Exonucleolytic Proofreading Enhances the Fidelity of DNA Synthesis by Chick Embryo DNA Polymerase-γ*

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The high fidelity of chick embryo DNA polymerase-γ (pol-γ) observed during in vitro DNA synthesis (Kunkel, T. A. (1985) J. Biol. Chem. 260, 12860–12874) has led us to examine this DNA polymerase for the presence of an exonuclease activity capable of proofreading errors. Highly purified chick embryo pol-γ preparations do contain exonuclease activity capable of digesting radiolabeled DNA in a 3' → 5' direction, releasing deoxyribonucleoside 5'-monophosphates. The polymerase and exonuclease activities cosediment during centrifugation in a glycerol gradient containing 0.5 M KCl. In the absence of dNTP substrates, this exonuclease excises both matched and mismatched primer termini, with a preference for mismatched bases. Excision is inhibited by the addition of nucleoside 5'-monophosphates to the digestion reaction. In the presence of dNTP substrates to permit competition between excision and polymerization from the mismatched primer, the exonuclease excises mismatched bases from preformed terminal mismatches with greater than 98% efficiency. The preference for excision over polymerization can be diminished by addition of either high concentrations of dNTP substrates or nucleoside 5'-monophosphates to the exonuclease/polymerase reaction. To determine if this exonuclease is capable of proofreading misinsertions produced during a normal polymerization reaction, a sensitive base substitution fidelity assay was developed based on reversion of an M13mp2 lacZa nonsense codon. In this assay using reaction conditions that permit highly active exonucleolytic proofreading, pol-γ exhibits a fidelity of less than one error for every 260,000 bases polymerized. As for terminal mismatch excision, fidelity is reduced by the addition to the synthesis reaction of high concentrations of dNTP substrates or nucleoside 5'-monophosphates, both hallmarks of exonucleolytic proofreading by prokaryotic enzymes. Taken together, these observations suggest that the 3' → 5' exonuclease present in highly purified chick embryo pol-γ preparations proofreads base substitution errors during DNA synthesis. It remains to be determined if the polymerase and exonuclease activities reside in the same or different polypeptides.

A wealth of genetic and biochemical observations have demonstrated that slow spontaneous mutation rates in prokaryotes result in part from proofreading of DNA synthesis errors by the 3' → 5' exonuclease activity associated with prokaryotic DNA polymerases (for review, see Ref. 1). Of the four classes of DNA polymerases found in higher eukaryotes (for review, see Ref. 2), DNA polymerase-δ has an associated 3' → 5' exonuclease activity (3, 4) that has recently been shown to proofread errors (5). Highly purified preparations of two other classes of higher eukaryotic DNA polymerases, α and β, have generally been found to lack associated nuclease activity (2). Although several reports do describe exonuclease activities associated with these DNA polymerases (6–10), a role for these exonucleases in proofreading has not been established.

The fourth class of DNA polymerases in animal cells is DNA polymerase-γ (pol-γ). This enzyme is responsible for replication of mitochondrial DNA and may be involved in DNA repair processes as well (2). It is found in both the mitochondrial and the nucleus and is present as only 1–10% of the total cell DNA polymerase activity. Because of this low abundance as well as its heterogeneity and instability during isolation, pol-γ has been difficult to purify to homogeneity for detailed characterization. Despite these difficulties the enzyme has been substantially purified from a variety of sources (11–19, and reviewed in Ref. 2), including to near homogeneity from chick embryos (20). The chick embryo pol-γ preparation, purified 1,500,000-fold to a very high specific activity, is both highly processive (21) and highly accurate for several different types of errors during in vitro DNA synthesis with natural DNA (22–24). The fidelity results have led us to examine chick embryo pol-γ preparations for an 3' → 5' exonucleolytic activity that could function as a proofreading error.

Such an activity, which fulfills the established criteria for a proofreading exonuclease, has been detected.

EXPERIMENTAL PROCEDURES

Bacteria and Bacteriophage

Escherichia coli strain MC1061 (hsdR*, hsdM*, araD139, Δara, leu2, galK, galU, galE, strA) was provided by Roel M. Schaper of this Institute. Other bacterial strains and wild type bacteriophage M13mp2 were as described (25–26). Mutant derivatives of M13mp2 containing single base changes were: A89, A103, G103, and (-)C106. To determine the exact nature of the base change for any mutant, a wealth of genetic and biochemical observations have demonstrated that low spontaneous mutation rates in prokaryotes result in part from proofreading of DNA synthesis errors by the 3' → 5' exonuclease activity associated with prokaryotic DNA polymerases (for review, see Ref. 1). Of the four classes of DNA polymerases found in higher eukaryotes (for review, see Ref. 2), DNA polymerase-δ has an associated 3' → 5' exonuclease activity (3, 4) that has recently been shown to proofread errors (5). Highly purified preparations of two other classes of higher eukaryotic DNA polymerases, α and β, have generally been found to lack associated nuclease activity (2). Although several reports do describe exonuclease activities associated with these DNA polymerases (6–10), a role for these exonucleases in proofreading has not been established.

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be published elsewhere? The eight mutants used for the heteroduplex expression experiments were blue revertants of the opal codon that were created by the A88 mutation and were obtained from several sources (5).

**DNA Polymerases**

The chick embryo DNA polymerase-γ used in this study was purified by Yamaguchi and co-workers as described (20). Briefly, this entails purification from a crude extract by phosphocellulose chromatography, ammonium sulfate fractionation, and successive column chromatography steps using phosphocellulose, Sephadex G-200, hydroxyapatite, and finally double-stranded DNA-cellulose. Assayed with the preferred template-primer poly(rA). oligo d(T)12.18, the final fraction, which was the fraction used in the present studies, was purified 1,500,000-fold to a specific activity of 570,000 units/mg protein. Over the course of these studies, three independently purified preparations of pol-γ were used. Similar results (i.e. mismatch excision, high fidelity) were obtained with all three preparations. The source and purity of both rat and chick embryo pol-γ (26, 28) have been described. T4 DNA polymerase, pol I, and AMV pol were from PL-Pharmacia. Pol I (Kf) was from either PL Pharmacia or Boehringer Mannheim. Restriction endonucleases AciI, KpnI, and PvuII as well as other reagents were from commercial sources as described (25, 26, 29).

**Construction of Radiolabeled Substrates for Exonuclease Assays**

To prepare the 3'-end-labeled substrate for 3'→5' exonuclease activity, M13mp2 DNA containing a 390-base gap (constructed as described below) was used. Polymerization to fill the gap was performed in a 200-μl reaction containing 20 mM Tris-HCl (pH 7.5), 2 mM dithiothreitol, 10 mM MgCl2, 1 μM each of dATP, dGTP, dTTP, and [α-32P]dCTP (1800 cpm/pmol), 5 μg of gapped M13mp2 DNA, and 5 units of T4 DNA polymerase. Incubation was at 37 °C for 20 min and the reaction was stopped by addition of EDTA to 15 mM. Unincorporated radioactively labeled dCTP was removed by two successive cycles of chromatography on Sephadex G-50 columns (Boehringer Mannheim) following the manufacturer's instructions. The DNA was precipitated with ethanol, resuspended in TE buffer (10 mM Tris-HCl [pH 8.0], 0.1 mM EDTA) and used as a substrate for the 3'-→5' exonuclease assays shown in Fig. 1A.

To prepare the 5'-end-labeled DNA substrate, an unphosphorylated 15-base oligonucleotide complementary to positions 177–191 of the lacZα-coding sequence in M13mp2 was phosphorylated at the 5'-end as described (31). The oligonucleotide was hybridized (as above) to a 5-fold excess of the lacZα sequence of M13mp2, to create a C(template)→G(primer) mispair at the 3'-OH end. This oligonucleotide was added 50 μg of single-stranded viral, circular M13mp2 DNA and standard sodium citrate (SSC) to a final 1× concentration (i.e. 150 mM NaCl, 15 mM sodium citrate). Hybridization was performed by placing the mixture (80 μl in a 1.5-ml microcentrifuge tube) into a 500-ml beaker of water at 70 °C and allowing the beaker to cool to 37 °C. To this was added 120 μl of polymerization mix to produce a 200-μl reaction containing the same components as for the α-32P-labeled DNA described above, except that the dNTP substrates (at 100 μM) were all unlabeled. Incubation to permit polymerization was at 37 °C for 15 min, and the reaction was terminated by addition of EDTA to 15 mM. Unincorporated [α-32P]dATP was removed and the DNA processed as described above. Analysis of the product DNA on a 8% polyacrylamide-sequencing gel demonstrated that all the 32P-label was in high molecular weight (>500 bases) material. This 5'-end-labeled DNA was used as a substrate for 5'→3' exonuclease activity (Fig. 1B).

**Pol-γ Proofreading**

**Exonuclease Assays with End-labeled DNA**

Exonuclease assays were performed in 50-μl reactions containing 20 mM Hepes (pH 7.8), 2 mM dithiothreitol, 10 mM MgCl2, and either 200 ng of 3'-end-labeled DNA (~550 cpm/ng), denatured just before use by incubating at 95 °C for 5 min, or 1.8 μg 5'-end-labeled DNA (34,000 cpm/μg) and the amount of DNA polymerase indicated in the legend to Fig. 1. At the indicated times aliquots were processed for determination of acid-insoluble radioactivity as described (30). At the final time point, 2 μl of the reaction were analyzed by polyethyleneimine cellulose chromatography as described (31).

**Polymerase Assay with Activated DNA**

Reactions (100 μl) included 20 mM Hepes (pH 7.8), 2 mM dithiothreitol, 10 mM MgCl2, 100 μM dATP, dTTP, dGTP, and [α-32P]dCTP (1800 cpm/pmol), 10 μg of activated DNA as described (30). At the final time point, 2 μl of the reaction were analyzed for polyethyleneimine cellulose chromatography as described (31).

**Electrophoretic Analysis of Terminal Mismatch Excision**

A 15-base oligonucleotide complementary to positions 106–120 of the lacZα sequence of M13mp2 was phosphorylated at the 5'-end as described above for the preparation of the 5'-end-labeled exonuclease substrate. This oligonucleotide was hybridized (as above) to a 3-fold molar excess of either wild type M13mp2 viral DNA, to create a correct C(template)→G(primer) base pair at the 3'-OH end, or to an M13mp2 mutant viral DNA containing a C from mismatch mutation at position 106, to create an incorrect G(template)→A(primer) mispair at the 3'-OH end. Terminal excision reactions (25 μl) contained 20 mM Hepes (pH 7.8), 2 mM dithiothreitol, 10 mM MgCl2, 300 ng of M13mp2 DNA (matched or mismatched) and 0.9 unit of pol-γ. Aliquots (5 μl) were removed after 0, 5, 15, 30, and 60 min of incubation at 37 °C, into 5 μl of dye mix (90% formamide, 10 mM EDTA, 0.1% bromphenol blue, 0.1% xylene cyanol). Electrophoretic analyses of 3-μl aliquots were performed in 20% polyacrylamide-sequencing gels (Fig. 2), which were dried and used to expose Kodak XAR film. The radioactivity of each band was quantitated by cutting out the bands from the gel and counting radioactivity in a Beckman LS7800 liquid scintillation counter.

**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 7.8), 1 mM dithiothreitol, 0.5 mM EDTA, and 0.5 mM 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 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 MgCl2, 0.5 mM EDTA, 150 mM KCl, 100 μM/ml activated DNA, and 15 μM each of dATP, dGTP, dCTP, and [H-dTTP (470 cpm/pmol). Exonuclease activity in a 6-μl aliquot of each fraction was determined using a 60-min incubation at 37 °C using the gel electrophoresis assay for terminal mismatch excision described above.

**Preparation of M13mp2 DNA Substrates**

Three types of M13mp2 DNA substrates were used: gapped heteroduplex molecules containing 3'-OH terminal mispairs (experiments shown in Fig. 3 and Table I), completely double-stranded heteroduplex molecules containing an internal single-base mispair (experiments shown in Tables I and II), and gapped molecules containing an opal codon within the single-stranded (template) DNA (experiments shown in Table III). For each of these constructions, single-stranded viral (plus DNA) and double-stranded replicative (minus DNA) RF DNA were obtained as described in Refs. 26 and 29, respectively.

**Construction of Gapped Heteroduplexes Containing 3' Terminal Mismatches**

The three types of M13mp2 DNA substrates were prepared: gapped heteroduplex molecules containing 3'-OH terminal mispairs (experiments shown in Fig. 3 and Table I), completely double-stranded heteroduplex molecules containing an internal single-base mispair (experiments shown in Tables I and II), and gapped molecules containing an opal codon within the single-stranded (template) DNA (experiments shown in Table III). For each of these constructions, single-stranded viral (plus DNA) and double-stranded replicative (minus DNA) RF DNA were obtained as described in Refs. 26 and 29, respectively.
individual reactions with each of these enzymes to determine the amount required to digest the DNA to completion (as determined by analysis in agarose gels). The reaction was then scaled up to digest 500 µg of DNA with both enzymes at once. This reaction produces two fragments, 6826 and 363 base pairs in length, each with one 4-base (5'-TGTG-) extended end and one 3-base (5' -TA- ) single-stranded end. These fragments are sufficiently different in size to permit separation by precipitation with polyethylene glycol by a variation (32) of the original procedure (33, 34) as follows. The digested DNA was phenol extracted, ethanol precipitated, and resuspended in TE buffer as described (29). The solution was adjusted to 0.05% PEG-8000, 0.55 M NaCl, and a final DNA concentration of 0.1 mg/ml. This mixture, in sterile 1.5-ml microcentrifuge tubes, was incubated at 37 °C overnight. The precipitated 6826-base pair fragment was pelleted by centrifugation for 10 min and the supernatant carefully removed. After resuspending the pellet in TE buffer, an aliquot of this sample and the supernatant was analyzed by agarose gel electrophoresis. The resuspended pellet fraction was highly enriched for the 6826-base pair fragment, containing only a trace of the 363-base pair fragment. Likewise, the supernatant was highly enriched for the small fragment and contained only a trace of the large fragment. The 6826-base pair fragment was precipitated with PEG a second time just as before, yielding a preparation lacking detectable small fragment as determined by agarose gel analysis. To remove residual PEG, the large fragment was precipitated with ethanol and then resuspended in TE buffer to a final concentration of 1 mg/ml. The final yield was ~90-95% of the starting material.

Using this 1:1 fragment to viral DNA ratio, typically about one-half eventually pure large fragment after a single precipitation step at 37 °C to produce uncontaminated large fragment, these blunt-ended fragments yielded essentially pure large fragment after a single PEG precipitation at 0 °C. After an incubation with RNase treatment, the DNA was simply phenol extracted, ethanol precipitated, and resuspended. Fragments required to produce gapped duplexes were purified not by gel electrophoresis to remove residual primer or residual single-stranded viral DNA to total biological activity are negligible relative to that of double-stranded circular DNA. This is true for cells made competent by either the CaCl2 (35) or Hanahan (37) procedures.

DNA Polymerase Reactions with M13mp2 Primer Templates

Reactions contained 20 mM Hepes (pH 7.8), 2 mM dithiothreitol, and 10 mM MgCl2. The variables, including reaction volume, time, amount of DNA polymerase, amount of gapped DNA (containing either primer or the opal codon), and the concentration of the deoxyxynucleoside triphosphates and deoxyguanosine monophosphate are given in the legends to the tables and figures. After incubation at 37 °C, reactions were terminated by addition of EDTA to a final concentration of 15 mM. Twenty µl of each reaction was analyzed by agarose gel electrophoresis as described (26). All polymerase reactions reported here generated products that migrated coincident with a replicative form II, fully double-stranded DNA standard.

Preparation of Competent Cells, Transfection, and Plating

The products from DNA polymerase reactions with M13mp2 primer templates were used to transfect competent cells prepared following the E. coli strain CH1 treatment by CaCl2 as described (35). This strain was chosen because of its high transfection efficiency relative to previously used strains. With this strain, one ng of untreated double-stranded M13mp2 DNA yields 10,000-50,000 (infec-
tive center) plaques. Single-stranded DNA is much less infective, yielding 100-400 plaques (not shown) demonstrated that even faint blue plaques can be quan-
titatively scored on confluent plates containing as many as 10,000 colorless plaques.

Comments on Improvements in M13mp2 Assay

Our efforts to probe fidelity mechanisms with natural DNA depend on the ability to construct precisely defined primer templates of high quality and in high yield. DNA must be prepared by methods least likely to generate cryptic damage to the template-primer, a concern for in vitro fidelity assays (23). High yield is important due to the expense of certain restriction endonucleases and since more DNA is required for reversion assays which focus on specific mutational pathways. For these reasons the following modifications of previously published procedures (36, 37) are worth stressing. During the purification of RF DNA by the Birnboim and Doly procedure (36), the 60 °C incubation with LiCl was omitted (to reduce denaturation) and, after RNase treatment, the DNA was simply phenol extracted, ethanol precipitated, and resuspended. Fragments required to produce gapped duplexes were purified not by gel electrophoresis but by selective PEG precipitation. This gives pure DNA in high yield without subsequent DNA to length measurements which could possibly introduce damage. Denaturation of the fragment at 70 °C (rather than 95 °C) was possible due to the low salt and DNA concentrations used. The template was not added until after the denaturation step so that the level of template cytosine deamination was reduced. Finally, the gapped duplex was not purified by gel electrophoresis to remove primer or residual single-stranded DNA, resulting in good yield while minimizing the possibility of damage. Gel purification is not required, since at the primer to template ratios used here (1:1), the contributions of either residual primer or residual single-stranded viral DNA to total biological activity are negligible relative to that of double-stranded circular DNA. This is true for cells made competent by either the CaCl2 (35) or Hanahan (37) procedures.

M13mp2 Mutant Color Discrimination

We have established a scale of blue color intensities for M13mp2 mutants relative to that of wild type M13mp2. Using the plating conditions described above, wild type M13mp2 generates dark blue plaques which, on a scale of 0-4, are assigned a value of 4. Mutants can then be readily described as 0+ (colorless), 1+ (faint blue), 2+ (medium blue), or 3+ (almost wild type). These subjective designations are assigned by directly comparing relative color intensities of two derivatives on the same plate, thus permitting subtle distinctions to be made reproducibly and with confidence.
RESULTS

3’ → 5’ Exonuclease Activity with End-labeled DNA—Chick embryo pol-γ was previously found to be accurate for certain mutational pathways during in vitro DNA synthesis (23, 24). This led us to examine pol-γ for the presence of an exonuclease that could proofread errors. We began by first determining if exonucleolytic activity capable of digesting radiolabeled DNA could be detected. As shown in Fig. 1A, nucleic activity capable of digesting the denatured 3’-end-labeled M13mp2 DNA is detectable in the pol-γ preparation. The products of this reaction were deoxynucleoside 5'-monophosphates, since upon analysis by thin layer chromatography using polyethyleneimine cellulose, all the released radioactivity migrated coincident with the appropriate deoxynucleoside 5'-monophosphate standard (dCMP). These observations indicate the presence of a 3’ → 5’ exonuclease. Further evidence that the exonuclease activity digested in the 3’ → 5’ direction but not in the 5’ → 3’ direction is provided by the observation that the pol-γ preparation digested little of the 5’-end-labeled substrate (Fig. 1B). The 3’ → 5’ exonuclease activity was not observed in the absence of MgCl₂ (data not shown).

Both E. coli pol I and T4 DNA polymerase were used as positive controls for 3’ → 5’ exonuclease activity. All three DNA polymerases were also concomitantly assayed under identical conditions for DNA polymerase activity using activated DNA. From these data the polymerase to exonuclease ratios were calculated (see legend to Fig. 1) to be 35:1 for T4 DNA polymerase, 88:1 for the pol-γ preparation, and 1700:1 for E. coli pol I.

The 3’ → 5’ proofreading exonuclease activities associated with E. coli pol I (38, 39) and pol III (40) and mammalian pol-α (3-5) are all inhibited in the presence of nucleoside monophosphates. We therefore examined the effect of monophosphates on the nuclease activity in the pol-γ preparation. Addition of 10 mM AMP inhibited this nuclease activity by 54% (Fig. 1A, open circle), while the 3’ → 5’ exonuclease activity of pol I was inhibited 80% (Fig. 1A, open triangle).

Terminal Mismatch Excision by Gel Electrophoresis—We next examined the ability of the 3’ → 5’ exonuclease to excise a 3’-terminal base from either a matched or mismatched end. Two substrates were prepared, by hybridizing a 5’-end-labeled 15-base oligonucleotide to two different single-stranded DNA templates, producing either a correct C-G base pair or an incorrect A-G mispair. Excision was performed over a 1-h time course and was monitored by gel electrophoresis in a 20% polyacrylamide-sequencing gel. The results (Fig. 2) confirm the presence of the exonuclease activity which excises...
in the 3'→5' direction, since 5'→3' excision would remove the label and the resulting products would not be detected. These results further demonstrate the absence of a 5'→3' exonuclease, since there was no loss of total radioactivity upon counting all the bands for any lane shown in Fig. 2. The presence of a series of oligonucleotides from 15 to 10 bases in length and differing by one base supports the previous product analysis demonstrating that single mononucleotides are excised. Both matched (Fig. 2, lanes 2–5) and mismatched (lanes 7–10) bases are removed, with a 2- to 6-fold preference for excision of the G from the A-G mispair over excision from the matched C-G base pair. Exonuclease activity is inhibited by addition of monophosphates (Fig. 2, lanes 12–15); the extent of inhibition using 10 mM dGMP ranges from 69% (lane 12 versus 7) to 42% (lane 15 versus 10).

Coedimentation in Glycerol Gradients—To determine if the polymerase and exonuclease activities could be separated, their sedimentation profiles during glycerol gradient centrifugation in 0.5 M KCl were determined. The two activities coedimented (Fig. 3), each as a single peak at 9.4 S (~180,000 daltons), and the ratio of polymerase to exonuclease remains relatively constant across the peak fractions.

Terminal Mismatch Excision in DNA Polymerase Reactions—We next examined the terminal mismatch excision capability under conditions where it is possible to establish a competition between excision and polymerization from the terminal mispair. To do this we constructed gapped, biologically active M13mp2 DNA substrates containing either a T-C or an A-C mispair, where the two bases of the mispair can be distinguished from each other by the intensity of blue color in an M13mp2 plaque produced upon transfection of the DNA. For example, for the T-C mismatch, polymerization to fill the gap without excision of the mismatched 3'-terminal cytosine will produce a double-stranded heteroduplex, which upon transfection will yield both 2+ and 4+ blue plaques: 2+ when the cytosine-containing minus strand is expressed and 4+ when the thymine-containing plus strand is expressed. These two phenotypes are easily distinguished from each other. However, if the mispaired cytosine is removed prior to extension by the polymerase, subsequent correct incorporation of adenine opposite the template thymine will yield a homoduplex molecule having exclusively a 4+ blue plaque phenotype. The proportion of 2+ and 4+ blue plaques obtained upon transfection of the reaction products thus describes the extent of terminal mismatch excision prior to polymerization. The logic is identical for the A-C mismatch, but the blue color intensities are different (see legend to Table I).

Both the T-C and A-C gapped, mismatched molecules were used as template-primers in polymerization reactions catalyzed by several DNA polymerases, including pol-γ. The products of an aliquot of each of these reactions were analyzed by electrophoresis in an agarose gel to monitor gap-filling DNA synthesis. The 363-base gap was filled by pol-γ to the extent that the product migrated coincident 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 each DNA polymerase and reaction condition used in these studies, both for terminal mismatch excision and for fidelity studies.

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 either AMV DNA polymerase or DNA polymerase-β, both of which lack associated 3'→5' exonuclease activity7, both of which lack associated 3'→5' exonuclease activity7,41,42, occurs without substantial excision of the T-C or A-C mismatches since the minus strand phenotype was observed at a frequency of 46–53%. In contrast, Kf removes 92% (T-C) or 88% (A-C) of the terminal mismatch prior to extension to fill the gap. Pol-γ removes the mismatches even more efficiently, excising 98% (T-C) and 99% (A-C) of the mispaired cytosine prior to filling the gap. In all cases, the extent of extension prior to polymerization is calculated (see legend to Table I) by comparison to two uncopied DNA controls (Table I). First, polymerization without excision creates an internal mispair within a complete heteroduplex. Transfection of such a heteroduplex yields a minus strand phenotype of 60%, a value similar to the results obtained by extension with non-exonuclease-containing pol-β and AMV DNA polymerase. Second, the opposite extreme is the background frequency of minus strand phenotype plaques. This value is 0.37%, the lowest value attainable upon complete excision of the terminal cytosine prior to polymerization.

Two variations in reaction conditions were examined for their effects on the efficiency of excision of cytosine from the terminal A-C mispair by the exonuclease. First, the ratio of exonuclease to polymerase activity may be reduced by increasing the concentration of dNTP substrates in the reaction. The expected result is an increase in the 2+ blue (minus strand) phenotype. This is exactly what is observed for both Kf and pol-γ (Fig. 4A). Comparing Kf reactions using 1 μM versus 1000 μM dNTPs, the increase in minus strand phenotype frequency (the next nucleotide effect) is 70-fold. Pol-γ is even more efficient at terminal mismatch excision since at all comparable dNTP concentrations the minus strand expression values are lower than for Kf. This remains so even at the highest dNTP concentrations used (2000 μM). The maximum obtainable next nucleotide effect, comparing values at 10 μM versus 2000 μM dNTPs, is 14-fold. Over the range of dNTP concentrations examined, there is no next nucleotide effect using AMV DNA polymerase, which lacks a 3'→5' exonuclease activity.

In the absence of DNA synthesis, the 3'→5' exonuclease activity in the pol-γ preparation can be inhibited by addition

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7 T. A. Kunkel, unpublished observations.

This value, as well as those shown in Table II, represents an approximate 2-fold increase in minus strand expression over our previous measurements.23 We attribute this to the improved method of construction of gapped DNA described in the text. The same increase over previous results has also been observed for other mispairs as well as for one-base frameshift heteroduplexes.
TABLE I

Terminal mismatch excision by pol-γ

| DNA Used                  | No. plagues scored* | Minus strand expression % |
|--------------------------|---------------------|---------------------------|
| T-C copied by            |                     |                           |
| AMV DNA polymerase       | 1170                | 1270                      | 48 |
| DNA polymerase-β         | 986                 | 1176                      | 46 |
| E. coli pol I (Kf)       | 100                 | 2276                      | 4.2|
| DNA polymerase-γ         | 21                  | 1605                      | 1.3|
| A-C copied by            |                     |                           |
| AMV DNA polymerase       | 222                 | 198                       | 53 |
| DNA polymerase-β         | 347                 | 358                       | 51 |
| E. coli pol I (Kf)       | 118                 | 1786                      | 6.2|
| DNA polymerase-γ         | 6                   | 677                       | 0.9|
| Uncopied                 |                     |                           |
| Complete heteroduplex    | 377                 | 248                       | 60 |
| Gapped heteroduplex      | 17                  | 4559                      | 0.37|

* The actual blue colors were scored 2+ for the minus strand (both mismatches) and either + (T-C) or + (A-C) for the plus strand.

Under some conditions, a substantial number of colorless mutants were observed. These mutants (including here) were analyzed and found to result from aberrant synthesis from the cytosine without excision and thus were scored as minus strand phenotype. The complete details of this analysis are beyond the scope of this study and will be described elsewhere.

The value shown is for the complete heteroduplex containing the internal T-C mispair. A similar value was obtained for the A-C mispair.

The gapped heteroduplex used here is a control containing a T-C mispair followed by a template expression for no excision. Thus, for the T-C mispair, Kf has shown that at 1000 μM dNTPs, Kf excision of cytidine from the A-C mispair is inhibited about 90%, while for pol-γ excision was only inhibited 50% even at 2000 μM dNTPs and 20 mM dGMP.

FIG. 4. Terminal A-C mismatch excision by pol-γ with increasing dNTP or dGMP concentrations. Reactions were performed as described under "Experimental Procedures." For A, the indicated concentrations of dNTP (all four in equimolar amounts) were used with either pol-γ (3 units, 37 °C for 30 min) (■); Kf (0.5 unit (manufacturer’s definition), 37 °C for 20 min (1 μM), 15 min (10 μM), 10 min (100 μM) or 5 min (1000 μM)) (▲); or AMV DNA polymerase (30 units (manufacturer’s definition), 37 °C for 60 min) (○). Open symbols represent the results from reactions using the indicated dNTP concentrations plus 20 mM dGMP. For B, reactions contained the indicated concentrations of dGMP and pol-γ (100 μM dNTPs, 37 °C for 30 min) (■); Kf (10 μM dNTPs, 37 °C for 15 min) (▲); and AMV DNA polymerase (10 μM dNTPs, 37 °C for 60 min) (○).
revertants upon transfection of the DNA polymerase reaction products, and the proportion of blue to total plaques (the reversion frequency) reflects the error frequency of DNA synthesis.

Table II lists the consequences of single base substitution errors at this codon, including the mispairs, codons, amino acids, and relative blue color intensities. Heteroduplex expression experiments similar to those described previously (23) demonstrate the efficiency of minus strand expression for each mutational pathway and indicate no strong bias against any particular error. Eight errors can be observed; the only exception is the transition mispair (T-G) at the middle position, which generates an ochre codon which is colorless. The assay is quite sensitive since the background reversion frequency of uncopied DNA is low (Table III).

Two experiments were performed to measure the fidelity of pol-\(\gamma\) in this base substitution assay. In the first experiment each DNA polymerase reaction was performed using an equal concentration of all four dNTP substrates. As expected from previous studies (26, 46), chick embryo pol-\(\beta\) is inaccurate, demonstrating the capability of the assay to detect in vitro polymerization errors. In contrast, Kf, used here as a positive control to detect proofreading, is substantially more accurate using a low concentration of dNTP substrates. The accuracy of Kf diminishes about 20-fold in reactions containing 1000 \(\mu\) M dNTPs and 20 mM GMP, conditions that reduce the contribution of proofreading to fidelity. The magnitude of this

### Table II

Opal codon reversion pathways and minus strand expression values

| Minus strand expression values were obtained by transfection of E. coli cells (made competent by the CaCl\(_2\) procedure) with complete heteroduplexes. The values shown are the average of either four or five determinations, in each case scoring 500 or more total plaques. The new procedure for preparing double-stranded molecules (either gapped or complete) does not remove residual single-stranded viral DNA remaining from hybridizations performed at a 1:1 primer to template ratio. In order to examine the influence of this residual single-stranded DNA on mutation frequencies and minus strand expression values, we systematically varied the primer to template ratio. Measurements were performed for 5 different ratios, both for the T-C and the A-C mispairs, and by transfection into cells made competent by both the CaCl\(_2\) and Hanahan procedures. In only the extreme case of 10-fold template excess did the minus strand expression value diminish significantly. This is consistent with our reproducible observation of a much higher transfection efficiency for double-stranded M13 DNA compared to single-stranded DNA. In competent (CaCl\(_2\)) cells, single-stranded viral M13mp2 DNA is reproducibly 30-fold less infective than is double-stranded DNA, and in Hanahan cells the difference is even greater (~100-fold). Because this double-stranded DNA: single-stranded DNA bias is greater than Hanahan cells, the results for minus-strand expression for the heteroduplexes shown in the table (shown for transfection of CaCl\(_2\) cells) are all slightly higher when transfecting Hanahan cells (ranging from 67% to 86%), but show no bias for any mispair.

### Table III

Base substitution fidelity of pol-\(\gamma\)

Reactions were performed as described under "Experimental Procedures," using 300 ng of gapped M13mp2 A89 (opal codon-containing) DNA per 50 \(\mu\)l of reaction. For experiment 1, the dNTP concentrations were equimolar and as indicated. The amounts of enzyme used per 50 \(\mu\)l of reaction and the incubation times were: chick pol-\(\beta\), 0.8 unit, 37 °C, 60 min; Kf, 0.5 unit, 37 °C, 20 min (1 \(\mu\)M) or 5 min (1000 \(\mu\)M plus 20 mM GMP); and chick pol-\(\gamma\), 3 units, 37 °C for 30 min (both conditions). 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. Only 4+ (wild type) blue mutants were scored, and all 4+ mutants were plaque purified and plated with wild type M13mp2 to confirm the phenotype. Sequence analysis of 10 of these revertants confirmed that all 10 had the predicted wild type TGG codon resulting from the misincorporation of C opposite A at position 89. Kf reactions used 0.5 unit of enzyme at 37 °C for 20 min. Pol-\(\gamma\) reactions were as for experiment 1. The average fidelity of pol-\(\gamma\) per base synthesized at the opal codon can be calculated (using as an example the reversion frequency for synthesis at 20 \(\mu\)M dNTPs from experiment 1, i.e. 8.3 \(\times\) 10^{-5}) by subtracting the background reversion frequency (1.3 \(\times\) 10^{-5}), divided by 0.6 (the approximate probability of expressing an error, see Table II) and then dividing by 3, since errors can be monitored at all three bases of the opal codon. At this codon with equimolar substrate concentrations, the pol-\(\gamma\) error frequency is one error for each 280,000 bases polymerized.

![Table III](https://example.com/table3.png)

| DNA polymerase | dNTP dGMP | Plaques Scored Total | Blue | Rev. Freq. |
|---------------|-----------|----------------------|------|-----------|
|               | \(\mu\)M | nM                   |      | x10^{-6}  |
| None          | —        | 3,000,000            | 4    | 1.3       |

**Experiment 1: equal dNTPs**

- **Chick pol-\(\beta\)**
  - 500 \(\mu\)l of 200,000 (107, 1,500)
  - 1,000 \(\mu\)l of 140,000 (33, 240)
- **Chick pol-\(\gamma\)**
  - 20 \(\mu\)l of 600,000 (5, 8.3)
  - 2,000 \(\mu\)l of 860,000 (30, 35)

**Experiment 2: 50 \(\times\) dCTP**

- **E. coli pol 1 (Kf)**
  - 100 \(\mu\)l of 190,000 (3, 16)
  - 500 \(\mu\)l of 210,000 (45, 230)
  - 100 \(\mu\)l of 130,000 (98, 750)
- **Chick pol-\(\gamma\)**
  - 500 \(\mu\)l of 590,000 (2, 3.4)
  - 2,000 \(\mu\)l of 440,000 (2, 4.5)
  - 2,000 \(\mu\)l of 550,000 (25, 46)

The proofreading effect is in fact similar to previous results with pol I in the \(\alpha\)X174am3 assay (39). Chick pol-\(\gamma\) is also highly accurate; at a dNTP concentration of 20 \(\mu\)M the reversion frequency of DNA copied by pol-\(\gamma\) is only slightly above the background. Using reaction conditions (2000 \(\mu\)M dNTPs, 20 mM GMP) that were demonstrated to partially reduce terminal mismatch excision by the 3' \(\rightarrow\) 5' exonuclease in the pol-\(\gamma\) preparation (Fig. 3A), the reversion frequency increases more than 4-fold, a result that is consistent with diminished proofreading. However, even under this extreme reaction condition, pol-\(\gamma\) remains more accurate than Kf.

The second experiment focuses exclusively on misincorporation of C opposite A in the TGA codon by utilizing a pool imbalance in which dCTP is in 50-fold excess over the other three dNTP substrates. This is intended to specifically increase the error frequency for A-C mispairs by two mechanisms. First, incorrect dCTP is present in 50-fold excess over correct dTTP, increasing the misincorporation frequency. Second, dCTP is the next correct nucleotide to be inserted after the error, opposite the template G of the TGA codon. Its presence in high concentration is 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, 4+ blue codon that is
easily scored and differentiated from the lighter colors generated by the other revertants (Table II). The reversion frequencies shown for experiment 2 therefore reflect only the 4+ phenotype. Kf is again used as a positive control for detection of proofreading activity. At a dCTP concentration of 100 \( \mu \text{M} \) (and 2 \( \mu \text{M} \) for the other three dNTPs), Kf reactions produce a 4+ reversion frequency of 16 \( \times 10^{-4} \). Either a 5-fold increase in next nucleotide (while keeping the 50 pool imbalance constant) or addition of dGMP to 20 mM substantially increases the reversion frequency, consistent with a reduced contribution of proofreading to fidelity. Again pol-\( \gamma \) is highly accurate, since at 500 \( \mu \text{M} \) dCTP the reversion frequency is only 2.1 \( \times 10^{-6} \). Even a 10-fold increase in dNTPs produced little effect on the reversion frequency. The absence of mutants under these conditions is not due to an inability to detect errors, since Kf generated mutants at high frequency with the same reagents under comparable conditions. Despite the high accuracy of pol-\( \gamma \), both a next nucleotide and a monophosphate effect are readily apparent. At 20 mM dGMP, a 10-fold increase in substrate concentration (from 500 to 5000 \( \mu \text{M} \)) produced a 5.7-fold increase in reversion frequency. At a constant 5000 \( \mu \text{M} \) dCTP concentration, addition of 20 mM dGMP increased the reversion frequency 14-fold.

**DISCUSSION**

We initiated this study to determine if the high fidelity of chick embryo DNA polymerase-\( \gamma \) (22–24) could result in part from 3' → 5' exonuclease proofreading. The results of this study show that three independently purified chick pol-\( \gamma \) preparations indeed contain a 3' → 5' exonuclease activity which releases nucleoside 5'-monophosphates. Each of the observations presented here is consistent with a proofreading function for this exonuclease since 1) hydrolysis proceeds in the 3' → 5' direction, 2) the products are nucleoside 5'-monophosphates, 3) the exonuclease exhibits a preference for mismatched rather than matched bases, 4) pol-\( \gamma \) synthesis is highly accurate and, most importantly, 5) both exonuclease activity and the fidelity of pol-\( \gamma \)-catalyzed DNA synthesis decrease in reactions containing high dNTP concentrations or dGMP. These conditions are known to inhibit proofreading by prokaryotic proofreading exonucleases, and their similar effect on pol-\( \gamma \) fidelity suggests a coordinated action of the 3' → 5' exonuclease with chick embryo DNA polymerase-\( \gamma \) to permit proofreading during DNA synthesis. Assuming that this interpretation is correct, chick embryo pol-\( \gamma \) represents the second animal cell DNA polymerase preparation, along with calf thymus pol-\( \delta \) II (5), to be shown to proofread errors by direct measurements of fidelity during DNA synthesis on biologically active templates.

The data in Fig. 1 suggest that the exonuclease to polymerase ratio is quite high, being intermediate between that of *E. coli* pol I and T4 DNA polymerase. The terminal mismatch excision studies with M13mp2 DNA also suggest this, since more excision prior to polymerization is observed with the exonuclease in the pol-\( \gamma \) preparation than for Kf at all dNTP or dGMP concentrations examined. Similarly, under all comparable conditions, pol-\( \gamma \) is more accurate for base substitution errors during DNA synthesis at the opal codon than is Kf. The difference can be as much as 100-fold, depending on the conditions that are compared (e.g. Table III, experiment 2, at 500 \( \mu \text{M} \) dCTP: after subtracting background) compare 229 \( \times 10^{-4} \) for Kf versus 2.1 \( \times 10^{-6} \) for pol-\( \gamma \)).

The data in Table III can also be used to calculate the average base substitution fidelity of pol-\( \gamma \) at the three template positions monitored by this reversion assay. At the lowest dNTP concentration examined (experiment 1, 20 \( \mu \text{M} \) dNTPs), a condition which permits the exonuclease to be highly active, the pol-\( \gamma \) error frequency at the opal codon is one error for each 260,000 bases polymerized (see legend to Table III for calculation). This represents the average frequency for all eight possible mispairs that can be detected at this codon (Table II). Considering only the A-C mispair at position 89 (experiment 2, Table III), pol-\( \gamma \) is even more accurate, with an error frequency that is at least 10-fold lower than the overall average. Thus, the chick embryo pol-\( \gamma \) used here exhibits accuracy which is similar to or even slightly higher than that of calf thymus pol-\( \delta \) II (5). The next nucleotide and monophosphate effects in the opal codon reversion assay further suggest that proofreading contributes at least 5- to 10-fold to the overall accuracy of pol-\( \gamma \). This is likely an underestimate since, in the terminal mismatch excision experiments (Fig. 4A), the exonuclease activity remains highly active for excision of A-C and T-C mispairs even under extreme reaction conditions.

It is interesting to compare these estimates for the base-substitution fidelity of pol-\( \gamma \), which are based on a reversion assay that detects eight mispairs at three template positions, to our previous results with pol-\( \gamma \) using a forward mutational assay, which detects over 200 mispairs at over 100 template positions. In the forward assay, using 500 \( \mu \text{M} \) dNTPs, the overall average base substitution fidelity of pol-\( \gamma \) was estimated to be only \( \approx 10^{-3} \), a value considerably less accurate than the values obtained here. The primary reason for these seemingly discordant observations is that pol-\( \gamma \) exhibits substantial differences in accuracy depending on the error being considered. In the previous studies, high fidelity was indeed observed for 9 of 12 possible mispairs (23) and for frameshift errors (24); it was this observation that led to the present study. However, pol-\( \gamma \) produced three specific mispairs, (C-A, G-A, and G-G), at a high frequency. These errors obviously escaped proofreading even under conditions (500 \( \mu \text{M} \) dNTPs) that do not fully eliminate exonuclease activity with other mispairs (our present results). Furthermore, the error frequency varied over more than 200-fold depending on the mispair (23) and over more than 16-fold for the same mispair, depending on its position (22). These observations, which are consistent with prokaryotic proofreading results (39, 40, 44, 45, 47, 48), demonstrate that the error frequency as well as the extent of proofreading depends on the composition and position of the mispair. Variations in reaction conditions as well as the mutational target DNA sequence should permit a further analysis of DNA context effects and of the parameters that determine pol-\( \gamma \) insertion and proofreading discrimination, not only for base substitution errors but for frameshift and deletion errors as well.

The presence of an exonuclease activity in preparations of highly purified chick embryo pol-\( \gamma \) leads to three questions. First, is the exonuclease activity physically associated with the DNA polymerization activity? Three observations suggest that this is likely. Pol-\( \gamma \) has been purified to very high specific activity through six purification steps based on different separation principles (solubility, size, charge, and affinity), yet the exonuclease is still present with a ratio of exonuclease to polymerase comparable to that observed for prokaryotic exonuclease-containing DNA polymerases. Also the exonuclease and polymerase activities cosediment in glycerol gradients containing high salt which should minimize nonspecific interactions, and the polymerase/exonuclease ratio remains relatively constant across the peak. Finally, the proofreading results imply a functional association of these activities, which act in a coordinated manner during DNA synthesis at the template-priming site to achieve high fidelity.
Pol-γ Proofreading

Are the two activities in the same polypeptide or do they reside on separate subunits? In prokaryotic systems, precedent exists for both possibilities, since E. coli pol I and T- phage DNA polymerases contain both activities within a single polypeptide (see Ref. 43 for review) while the two activities reside on different subunits of E. coli pol III (49). Denaturing polyacrylamide gel electrophoresis of the pol-γ preparations used here demonstrates the presence of a major polypeptide species of 47,000 daltons (86% of the total protein) and a second polypeptide of 135,000 daltons (12% of the total protein), both of which correlate with DNA polymerase activity (20). Based on native molecular weight, stoichiometry, and specific activity, Yamaguchi et al. (20) suggest that the native chick DNA polymerase-γ may be a tetramer of four identical 47,000-dalton subunits. However, we do not exclude the possibility that the 135,000-dalton polypeptide has DNA polymerase activity. The exonuclease activity could reside in either of these two polypeptides or even in a minor species of polypeptide (although specific activity considerations make this last possibility seem less likely). Clearly, further attempts to purify and/or dissociate the exonuclease and polymerase activities are required.

Thirdly, are the observations presented here peculiar to preparations of chick embryo pol-γ or do comparably purified preparations of pol-γ isolated from other systems contain 3′ → 5′ exonucleolytic activity? First purified from rat liver tissue as the mitochondrial DNA polymerase in 1968 (11, 12), DNA polymerase-γ has since been purified and characterized from a variety of sources (for review see Ref. 2). In most cases these preparations were not examined for nuclease activity. Three early reports, two for rat liver (11, 12), and one for mouse myeloma cells (14), describe partially purified pol-γ preparations devoid of detectable nuclease activity. These negative results should be interpreted with caution due to the sensitivity limitations imposed by the substrates and reaction conditions employed and the methods of detection available. Partially purified DNA polymerase-γ from human placenta has been reported to contain detectable 3′ → 5′ exonuclease activity that could be removed by glycerol gradient sedimentation (17). This exonuclease neither removed terminal-matched bases from synthetic polynucleotides under polymerization conditions, nor affected the fidelity of DNA synthesis with such templates. This latter observation on fidelity may not be particularly instructive, however, since with E. coli pol I, proofreading contributes little to the fidelity of synthesis on synthetic substrates (50).

Partially purified pol-γ fidelity studies with natural DNA have been reported. HeLa cell pol-γ was shown to be inaccurate for base substitution errors (A-C, A-A, and/or A-G) at position 587 in OX174ram3 DNA (46), implying that either exonuclease proofreading was not present, or, if present, was not particularly active at this site with these mispairs. In our initial fidelity studies of pol-γ (22), a crude preparation of fetal calf liver pol-γ was even more accurate in the M13mp2 forward mutagenesis assay than was the same preparation of chick embryo pol-γ used here (22–24). This accuracy could reflect either high insertion fidelity or proofreading activity. As expected for an enzyme that has been difficult to purify due to low abundance, microheterogeneity and instability, no clear understanding emerges from the limited data available to date. Further work will be required to relate these chick embryo pol-γ results to results obtained with pol-γ isolated from additional sources.

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