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DNA polymerase α and models for proofreading

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ABSTRACT

Using a modified system to measure fidelity at an amber site in ØX174, we have employed DNA polymerase α to test different mechanisms for proofreading. DNA polymerase α does not exhibit the characteristics of "kinetic proofreading" seen with procaryotic polymerases. Polymerase α shows no evidence for a "next nucleotide" effect, and added deoxynucleoside monophosphates do not alter fidelity. Pyrophosphate, which increases error rates with a procaryotic polymerase, appears to weakly improve polymerase α fidelity. DNA polymerase α does exhibit a dramatic increase in error rate in the presence of a deoxycytidine thiotriphosphate (dCTPαS), but this enhanced mutagenesis also occurs under conditions where kinetic proofreading should be otherwise defeated. This particular effect with dCTPαS appears specific for DNA polymerase α and is not seen with the other polymerases tested.

INTRODUCTION

Many homogeneous procaryotic DNA polymerases possess a 3'→5' exonuclease activity (1) which excises noncomplementary nucleotides and contributes substantially to DNA replication fidelity. Four general conditions have been identified which either inhibit this exonucleolytic proofreading activity or defeat it through kinetic mechanisms: added deoxynucleoside monophosphates, pyrophosphate, deoxynucleoside thiotriphosphates, and high concentrations of the "next nucleotide." Any of these agents will increase the error rate of a procaryotic DNA polymerase by one to two orders of magnitude (2).

In contrast, purified eucaryotic polymerases, with a few notable exceptions, lack 3'→5' exonuclease activities (3). Since their accuracy is greater than that predicted by base pairing alone (2) they must have other mechanisms for enhancing fidelity. Several hypothetical models for replication fidelity might be relevant to eucaryotic polymerases: in those that invoke "kinetic proofreading," (4,5) incorrect substrates are rejected as monophosphates either before or after incorporation. In other models, incorrect substrates could be rejected as triphosphates via pyrophos-

phate exchange (6) or by an "energy-relay" mechanism (7). Alternatively, it could be the case that eucaryotic DNA polymerase preparations contain proofreading exonuclease activities at levels below those which are detectable with synthetic templates.

We have previously developed a system to examine replication fidelity using a natural ØX174 am3 DNA template primed with ØX restriction fragments (8,9). With this system, a DNA polymerase copies the ØX viral DNA template in vitro. The copied DNA is transfected into spheroplasts, which complete replication and produce progeny phage. Fidelity is assessed by plating phage on Escherichia coli strains permissive or nonpermissive for the amber codon and measuring reversion frequency to wild type. Using this system, we have reported an error rate of 1/30,000 for DNA polymerase α, and 1/7,000 for DNA polymerase β --values which are considerably less faithful than those one would expect for a eucaryotic cell (10). Because mammalian DNA polymerases are generally inefficient in copying long single-stranded regions of template, in these studies it was necessary to employ high concentrations of deoxynucleoside triphosphates for synthesis to proceed past the amber site. These concentrations, however, inhibit proofreading in procaryotic enzymes by the "next nucleotide" effect (2,10).

We have modified the ØX fidelity system by priming the template with a synthetic oligodeoxynucleotide which hybridizes with its 3'-hydroxyl group a few nucleotides from the amber site. Characterizing this modified system indicated that under identical conditions, the percentage of molecules copied by DNA polymerase and the percentage of time the newly synthesized DNA is expressed ("penetrance") are similar, whether the template is primed with either a restriction fragment or a synthetic oligodeoxynucleotide (29). Because a polymerase need synthesize only a few nucleotides to proceed through the amber site, reactions can be conducted with eucaryotic enzymes under conditions where the "next nucleotide" effect would be minimized. These circumstances allowed us to probe mechanisms of fidelity with DNA polymerase α.

MATERIALS AND METHODS

Materials

Unlabeled deoxynucleoside triphosphates (dNTPs) and dCMP were obtained from Sigma. Other deoxynucleoside monophosphates (dNMPs) and a synthetic oligodeoxynucleotide were obtained from P-L Biochemicals. Labeled dNTPs

were obtained from New England Nuclear. Unlabeled 2'-deoxynucleoside 5'-O-([1-thio] triphosphate) derivatives (dNTPαS) were prepared as described (11) and were a generous gift from F. Eckstein (Max Planck Institut für Experimentelle Medizin, Göttingen). Preparations of dATPαS and dGTPαS contained the A isomer; preparations of dCTPαS contained both the A and B isomers.

Enzymes

E. coli DNA polymerase I (Pol I) was purified by the method of Jovin et al. (12) as modified by Slater et al. (13). DNA polymerase α forms designated C (7.3 S) and D (6.8 S) were purified from calf thymus as described by Holmes et al. (14,15). DNA polymerase α "holoenzyme" was purified from calf thymus as described by Hübscher et al. (16). DNA polymerase β was purified from rat hepatoma cells (Novikoff) (17) and was a generous gift from D.W. Mosbaugh and R.R. Meyer (University of Cincinnati). Avian myeloblastosis virus (AMV) DNA polymerase was obtained from J.W. Beard at Life Sciences Research Laboratories.

Hybridization

^{3}H-labeled ØX174 am3 viral DNA (6.7 x 10^4 dpm/μg) was hybridized at a primer:template molar ratio of 10:1 with a synthetic oligodeoxynucleotide, 15 nucleotides long, which has its 3'-hydroxyl group three nucleotides from position 587. Hybridizations were carried out as previously described; DNA sequencing has shown that this oligomer hybridizes with ØX viral DNA at the expected region (18).

In Vitro Copying of ØX DNA

Reaction mixtures (25 μl) contained 0.05 μg of primed ØX DNA, 20 mM Tris-HCl (pH 8.0), 2 mM dithiothreitol, 20 μM to 1 mM concentration each of dATP, dCTP, dGTP, and [α-^{32}P] dTTP (500-1250 dpm/pmol), $MgCl_2$ as divalent metal activator, and polymerase. $MgCl_2$ concentration was 5 mM for AMV polymerase and all forms of DNA polymerase α, or 9 mM for DNA polymerase β and Pol I. Enzyme amounts in each reaction mixture were approximately 0.05 units of either form of DNA polymerase α, 0.04 units of DNA polymerase β, 10 units of AMV polymerase, or a 25:1 molar ratio of enzyme:template for homogeneous Pol I. Reactions were conducted at 30° to ensure that primers remained hybridized. Incorporation was calculated from acid-insoluble radioactivity (19). Unless otherwise indicated, in all experiments, average incorporation was more than sufficient to proceed past the amber site. Copied DNA was transfected and reversion frequency determined by the progeny phage method as was previously described (8,9). Back-

ground reversion frequency was determined with primed ØX DNA which was not incubated with DNA polymerase ("uncopied DNA").

Polymerase assay

DNA polymerase assays were performed as previously described (18). One unit of polymerase activity represents the incorporation of 1 nmol of labeled nucleotide into activated DNA in 60 min at 37°C.

RESULTS

Next Nucleotide Effect

In previous work, including sequencing data, viable revertants from the am3 phenotype have been produced only through misincorporation at position 587, opposite the viral template A in the middle of the amber codon (20). Exonucleolytic proofreading by procaryotic enzymes involves competition between excision and polymerization. When a mistake is inserted, it can be removed by excision or locked into the growing DNA chain by the subsequent incorporation of the next nucleotide (2, 21). The sequence of the am3 template, in order of synthesis from the synthetic oligomer primer, is -G-A-T-. Thus, an incorrect substitution at position 587 would be locked in by the next correct nucleotide, an A, opposite the template T at position 586.

Table I indicates this next nucleotide effect on the fidelity of Pol I. An increased concentration of dATP, which itself is incorrect for position 587, produces a reversion frequency which is only slightly above background. With increased concentrations of both dCTP and dATP, the reversion frequency using Pol I is greater than the additive effects of either individual incorrect substrate. This enhanced reversion frequency has been shown to be due to dCTP misincorporation, and has been attributed to the effect of the next nucleotide substrate, dATP (20). Analysis of the next nucleotide effect for DNA polymerase α is made more difficult by the fact that this enzyme, unlike Pol I, appears to misinsert dATP more frequently than any other incorrect substrate (2). Nonetheless, the reversion frequency with elevated levels of all three incorrect dNTPs is a value no greater than the additive effects of individual biased dNTPs. Table I thus provides no evidence for the next nucleotide effect, a hallmark of exonucleolytic proofreading, with DNA polymerase α form C; nor is there evidence for such an effect with form D (data not shown).

Effect of Deoxynucleoside Monophosphates

The effect of deoxynucleoside monophosphates on the fidelity of Pol I

Table I: Effect of Incorrect Substrate Concentration on Fidelity

Non-complementary dNTP Substrates	Reversion Frequency ($\times 10^{-6}$)
Polymerase α, form C	
1 mM dATP	3.44
1 mM dCTP	1.98
1 mM dGTP	2.80
1 mM dATP + dCTP + dGTP	5.76
Pol I	
1 mM dATP	0.94
1 mM dCTP	4.50
1 mM dATP + dCTP	22.8

In vitro copying reactions with ØX DNA were carried out as described under Materials and Methods. "Baseline" substrate concentrations were 20 μM dATP and dTTP, and 50 μM dCTP and dGTP; concentrations of individual substrates were increased to the values indicated for each experiment. Reversion frequencies were the averages of duplicate samples and were calculated after subtracting the reversion frequency of uncopied ØX am3 DNA, which was 2.80×10^{-6}.

and DNA polymerase α is shown in Table II. As previously observed, dNMPs, which inhibit the 3'→5' exonuclease activity of Pol I, significantly increase reversion frequency of this enzyme; all four monophosphates are mutagenic, with dCMP showing the least effect (20). In contrast, dNMPs produce no significant effect on the fidelity of either form of calf thymus polymerase α, at elevated levels of dCTP (Table II) nor at 1 mM of both dCTP and dGTP (data not shown).

Effect of Pyrophosphate

Pyrophosphate also increases the error rate of Pol I in copying ØX174 am3 DNA. This effect is seen in reactions containing saturating amounts of the next nucleotide. Furthermore, pyrophosphate does not enhance misincorporations with AMV DNA polymerase, an enzyme devoid of a 3'→5' deoxyexonuclease. These results suggest that pyrophosphate-induced misincorporation occurs by inhibition of the exonuclease proofreading activity of Pol I, but by a different pathway than does the next nucleotide effect (T.A. Kunkel, R.A. Beckman and L.A. Loeb, unpublished experiments). This observation is confirmed with Pol I in Table III. Pyrophosphate

Table II: Effect of deoxynucleoside monophosphates on fidelity

	Reversion frequency ($\times 10^{-6}$)		
Addition	Pol α, form C	Pol α, form D	Pol I
None	4.68	3.80	4.35
9 mM dAMP	4.14	1.95	29.1
10 mM dCMP	4.65	2.35	7.77
10 mM dGMP	3.12	2.18	43.8
10 mM dTMP	---	---	38.7

DNA copying reactions were carried out as described in the legend to Table I. Reaction mixtures contained 20 μM dATP and dTTP, 50 μM dGTP, 1 mM dCTP, and added dNMP as indicated. Reversion frequencies for polymerase α were the averages of duplicate samples and were calculated after subtracting the reversion frequency of uncopied ØX am3 DNA, which was 1.09×10^{-6}. Reversion frequencies for Pol I were also calculated after subtracting background, but were single samples.

inhibits nucleotide incorporation and increases the reversion frequency of ØX DNA under conditions in which the next nucleotide effect is saturated. Pyrophosphate has quite a different effect on DNA polymerase α. Increasing concentrations of pyrophosphate produce no significant increase in reversion frequency, and 7 mM pyrophosphate apparently enhances fidelity. Higher concentrations of pyrophosphate severely inhibited incorporation by DNA polymerase α and thus could not be analyzed in this system. Under the conditions of Table III, pyrophosphate appears to enhance fidelity by as much as 5-fold. With copying reactions containing 1 mM dCTP and 20 μM dATP, pyrophosphate shows a similar fidelity-enhancing effect with either form of DNA polymerase α. However, since the baseline reversion frequency in the absence of pyrophosphate is lower than in Table III, it was more difficult to determine the magnitude of the pyrophosphate effect under these conditions (data not shown).

Effect of [1-thio] triphosphates

Table IV indicates the effect of dNTPαS on the fidelity of DNA polymerase α. When dATP or dGTP is replaced by its thiol derivative, there is no significant effect on reversion frequency. Under conditions where dCTP is replaced by dCTPαS, however, reversion frequency increases by an order of magnitude or more. The ability of the dCTP derivative to increase

Table III: Effect of pyrophosphate on fidelity

PPi,mM	Incorporation, Nucleotides Per Template	Reversion Frequency (x 10^{-6})
Polymerase α, form C		
0	74	13.3
0.1	89	22.6
0.5	91	9.22
1.0	88	9.42
3.0	148	4.82
5.0	131	9.92
7.0	58	2.23
Polymerase α, form D		
0	108	14.9
0.1	123	24.5
0.5	168	24.0
1.0	168	10.0
3.0	127	11.8
5.0	90	9.02
7.0	54	2.79
Pol I		
0	945	15.1
1.0	739	16.9
3.0	541	20.1
5.0	211	56.4
7.0	61	66.6

DNA copying reactions were carried out as described in the legend to Table I. Reaction mixtures contained 1 mM dATP, 1 mM dCTP, 50 μM dGTP, and 20 μM dTTP. Reversion frequencies were calculated after subtracting the reversion frequency of uncopied ØX am3 DNA, which was 3.28 x 10^{-6}. Values for samples with no pyrophosphate added were the average of duplicate samples; other values are for single samples.

reversion frequency is also seen with elevated levels of dATP, suggesting that the effect is independent of proofreading by an exonuclease pathway.

This effect appears to be specific for DNA polymerase α. Table V indicates that both the C and D forms, as well as a putative polymerase α "holoenzyme" purified from calf thymus by the method of Hübscher et al. (16), show dramatically higher reversion frequencies when dCTP is replaced by dCTPαS. With DNA polymerase β, however, the thiol derivative is antimutagenic, apparently because it is inserted inefficiently by this polymerase. When dCTP is replaced by dCTPαS, measured incorporation goes to zero, and reversion frequency returns to values near background. The

Table IV: Effect of dNTPαS on DNA polymerase α

dNTP Concentrations	Reversion Frequency (x 10^{-6}) Polymerase α, Form C	Polymerase α, Form D
1 mM dATP	5.75	1.86
1 mM dATPαS	6.10	2.79
1 mM dCTP	4.13	2.42
1 mM dCTPαS	79.4	48.2
1 mM dGTP	1.78	0.85
1 mM dGTPαS	1.60	0.22
1 mM dATP+dCTP+dGTP	9.95	3.25
1 mM dATPαS+dCTPαS+dGTPαS	128.	39.8

DNA copying reactions were carried out as described in the legend to Table I. "Baseline" substrate concentrations were 20 μM dATP and dTTP, and 50 μM dCTP and dGTP; individual substrates were increased to the concentrations indicated, or were replaced by the appropriate dNTPαS, as indicated, for each experiment. Reversion frequencies were the averages of duplicate samples and were calculated after subtracting the reversion frequency of uncopied ØX am3 DNA, which was 2.75 x 10^{-6}.

ØX viral DNA sequence from the 3'-hydroxyl end of the synthetic oligomer to the amber site is -G-G-A-T. DNA polymerase β apparently incorporates dCTPαS poorly and therefore does not proceed through the amber site, so few revertants are produced, even under conditions where polymerase β with normal substrates is much more mutagenic than α. Our experiments with AMV polymerase and Pol I are consistent with previous observations (22, and data not shown): First, dCTPαS has no effect on the fidelity of AMV polymerase. Secondly, this thiol derivative does increase reversion frequency with Pol I, but it also abolishes the next nucleotide effect. That is, either dCTPαS, which is incorporated but not excised; or high concentrations of dATP, which defeat proofreading, increase the Pol I error rate similarly. However, there is no additive effect when these two agents are combined.

DISCUSSION

The data in this paper reinforce the concept that most purified DNA polymerases α lack proofreading mechanisms that are available to procaryotic polymerases (23). Even though DNA polymerase α does not catalyze

Table V: Effect of dCTPαS on fidelity

dNTP Concentration	Incorporation, Nucleotides per Template	Reversion Frequency (x 10^{-6})
Polymerase α, form C		
1 mM dCTP	86	5.42
1 mM dCTPαS	24	146
1 mM dATP + dCTP	70	28.4
1 mM dATP + dCTPαS	50	234
Polymerase α, form D		
1 mM dCTP	75	3.89
1 mM dCTPαS	30	141
1 mM dATP + dCTP	116	32.2
1 mM dATP + dCTPαS	42	233
Polymerase α "holoenzyme"		
1 mM dCTP	26	3.98
1 mM dCTPαS	18	212
1 mM dATP + dCTP	58	21.2
1 mM dATP + dCTPαS	31	170
Polymerase β		
1 mM dCTP	35	1.37
1 mM dCTPαS	0	0.20
1 mM dATP + dCTP	29	160
1 mM dATP + dCTPαS	0	0.13

DNA copying reactions were carried out as described in the legend to Table I. Concentrations of dATP or dCTP were increased as indicated, or dCTP was replaced by dCTPαS. Enzyme sources were as indicated in Materials and Methods. Copying reactions with DNA polymerase α holoenzyme contained 0.08 unit of this species. Experiments with the polymerase α holoenzyme and polymerase β were conducted on different days; however, control samples with both polymerase forms C and D showed similar increases in reversion frequency in the presence of dCTPαS on both days. Reversion frequencies were the averages of duplicate samples and were calculated after subtracting the reversion frequency of uncopied ØX am3 DNA, which was 1.37 x 10^{-6}.

the overall generation of deoxynucleoside monophosphates (24) a small amount of uncomplementary monophosphate generation would be particularly difficult to detect yet might have profound effects on the fidelity of DNA polymerization. The lack of enhanced misincorporation by addition of deoxynucleoside monophosphates and the complementary next nucleoside triphosphate (the "next nucleotide" effect) provide independent evidence against proofreading by any putative exonucleolytic activity. In order to account for the lower error rate of polymerase α ($\leq$1/30,000) compared to non-enzyme mediated base pairings (1/100), one can invoke an enzyme-

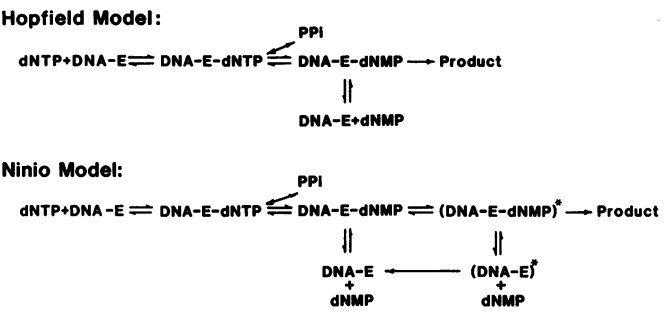

Figure 1: Models of kinetic proofreading. The features of Hopfield's kinetic proofreading (4) and Ninio's kinetic amplification (5) models are represented. E = DNA polymerase, * indicates an unspecified change in the DNA-E-dNMP intermediate.

mediated change in the conformation of the complementary substrate into a correct fixed position for insertion at the primer terminus of the growing DNA chain (25).

In addition to exonucleolytic proofreading, other kinetic mechanisms have been formulated for increasing accuracy. In Hopfield's model for kinetic proofreading (4) or Ninio's model for kinetic amplification (5), enzymes can achieve accuracy greater than that obtained by differences in free energies between base pairings by utilizing a branched catalytic pathway (Figure 1). In Hopfield's model, the driving force of pyrophosphate release is coupled with a second step at which incorrect substrates can be rejected as monophosphates. In Ninio's model, the enzyme-template-monophosphate intermediate is subject to a time delay during which the monophosphate may be rejected, but may not be incorporated. Even though these two models differ in topology, the end results are nearly identical (26): the branched pathway produces an overall error rate which is the product of the error rates at individual steps. With either model, rejection of incorrect substrates as monophosphates could occur before or after incorporation. Procaryotic enzymes use their 3'→5' exonuclease activity to excise errors after incorporation; eucaryotic polymerases might reject errors prior to phosphodiester bond formation. In both models, exogenous pyrophosphate makes the triphosphate to monophosphate reaction reversible, which has the effect of decoupling the steps at which enzyme can discriminate between correct and incorrect base pairs, and thus increases overall error rate. With our results here, DNA polymerase α shows none of the characteristics of

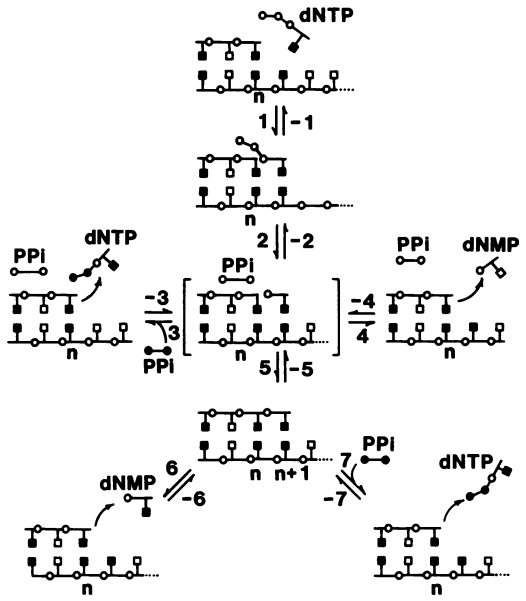

Figure 2: Model of Doubleday, Lecomte, and Radman (6). The individual steps are: 1) Formation of the DNA polymerase-DNA-dNTP complex, 2) Cleavage of pyrophosphate from the incoming dNTP, 3) Release of a dNTP which has undergone pyrophosphate exchange, 4) Release of free dNMP, 5) Formation of the phosphodiester bond, 6) Excision of an incorporated nucleotide, 7) Pyrophosphorolysis. Reprinted with permission from Alan R. Liss, Inc.

kinetic proofreading models: There is no evidence for the next nucleotide effect, and deoxynucleoside monophosphates have no effect on fidelity. This latter observation indicates that rejection of monophosphates, either before or after incorporation, is not important for the fidelity of polymerase α. Pyrophosphate, which decreases fidelity for the proofreading enzyme Pol I, appears to modestly enhance fidelity of polymerase α. DNA polymerase α does exhibit dramatically increased error rates with the thiol derivative dCTPαS. However, even at high concentrations of the "next nucleotide", conditions which defeat proofreading in procaryotic enzymes, dCTPαS still significantly increases mutagenesis with polymerase α.

Doubleday, Lecomte, and Radman (6) have combined the salient features of the Hopfield and Ninio kinetic models, and have postulated additional steps at which incorrect substrates can be rejected as triphosphates through pyrophosphate exchange or pyrophosphorolysis (Figure 2). A prediction of their scheme is that the addition of pyrophosphate should enhance replication fidelity and these workers have indeed reported that pyro-

phosphate enhances the fidelity of DNA polymerase α on a synthetic template (6). Our results in Table III with a natural DNA template show a modest enhancement in fidelity by pyrophosphate and tend to support these workers' hypothesis that pyrophosphate exchange improves fidelity. In an attempt to better quantify the pyrophosphate effect, we conducted an experiment with DNA polymerase α using dCTPαS, so that the mutagenesis baseline might be significantly increased. Under these conditions, even pyrophosphate concentrations as high as 10 mM, which severely inhibited incorporation, reduced reversion frequencies by no more than 4-fold (data not shown). Thus, whatever the mechanisms by which dCTPαS and pyrophosphate act, pyrophosphate shows little ability to "cure" the mutagenic effect of the thiol derivative. These observations and the results in Table III suggest that the effect of pyrophosphate on replication fidelity in vivo may be small at best.

An alternative model in which incorrect substrates are rejected as triphosphates is Hopfield's energy relay mechanism (7); the energy released by phosphate bond cleavage is applied to proofread the insertion of the following nucleotide. The most straightforward prediction of this hypothesis is that the incorporation of the first nucleotide should be more error-prone than the incorporation of subsequent nucleotides. Our previous work found no support for this prediction with DNA polymerase α or β, indicating that the most direct form of energy relay proofreading is not an important contributor to the fidelity of these eucaryotic polymerases (18). This does not rule out the possibility that auxiliary proteins or interaction with an unincorporated high energy compound might impart the ability to proofread by an energy relay mechanism.

The physiological significance of the effect of dCTPαS is not clear, since one would not expect cells to often be exposed to thiol nucleoside derivatives. Kinetic studies and studies with aphidicolin have suggested that DNA polymerase α may contain at least two separate dNTP binding sites (27,28). A direct interpretation of the data in Tables IV and V is that one or both isomers of dCTPαS fits into the pyrimidine binding site of polymerase α in such a way that it is read as dTTP, and is misinserted in the amber site of ØX DNA. This necessarily implies that at least the α-phosphate group is involved in the recognition of correct substrate by DNA polymerase α. It is conceivable that a contaminant is responsible for the mutagenesis observed with dCTPαS. Such a contaminant, however, would still be specific for DNA polymerase α, since it does not produce the same

effect on other enzymes tested. Moreover, two separate preparations of dCTPαS have produced similar enhanced reversion frequencies with polymerase α (data not shown).

The most significant consequences of the dCTPαS experiments may be diagnostic: Further experiments with this derivative might elucidate mechanisms of fidelity in eucaryotes. In addition, since the measured error rates of purified eucaryotic polymerases are considerably greater than one would expect for the cell, the implication is strong that auxiliary factors must enahnce eucaryotic fidelity. By producing dramatically mutagenic conditions, dCTPαS might make detection of fidelity-enhancing agents easier.

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