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Proofreading by DNA polymerase III of *Escherichia coli* depends on cooperative interaction of the polymerase and exonuclease subunits

(fidelity of DNA replication/protein–protein interactions/mutator genes/*dnaE*/*dnaQ*)

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ABSTRACT **The polymerase subunit (α) of *Escherichia coli* DNA polymerase III holoenzyme and the 3′→5′ exonuclease subunit (ε) are each less active separately than together in the holoenzyme core (an assembly of α, ε, and θ subunits). In a complex formed from purified α and ε subunits, polymerase activity increased 2-fold, and that of the 3′→5′ exonuclease increased 10- to 80-fold. The α–ε complex contains one each of the subunits as does the core. Stimulation of 3′→5′ exonuclease activity is due mainly to a greatly increased affinity of the ε subunit for the 3′-hydroxyl terminus, resulting from DNA binding by the α subunit. Proofreading in the course of DNA synthesis by the α–ε complex was indistinguishable from that of the core. These findings identify the participation of the α subunit in proofreading by polymerase III holoenzyme and suggest that the fidelity of DNA replication may be influenced by the relative levels of the α and ε subunits in the cell.**

Spontaneous mutations in *Escherichia coli* result mainly from errors during replication of the chromosome (1, 2). These errors are reduced to low frequencies (10^{-9} to 10^{-10} per base replicated) by (*i*) proofreading built into the replication apparatus to remove mismatches immediately and (*ii*) postreplicational recognition and repair of mismatches in the nascent strand of duplex DNA (3, 4). Genes involved in both proofreading and repair processes have been identified as mutator genes (5). Extensive genetic and biochemical studies of the DNA polymerase of phage T4 have identified it with a major role in replication fidelity (5, 6). Incorporation of an incorrect nucleotide due to a rare tautomeric form of the base in the template or substrate is eliminated by the proofreading function of a 3′→5′ exonuclease domain of the polymerase molecule. The relative rates of polymerization and proofreading at the growing end of the chain can account for the spontaneous mutation frequency of T4 phage (6).

In *E. coli*, DNA polymerase III (pol III) holoenzyme, a complex of 10 polypeptides, is responsible for elongation and proofreading of DNA chains during chromosome duplication (7, 8). Both the polymerase and 3′→5′ exonuclease activities are contained within the pol III core, a tight subassembly of the α, ε, and θ subunits (9). Recently, the α subunit (encoded by the *dnaE* gene) and the ε subunit (product of the *dnaQ* gene) were purified to homogeneity from strains overproducing the gene products (10, 11). The α subunit proved to be the polymerase, and the ε subunit proved to be the 3′→5′ exonuclease. These findings help to explain earlier genetic observations that (*i*) all *dnaE* mutants are defective in DNA synthesis (12), (*ii*) all *dnaQ* mutants show a strong mutator phenotype (10^3- to 10^5-fold increase in mutation frequency) (13, 14), and (*iii*) a decreased level of *dnaQ* gene expression can be responsible for the mutator phenotype (15). Thus, it seems likely that the spontaneous mutation frequency in *E. coli* is determined mainly by the editing function of the ε subunit, the level of which depends on *dnaQ* gene expression. The physically discrete nature of the polymerase and 3′→5′ exonuclease activities of pol III holoenzyme, unique among prokaryotic DNA polymerases, may make it possible to alter their relative levels. Thus, reduction of the proofreading function may be achieved without seriously affecting polymerization, thereby enabling the cell to exercise more control over the fidelity of replication.

The efficiency and substrate specificity of the 3′→5′ exonuclease activity of the ε subunit are profoundly affected when the subunit is complexed in the pol III core. Compared to that of the free ε subunit, the 3′→5′ exonuclease activity of the pol III core on single-stranded DNA is 10 times faster; unlike the ε subunit, the core can act on double-stranded DNA. These effects suggest an involvement of the α or θ subunits in proofreading. In the present studies, we explored the functional and physical interactions between the α and ε subunits by reconstituting an α–ε complex from the purified subunits. Our findings account not only for the functions of the pol III core but also for genetic results that implicate the *dnaQ* gene in synthesis and the *dnaE* gene in fidelity.

MATERIALS AND METHODS

Chemicals and Buffers. Unlabeled and labeled nucleotides were from Pharmacia and Amersham, respectively. Buffer A is 120 mM potassium phosphate, pH 6.9/25% (vol/vol) glycerol/1 mM EDTA/5 mM dithiothreitol; buffer B is 50 mM imidazole·HCl, pH 6.8/10% glycerol/50 mM NaCl/5 mM $MgCl_2$/0.1 mM EDTA/5 mM dithiothreitol.

Proteins and Nucleic Acids. The α subunit and pol III core were prepared from a *dnaE* overproducer (10); the ε subunit was kindly provided by H. Echols (University of California, Berkeley). Terminal deoxynucleotidyltransferase was purchased from Worthington. A hook-like DNA, a 56-mer with a 5′ template tail of 22 nucleotides synthesized by the solid-phase triester method, was generously furnished by M. Urdea (Chiron, Emeryville, CA). Poly(dA) and oligo$(dT)_{10}$ were from Pharmacia.

Polymerase Assay. The reaction was initiated by addition of the test sample to a mixture (25 μl) containing 20 mM Tris·HCl, pH 7.5/4% sucrose/8 mM $MgCl_2$/8 mM dithiothreitol/50 μg of bovine serum albumin per ml/0.16 μM (as molecules) "hook" DNA/100 μM each dCTP, dGTP, and [α-^{32}P]dATP (≈800 cpm/pmol). After incubation at 30°C for 5 min, acid-insoluble radioactivity was measured as described (9). Deoxyribonucleotide incorporation was estimated by multiplying the amount of acid-insoluble dAMP by 2 because the template tail of 22 bases contains 11 thymines.

3′→5′ Exonuclease Assay. Substrates for the exonuclease were prepared by adding either dAMP or dTMP to the

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Abbreviation: pol III, DNA polymerase III.

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Table 1. Activities of pol III core and its subunits

	Activity, dnt per min per molecule		
		3′→5′ Exonuclease	
	Polymerase	Mispaired	Paired
pol III core	1200	140	14
α subunit	460	—	—
ε subunit	—	3.8	0.084

Polymerase and 3′→5′ exonuclease activities were determined in a reaction mixture containing 0.16 μM 3′-hydroxyl termini. dnt, Deoxynucleotides.

3′-hydroxyl terminus of the hook DNA by terminal transferase and [α-^{32}P]dATP or [α-^{32}P]dTTP. DNA extended by an average of 1.2 dAMP residues is paired at the 3′-hydroxyl terminus, whereas that with 1.3 residues of dTMP is mispaired. The labeled DNAs were purified by filtration through a Sephadex G-150 column equilibrated with 10 mM Tris·HCl, pH 7.5/1 mM EDTA. Exonuclease assays were initiated by addition of enzyme to a reaction mixture (25 μl) containing 20 mM Tris·HCl (pH 7.5), 4% sucrose, 8 mM $MgCl_2$, 8 mM dithiothreitol, 50 μg of bovine serum albumin per ml, and a labeled DNA and were carried out at 30°C for 5 min. Reactions were stopped by addition of 50 mM EDTA. Released [^{32}P]dAMP or [^{32}P]dTMP was separated from the substrate DNA by PEI-cellulose thin-layer chromatography in 1 M LiCl.

dTMP Turnover Assay. The assay for conversion of dTTP to dTMP was performed in a mixture (25 μl) containing 20 mM Tris·HCl (pH 7.5), 4% sucrose, 8 mM $MgCl_2$, 8 mM dithiothreitol, 50 μg of bovine serum albumin per ml, 50 μM [α-^{32}P]dTTP (2×10^4 cpm/pmol), and poly(dA)–oligo(dT)$_{10}$ (50 μM as nucleotides, dA:dT = 2:1). Reactions, started by the addition of enzyme, were incubated at 30°C for 2.5 or 5 min and were quenched by addition of 50 mM EDTA. Free dTMP and DNA were fractionated by PEI-cellulose thin-layer chromatography of a 2-μl sample, after which the PEI-cellulose was sliced and radioactivity was determined. The chromatography solvent was 1 M LiCl/1 M formic acid.

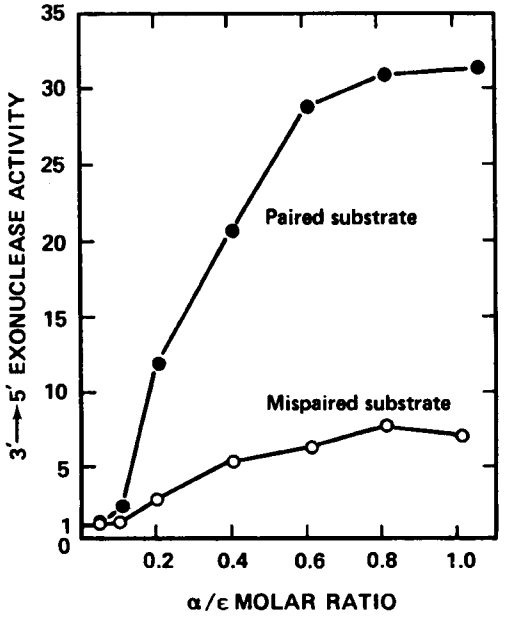

FIG. 1. The α subunit stimulates the 3′→5′ exonuclease activity of the ε subunit. Assays were with 0.16 μM 3′-hydroxyl termini of paired and mispaired hook DNA, 1 ng of ε subunit (activity set at 1 on the ordinate), and various amounts of α subunit.

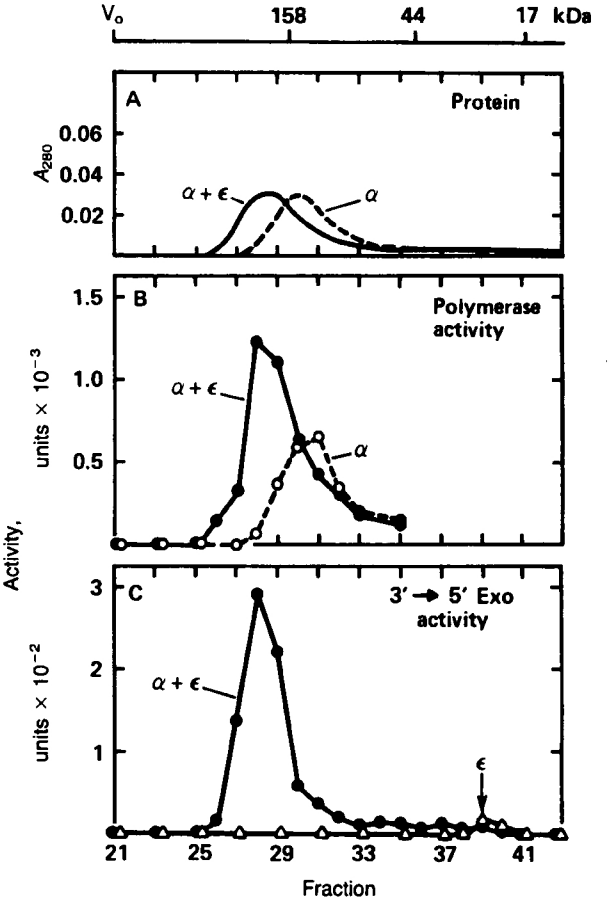

FIG. 2. Isolation of the α–ε complex by HPLC gel filtration. The α and ε subunits were mixed in 100 μl at a molar ratio of 1:4 and, after 5 min at 0°C, were filtered through a TSK 300 column (Bio-Rad) equilibrated with buffer A at 0°C (●). In separate experiments, either free α (○) or free ε subunit (△) was also filtered. The UV absorbance of the ε subunit was not detected presumably because of its low content of tryptophan and tyrosine. Polymerase and 3′→5′ exonuclease (Exo) activities in fractions (0.35 ml) were determined as described with 0.08 μM mispaired hook DNA in the exonuclease assay.

Other Methods. Sodium dodecyl sulfate gel electrophoresis and silver-staining of gels were as described (10). Protein concentration was determined by the Bradford method (16) with bovine serum albumin as a standard.

RESULTS

Polymerase and 3′→5′ Exonuclease Activities of pol III Core and Its Separated Subunits. The polymerase activity of pol III core was about 3 times that of the free α subunit (Table 1). In contrast, the exonuclease activity of core on the mispaired substrate was nearly 40-fold greater than that of the ε subunit. On a paired substrate, the disparity between the core and ε subunit activities was far greater. Thus, the single-strand preference of the ε subunit is profoundly altered in the core.

Stimulation of 3′→5′ Exonuclease Activity of ε Subunit by the α Subunit. The α subunit stimulated the exonuclease activity of the ε subunit 8-fold with the mispaired and 32-fold with the paired substrate (Fig. 1), mimicking the effect seen in the core. The optimal molar ratio of α to ε was near 1.0. The polymerase activity of the α subunit was also stimulated about 2-fold in such mixtures (data not shown). These findings clearly indicate that the α subunit interacts with the ε subunit, strikingly improving its exonuclease activity and

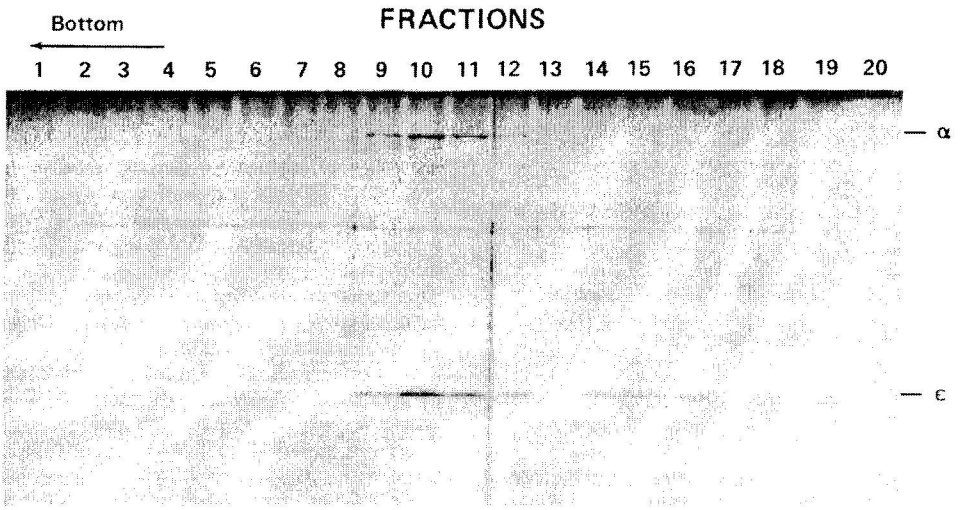

FIG. 3. Sedimentation profile of the α–ε complex. The α subunit (18 pmol) and ε subunit (36 pmol) were mixed and sedimented through 2 ml of a 10–40% sucrose gradient in buffer B at 4°C (TLS 55 rotor, 55,000 rpm, 16 hr). Fractions (100 μl) were collected from the bottom of the tube and 25 μl of each was applied to a sodium dodecyl sulfate polyacrylamide gel (12.5%). Proteins were visualized by silver staining.

altering its substrate specificity to include a paired as well as a mispaired 3′-hydroxyl terminus.

Isolation of an α–ε Complex Reconstituted from Purified α and ε Subunits. A tight complex of the α and ε subunits was isolated away from the free subunits by HPLC gel filtration or sucrose gradient sedimentation. When a mixture of α and ε subunits in a molar ratio of 1 to 4 was applied to an HPLC gel filtration column, the α–ε complex was eluted ahead of where the free α subunit might appear (Fig. 2). The polymerase activity of the purified α–ε complex was twice that of the free α subunit; the 3′→5′ exonuclease activity, coeluting with the polymerase activity, was increased 80-fold compared with that of the free ε subunit.

When the α and ε subunits, mixed in a molar ratio of 1 to 2, were sedimented through a 10–40% sucrose gradient, about half of the applied ε subunit cosedimented with the α subunit (Fig. 3). Increasing the amounts of ε subunit did not increase its content in the α–ε complex. The molar ratio of α and ε in the complex was estimated to be 1.05, based on silver-stain densities compared with those of the purified subunits applied to other lanes on the gel. The molar ratio of α and ε subunits observed was 0.93 in the pol III core and 1.10 in the pol III holoenzyme. Association of one α subunit with one ε subunit is in keeping with sizes estimated for the α–ε complex and that of the pol III core (Table 2).

Affinity of the α Subunit for 3′-Hydroxyl Terminus Influences the 3′→5′ Exonuclease Activity of the ε Subunit. The reaction rates for polymerase and exonuclease activities of the α and ε subunits versus the concentrations of the 3′-hydroxyl termini of hook DNA obeyed Michaelis–Menten kinetics, yielding a K_m value for the α subunit of 0.67 μM (3′-hydroxyl termini) and immeasurable for the ε subunit (>10 μM) (Table 3). This feeble affinity of the ε subunit for the 3′-hydroxyl terminus was enormously improved in the α–ε complex; the K_m value was 0.38 μM for both the paired and mispaired 3′-hydroxyl termini. This K_m value for the exonuclease activity of the α–ε complex approximates that of the pol III core (0.14 μM). The low K_m values of the α–ε complex and pol III core are likely attributable to the relatively strong affinity of the α subunit for the 3′-hydroxyl terminus, which enables the complexed ε subunit to gain access to the 3′-hydroxyl terminus in the ternary complex with DNA.

Reconstitution of Proofreading in the α–ε Complex. Proofreading by the pol III core and the α–ε complex were compared by measuring the turnover of dTTP to dTMP during DNA synthesis on poly(dA), primed by oligo(dT)$_{10}$ (Fig. 4). Generation of dTMP during synthesis is due to release of newly incorporated dTMP by 3′→5′ exonuclease action and reflects proofreading in that both correct and incorrect base-pairing are examined by the same mechanism. Whereas the free α subunit released no dTMP, addition of the ε to the α subunit caused the production of free dTMP in accordance with the formation of the α–ε complex. The α–ε complex and pol III core each generated about 5 dTMPs for every 100 incorporated (Fig. 4).

DISCUSSION

The ε subunit of pol III holoenzyme, encoded by the *dnaQ* (*mutD*) gene, is a 3′→5′ exonuclease, specific for single-stranded DNA (11). In duplex DNA, a mispaired 3′-hydroxyl terminus is excised by the ε subunit 50 times more rapidly than a base-paired terminus. By contrast, the 3′→5′ exonuclease activity of the core of holoenzyme (consisting of α, ε, and θ subunits) is far more efficient than the free ε subunit catalytically and differs in substrate specificity. The exonuclease activity of the core was greater by 18-fold on mispaired termini and was also highly active on paired termini. When complexed with the α subunit, the ε-subunit was stimulated to a similar extent as in the core. Thus, the ε subunit, relatively feeble by itself, depends on the α subunit to bring its exonuclease activity state close to that of the pol

Table 2. Sizes of the α subunit, α–ε complex, and pol III core

	Stokes' radius, Å	$S_{20,w}$, $\times 10^{-13}$ sec	Molecular mass, kDa
α subunit	50	6.3	132
α–ε complex	56	6.6	155
pol III core	57	6.7	160

Stokes' radius and sedimentation coefficient determinations were by gel filtration [TSK-300 (Bio-Rad) or Suparose 12 (Pharmacia)] and by sedimentation in a 10–40% sucrose gradient, respectively, as described in the legends for Figs. 2 and 3. Size markers were thyroglobulin (85.0 Å, 19.2 S), ferritin (61.0 Å, 17.6 S), catalase (52.2 Å, 11.3 S), aldolase (48.1 Å, 7.4 S), and bovine serum albumin (35.5 Å, 4.2 S). Molecular mass was calculated from the Stokes' radius and sedimentation coefficient (17) assuming a partial specific volume of 0.73.

Table 3. Affinity of ε subunit for the 3'-hydroxyl terminus is increased by the α subunit

	$K_m \times 10^6$ of 3'-hydroxyl termini		
		3' → 5' Exonuclease	
	Polymerase	Mispaired	Paired
pol III core	0.21	0.14	0.14
α subunit	0.67	—	—
ε subunit	—	>10	>10
α–ε complex	ND	0.38	0.38

Rates of polymerization and hydrolysis were determined at concentrations of DNA from 0.02 to 3.0 μM; K_m values were calculated from Lineweaver–Burk plots. ND, not determined.

III core or holoenzyme. The contribution of the θ subunit appears to be relatively minor and may account for a 2-fold increase.

One molecule of each of the α and ε subunits binds to form an isolable complex in which the 3'→5' exonuclease activity is remarkably elevated. Kinetic studies of polymerase and exonuclease activities reveal that the very weak affinity of the free ε subunit for the 3'-hydroxyl terminus is vastly improved in the α–ε complex and pol III core, presumably due to the strong affinity of the α subunit for the terminus. Inasmuch as the α–ε complex and the pol III core have similar K_m values for 3'-hydroxyl termini of DNA in both the polymerase and 3'→5' exonuclease reactions, the binding of DNA by the α subunit qualifies as the major factor influencing both polymerase and exonuclease activities.

Relaxation in substrate specificity, enabling exonuclease to act on both paired and mispaired 3'-hydroxyl termini by the α–ε complex and pol III core, can be explained by destabilization of the paired terminus, rendering it more susceptible to the exonuclease activity of the ε subunit. To verify this interpretation, additional information is needed regarding the structure of primer-template DNA bound by various forms of polymerase.

As judged by the generation of dTMP during synthesis, reconstitution of a proofreading complex from purified α and ε subunits has been achieved. The α–ε complex excises

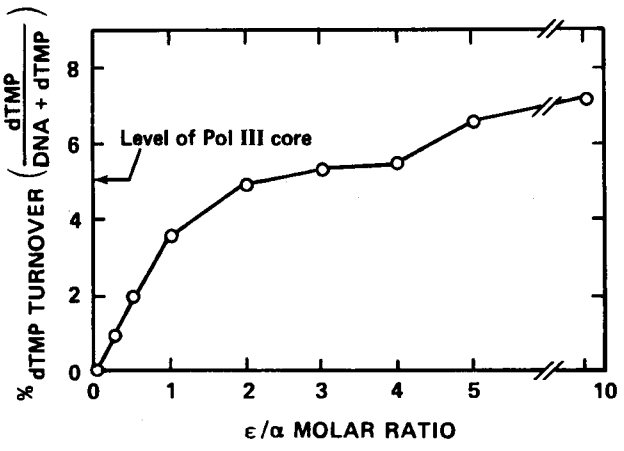

FIG. 4. Reconstitution of proofreading with α and ε subunits. The turnover was determined by dTMP release in reactions containing 10 ng of the α subunit and various amounts of the ε subunit. Conditions for the assay are described. The dTMP turnover of pol III core is near 5.0%.

incorporated dTMP to the same extent as does the pol III core, indicating that the complex suffices for proofreading. The ratio of release of dTMP to its incorporation with the α–ε complex is 0.05, a value 5 times that calculated from the ratio of polymerase activity to the 3'→5' exonuclease activity in the absence of synthesis. Thus, the exercise of proofreading—that is, the exonuclease activity during synthesis—represents a 5-fold stimulation compared to the exonuclease activity uncoupled from synthesis. A pertinent question is whether the fidelity of DNA replication might be controlled by the relative cellular abundance of the ε and α subunits. This possibility is supported by observations that a decreased level of *dnaQ* gene expression in cells leads to a mutator phenotype in these mutants (15).

All of our findings point to an involvement of the α subunit in proofreading. By greatly stimulating the 3'→5' exonuclease activity of the ε subunit and altering its specificity, the α subunit makes an important contribution to the fidelity of DNA replication. In this light, some genetic observations of the *dnaE* and *dnaQ* mutator mutants are better understood. For one, the strong mutator phenotypes of two alleles of the *dnaE* gene (18, 19) are likely due to defective associations of the α subunit with an intact ε subunit. For another, the dominant mutator phenotype of the *dnaQ* mutant, *mutD5* (20, 21), may be attributable to an increased affinity of an enzymatically inactive ε subunit for the α subunit.

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