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# Exonucleolytic Proofreading by a Mammalian DNA Polymerase $\gamma$†

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ABSTRACT: Porcine liver DNA polymerase $\gamma$ contains exonuclease activity capable of digesting DNA in the 3′ → 5′ direction, releasing deoxyribonucleoside 5′-monophosphates. The exonuclease activity excises 3′-terminal bases from both matched and mismatched primer termini, with a preference for mismatched bases. Under polymerization conditions, mismatch excision by the exonuclease occurs prior to polymerization by polymerase $\gamma$, and this excision can be inhibited by adding to the reaction a high concentration of dNTP substrates and/or nucleoside 5′-monophosphates. In an M13mp2-based reversion assay for detecting single-base substitution errors, porcine liver polymerase $\gamma$ is highly accurate; the estimated base substitution error rate is less than one error for each 500 000 bases polymerized. Lower fidelity is observed using reaction conditions that inhibit the exonuclease activity, strongly suggesting that the exonuclease proofreads errors during polymerization. However, in a forward mutation assay capable of detecting all 12 mispairs at a variety of template positions, certain base substitution errors are readily detected even using unperturbed polymerization conditions. Thus, for some errors, polymerase $\gamma$ is not highly accurate, suggesting that proofreading is not equally active against all mispairs. To examine if the polymerase and exonuclease activities are physically as well as functionally associated, both activities were monitored during purification by four procedures, each based on a different separation principle. The two activities copurify during chromatography using phosphocellulose, heparin–agarose, or double-strand DNA–cellulose, and during velocity sedimentation in a glycerol gradient containing 0.5 M KCl. These results suggest that the polymerase and exonuclease activities are physically associated. It remains to be determined if they reside in the same subunit.

In vertebrate cells, mitochondrial DNA replication is catalyzed by DNA polymerase $\gamma$ (Pol-$\gamma$).[1] This DNA polymerase has been partially purified from a variety of sources, and several of its catalytic properties have been described [for a review, see Fry and Loeb (1987)]. One of the least studied properties of Pol-$\gamma$, but one which is critical for a replicative DNA polymerase, is the fidelity of polymerization. The fidelity of DNA synthesis by any DNA polymerase reflects its ability to discriminate against errors as nucleotides are being incorporated onto a primer-template. In addition, certain DNA polymerases contain associated 3′→5′ exonuclease activity capable of removing nucleotides from a 3′-OH primer prior to further elongation [for a review, see Kunkel (1988)]. We have recently shown that highly purified preparations of chick embryo Pol-$\gamma$ contain a proofreading exonuclease activity that substantially enhances the single-base substitution fidelity of this polymerase (Kunkel & Soni, 1988). This observation led to the present study in which we address three questions. (i) Does Pol-$\gamma$ isolated from a mammalian source contain associated 3′→5′ exonuclease activity? (ii) If so, does the exonuclease enhance fidelity by proofreading? (iii) Are the two activities physically associated? The results presented here suggest an affirmative answer to all three questions. In addition, the fidelity measurements required to answer these questions demonstrate that particular base substitution errors occur frequently even under conditions which should permit active proofreading, suggesting that the exonuclease does not efficiently proofread certain mispairs.

## Experimental Procedures

### *Materials*

Porcine liver DNA polymerase $\gamma$ was purified as described (Mosbaugh, 1988; see Results). Rat Novikoff hepatoma DNA polymerase $\beta$, fraction VI, was purified as described (Stalker et al., 1976). *Escherichia coli* DNA polymerase I, large (Klenow) fragment, was purchased from Boehringer Mannheim Biochemicals, and avian myeloblastosis virus (AMV) reverse transcriptase was obtained from Pharmacia. The

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[1] Abbreviations: Pol-$\beta$, DNA polymerase $\beta$; Pol-$\gamma$, DNA polymerase $\gamma$; Pol-$\delta$, DNA polymerase $\delta$; Pol I, *E. coli* DNA polymerase I; AMV Pol, avian myeloblastosis virus reverse transcriptase; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; monophosphate, nucleoside 5′-monophosphate; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetate.

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source of all other materials has been described (Kunkel, 1985a; Kunkel & Soni, 1988).

*Methods*

Preparation of M13mp2 DNA substrates, product analysis, transfection of competent cells, plating and scoring of α-complementation mutants, and DNA sequence analysis were performed as described (Kunkel, 1985a; Kunkel & Soni, 1988).

*Electrophoretic Analysis of Terminal Mismatch Excision.* This assay for 3′→5′ exonuclease activity is described in detail in Kunkel and Soni (1988). Briefly, a $^{32}$P-5′-end-labeled oligonucleotide complementary to positions 106 through 120 of the *lacZα* sequence of M13mp2 was hybridized to an M13mp2 mutant viral DNA to create an incorrect A(template)·G(primer)[2] mispair at the 3′-OH end. Terminal nucleotide excision reactions (25 μL) contained 20 mM Tris-HCl (pH 8.0), 2 mM dithiothreitol, 10 mM $MgCl_2$, 300 ng of M13mp2 DNA, and the amount of Pol-γ indicated in the legends to Figures 1–3. After incubation at 37 °C for the times indicated, aliquots (5 μL) were removed into 5 μL of dye mix (90% formamide, 5 mM EDTA, 0.1% bromophenol blue, and 0.1% xylene cyanol). Electrophoretic analyses of 3-μL samples were performed in 20% polyacrylamide sequencing gels, which were then dried and used to expose Kodak XAR film. The radioactivity in each band was quantitated by excising the bands from the gel and counting radioactivity in a Beckman LS7800 liquid scintillation counter.

*DNA Polymerase Reactions with M13mp2 Template-Primers.* Reactions contained 20 mM Hepes–KOH (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 terminal mismatch, the opal codon, or wild-type DNA), and the concentrations of the deoxynucleoside triphosphates and deoxyguanosine monophosphate, are given in the legends to Tables II and III. After incubation at 37 °C, reactions were terminated by addition of EDTA to a final concentration of 15 mM. Aliquots (15 μL) of each reaction were analyzed by electrophoresis in 0.8% agarose gels as described (Kunkel, 1985a). All polymerase reactions reported here generated products that migrated coincident with a replicative form II, fully double-stranded DNA standard.

*3′→5′ Exonuclease Activity under Polymerization Conditions.* The assay (Kunkel & Soni, 1988) measures the 3′→5′ exonucleolytic removal of a mismatched base from a primer terminus prior to polymerization to fill a single-strand gap. DNAs from two mutant derivatives of bacteriophage M13mp2, containing a single-base difference at position 103 in the *lacZα* gene, were used to construct a double-stranded heteroduplex with a 363-nucleotide gap of single-strand DNA. This molecule contains a 3′-terminal cytosine residue in the primer (minus) strand opposite an adenine residue in the template (plus) strand. The minus-strand cytosine results in a medium blue plaque phenotype whereas expression of the plus-strand adenine yields faint blue plaques. Polymerization to fill the gap without excision of the terminal cytosine will produce a complete heteroduplex that, upon transfection, has been shown to yield approximately 50% medium blue and 50% faint blue plaques. Alternatively, if the mispaired cytosine is excised prior to gap-filling synthesis by a polymerase, subsequent correct incorporation of thymidine opposite the template adenine will yield a homoduplex molecule that will produce exclusively faint blue plaques. For any given gap-filling reaction condition, the proportion of medium blue and faint blue plaques observed upon transfection of the reaction products and plating to score α-complementation is a measure of terminal mismatch excision activity.

*Reversion Assay for Single-Base Substitution Errors.* Single-base substitution errors occurring during in vitro DNA synthesis were measured by using the previously described (Kunkel & Soni, 1988) opal codon reversion assay. An M13mp2 DNA molecule was constructed having a 361-nucleotide gap containing a single-base change [G → A in the viral (plus) template strand at position 89 of the *lacZα* coding sequence]. This change creates an opal (TGA) codon, resulting in a colorless plaque phenotype under the appropriate plating conditions. The gap was filled by a single cycle of in vitro DNA synthesis, a portion of the product was analyzed to confirm gap-filling synthesis (Kunkel, 1985a), and the remainder was used to transfect competent α-complementation host cells to score the colors of the resulting M13mp2 plaques. Base substitution errors were detected as blue plaques, and the reversion frequency (the proportion of blue to total plaques) reflected the base substitution error rate. Eight of nine possible base substitution errors at the TGA opal codon yield a detectable blue plaque phenotype.

*Forward Mutation Assay.* Fidelity for a large variety of errors produced during in vitro DNA synthesis was determined by using the M13mp2 forward mutation assay (Kunkel, 1985a). As described above, a gapped DNA substrate was used (in this case, the single-strand gap was 390 nucleotides), but the target for mutations was the wild-type M13mp2 DNA sequence, and the assay scored the loss of α-complementation of β-galactosidase activity; i.e., mutants were identified as light blue or colorless plaques. Following confirmation of the mutant phenotype, the precise nature of the sequence change for a collection of mutants was determined by DNA sequence analysis (Kunkel, 1984). Since α-complementation activity is not essential for M13mp2 plaque production, over 200 different base substitution errors at 110 different sites (as of this writing) can be scored within the 250-base *lacZα* sequence. In addition, frameshift mutations, deletions, and more complex errors can be scored. The mutant frequency, i.e., the number of light blue and white plaques relative to the total number of plaques scored, reflected the error rate, which was calculated on a per detectable nucleotide polymerized basis, as described (Kunkel & Alexander, 1986; Kunkel, 1986).

*Glycerol Gradient Centrifugation.* DNA polymerase γ, in a volume of 200 μL, was layered onto a 4.8-mL linear 10–30% (v/v) glycerol gradient containing 50 mM Tris-HCl (pH 7.6), 1 mM dithiothreitol, 0.5 mM EDTA, and 0.5 M KCl. Centrifugation in polyallomer tubes was at 50 000 rpm for 13 h at 2 °C in a Beckman SW 55 Ti rotor. For comparison, rabbit aldolase (8.3 S, 158 000 daltons) was used as a standard in a parallel gradient. Seven-drop (~140 μL) fractions were collected from the top using a Buchler Auto Densi-flow IIC fraction collector. Polymerase activity for a 60-min incubation at 37 °C was determined by using 15 μL of each fraction in a 60-μL reaction containing 50 mM Tris-HCl (pH 8.0), 5 mM 2-mercaptoethanol, 7.5 mM $MgCl_2$, 0.5 mM EDTA, 150 mM KCl, 100 μg/mL activated DNA, and 15 μM each of dATP, dGTP, dCTP, and [$^3$H]dTTP (470 cpm/pmol). Exonuclease activity in a 6-μL aliquot of each fraction was determined during a 60-min incubation at 37 °C using the gel electrophoresis assay for terminal mismatch excision described above.

*Phosphocellulose Chromatography.* Porcine liver DNA polymerase γ (fraction II), derived from 170 g (wet weight)

[2] Base pairs will be described by listing the base in the template strand first and then the base in the primer strand.

of mitochondria, was purified as previously described (Mosbaugh, 1988). After dialysis against buffer containing 50 mM Tris-HCl (pH 7.5), 5 mM 2-mercaptoethanol, 1 mM EDTA, 10% (w/v) glycerol, and 150 mM NaCl, fraction II was loaded onto a phosphocellulose column (4.9 $cm^2 \times 30$ cm) equilibrated in the same buffer. DNA polymerase $\gamma$ was eluted with a 500-mL linear gradient from 150 to 1000 mM NaCl, at a flow rate of about 20 mL/h. Fractions (5.4 mL each) containing both DNA polymerase and 3′→5′ exonuclease activities were pooled as fraction III.

*Heparin–Agarose Chromatography.* Fraction III was dialyzed against TMEG buffer [50 mM Tris-HCl (pH 8.0), 5 mM 2-mercaptoethanol, 1 mM EDTA, and 10% (w/v) glycerol] containing 100 mM NaCl and loaded onto a heparin–agarose column (1.8 $cm^2 \times 9$ cm) equilibrated in the same buffer. A 150-mL linear gradient from 100 to 800 mM NaCl was used to elute the enzyme activities. The flow rate was 35 mL/h, and 2.7-mL fractions were collected. Fractions containing both polymerase and exonuclease activities were pooled (fraction IV).

*Double-Stranded DNA–Cellulose Chromatography.* After diluting 1 mL of fraction IV enzyme with 32 mL of TMEG buffer containing 100 mM NaCl, the sample was loaded onto a double-stranded DNA–cellulose column (0.5 $cm^2 \times 4.5$ cm) equilibrated in the same buffer. A 100-mL linear gradient from 100 to 500 mM NaCl was applied at 20 mL/h, and fractions (2.7 mL each) were collected and assayed for both DNA polymerase and exonuclease activity.

## Results

Porcine liver DNA polymerase $\gamma$ was purified from isolated mitochondria by using a procedure which included preparation of a soluble extract followed by chromatography on DEAE-Sephadex, phosphocellulose, and heparin–agarose (Mosbaugh, 1988). This procedure results in a 9500-fold purification,[3] yielding a Pol-$\gamma$ preparation that has catalytic properties characteristic of other mitochondrial DNA polymerases [for a review, see Fry and Loeb (1987)]. The most purified preparation of this polymerase contained exonuclease activity capable of generating deoxynucleoside 5′-monophosphates during polymerization (Mosbaugh, 1988).

*Detection of 3′→5′ Exonucleolytic Activity.* We began the present study by examining DNA polymerase $\gamma$ (fraction IV) for the presence of exonuclease activity capable of excising a 3′ terminally mismatched nucleotide. A mismatched substrate was prepared by hybridizing a $^{32}$P-5′-end-labeled 15-base oligonucleotide to a single-strand DNA template to produce an A·G mispair. Exonuclease reactions were performed over a 2-h time course, and the products were analyzed by gel electrophoresis. The results (Figure 1) demonstrated the existence of an exonucleolytic activity in the porcine liver Pol-$\gamma$ preparation which excised in the 3′ → 5′ direction. These results also suggested the absence of a 5′→3′ exonuclease, since 5′ → 3′ excision would have removed the 5′-end-label and the resulting products would not be detected, yet there was no loss of total radioactivity for any time point (data not shown). The presence of bands at the position of a 14-mer, a 13-mer, and a 12-mer (see inset to Figure 1) demonstrates that both mismatched and matched bases were removed, but with a preference for excision of the G from the A·G terminal mispair over excision from the two subsequent matched terminal C·G base pairs (compare open versus closed circles). The presence of a series of oligonucleotides differing in length by one nucleotide suggests that single mononucleotides were excised. This was confirmed by dTMP turnover measurements and product analysis using PEI–cellulose [data not shown, but see Mosbaugh (1988)].

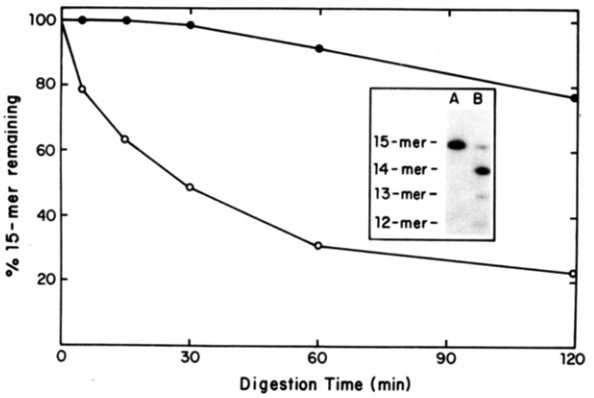


FIGURE 1: Terminal mismatch excision activity in preparations of porcine liver DNA polymerase $\gamma$. Excision reactions were performed as described under Methods, using 3.0 units of porcine liver Pol-$\gamma$ per 25-$\mu$L reaction. Open circles represent excision of the mismatched based, and closed circles reflect excision of a correctly paired base, which in this experiment represents disappearance of radioactivity from the position of the 14-mer. This method of determining the amount of incorrect versus correct base excision using a single substrate yields results similar to experiments using separate incorrect and correct substrates (Kunkel & Soni, 1988). The inset shows an autoradiogram of reaction products resulting from incubation for 120 min without enzyme (lane A) or with porcine liver Pol-$\gamma$ (lane B).

Table I: Excision versus Extension from a Terminal Mispair[a]

| DNA polymerase | [dNTP] ($\mu$M) | [dGMP] (mM) | medium blue[b] (%) | excision[c] (%) |
|---|---|---|---|---|
| AMV polymerase | 10 | 0 | 54 | 0 |
| Pol I, Klenow | 10 | 0 | 6.5 | 88 |
| | 1000 | 20 | 34 | 33 |
| porcine liver Pol-$\gamma$ | 50 | 0 | 1.9 | 97 |
| | 50 | 20 | 10 | 81 |
| | 1000 | 0 | 2.7 | 95 |
| | 1000 | 20 | 13 | 75 |

[a] Reactions (30 $\mu$L) were performed as described under Methods using the indicated concentration of each dNTP (in equimolar amounts), dGMP where indicated, and 150 ng of M13mp2 gapped DNA substrate containing the A·C terminal mispair and either AMV DNA polymerase (20 units, 37 °C, for 60 min), Klenow polymerase [0.5 unit, 37 °C, for either 15 min (for 10 $\mu$M reaction) or 5 min (for 1000 $\mu$M reaction)], or Pol-$\gamma$ (all conditions, 1 unit, 37 °C for 30 min). [b] In all cases, this value reflects the percent of medium blue plaques among at least 3000 total plaques scored. [c] To calculate the percent cytosine excised, we have used a background of 0.37% for complete excision and a value of 50% minus-strand expression for no excision [see Kunkel and Soni (1988)]. Thus, for example, at 10 $\mu$M dNTPs, the Klenow polymerase excises $[1 - [(6.5 - 0.37)/(50 - 0.37)]] \times 100 = 88\%$ of the cytosine prior to gap-filling synthesis.

*Excision versus Polymerization from a Terminal Mispair.* To examine the competition between excision versus extension from the terminal mismatch, we performed polymerase reactions with a gapped M13mp2 DNA substrate containing a 3′-terminal A·C mispair. The two bases of the mispair are distinguished as medium or faint blue plaques, and the proportion of each obtained upon transfection of the reaction products describes the extent of terminal mismatch excision prior to polymerization (see Methods). The gapped, mismatched molecule was used as template-primer in polymerization reactions catalyzed by three DNA polymerases (Table I). In order to monitor gap-filling DNA synthesis, reaction products were examined by analyzing an aliquot of each re-

[3] The value for the fold purification is expressed relative to the mitochondrial soluble extract rather than to the intact mitochondria or to liver cells.

action by electrophoresis in an agarose gel. The 363-nucleotide gap was filled by each enzyme to the extent that the product comigrated with the completely double-stranded DNA standard (data not shown). Within the limits of detection of this analysis, no uncopied DNA was observed. Similar results were obtained for all DNA polymerase reactions reported here.

The remaining gap-filled DNA was used for transfection of competent cells to score the colors of the resulting plaques (Table I). Gap-filling polymerization by AMV reverse transcriptase, which lacks associated 3′→5′ exonuclease activity (Battula & Loeb, 1976), occurred without substantial excision of cytosine from the A·C mismatch, since the minus-strand phenotype (medium blue plaques) was observed at a frequency of 54%, similar to the complete heteroduplex control (see legend). As expected for a polymerase containing a proofreading exonuclease, the Klenow fragment of *Escherichia coli* DNA polymerase I removed 88% of the terminal mismatch prior to extension to fill the gap. This occurred using reaction conditions that allowed the exonuclease to function (low dNTPs, no monophosphate added). Excision was reduced to 33% by using conditions that inhibited the exonuclease. When the exonuclease in the Pol-γ preparation was examined, we found that it removed the mismatches even more efficiently, excising 97% of the mispaired cytosine residues prior to filling the gap using 50 μM dNTPs. Next, three variations in reaction condition were employed, each of which has been shown to inhibit proofreading exonucleases. The first, addition of a 40-fold higher concentration of dNTPs to favor polymerization over excision (Ninio, 1975), only slightly decreased the frequency of mismatched nucleotide excision. Excision was only reduced from 97% to 95% (without dGMP) or from 81% to 75% (with dGMP). The second, addition of 20 mM dGMP, which binds to the active site of proofreading exonucleases and inhibits catalysis (Que et al., 1978), was more effective. Under this condition, extension from the terminal mispair by Pol-γ was increased 5-fold (e.g., compare 5% versus 25% extension at 1000 μM dNTPs). However, even the third condition, employing a high concentration of both dNTPs and dGMP, was again only partially effective in reducing mismatch excision.

*Fidelity of Pol-γ and Proofreading during Synthesis.* If the exonuclease functions to proofread base substitution errors during polymerization, porcine liver Pol-γ should exhibit high fidelity. To examine this, we measured the fidelity of Pol-γ in the highly sensitive (i.e., low background) opal codon base substitution reversion assay. Under conditions that allowed active proofreading (low dNTPs), Pol-γ was indeed highly accurate, since gap-filling synthesis using an equal concentration of all four dNTPs produced a reversion frequency less than 3-fold above the spontaneous background (Table II, experiment 1). From this result, the average error rate at the three-base opal codon was calculated (see legend) to be one base substitution error for each 560 000 nucleotides polymerized. Consistent with a proofreading function for the exonuclease, this fidelity is 10-fold to more than 100-fold higher than that of non-exonuclease-containing DNA polymerases (Kunkel & Alexander, 1986), and similar to that of other exonuclease-containing DNA polymerases known to proofread base substitution errors (Loeb & Reyland, 1987).

In order to further examine a proofreading function for this exonuclease, we determined the next nucleotide and monophosphate effects on fidelity. At equal substrate concentrations, the reversion frequency using 2 mM dNTPs and 20 mM dGMP was increased 3-fold (after subtracting the background). Although this effect was small, it was nevertheless consistent with a proofreading activity. However, fidelity remains high, consistent with the inability of these reaction conditions to completely diminish mismatch excision (Table I).

Table II: Fidelity of Pol-γ in Base Substitution Reversion Assay[a]

| DNA polymerase | [dNTP] (μM) | [dGMP] (mM) | plaques scored (×10⁵) total | plaques scored (×10⁵) blue | reversion frequency (×10⁻⁶) |
|---|---|---|---|---|---|
| Experiment 1: Equal dNTPs | | | | | |
| Pol-γ | 20 | 0 | 61 | 32 | 5.2 |
| | 2000 | 20 | 32 | 35 | 11 |
| Experiment 2: 50× dCTP | | | | | |
| Pol I (Klenow)[b] | 100 | 20 | 1.3 | 98 | 750 |
| Pol-γ | 500 | 0 | 23 | 7 | 3.0 |
| | 2000 | 20 | 26 | 19 | 7.3 |
| Experiment 3: 10× dGTP, dTTP, and dCTP | | | | | |
| Pol-γ | 50 | 0 | 53 | 20 (3) | 3.8 (0.6) |
| | 50 | 5[c] | 48 | 32 (12) | 6.7 (2.5) |
| | 2000 | 5[c] | 40 | 32 | 8.0 |

[a] Reactions were performed as described under Methods, using 300 ng of gapped M13mp2 A89 (opal codon-containing) DNA and 3 units of Pol-γ per 50-μL reaction. For experiment 1, the dNTP concentrations were equimolar. In experiment 2, a constant 50-fold excess of dCTP over dATP, dTTP, and dGTP was employed, and the concentration listed is that of dCTP. All blue revertants were plaque purified and plated with wild-type M13mp2 to confirm the phenotype. Only wild-type dark blue mutants resulting from misincorporation of dCMP opposite the template A of the TGA codon are shown. The Kf reaction used 0.5 unit of enzyme at 37 °C for 20 min. Pol-γ reactions were as described for experiment 1. In experiment 3, a constant 10-fold excess of dGTP, dTTP, and dCTP over dATP was used, and the concentration listed is that of the incorrect nucleotides. Reactions were performed as before. In this experiment, 5 mM AMP was used rather than dGMP. The number of revertants listed includes medium blue mutants only, as defined by plaque purification and plating with dark blue wild-type phage. The analysis is thus limited to revertants potentially created by the pool bias employed. For the low dNTP reactions, either with or without AMP, the sequence of the medium blue revertants was determined. The number of mutants identified by sequencing to have a single-base change consistent with the pool bias used (i.e., those having a change at the first position, a template T) are shown in parentheses. The average fidelity of Pol-γ can be calculated by subtracting the background reversion frequency (which for these experiments was $2 \times 10^{-6}$), dividing by 0.6 [the approximate probability of expressing an error, see Table II in Kunkel and Soni (1988)], and then dividing by three, since errors can be monitored at all three bases of the opal codon. [b] Data taken from Kunkel and Soni (1988). [c] AMP not dGMP.

In an attempt to increase the sensitivity of the analysis, we performed fidelity measurements with imbalanced dNTP substrate pools. Experiment 2 in Table II focuses exclusively on misincorporation of C opposite A in the TGA codon by utilizing a pool imbalance in which dCTP was in 50-fold excess over the other three dNTP substrates. This should increase the error frequency for A·C mispairs by two mechanisms. First, incorrect dCTP was present in 50-fold excess over correct dTTP, which should increase the misinsertion frequency. Second, dCTP was the next correct nucleotide to be inserted after the error, opposite the template G of the TGA codon. Its presence in high concentration was expected to increase the rate of polymerization from the mismatch, thus decreasing the likelihood of exonucleolytic removal. Stable misincorporation of C opposite A also produces a TGG, dark blue codon that is easily scored and differentiated from the lighter colors generated by the other revertants (Kunkel & Soni, 1988). The reversion frequencies shown for experiment 2 therefore reflect only the dark blue phenotype. The Klenow polymerase, used as a positive control, was highly inaccurate under this pool bias condition. In contrast, porcine liver Pol-γ was 100-fold more accurate. In fact, even under extreme conditions with 2 mM

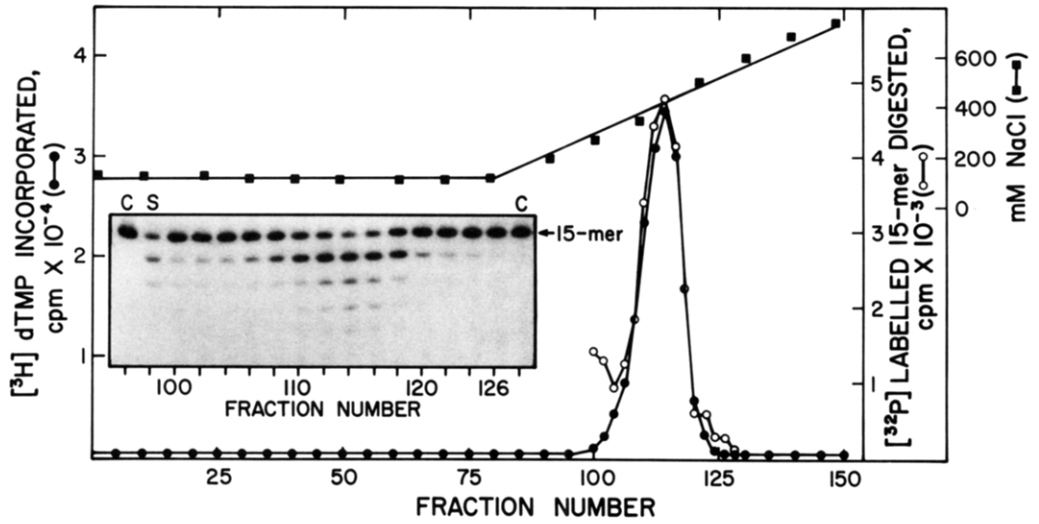


FIGURE 2: Phosphocellulose column elution profile for porcine liver DNA polymerase γ and 3′→5′ exonuclease. Phosphocellulose column chromatography and the assay for DNA polymerase activity with activated calf thymus DNA were performed as described (Mosbaugh, 1988). Assays for exonuclease activity were performed as described under Methods, using samples (5 μL) of the indicated fractions in reactions (25 μL) incubated for 60 min. The inset shows the autoradiogram. C = control containing column equilibration buffer only; S = 5 μL of the porcine liver Poly-γ (fraction II) applied to the column.

dCTP and 20 mM dGMP, Pol-γ was so accurate that we could only demonstrate a minor next nucleotide or monophosphate effect. We therefore attempted a different pool imbalance (experiment 3 in Table II), for errors opposite the T of the TGA opal codon, since this condition previously permitted detection of next nucleotide and monophosphate effects on proofreading by highly accurate calf thymus Pol-δ (Kunkel et al., 1987). Again, Pol-γ was highly accurate, and varying the reaction conditions produced only a small effect on the reversion frequency. Since in this experiment it was not possible to distinguish by color alone those errors consistent with the pool bias employed, the DNA sequence of the revertants from the first two reactions for experiment 3 (Table II) was determined. This effort (values shown in parentheses) revealed that the addition of 5 mM AMP to the Pol-γ reactions performed with a low concentration of dNTPs produced approximately a 4-fold increase in reversion frequency for errors opposite the template T. Such an effect is a hallmark of exonuclease proofreading (Que et al., 1978).

*Inefficient Proofreading of Certain Mispairs.* The sequencing of mutants from experiment 3 (Table II) revealed a surprising specificity. Among the 52 mutants analyzed were 37 that were not consistent with misinsertions driven by the pool imbalance used (i.e., they were not opposite the T of the TGA codon). Rather, 28 of the mutants contained a second-position G → T or G → C transversion. While this result was only for one site, this specificity, transversions at a template G residue, was reminiscent of our previous observations with chick embryo Pol-γ in the forward mutation assay. In that study, while fidelity was generally high, three specific errors (G → T and G → C and C → T) were produced at a high frequency. The responsible mispairs (G·dATP, G·dGTP, and C·dATP) obviously escaped proofreading under conditions that permit active proofreading against other mispairs. In order to determine if this effect was peculiar to the chick Pol-γ or reflects a more generaly proofreading specificity response, we examined the fidelity of the porcine liver Pol-γ in the forward mutation assay. The results are shown in Table III. As expected from previous studies (Kunkel, 1985a), Novikoff hepatoma Pol-β, which lacks associated exonuclease activity and was included here as a positive control, was highly inaccurate in the forward mutation assay. Consistent with the presence of an active proofreading exonuclease, porcine liver

Table III: Fidelity of Pol-γ in Forward Mutation Assay[a]

| DNA polymerase | [dNTP] (μM) | [dGMP] (mM) | plaques scored | | mutant frequency (×10⁻⁴) |
|---|---|---|---|---|---|
| | | | total | mutant | |
| Pol-β | 500 | 0 | 28038 | 1495 | 530 |
| Pol-γ | 20 | 0 | 40855 | 80 | 20 |
| | 2000 | 20 | 36043 | 125 | 35 |

[a] Reactions were performed as described under Methods and in the legend to Table II, using the wild-type M13mp2 gapped DNA substrate and equimolar concentrations of all four dNTPs. The control mutant frequency for uncopied DNA was 6.7 × 10⁻⁴, determined as described (Kunkel, 1985).

Pol-γ was much more accurate than Pol-β, particularly in reactions containing 20 μM dNTPs, and less so when the reaction contains 2000 μM dNTPs and dGMP. However, even with a low concentration of dNTPs, a condition which permitted highly active mismatch excision (Table I) and high fidelity for certain mispairs at the opal codon (Table II), the forward mutant frequency was 3-fold above the spontaneous background mutant frequency, demonstrating that some errors were produced at an easily detectable frequency even in the relatively insensitive forward assay. DNA sequence analysis demonstrated that 9 of 10 light blue mutants derived from the 20 μM reaction contained either a C → T transition or a G → T transversion. This specificity was remarkably similar to the results with chick embryo Pol-γ. This suggests that the error rate for (at least some) C·dATP and G·dATP mispairs was much higher than for other mispairs, perhaps because they were not proofread efficiently.

*Copurification of Exonuclease and Polymerase Activity.* To determine if the exonuclease is physically as well as functionally associated with porcine liver Pol-γ, we assayed for both activities during enzyme purification. DEAE-Sephadex chromatography yielded a single peak of polymerase (Mosbaugh, 1988), and exonuclease activity was observed throughout these column fractions (not shown). Further chromatography on phosphocellulose chromatography provided a substantial purification of the polymerase which eluted as a symmetrical peak (Figure 2). When fractions across the peak were monitored for exonuclease activity using the gel assay (Figure 2, inset), 3′→5′ exonuclease activity coeluted with the polymerase. In addition, the ratio of the two activities was constant across the peak fractions (Table IV). The two

Table IV: Ratio of 3′→5′ Exonuclease to DNA Polymerase γ Activity across Peak Fractions during Purification[a]

| purification method | fraction no. | | | | | | |
|---|---|---|---|---|---|---|---|
| | −3 | −2 | −1 | peak | +1 | +2 | +3 |
| phosphocellulose | 1.0 | 1.1 | 1.0 | 1.0 (114) | 1.0 | 1.0 | 0.8 |
| heparin–agarose | 1.9 | 1.3 | 1.1 | 1.0 (74) | 1.2 | 1.1 | 1.7 |
| glycerol gradient | 2.5 | 2.2 | 1.3 | 1.0 (15) | 1.0 | 1.1 | 1.4 |
| dsDNA–cellulose | 0.4 | 0.8 | 0.9 | 1.0 (31) | 0.9 | 0.8 | 0.9 |

[a] For each method of purification, the exonuclease to polymerase ratio was calculated for seven column fractions across each peak. Values are expressed relative to the peak fraction, which is assigned an arbitrary value of 1.0 to describe the ratio of cpm of $^{32}$P-labeled 15-mer digested in the exonuclease assay to cpm of [$^3$H]dTMP incorporated in the polymerase assay. The peak fraction numbers are shown in parentheses. For the first two methods, every other fraction across the peak was assayed, so that, for example, the +1 phosphocellulose fraction is fraction 116. For the final two methods, every fraction across the peak was assayed. The number of cpm (digested versus incorporated) by the peak fraction for each method listed from top to bottom were, respectively, the following: 4770 versus 25 500; 2100 versus 17 500; 4000 versus 19 500; and 1080 versus 1450. The data for the first three methods come from Figures 1 and 2.

enzyme activities also coeluted as a single peak during heparin–agarose chromatography (Figure 3A) and cosedimented in a glycerol gradient containing 0.5 M KCl (Figure 3B). The latter technique suggests that the native molecular weight of both activities in high salt is 160 000. Again, the ratio of the two activities was relatively constant across the peak fractions (Table IV). The variations from a value of 1.0 may reflect experimental error in the enzyme assays, particularly for those fractions well removed from the peak fraction that contains small amounts of activity.

Further attempts at purification using hydroxyapatite or double-strand DNA–cellulose columns resulted in a significant loss of DNA polymerase activity, and the activity that was recovered was highly unstable. Such effects with Pol-γ have been noted before (Yamaguchi et al., 1980). Nevertheless, when the double-stranded DNA–cellulose column fractions were assayed immediately after elution, we detected both polymerase and exonuclease activities in the peak fractions (Table IV).

## Discussion

These results demonstrate that preparations of porcine liver DNA polymerase γ contain a 3′→5′ exonuclease activity that copurifies using several procedures based on different separation principles. That this exonuclease functions to proofread base substitution errors during DNA synthesis is suggested by the following observations: (i) the exonuclease excises nucleotides from a 3′-OH primer, with a preference for excision of mismatched bases; (ii) terminal mismatch excision is reduced in reactions which allow the polymerase to compete with the exonuclease; (iii) Pol-γ is highly accurate for certain base substitution errors; and (iv) fidelity is lower using reaction conditions known to reduce proofreading by other DNA polymerase associated exonucleases. The last point has proven difficult to establish with highly accurate porcine Pol-γ and is based on small (2–5-fold) next nucleotide and monophosphate effects. Nevertheless, the results are qualitatively similar to those obtained with chick embryo Pol-γ, where convincing 6- and 14-fold next nucleotide and monophosphate effects on fidelity were seen (Kunkel & Soni, 1988). The observation that a second, in this case mammalian, γ-polymerase proofreads base substitution errors suggests that this may be a common property of this class of higher eukaryotic DNA polymerases.

Compared under identical reaction conditions, porcine liver Pol γ is much more faithful than either *E. coli* DNA polymerase I or calf thymus DNA polymerase δ (Kunkel et al., 1987), and the porcine enzyme is somewhat more accurate than its chick embryo counterpart (Kunkel & Soni, 1988). In fact, the results in Table II suggest that porcine liver Pol-γ is one of the most accurate DNA polymerases described to date. Conservative error rate calculations (as described in the

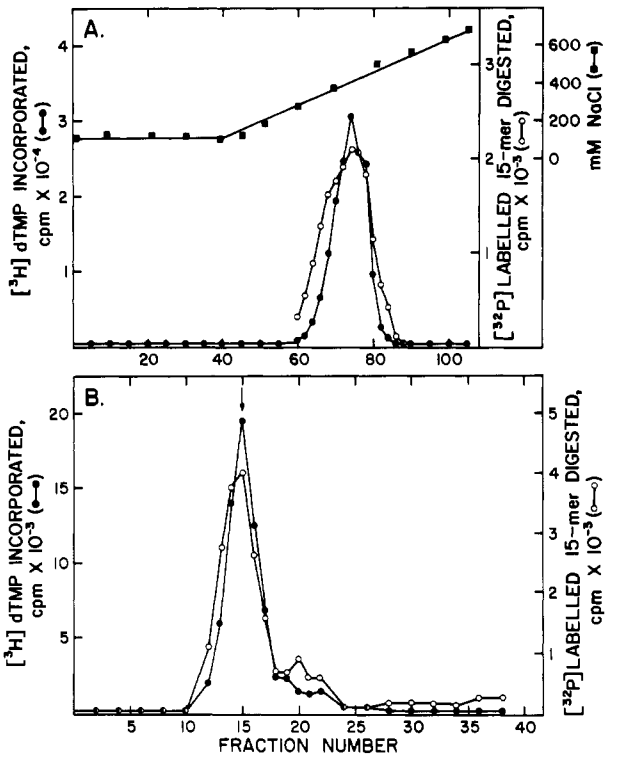


FIGURE 3: Heparin–agarose column elution profile and glycerol gradient sedimentation profile for porcine liver DNA polymerase γ and 3′→5′ exonuclease. The assays were described in the legend to Figure 2, but for exonuclease activity used 4 μL of each fraction. Heparin–agarose column chormatography (A) and glycerol gradient sedimentation analysis (B) were as described under Methods. Sedimentation was from right to left. The arrow indicates the position of the rabbit aldolase standard ($M_r$ 158 000) run in a parallel gradient.

legend to Table II) suggest that, under conditions which permit proofreading to function, Pol-γ stably misincorporates less than one error for each million detectable nucleotides polymerized, when considering A·C mispairs (experiment 2 in Table II) or T·G, T·C, and T·T mispairs (experiment 3 in Table III) at the opal codon. The high base substitution fidelity for some mispairs is in striking contrast to the low fidelity of Pol-γ observed for other base substitution errors (Table III and see text). Fidelity differences greater than 100-fold have also been described for chick Pol-γ (Kunkel & Soni, 1988). Such differential proofreading phenomena are not limited to the γ-polymerases, as they have been observed in studies with DNA polymerase III holoenzyme (Fersht & Knill-Jones, 1983), with *E. coli* DNA polymerase I (Kunkel et al., 1981) and the large (Klenow) fragment of this polymerase,[4] with T4

[4] K. Bebenek, M. P. Fitzgerald, and T. A. Kunkel, manuscript in preparation.

DNA polymerase (Sinha, 1987), and with the *Drosophila* DNA polymerase–α-primase complex (Reyland et al., 1988). These effects presumably reflect the balance of the rate constants for the misinsertion of a nucleotide, for polymerization from the mispair, and for the exonucleolytic removal of a terminal nucleotide. Each rate is expected to vary not only for correct versus incorrect bases but also for each different mispair and by position as well, because each template-primer position provides a new set of protein–DNA contacts and a new set of base stacking interactions. A thorough understanding of proofreading depends on a kinetic description of each of these competing reactions and information on the structure of mispairs in different DNA sequence contexts.

Prokaryotic DNA polymerases that have polymerase and exonuclease active sites in the same polypeptide include *E. coli* DNA polymerase I (Setlow et al., 1972; Derbyshire et al., 1988) and T-phage DNA polymerases (Das & Fujimura, 1976; Adler & Modrich, 1979; Hori et al., 1979; Lin et al., 1987). For the replicative polymerase of *E. coli*, DNA polymerase III holoenzyme, the polymerase and exonuclease activities are on separate subunits (Scheuermann et al., 1983; Scheuermann & Echols, 1984), designated α and ε, respectively. In eukaryotes, exonuclease activities that are physically associated with DNA polymerases have now been described [for reviews, see Fry and Loeb (1987) and Kunkel (1988)] for representatives of all four classes of DNA polymerases (α, β, δ, and γ). Among these are examples of DNA polymerases from which the exonuclease can be dissociated from the polymerase (Skarnes et al., 1986; Crute et al., 1986; Mosbaugh & Meyer, 1980; Mosbaugh & Linn, 1983) and other examples of these polymerases where the two activities have not been separable (Chen et al., 1979; Cotterill et al., 1987; Byrnes et al., 1976; Lee et al., 1980). While it is possible that the two porcine liver enzymatic activities are not associated but have the same binding and sedimentation behavior, the copurification results with four different procedures suggest that the two activities are physically associated. Whether they reside in the same polypeptide or on different subunits remains to be established.

Just as for eukaryotic DNA polymerases α and δ, the subunit composition of DNA polymerase γ has not yet been clearly defined. The heparin–agarose fraction of porcine liver Pol-γ contains four polypeptides that cosediment in the glycerol gradient with both polymerase and exonuclease activity. Their molecular weights are 120 000, 55 000, 50 000, and 48 000. Several possible combinations of these polypeptides could explain the native molecular weight of the polymerase–exonuclease (160 000) observed upon sedimentation (Figure 3B).[5] On the basis of native molecular weight and specific activity, Yamaguchi et al. (1980) suggest that the native chick Pol-γ may be a tetramer of four identical 47 000-dalton subunits, although the possibility that the DNA polymerase activity resided in a 135 000 molecular weight polypeptide was not excluded. Similarly, Pol-γ from mouse myeloma was suggested to consist of a tetramer of four 47 000 molecular weight subunits (Matsukage et al., 1981). For the enzyme purified from *Drosophila melanogaster* embryos, in situ detection of DNA polymerase γ activity after SDS–polyacrylamide gel electrophoresis demonstrated activity associated with a polypeptide having a molecular weight of 125 000 (Wernette & Kaguni, 1986), although a second 35 000 molecular weight polypeptide was also present. That Pol-γ can exist as a multisubunit complex is suggested by the observation that partially purified preparations of Pol-γ from human KB cells (Wong & Clayton, 1985) and rat liver clels (Ledwith et al., 1986) contain associated DNA primase activity that can be separated by sedimentation in glycerol gradients or by heparin–agarose chromatography, respectively. The observation in the present study that neither of these methods resolved the polymerase and exonuclease activities suggests either that the two active sites are contained within the same polypeptide or that, if they are on separate subunits, the exonuclease subunit is more tightly associated with the polymerase than is primase.

[5] We are presently attempting to elucidate the subunit composition of porcine liver DNA polymerase γ and its associated exonuclease by in situ detection of enzymatic activities after SDS–polyacrylamide gel electrophoresis.

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**Registry No.** DNA polymerase, 9012-90-2; exonuclease, 79393-91-2.

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# Topological Complexes between DNA and Topoisomerase II and Effects of Polyamines

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ABSTRACT: The polyamines spermine and spermidine were found to enhance the formation of a stable noncovalent complex between mammalian topoisomerase II and DNA. This complex is not associated with DNA strand breaks and forms to a greater extent with supercoiled than with relaxed circular or with linear DNA. Polyamine-induced complex formation is associated with a stimulation of the enzymatic relaxation of DNA supercoils. In these respects, the polyamine-enhanced complex differs from the covalent cleavable complexes stabilized by DNA intercalators such as amsacrine (*m*-AMSA) or epipodophylotoxins such as teniposide (VM-26). In the polyamine-enhanced complex, the topoisomerase II may be a donutlike structure topologically bound to the DNA and able to migrate and dissociate from the ends of linear DNA molecules. At relatively high concentrations, spermine (1 mM) enhances topoisomerase II induced cleavage at certain sites on the SV40 genome that could have regulatory significance.

Because the polyamines spermine and spermidine and topoisomerase II are both normal constituents of chromatin and may have important roles in the regulation of genetic transcription and replication, it appeared useful to determine their interaction in a purified enzyme system.

Polyamines are found at millimolar concentrations in mammalian cells and are present in the nucleus in quantities sufficient to neutralize 15–30% of the DNA negative charge (McCormick, 1978). Crystallographic, NMR, and molecular modeling studies suggest that polyamines bind to the DNA grooves with their charged amines in close proximity to the phosphates of the DNA backbones (Marton & Morris, 1987). Polyamine binding to DNA facilitates DNA condensation and aggregation (Hoopes & McClure, 1981; Baeza et al., 1987) and increases the stability of nucleosome core particles (Morgan et al., 1987).

Two types of DNA topoisomerases have been isolated from mammalian cells. Type I topoisomerase opens and seals reversibly DNA single-strand breaks, while type II topoisomerase opens and seals reversibly DNA double-strand breaks in the presence of ATP (Wang, 1985, 1987). Both topoisomerases can relax DNA supercoiling, and the regulation of DNA supercoiling may affect gene expression and DNA replication (Menzel & Gellert, 1987). DNA decatenation at the end of chromosome replication can be carried out only by topoisomerase II (Holm et al., 1985; Uemura et al., 1987). Topoisomerase II is inhibited by certain DNA intercalators, such as amsacrine (Minford et al., 1986; Nelson et al., 1985; Pommier et al., 1985), and by demethylepipodophyllotoxins, such as teniposide (Long et al., 1984; Chen et al., 1985). The drugs inhibit the enzyme primarily by stabilizing an enzyme–DNA complex in which one or both DNA strands are cleaved and the 5′ terminus of each strand break is covalently linked to a tyrosine residue on the enzyme (Kohn et al., 1987).

Spermidine has been shown to stimulate the activity of mammalian topoisomerase I and to inhibit the activity of the bacterial enzyme (Srivenugopal & Morris, 1985; Srivenugopal et al., 1987). Spermidine has been reported to stimulate the activity of bacterial DNA gyrase (Srivenugopal et al., 1987), a type of topoisomerase II quite different from that found in eukaryotic cells. Polyamines shift the reversible DNA ca-

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