Proofreading by the ϵ subunit of *Escherichia coli* DNA polymerase III increases the fidelity of calf thymus DNA polymerase α

(nucleotide misinsertion/lack of mispair extension/3'→5' exonuclease/mispair hydrolysis/proofreading mechanism)

FRED W. PERRINO AND LAWRENCE A. LOEB*

The Joseph Gottstein Memorial Cancer Research Laboratory, Department of Pathology, University of Washington, Seattle, WA 98195

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ABSTRACT Addition of the 3'→5' proofreading exonuclease, ϵ subunit of *Escherichia coli* DNA polymerase III, to DNA polymerase α from calf thymus has been studied. Alone, calf thymus DNA polymerase α terminates *in vitro* DNA synthesis upon insertion of noncomplementary nucleotides. Upon addition of the ϵ subunit, DNA polymerase α elongates the newly synthesized DNA as a result of hydrolysis of the 3'-terminal mispair. The fidelity of DNA polymerase α *in vitro* is increased 7-fold by addition of the exonuclease. The functional interaction between DNA polymerase α and the ϵ subunit is independent of any detectable physical association. This suggests that a mechanism for proofreading could exist in mammalian cells involving sequential catalysis by DNA polymerase α excision of errors by a separate 3'→5' exonuclease, and further elongation onto correctly base-paired 3' termini by DNA polymerase α .

DNA replication is an exceptionally accurate process. Measurements of spontaneous mutation rates suggest that the average frequency of errors in prokaryotes and eukaryotes is $\approx 10^{-8}$ to 10^{-11} (1). In bacteria, a major contribution to this high accuracy is a 3'→5' exonuclease activity that is either an integral part of the DNA polymerase or an associated polypeptide that is tightly complexed with the DNA polymerase (2–6). In eukaryotes, proofreading by a 3'→5' exonucleolytic activity during DNA replication has been difficult to establish. The DNA polymerase α is thought to be the primary enzyme responsible for DNA replication in eukaryotic cells (7) and, in most cases, when purified is devoid of exonuclease activity (8–12). A cryptic 3'→5' exonuclease is associated with *Drosophila* DNA polymerase α -primase (13, 14), but, so far, this association has been restricted to *Drosophila*. Even though a multiprotein form of DNA polymerase α with exonuclease activity has been purified from several tissues, the establishment of this activity as a proofreading component of the replicative enzyme is lacking (15–17). The DNA polymerase δ has a tightly associated 3'→5' exonuclease that enhances the fidelity of DNA synthesis by this enzyme (18–21). Thus, even though DNA polymerase α is characterized by lack of exonucleolytic activity, a separate 3'→5' exonuclease could act in concert with DNA polymerase α .

We purified calf thymus DNA polymerase α -primase (referred to as DNA polymerase α) and the ϵ subunit of *Escherichia coli* DNA polymerase III and used these two enzymes *in vitro* to determine whether proofreading might play a role in eukaryotic DNA synthesis. The antibody-purified DNA polymerase α complex from calf thymus consists of four polypeptides: polymerase activity is associated with the high molecular mass polypeptide (140–180

kDa), primase activity is associated with the 50- and 60-kDa subunits, and the function of the 70-kDa subunit is unknown (8–10). The four subunit complex is devoid of exonuclease activity. The ϵ subunit of DNA polymerase III is a single polypeptide of 25 kDa that preferentially hydrolyzes single-stranded DNA in the 3'→5' direction (22). This exonuclease has been identified as the 3'→5' proofreading component of the seven-subunit *E. coli* DNA polymerase III holoenzyme, considered to be responsible for replication of the *E. coli* chromosome (23). Molecular cloning of the *dnaQ* gene, which codes for the ϵ polypeptide, and overexpression of this polypeptide have facilitated the rapid purification of large quantities of the exonuclease free from the DNA polymerase III complex (22).

We determined whether the ϵ subunit of *E. coli* DNA polymerase III could enhance the accuracy of DNA polymerase α and thus substitute for a putative proofreading component in animal cells. Nucleotide misinsertions by the antibody-purified DNA polymerase α from calf thymus resulted in 3'-terminal mispairs that impeded further DNA synthesis. The ϵ subunit hydrolyzed these mispairs and permitted elongation by the DNA polymerase α . The interaction of these two enzymes shows that a separate 3'→5' proofreading exonuclease could functionally interact with DNA polymerase α to achieve highly accurate DNA synthesis.

MATERIALS AND METHODS

Primed DNA Templates. Template ϕ X174 *am3* DNA was primed at a 2:1 molar ratio (primer:template) with a 15-base oligonucleotide, the 3' terminus of which was 3 nucleotides from position 587 (*am3*). The 30-base oligonucleotide, identical to ϕ X174 *am3* position 581–610, was primed at a 1:1 molar ratio with a 16-base oligonucleotide to produce a 3'-terminal adenine:adenine mispair at position 587. Primers were labeled with 32 P at the 5' position and hybridized as described (24, 25).

Enzymes and Reactions. Calf thymus DNA polymerase α -primase and the ϵ subunit of *E. coli* DNA polymerase III were purified as described (26, 27). Reactions contained 20 mM Tris-HCl, pH 7.5, 2 mM dithiothreitol, 10 mM MgCl₂, bovine serum albumin at 0.1 mg/ml, primed DNA at 2.2 nM of 3' termini, and dNTPs as indicated. When present, calf thymus DNA polymerase α was at 0.12 unit/ μ l; 1 unit catalyzes the incorporation of 1 pmol of [α - 32 P]dTMP into 120 pmol (as nucleotide) of 15-mer-primed ϕ X174 *am3* DNA in 30 min at 30°C with all four dNTPs at 50 μ M. The ϵ subunit was at the concentrations indicated in the figure legends. Reactions were eluted through 0.5-ml Sephadex G-100 columns to remove unincorporated deoxynucleoside triphosphates (28), fractionated on 12% polyacrylamide sequencing gels, and

visualized by autoradiography. For quantitation, bands were cut and counted in scintillation fluid.

ϕ X174 *am3* Reversion Analysis. Reaction products (100 ng) were transfected into *E. coli* KT-1 spheroplasts, and reversion frequencies were determined by plating on *E. coli* HF4714 (Sup⁺) and HF4704 (Sup⁻) to determine total and revertant progeny phage, respectively (29, 30). The percent of nascent strand expressed was quantitated using ϕ X174 *am3* template to which an oligonucleotide primer that spanned the *am3* site and contained the wild-type sequence was hybridized. This oligonucleotide was extended in copying reactions identical to those used for reversion-frequency determinations. The percent of nascent strand expressed was calculated as the wild-type titer divided by the *am3* titer (29, 30).

RESULTS AND DISCUSSION

We first studied the interactions between calf thymus DNA polymerase α and the ϵ subunit of DNA polymerase III by analyzing the products of a DNA synthesis reaction. A time course of DNA synthesis by calf thymus DNA polymerase α with a 15-base oligonucleotide primer (15-mer) hybridized to position 604–590 on ϕ X174 *am3* DNA and only three deoxynucleoside triphosphate substrates (no dTTP) is shown in Fig. 1. Even before incubation at 30°C was initiated, 70% of the 15-mer was elongated by one or two nucleotide additions, resulting in 16-mer and 17-mer oligonucleotide bands at positions 589 and 588, respectively (≈ 20 sec at 0°C; lane 2). After 5 min at 30°C, four bands in addition to the 15-mer primer were detected (lane 3). The 17-mer and 24-mer bands at positions 588 and 581 correspond to extended primers with 3' termini that are located one nucleotide before template adenines. The 18-mer and 25-mer bands at positions 587 and 580 correspond to extended primers that contain 3'-terminal mispairs opposite template adenines. By 30 min, >90% of the extended primers were detected as 18-mer and 25-mer oligonucleotides with 3'-terminal mispairs at positions 587 and 580. Limited extension from the mispair at position 580 was detected as the accumulation of 32-mer and 37-mer at positions 573 and 568, respectively (lanes 4 and 5). Thus, DNA polymerase α terminates synthesis by inserting a single noncomplementary nucleotide and only infrequently adds further nucleotides onto the mismatched primer terminus.

When the ϵ subunit of DNA polymerase III and dTTP were added to the reaction, DNA polymerase α catalyzed the incorporation of >100 nucleotides onto the primers; presumably, the 3'-terminal mispairs that acted to impede further DNA synthesis were excised by the *E. coli* exonuclease (lanes 6–8). This result is indicated by the loss of the 18-mer and 25-mer products and generation of bands at positions that correspond to >100 nucleotides. These data suggest that terminal mismatches inserted by calf thymus DNA polymerase α are excised by the proofreading *E. coli* exonuclease before further elongation by DNA polymerase α .

Rapid extension of oligonucleotides with 3'-terminal mispairs produced by DNA polymerase α depended on the addition of both ϵ subunit and dTTP. The addition of dTTP provided the full complement of all four dNTPs, but in the absence of the ϵ subunit >90% of the 18-mer, 25-mer, 32-mer, and 37-mer oligonucleotides remained unextended (lanes 9–11). When only the ϵ subunit was added (lanes 12–14), exonucleolytic removal of the 3'-terminal mispairs resulted in the generation of bands corresponding to 17-mer, 24-mer, 31-mer, and 36-mer oligonucleotides. Within 5 min after addition of the ϵ subunit, approximately equal amounts of oligonucleotides were detected at the 17/18-mer, 24/25-mer, 31/32-mer, and 35/36-mer and maintained over the 30-min reaction time. This suggests an "idling" reaction, such that upon hydrolysis of the 3'-terminal mispairs by the exonu-

FIG. 1. The 3'-terminal mispairs generated by calf thymus DNA polymerase α are hydrolyzed by the ϵ subunit of *E. coli* DNA polymerase III. A single reaction (400 μ l) was prepared, as described, containing 1 mM dATP, dCTP, dGTP, primed ϕ X174 *am3* DNA, and calf thymus DNA polymerase α . Samples (20 μ l) were removed before DNA polymerase α addition (lane 1), just after DNA polymerase α addition (lane 2), and at the indicated times after incubation at 30°C (lanes 3–5). At 30 min the reaction was aliquoted for three separate time course reactions ($3 \times 80 \mu$ l): (i) (lanes 6–8) ϵ subunit at 0.6 ng/ μ l and 0.1 mM dTTP, (ii) (lanes 9–11) only 0.1 mM dTTP, and (iii) (lanes 12–14) only ϵ subunit at 0.6 ng/ μ l. Samples were removed from each reaction at the indicated times. Lane 15 was a sample of the initial reaction after 60 min. Positions of the primer, extended oligonucleotides, and template adenines are indicated.

lease, DNA polymerase α , in the absence of correct nucleotide, again inserts noncomplementary nucleotides. Interestingly, the total amount of both 17-mer and 18-mer oligonucleotides decreased over the 30-min reaction time (lanes 12–14). This is consistent with a reaction in which DNA polymerase α and the ϵ subunit compete for the 3' terminus, such that extension of the mispair by the DNA polymerase is more rapid than removal by the exonuclease, resulting in a net movement in the forward direction. These results demonstrate that upon mispair formation by calf thymus DNA polymerase α rapid elongation rates can only be restored when (i) the 3'-terminal mispair is excised and (ii) when correct nucleotide substrate is provided.

The calf thymus DNA polymerase α and the ϵ subunit exonuclease could physically and cooperatively interact, or they could act independently and sequentially. To determine whether DNA polymerase α might possess a domain similar

to that of *E. coli* DNA polymerase III α subunit, which associates very tightly with the ϵ subunit (3), we studied a potential physical association between calf thymus DNA polymerase α and the ϵ subunit. The DNA polymerase α activity and the ϵ subunit exonuclease activity sedimented independently through glycerol gradients, the thermolability of each enzyme was unaffected by the presence of the other, and the 3'→5' exonuclease activity of the ϵ subunit was not influenced by the presence of DNA polymerase α (data not shown). These combined results suggest that calf thymus DNA polymerase α and the ϵ subunit do not physically associate into a stable complex. We cannot dismiss a possible weak-transient interaction that may be detectable by protein-affinity chromatography (27). The most logical interpretation of the interaction between DNA polymerase α and ϵ subunit is that nucleotide misinsertion results in DNA polymerase α dissociating from the template-primer, leaving the 3'-terminal mispair accessible to the ϵ subunit. Upon excision of the mispair the exonuclease dissociates allowing the DNA polymerase α to reassociate with the 3' terminus and resume DNA synthesis. Kinetic studies indicate that DNA polymerase α fails to bind a mismatched terminus (31), but it remains to be determined whether nucleotide misinsertion results in dissociation of DNA polymerase α . Therefore, we must consider the possibility that DNA polymerase α remains associated and undergoes a conformational change to position the misincorporated nucleotide for hydrolysis by the independent 3'→5' exonuclease. CocrySTALLIZATION of Klenow fragment of *E. coli* DNA polymerase I with DNA suggests that the primer terminus is moved a distance of 8 base pairs to position the 3' terminus at the exonuclease active site, resulting in melting of the 3 or 4 terminal base pairs (32).

The sequential and independent catalytic steps by the ϵ subunit and calf thymus DNA polymerase α were shown using a preformed 3'-terminal mispair (Fig. 2). A time course

FIG. 2. Hydrolysis of the 3'-terminal mispair by the ϵ subunit is required for extension by calf thymus DNA polymerase α . A single reaction (100 μ l) was prepared, as described, containing the oligonucleotide template (A) and the ϵ subunit at 0.1 ng/ μ l. Two samples (5 μ l) were removed before ϵ subunit addition (t_0) and at the indicated times after incubation at 30°C (B). One sample showed the products of ϵ subunit hydrolysis [(-)Pol α]. The second sample was used in a DNA synthesis reaction (90 sec, 30°C) by addition of calf thymus DNA polymerase α and 1 mM of all four dNTPs [(+)Pol α]. Positions of the 16-mer primer and product oligonucleotides are indicated.

for the hydrolysis of a 3'-terminal mispair by the ϵ subunit was established using a 16-mer oligonucleotide primer that, after hybridization to a 30-mer oligonucleotide template, formed a 3'-terminal adenine:adenine mispair (Fig. 2A). Hydrolysis of the mispaired 3' terminus was evident from the formation of 15-mer and 14-mer oligonucleotide bands [Fig. 2B, (-)Pol α]. The product oligonucleotides containing correctly base-paired 3' termini were subsequently extended by calf thymus DNA polymerase α [Fig. 2B, (+)Pol α]. Elongation from the 15-mer and 14-mer oligonucleotides by DNA polymerase α resulted in decreased intensities of the 15-mer and 14-mer bands coordinate with the increased intensity of the 22-mer product band. Similar results were obtained with an adenine:guanine mispaired template (data not shown). To further demonstrate the independent nature of the hydrolysis and synthesis steps, the ϵ subunit was heat-inactivated (100°C, 3 min) after hydrolysis of the 3' mispair. Again, synthesis by DNA polymerase α from the newly formed, correctly base-paired 3' termini was proportional to hydrolysis of the 3'-terminal mispair (data not shown). The correlation between removal of the mispair and elongation from the correctly base-paired 3' termini demonstrates that hydrolysis of the mispair is required to provide a 3' terminus that is efficiently extended by DNA polymerase α .

The contribution of exonucleolytic proofreading to the fidelity of *in vitro* DNA synthesis by calf thymus DNA polymerase α was determined by copying ϕ X174 *am3* DNA in the presence of both DNA polymerase α and the ϵ subunit (Fig. 3). The lack of mispair extension by calf thymus DNA polymerase α was detected as the appearance of bands corresponding to positions opposite template adenines in reactions with reduced concentrations of dTTP (Fig. 3A, ϵ subunit = 0 ng/ μ l). The subsequent excision of the 3'-terminal mispairs by the ϵ subunit was detected as decreased band intensities at these sites as a function of the amount of exonuclease added to the reaction (Fig. 3A, ϵ subunit = 0.1–2 ng/ μ l). The products of these reactions were then used in ϕ X174 *am3* reversion assays to determine the effect of the ϵ subunit on the fidelity of calf thymus DNA polymerase α . Errors were detected at the *am3* site when DNA polymerase α misinserted and then extended the mispair before the exonuclease could remove it. The high reversion frequency exhibited by DNA polymerase α was due to the nucleotide pool bias in the reaction mixture. The ratio of noncomplementary nucleotides to complementary nucleotide was 1000:1. The reversion frequencies of the copied ϕ X174 *am3* DNA decreased linearly upon addition of increasing amounts of the ϵ subunit, and saturation was achieved at a concentration of 0.5 ng/ μ l of the exonuclease (Fig. 3B); a 7-fold decrease in error frequency was detected with no apparent decrease in the expression of the nascent strands. At concentrations of ϵ subunit >4 ng/ μ l the oligonucleotide primer was hydrolyzed and no longer hybridized to the ϕ X174 *am3* template; as a result the expression of the nascent strand was dramatically reduced (data not shown). The 7-fold effect on fidelity is likely to be a minimum estimate because high nucleotide concentrations, which favor the forward polymerization reaction and reduce the effect of proofreading, are required to detect errors by calf thymus DNA polymerase α . Thus, we have demonstrated that exonucleolytic proofreading can contribute to high-fidelity DNA synthesis by DNA polymerase α .

The functional interaction between the ϵ subunit proofreading exonuclease and the mammalian DNA polymerase α is valuable in two respects. (i) A mechanism can now be proposed that provides a role for exonucleolytic proofreading for DNA polymerase α . When an infrequent nucleotide misinsertion event does occur, the DNA polymerase α dissociates from the growing strand, a separate 3'→5' exonuclease removes the misinserted base, and DNA synthesis

FIG. 3. Mutagenesis by calf thymus DNA polymerase α is decreased by ϵ subunit of *E. coli* DNA polymerase III. Reactions (50 μ l, 30 min, 30°C) were prepared, as described, containing 1 mM dATP, 1 mM dCTP, 1 mM dGTP, 1 μ M dTTP, primed ϕ X174 *am3* DNA, calf thymus DNA polymerase α , and the indicated concentration of ϵ subunit. Samples (25 μ l) were fractionated on 12% polyacrylamide gels and visualized by autoradiography (A). The positions of the primer and template adenines are indicated. The remaining portions of the reactions (25 μ l) were transfected into spheroplasts for ϕ X174 *am3* reversion analysis as described. (B) Decrease in reversion frequency (●) as a function of added ϵ subunit is shown. A background reversion frequency of 5.4×10^{-6} is not subtracted. The percent of nascent strand expressed (○) was quantitated as described in text.

resumes. Thus, proofreading could occur by an exonuclease that is neither an integral part of the DNA polymerase α nor a tightly associated polypeptide. The initial mechanism for proofreading proposed by Brutlag and Kornberg (2) for prokaryotic DNA polymerases can now be extended to the eukaryotic DNA polymerase α . (ii) The resumption of DNA synthesis upon mispair hydrolysis provides a sensitive biochemical assay to test mammalian 3'→5' exonucleases for their ability to proofread for DNA polymerase α . A DNA primer with a 3'-terminal guanine opposite a template adenine is elongated inefficiently by calf thymus DNA polymerase α unless the terminal mismatch is first excised by a 3'→5' exonuclease. A search for this activity might begin with the replicating complex purified by Ottiger *et al.* (16) containing DNA polymerase α in association with a 3'→5' exonuclease.

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