all four normal substrates with [α-^{32}P]dTTP. Where indicated, dNTPαS was used in place of the corresponding normal dNTP. The 100% incorporation values were as follows: AMV DNA polymerase, 105 nucleotides per template (2.1 pmol); Pol I, 686 nucleotides per template (13.0 pmol); and T4 DNA polymerase, 339 nucleotides per template (7.0 pmol).

Fidelity Measurements with dNTPαS. The normal rates of synthesis and lack of hydrolysis led us to measure the effect of the phosphorothioated substrates on fidelity.

The assay (21) uses a single-strand circular ϕX174 DNA containing a TAG amber codon (amber 3, in gene *E*) in place of the TGG wild-type codon. Because the same sequence codes for the gene *D* protein in a different reading frame, the number of possible substitutions is limited. Synthesis was initiated at a single fixed point on the template, using as a primer a DNA restriction endonuclease fragment whose 3′-OH terminus is 83 nucleotides away from the *am*3 site. The accuracy of *in vitro* DNA synthesis was quantitated by transfecting the copied DNA into *E. coli* spheroplasts and measuring the titer of the resultant progeny phage on permissive and nonpermissive indicator bacteria. Because the mRNA and gene E proteins are coded for by the *in vitro* synthesized minus strand, an error rate for the DNA polymerase *in vitro* at the *am*3 site can be calculated from the reversion frequency of the phage (21). We have shown by DNA sequencing that those substitutions which produce wild-type phage occur at position 587, opposite the template A of the TAG amber codon (21). The most frequent error in Mg^{2+}-activated Pol I reactions is misincorporation of C, which produces the original wild-type DNA sequence (21). Therefore, we carried out polymerization reactions with purified DNA polymerases using either normal dCTP or dCTPαS in the presence of the other three normal dNTP substrates. The error rates of AMV DNA polymerase and DNA polymerase β, which lack associated 3′→5′ exonuclease activity (15, 16, 25), were the same with either substrate (Table 2). Two conclusions are evident from this result. First, the DNA synthesis-dependent increase in reversion frequency observed with these enzymes shows that the phosphorothioate-substituted minus strand DNA is biologically active (i.e., it is expressed in the transfection assay). Second, because dCTPαS is not more mutagenic than normal dCTP, the

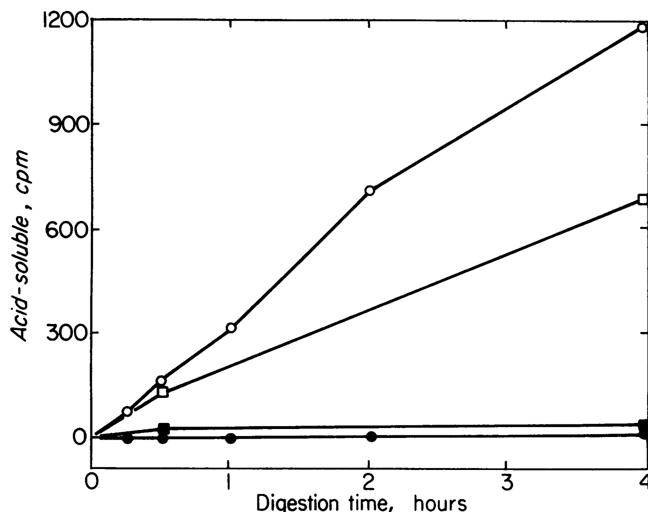

FIG. 1. DNA polymerase-associated exonuclease digestion of normal versus phosphorothioate-substituted ϕX174 DNA. ○, Pol I with dNTP; □, phage T4 DNA polymerase with dNTP; ●, Pol I with dNTPαS; ■, T4 polymerase with dNTPαS. In digestion experiments with ϕX174 DNA, in which only one of four deoxynucleotide substrates was a phosphorothioate (dCTPαS), we did observe some degradation, although at a slower rate than with unmodified DNA. Presumably, this represents cleavage of normal phosphodiester bonds releasing oligonucleotides and is not hydrolysis of phosphorothioate diester bonds.

analogue is not mutagenic by any unexpected mechanism, such as a change in base-pairing specificity.

The critical experiments were those with Pol I and phage T4 DNA polymerase (Table 2). Both of these enzymes contain associated 3′→5′ exonuclease activities, and both showed substantially reduced accuracy with dCTPαS. As previously reported (21), Pol I is highly accurate; in this experiment (with 5 μM dNTP), the error rate was 1/2,000,000 as determined from the reversion frequency obtained by increasing the normal dCTP concentration 50-fold over the other three dNTPs. However, with dCTPαS, a significant increase in reversion frequency of copied DNA was seen, even without any pool bias, and the effect was approximately 10-fold greater with a 10-fold dCTPαS bias (50 μM). This calculates to an error rate of approximately 1/100,000 and represents a 20-fold decrease in accuracy. The mutagenic effect of dCTPαS was even greater with T4 DNA polymerase. With normal dCTP, no enhancement in mutagenicity was observed, even with a 500-fold bias. However, with dCTPαS, even with an unbiased substrate condition, the reversion frequency of copied DNA was several-fold greater than background. The increase in the reversion frequency was in proportion to the concentration of dCTPαS in the reaction mixture. Therefore, the change in fidelity was from <1/10,000,000 with dCTP to approximately 1/20,000 with dCTPαS, or >500-fold.

Absence of "Next-Nucleotide Effect" with dCTPαS. A recent study of the *E. coli* DNA polymerase III holoenzyme (17) has provided kinetic evidence that proofreading activity is accompanied by what may be referred to as the "next nucleotide effect." Intuitively, the amount of time available to excise a misinserted base at the primer terminus can be decreased by increasing the rate of incorporation of the next correct nucleotide after the mistake. This will move the enzyme forward along the template and remove the incorrect nucleotide from the catalytic site for excision. In our assay, the relevant template DNA sequence, in order of synthesis from the provided Z-5 primer is ...GAT... Thus, an incorrect substitution at position 587 is followed by the next correct nucleotide, an A, opposite the template T at position 586. Once incorporation of A occurs at 586, any mistake made at position 587 will be less accessible to excision by a 3′→5′ exonuclease and more likely to remain as a stably misincorporated base. Thus, for an enzyme that is actively proofreading mistakes, the error rate should show a dependence on the dATP concentration in the polymerization reaction such that, at low dATP, few mistakes are stably misincorporated, whereas at high dATP, stable misincorporation increases.

We have shown that in Mg^{2+}-activated Pol I reactions C is misincorporated at least 10 times more frequently than A (21). Furthermore, DNA sequence analysis has shown that the increase observed with increasing dATP is, in fact, due to misincorporation of C at position 587 (18). The control experiment to demonstrate this next nucleotide effect is shown in Fig. 2. With normal dCTP, the reversion frequency increased as much as 25-fold with increasing dATP concentration (open circles). When a similar experiment was performed with dCTPαS rather than dCTP (Fig. 2, closed circles), the dATP-dependent enhancement in mutagenesis was not observed. These results support the conclusion that the use of dNTPαS substrates reduces or eliminates proofreading during polymerization.

DISCUSSION

In this paper, we describe experiments to quantitate the contribution of proofreading to the fidelity with which purified polymerases synthesize DNA *in vitro*. The approach is unique in that natural DNA is used as a template for directly measuring the stable misincorporation of a substrate with normal base-pairing properties. The analogue used is one of the well-characterized dNTPαS, which have been used previously to probe the stereochemistry of the reaction catalyzed by Pol I (23). Pol I has been shown to incorporate the *S*-diasteroisomer of dATPαS (isomer A) with normal kinetics *in vitro* (23) and with stereochemical inversion at phosphorus to yield a phosphorothioate diester with the *R*-configuration. When used in permeabilized *E. coli* cells or crude cell extracts, these modified dNTPs are incorporated into ϕX174 DNA (22). Similarly, in the experiments shown here, *in vitro* incorporation is relatively normal for three different DNA polymerases (Table 1). Moreover, AMV polymerase and phage T4 DNA polymerase, like Pol I, use the S_p-diastereoisomer of dCTPαS.

Table 2. Effect of normal dCTP versus dCTPαS on fidelity of DNA polymerases *in vitro*

DNA polymerase	dCTP or dCTPαS, μM	dCTP $\nu_{rev}\times 10^{-6}$	dCTP Unbiased error rate	dCTPαS $\nu_{rev}\times 10^{-6}$	dCTPαS Unbiased error rate
AMV	10	11.4	1/17,100	11.0	1/17,700
β	500	18.9	1/10,300	17.3	1/11,300
Pol I	5	0.11	—	1.61	1/121,000
	50	—	—	17.6	1/111,000
	250	4.47	1/2,180,000	—	—
T4	5	<1.50	—	6.69	1/29,100
	50	<1.50	—	82.2	1/23,700
	250	<1.50	—	588.0	1/16,600
	2500	<1.50	$<1/10^7$	—	—

Duplicate DNA polymerase reactions were performed with dATP, dGTP, and [α-^{32}P]dTTP at 10 μM (AMV DNA polymerase), 500 μM (DNA polymerase β), or 5 μM (Pol I and phage T4 DNA polymerase) and the indicated concentration of either dCTP or dCTPαS. Error rates were calculated as described (21) and unbiased (Pol I and T4 polymerase) by dividing the calculated error rate by the ratio of the incorrect (dCTP) nucleotide to the correct (dTTP) nucleotide in the reaction mixture. ν_{rev}, Reversion frequency.

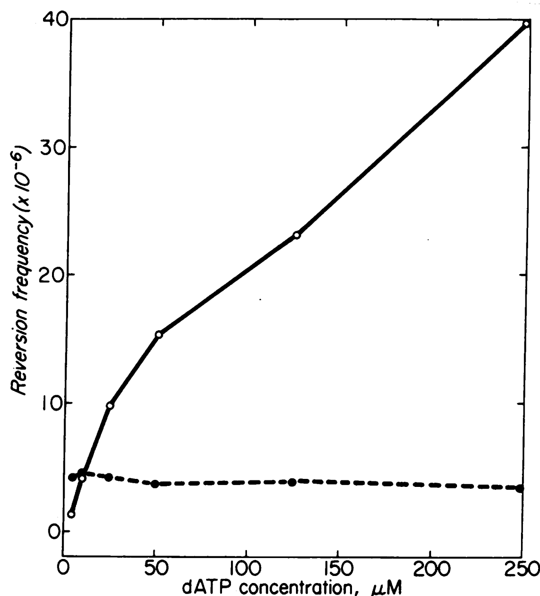

FIG. 2. Effect of concentration of "next nucleotide" on the error rate of Pol I with normal and phosphorothioated substrates. Duplicate DNA polymerase reactions were performed with Pol I and the following substrate concentrations: ○——○, 5 μM dGTP and TTP, 250 μM dCTP, and increasing dATP; ●---●, 5 μM dGTP and dTTP, 25 μM dCTPαS (A isomer), and increasing dATP. The reversion frequencies shown were determined in the transfection assay and are minus the background for uncopied DNA (1.79×10^{-6}).

Although incorporation of this analogue is normal, excision by exonucleases clearly is not. This was first demonstrated with Pol I-synthesized phosphorothioate-substituted poly (dA). The rate of snake venom phosphodiesterase-catalyzed hydrolysis of the R_p-configuration of the phosphorothioate polymer is approximately 10-fold less than that of the S_p-configuration, which is estimated to be 20,000-fold less than that of unsubstituted poly (dA) (23). More importantly, when an alternating copolymer containing normal T and phosphorothioate A is subjected to hydrolysis by the exonuclease activities of Pol I, only dinucleotides are obtained, and these retain the phosphorothioate-diester linkage intact (26). This bond in the R_p-configuration is thus refractory to the proofreading exonuclease of Pol I. The digestion experiment described here (Fig. 1) supports this conclusion and extends the observation to natural DNA and to the 3'→5' exonuclease of wild-type phage T4 DNA polymerase. Because no degradation of the αS-substituted DNA is observed with either prokaryotic polymerase, there is—at the very least—a large difference in the rate of hydrolysis of the phosphorothioate-diester bond.

The lack of hydrolysis of substituted DNA by proofreading exonucleases led us to perform the fidelity measurements with the φX assay. When dCTPαS is used as an incorrect nucleotide, enhanced mutagenesis is observed specifically for those enzymes with an associated proofreading exonuclease activity. A comparison of error rates with the two different substrates demonstrates that the accuracy of Pol I and phage T4 DNA polymerase is increased 20-fold and greater than 500-fold, respectively, due to proofreading of misinserted bases. The contribution of proofreading by Pol I, assessed with the [1-thio]triphosphate, is in accord with an estimate of 25-fold using the next-nucleotide effect (18). The absence of the next-nucleotide effect with dCTPαS (Fig. 2) adds further support to the conclusion that the phosphorothioate analogue is not proofread.

A final estimate of the fidelity of phage T4 DNA polymerase with normal substrates will require further analysis of the product of the reaction. However, the higher ratio of 3'→5' exonuclease to polymerase in phage T4 DNA polymerase (1, 17) compared to Pol I argues that T4 DNA polymerase is more accurate. It should be noted that our estimate of accuracy with purified wild-type T4 DNA polymerase is the same or less than that reported by Hibner and Alberts (27) for the entire T4 replication complex. This similarity may result from the methods of assay employed or may reflect the nature of the mismatch being measured. Alternatively, the DNA polymerase may be the primary contributor to accuracy by the T4 replicating complex. With either enzyme, the absolute values (20-fold and >500-fold) are with respect to a single mismatch at a single position and may differ in other situations, since proofreading may be affected by position or the nature of the mismatch (18). If we assume that proofreading is negligible when the analogue is misinserted, then the misinsertion frequency of these enzymes becomes equivalent to the misincorporation frequency. Thus, the error rate with the phosphorothioate analogues is a direct measure of the error prevention (base-selection) capabilities for these DNA polymerases. With dCTPαS, the error rates for T4 DNA polymerase and Pol I are, respectively, 2 and 3 orders of magnitude lower than predicted by Watson–Crick base-pairing alone (28)—a value possibly obtained due to an active role of the enzyme in base discrimination.

We have suggested a structural mechanism for error prevention, based on NMR studies of the conformation of bound purine and pyrimidine substrates on Pol I (29, 30) and on fluorescence polarization studies of the moblity of the bound substrate (28, 30). Pol I was found to change the conformation of the bound nucleotide substrate to one that fits more precisely into double helical B DNA (29) and to immobilize the purine ring (30). Such orientation and immobilization of the substrate by the enzyme could prevent errors to the extent observed with dCTPαS. This fidelity is then further increased 20-fold due to correction of misinserted bases at the primer terminus during ongoing polymerization. Interestingly, the phage T4 DNA polymerase is less accurate than Pol I for error prevention (error rate approximately 1/20,000), but the overall accuracy of this enzyme is greater than that of Pol I, presumably due to a much more highly active proofreading activity for correcting errors (1).

The phosphorothioated dNTPαS can be used to probe the contribution of proofreading in a number of systems. For example, those mutator and antimutator phage T4 DNA polymerases that have altered exonuclease-to-polymerase ratios (7) should exhibit similar fidelity with the phosphorothioate substrates. The analogue can be used to address questions on the frequency of misinsertions of different incorrect bases and of excision of different mismatches during proofreading. Finally, these substrates should be a powerful probe to search for proofreading activities in eukaryotic cells.

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