Dysfunctional proofreading in the *Escherichia coli* DNA polymerase III core

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The ϵ -subunit contains the catalytic site for the 3' → 5' proofreading exonuclease that functions in the DNA pol III (DNA polymerase III) core to edit nucleotides misinserted by the α -subunit DNA pol. A novel mutagenesis strategy was used to identify 23 *dnaQ* alleles that exhibit a mutator phenotype *in vivo*. Fourteen of the ϵ mutants were purified, and these proteins exhibited 3' → 5' exonuclease activities that ranged from 32 % to 155 % of the activity exhibited by the wild-type ϵ protein, in contrast with the 2 % activity exhibited by purified MutD5 protein. DNA pol III core enzymes constituted with 11 of the 14 ϵ mutants exhibited an increased error rate during *in vitro* DNA synthesis using a forward mutation assay. Interactions of the purified ϵ mutants with the α - and θ -subunits were examined by gel filtration chromatography and exonuclease stimulation assays, and by measuring polymerase/exonuclease ratios to identify the catalytically active ϵ 511 (I170T/V215A) mutant with dysfunctional proofreading in the

DNA pol III core. The ϵ 511 mutant associated tightly with the α -subunit, but the exonuclease activity of ϵ 511 was not stimulated in the α - ϵ 511 complex. Addition of the θ -subunit to generate the α - ϵ 511- θ DNA pol III core partially restored stimulation of the ϵ 511 exonuclease, indicating a role for the θ -subunit in coordinating the α - ϵ polymerase–exonuclease interaction. The α - ϵ 511- θ DNA pol III core exhibited a 3.5-fold higher polymerase/exonuclease ratio relative to the wild-type DNA pol III core, further indicating dysfunctional proofreading in the α - ϵ 511- θ complex. Thus the ϵ 511 mutant has wild-type 3' → 5' exonuclease activity and associates physically with the α - and θ -subunits to generate a proofreading-defective DNA pol III enzyme.

Key words: DNA polymerase, DNA replication, exonuclease, fidelity, mutator, proofreading.

INTRODUCTION

The DNA pol III (DNA polymerase III) holoenzyme of *Escherichia coli* is a multi-subunit replicase consisting of at least 10 different polypeptides [1]. In the holoenzyme complex there are two heterotrimeric catalytic cores, each composed of one α -, one ϵ - and one θ -subunit. The α -subunit encoded by *dnaE* contains the catalytic site for DNA polymerization [2], and the ϵ -subunit encoded by *dnaQ* contains the 3' → 5' proofreading exonuclease [3,4]. The θ -subunit encoded by the *holE* gene has no catalytic activity [5,6]. The three-subunit α - ϵ - θ DNA pol III complex is the minimal active polymerase form purified from the DNA pol III holoenzyme complex [7], indicative of the intimate association of these three polypeptides. Constitution of the three-subunit complex *in vitro* using recombinant proteins demonstrates tight physical association between α and ϵ and between ϵ and θ , but not between α and θ [5,8–10]. The interactive nature of the α -, ϵ -, and θ -subunits within the catalytic core is indicated by the stimulation of α -subunit DNA pol activity by addition of ϵ , and by the stimulation of the ϵ -subunit exonuclease activity by the addition of α or θ [8,9,11,12]. The structural and functional interactions between the core subunits in DNA pol III are necessary for high-fidelity DNA synthesis by this enzyme.

The overall fidelity of DNA pol III is determined by the selection of correct nucleotides during polymerization by the α -subunit and by removal of incorrectly selected nucleotides during proofreading by the ϵ -subunit 3' → 5' exonuclease. A functional *dnaQ* allele encoding the ϵ subunit is required to achieve high-fidelity replication in *E. coli*. Mutations in *dnaQ* increase the spontaneous mutation rates in cells, resulting in a mutator phenotype [13–18]. Targeted mutagenesis of the *dnaQ* gene and subsequent detection of mutators has been used to identify *dnaQ* alleles encoding defective ϵ proteins [19–23]. The specific amino acid changes

in *dnaQ* mutator alleles have provided genetic evidence for the location of catalytic residues in the N-terminal region of the ϵ protein and suggested a specialized function for the C-terminal region of ϵ in binding to the α -subunit [22]. A fragment of the ϵ protein containing the N-terminal 186 amino acids (ϵ 186) was shown to contain the structural domain for full catalytic activity, but to lack interaction with the α -subunit [9]. Structural studies using the N-terminal catalytic domain of ϵ showed that nearly all of the mutated amino acids identified in the *dnaQ* mutator alleles in genetic studies are located in or near the region of the ϵ protein involved in DNA binding and catalysis [24,25]. The N-terminal ϵ catalytic domain is tethered to the α -subunit DNA pol by a region of the ϵ protein contained within the C-terminal 40 amino acids of ϵ [9,26]. Thus a two-domain structure for the ϵ protein has been proposed in which the N-terminal 186 amino acids containing the 3' → 5' exonuclease activity are bound to the α -subunit through the C-terminal region of ϵ , generating a tightly coupled exonuclease–polymerase complex to facilitate the proofreading function of the ϵ exonuclease for the α -subunit DNA pol. Structural studies indicated that the θ -subunit binds to the ϵ -subunit at a position near the exonuclease active site, suggesting that the θ -subunit might stabilize ϵ and modulate the polymerase–exonuclease interaction in the DNA pol III core [10].

The ϵ -subunit, as an isolated polypeptide or as a subunit integrated into the DNA pol III core or holoenzyme complex, hydrolyses mispaired nucleotides at a higher rate than correctly paired nucleotides [4,8]. The activity of the ϵ -subunit shows a greater dependence on temperature when using duplex DNA substrates compared with single-stranded DNA substrates [27,28], supporting the hypothesis that proofreading specificity results from the melting capacity of the 3' terminus, which is higher for mispaired than for paired DNA [29,30]. Analysis of the ϵ -catalysed reaction suggests that the physiologically relevant

Abbreviations used: pol, polymerase; HSV-tk, herpes simplex virus type 1 thymidine kinase gene.

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substrate for the ϵ subunit within the holoenzyme complex is probably single-stranded DNA of at least three nucleotides in length [28], but the precise mechanism of DNA melting in the DNA pol III enzyme has not been established. Since melting of DNA at the 3' terminus might be the rate-limiting step in the proofreading process, a detailed understanding of the interaction between the α -subunit polymerase and the ϵ -subunit exonuclease, and how the θ -subunit might affect this interaction, is necessary to provide insights into the mechanism of DNA pol III.

Previous mutagenesis strategies with *dnaQ* identified mutator alleles that were predominantly ϵ catalytic mutants or ϵ proteins lacking interactions with the α -subunit. We sought a strategy to identify ϵ mutants that contain exonuclease activity and maintain physical association with the α -subunit, yet display a mutator phenotype, presumably resulting from a dysfunctional polymerase–exonuclease interaction. Random mutagenesis targeting the C-terminal region of ϵ was coupled with a genetic screen that identifies ϵ mutators that are catalytically active. Focusing mutagenesis to the C-terminal region reduced the likelihood of detecting dominant catalytic mutants, and introducing the ϵ mutants into cells on a plasmid limited the detection of recessive mutants that do not associate with the α -subunit. Using this strategy, the ϵ mutant protein expressed from the plasmid is required to compete with wild-type ϵ expressed from the chromosomal *dnaQ* allele for incorporation into the holoenzyme complex in order to elicit a mutator phenotype. Catalytically active ϵ mutants exhibiting a mutator phenotype *in vivo* were identified, and the fidelity of DNA synthesis catalysed by DNA pol III core enzymes constituted with the ϵ mutant proteins was measured using a forward mutation assay. Physical interactions between ϵ mutants and the α - and θ -subunits were assessed by gel filtration, and functional interactions were measured in exonuclease and polymerase assays. These experiments have identified the *dnaQ511* allele, encoding the catalytically competent ϵ 511 protein with dysfunctional proofreading capacity within the DNA pol III core.

EXPERIMENTAL

Materials

[γ - 32 P]ATP, heparin–Sepharose and Q-Sepharose resins, and Mono Q, Superdex 200 and phenyl–Superose columns were from Amersham Pharmacia Biotech. Phosphocellulose (P-11) was from Whatman, and hydroxyapatite was from Bio-Rad. The pGem-T Easy and pBR322 vectors were from Promega. All restriction enzymes were from Promega, except *Dra*III (from New England Biolabs). Oligonucleotides were synthesized and purified and all DNA sequencing was performed in the Cancer Center of Wake Forest University. QIAprep[®] Spin Mini-preps (Qiagen Inc.) were used to purify all plasmids. The *E. coli* strains BL21(DE3) (Novagen) and XL-1 Blue (Stratagene) were used for protein expression. Strain NR9360 was a gift from Dr R. M. Schaaper (National Institute of Environmental Health Sciences, Research Triangle Park, NC, U.S.A.). Strain FT334 and the HSV-*tk* (herpes simplex virus type 1 thymidine kinase gene) assay components were generously provided by Dr K. Eckert (The Pennsylvania State University College of Medicine, Hershey, PA, U.S.A.).

Plasmid construction

For *in vivo* studies using the pBRQ plasmid, transcription and translation of *dnaQ* were under the control of the chromosomal promoter and ribosome binding site respectively. The *dnaQ* regulatory sequence was recovered from *E. coli* (XL-1) genomic

DNA using PCR. A 38-nucleotide primer containing *Hpa*I and *Eco*RI sites and the sequence of the first 15 nucleotides of the *dnaQ* promoter region was synthesized as the forward primer, and a 21-nucleotide primer complementary to *dnaQ* and including the unique *Pvu*I site was synthesized as the reverse primer. The PCR product that was generated using the 38mer and 21mer primers was digested with *Hpa*I and *Pvu*I and cloned into the pEXO5 plasmid [9] to generate pQPRBS. Two silent mutations were introduced into the *dnaQ* gene at nucleotides 441 and 442, encoding amino acids 147 and 148 of ϵ , in the pQPRBS plasmid using PCR [31] to generate a unique *Dra*III restriction site at this position. The complete *dnaQ* gene was cloned into the *Eco*RI site of pBR322 to generate the pBRQ plasmid that was used to prepare the *dnaQ* mutator library (pdnaQ#). The *mutD5* (*dnaQ*;Thr15Ile) allele was generated by mutagenesis of codon 15 from Thr → Ile by PCR [31] using the pQPRBS plasmid as template, and was cloned into the *Eco*RI site of pBR322 to generate the pBRmutD5 plasmid.

For expression and purification of the MutD5 ϵ protein, the pmutD5 plasmid was generated by mutagenesis [31] using the pEXO5 plasmid [9] as template. For the mutant ϵ proteins identified in this study, the *Bam*HI–*Sac*I fragments of the *dnaQ* mutator alleles were recovered from the pdnaQ# plasmids and cloned into the pEXO5 plasmid to generate the p ϵ # plasmids. All other plasmids used for protein expression have been described [9]. Plasmids were sequenced to confirm the presence of the desired mutations.

Random mutagenesis and the plasmid library

PCR conditions permitting adequate product generation and a sufficiently high frequency of polymerization errors to generate randomly mutagenized *dnaQ* encoding the C-terminal region of ϵ were determined in test reactions (results not shown). Ultimately, an 18mer (primer1) complementary to nucleotides 403–420 of *dnaQ* and a 21mer (primer2) complementary to the pBRQ plasmid downstream of *dnaQ* were used in four separate PCRs containing the four dNTPs under biased concentrations such that three of the dNTPs were present at 2 mM and the fourth (biased) dNTP was present at 20 μ M. The products of these four PCRs were pooled and amplified in the presence of equal dNTP concentrations, and the product was digested with *Sac*I and *Dra*III to generate the randomly mutagenized *dnaQ* encoding the C-terminal region of ϵ . The *Sac*I–*Dra*III fragment was ligated into pBRQ to produce the pBRQ plasmid library (pdnaQ#).

Genetic screen of *dnaQ* mutator alleles

The pBRQ plasmid library was transformed into strain NR9360 [32] by electroporation and plated on Luria–Bertani broth containing tetracycline and kanamycin. The next day, plates were replicated to MacConkey Agar containing galactose and antibiotics. Colonies exhibiting the mutator phenotype were identified as described [19,32]. Briefly, the MacConkey Agar plates were incubated at 37 °C for 3 days and inspected for the appearance of red (gal⁺; able to ferment galactose) papillae growing on the surface of the colourless (gal[−]; unable to ferment galactose) colonies. Colonies with increased numbers of papillae relative to the number of papillae observed in untransformed NR9360 colonies were considered as containing candidate mutator alleles. Candidate mutators were sequenced to identify the specific nucleotide and deduced amino acid changes in the *dnaQ* allele. Mutants identified in the initial screen were confirmed as mutators in a second papillation screen. The plasmids of candidate *dnaQ* mutators were purified, and each plasmid (50 ng) was transformed

into 40 μ l of RbCl₂-competent NR9360 cells. Cells were heat-shocked at 42 °C for 45 s and incubated on ice for 2 min; 1 ml of Luria–Bertani broth was then added, and cells were incubated at 37 °C for 45 min. An aliquot of 5 μ l of each transformation was spotted on to a MacConkey Agar plate containing galactose and incubated at 37 °C for 1 day. Two independent transformations and platings were performed with each plasmid, and the average number of papillae/colony was determined for each *dnaQ* allele.

Purification of DNA pol III α -, ϵ - and θ -subunits

The α -subunit, θ -subunit and the ϵ 186 proteins were prepared as described [9]. The wild-type, mutD5 and ϵ mutant proteins identified in this study were expressed and purified from *E. coli* BL21(DE3) cells containing the pEXO5 plasmid [9], the pmutD5 plasmid and the p ϵ # plasmids respectively, using the described procedure [28]. Protein concentrations were determined at A_{280} using the following molar absorption coefficients: α -subunit, $\epsilon = 95\,800\text{ M}^{-1}\cdot\text{cm}^{-1}$; θ -subunit, $\epsilon = 8250\text{ M}^{-1}\cdot\text{cm}^{-1}$; ϵ -subunits, $\epsilon = 13\,430\text{ M}^{-1}\cdot\text{cm}^{-1}$; ϵ 186 protein, $\epsilon = 7740\text{ M}^{-1}\cdot\text{cm}^{-1}$. (SDS/PAGE analysis of the purified α , ϵ and θ proteins is shown in the Supplementary Figure 1 at <http://www.BiochemJ.org/bj/384/bj3840337add.htm>.)

Exonuclease assays

Reactions (10 μ l) contained 20 mM Tris/HCl, pH 7.5, 2 mM dithiothreitol, 5 mM MgCl₂, 100 μ g/ml BSA, 100 nM 5'-³²P-labelled 23mer or partial duplex DNA (50mer:23mer), and the amount of enzyme indicated in the Figure legends. Enzyme dilutions were performed on ice in 1 mg/ml BSA. Protein mixtures were incubated at room temperature for 10 min prior to addition to reactions. Reactions were for 20 min at 37 °C and were stopped by addition of 30 μ l of cold 95 % (v/v) ethanol. Reactions were dried *in vacuo* and resuspended in 8 μ l of 100 % (v/v) formamide. Samples were heated at 95 °C for 5 min and subjected to gel electrophoresis on a 23 % (w/v) polyacrylamide denaturing gel. The radiolabelled products were quantified by phosphorimaging (Molecular Dynamics) as described in [9]. The amount of nucleotides excised in a reaction was determined by calculating the percentage of the total radiolabelled oligomer that was present at each band within a specific lane. The percentage of oligomer at each band position was multiplied by the total number of nucleotides excised from the 23mer and by 1000 fmol. The sum of these values yielded the total amount (fmol) of 3' terminal nucleotides excised in the reaction.

Forward mutation assay

The accuracy of DNA synthesis *in vitro* by the DNA pol III core enzymes constituted with wild-type α and θ and mutant ϵ proteins was determined using the HSV-*tk* forward mutation assay [33]. The DNA synthesis reactions (50 μ l) contained 20 mM Tris/HCl, pH 7.5, 10 mM MgCl₂, 8 mM dithiothreitol, 10 % (v/v) glycerol, 2 pmol of the oligonucleotide-primed HSV-*tk*-containing single-stranded DNA template, and dNTPs. Four separate reactions were performed for 1 h at 37 °C with 10 pmol of each DNA pol III core in the presence of three dNTPs at 2 mM and the fourth dNTP at 200 μ M (final concentrations), and the reaction products were pooled. The DNA pol III cores were constituted by incubating wild-type α and θ with ϵ or with a mutant ϵ protein (10 pmol of each protein) for 10 min at room temperature. The reaction products were digested with *EcoRV* and *MluI*, hybridized to a gapped duplex molecule as described by Eckert et al. [33] and transformed into *E. coli* strain FT334. Mutation

frequencies are defined as the number of colonies resistant to both chloramphenicol and 5-fluoro-2'-deoxyuridine divided by the total number of chloramphenicol-resistant colonies.

Gel filtration

Mixtures containing α (0.9 nmol) and one of the ϵ proteins (1.8 nmol), or mixtures containing α (0.9 nmol), one of the ϵ proteins (1.8 nmol) and θ (3.6 nmol), were incubated at 15 °C for 30 min. The mixtures were applied to a Superdex 200 column equilibrated in 20 mM Tris/HCl, pH 7.5, 0.5 mM EDTA, pH 8.0, 100 mM NaCl and 10 % (v/v) glycerol at a flow rate of 0.5 ml/min. After collecting the initial 12 ml, 200 μ l fractions were collected. The 200 μ l fractions were concentrated in Microcon concentrators (Amicon), suspended in SDS-sample buffer, and analysed on SDS/15%-polyacrylamide gels stained with Coomassie Brilliant Blue.

Polymerase/exonuclease ratio assays

The polymerase/exonuclease ratios in the DNA pol III subunit mixtures were determined by measuring the ratio of single-nucleotide addition to the 23mer primer in a forward polymerization reaction relative to the excision of nucleotides from the 23mer of the partial duplex DNA substrate. Reactions (20 μ l) contained 20 mM Tris/HCl, pH 7.5, 2 mM dithiothreitol, 5 mM MgCl₂, 5 nM 5'-³²P-labelled partial duplex DNA and 100 μ M dCTP. The protein mixtures were prepared using equal molar ratios of α , ϵ and θ as described above and incubated at room temperature for 10 min. Reactions containing the protein mixtures at a final concentration of 1 nM were incubated for 5 min at 37 °C in the presence of the partial duplex 50mer:23mer DNA and dCTP substrates in the absence of the required bivalent metal Mg²⁺. The polymerase/exonuclease reactions were initiated upon addition of MgCl₂ and incubated at 37 °C for 2 min. The reactions were quenched by addition of 50 μ l of cold 95 % (v/v) ethanol, and the samples were processed as described above. The amount of 23mer primer extended by the α -subunit polymerase was determined by calculating the percentage of total radiolabelled oligomer detected at the 24mer position and multiplying this by 1000 fmol. The amount of 3' excision by the ϵ -subunit exonuclease was determined by calculating the percentage of the total radiolabelled oligomer that was present in each band migrating to a position smaller than 23 nucleotides in length within a specific lane. The percentage of oligomer at each band position was multiplied by the total number of nucleotides excised from the 23mer and by 1000 fmol. The sum of these values yielded the total amount (fmol) of 3' terminal nucleotides excised in the reaction. The polymerization/exonuclease ratio was calculated by dividing the total fmol of DNA extended by the total fmol of nucleotides excised.

RESULTS

dnaQ mutator alleles

PCR was used to mutate the *dnaQ* gene in the region encoding amino acids 150–243 of ϵ (Figure 1). A 453-nucleotide product containing polymerization errors was generated using the pBRQ plasmid as template and oligomer primer1 (complementary to nucleotides 403–420 in the *dnaQ* gene) and primer2 (complementary to the pBRQ plasmid sequence 106 nucleotides downstream from *dnaQ*). The nucleotide concentrations in four separate PCRs were biased as described in the Experimental section to ensure polymerization errors and to promote all possible nucleotide misincorporations. The mutagenized products were pooled and

Figure 1 Strategy for preparing the *dnaQ* mutator allele plasmid library

PCRs were performed using primer1 and primer2 under biased nucleotide conditions as described in the Experimental section to ensure incorporation of base substitution errors in the targeted region of ϵ (step 1). The products of the biased PCRs were pooled and PCR-amplified under unbiased nucleotide conditions (2). The PCR products were digested with *Dra*III and *Sac*I and ligated into the *Dra*III–*Sac*I sites of the pBRQ plasmid to generate the plasmid library containing *dnaQ* alleles randomly mutagenized from amino acid positions 150–243 of ϵ (*pdnaQ*#). The positions of the ϵ catalytic core, linker and α -association regions are indicated. The relative positions of the ϵ active-site residues in motifs ExoI (DxE), ExoII (D) and ExoIIIe (HxAxxD) are indicated.

amplified further by PCR in the presence of equal nucleotide concentrations, resulting in sufficient quantities of the randomly mutated product of *dnaQ* encoding the C-terminal region of ϵ . The PCR-generated mutations were cloned into the *Dra*III–*Sac*I site of the pBRQ plasmid, and the ligation products were amplified in *E. coli* to generate the *dnaQ* mutant plasmid library. Twelve clones were selected at random and sequenced to confirm the presence of mutations in the targeted region of *dnaQ* in the plasmid library. Six of the twelve clones contained PCR-generated mutations, with an average of 2.8 point mutations and 1.7 amino acid substitutions detected per mutated clone (results not shown).

A total of 23 *dnaQ* mutants that exhibited a mutator phenotype *in vivo* were identified from the mutagenized *dnaQ* plasmid library using a papillation assay. The specific nucleotide changes in the *dnaQ* mutator alleles resulting in the deduced amino acid changes are summarized in Table 1. The mutagenized *dnaQ* plasmids were introduced into cells containing a wild-type chromosomal *dnaQ* allele. A dominant *dnaQ* mutator allele was detected if the mutant ϵ protein expressed from the plasmid competed successfully with the wild-type ϵ protein expressed from the chromosomal *dnaQ* allele to effect a detectable increase in the mutation frequency during chromosomal replication. A papillation assay was used to screen for ϵ mutants that confer a mutator phenotype by scoring for mutations that revert the chromosomal *galK2* gene to the wild-type sequence, restoring galactose utilization [32,34–36]. In this assay, ‘white’ colonies that are unable to ferment galactose (*gal*[−]) become spotted with red papillae microcolonies capable of galactose fermentation (*gal*⁺) as a result of the *galK2* mutation that occurs during colony growth. The density of papillae appearing in a colony is a measure of the mutation frequency. The randomly mutagenized *dnaQ* pBRQ plasmid library was transformed into the mismatch repair-defective strain NR9360 to minimize the indirect effects of repair saturation that proofreading defects have in mismatch repair-competent strains [37]. The plasmid *dnaQ* allele was cloned under control of the chromosomal regulatory sequences in the multicopy pBR322 plasmid for expression of the mutant *dnaQ* plasmid allele to provide sufficient copies of the candidate mutators [38]. Candidate mutators were initially identified by visual scoring of the density of papillae in colonies.

Mutator *dnaQ* alleles were confirmed by transformation of the *pdnaQ*# plasmids into NR9360 and spotting of the resultant culture on to plates (results not shown). The papillae densities in cells containing the *pdnaQ*# plasmids confirmed the presence of a mutator phenotype (Table 1). The NR9360 strain transformed with wild-type ϵ exhibited an average of 16 papillae. The papillae in the NR9360 strain transformed with the *pdnaQ*# plasmids showed 1.3–9.2-fold higher density. Mutants contained from one to five amino acid changes: nine mutants were identified with one amino change, nine with two amino acid changes, one with three amino acid changes, three with four amino acid changes, and one with five amino acid changes. A total of 47 amino acid substitutions were detected at 36 different positions within the 94-amino-acid target region. The mutation L171F and mutations at Gly¹⁸⁰ identified in three of the *dnaQ* mutator alleles were also detected in a study by Taft-Benz and Schaaper [22]. Approx. 25000 colonies were screened to identify the 23 *dnaQ* mutator alleles. The library screen was discontinued when several mutants containing identical mutations were detected more than once.

ϵ mutant proteins

Fourteen of the ϵ mutant proteins and the MutD5 ϵ protein [20,39] were purified, and the activities of these enzymes were measured to determine if the ϵ mutant proteins contained functional exonucleases. For each enzyme preparation, a predominant band corresponding to the correct molecular mass of approx. 27 kDa was detected. For several of the ϵ mutant preparations, including the MutD5 protein, smaller protein bands of approx. 20 kDa were detected, suggesting possible instability and proteolytic degradation of some of the ϵ mutant proteins. The catalytic activities of the 14 purified ϵ mutants were compared with the activity of the wild-type ϵ and the MutD5 ϵ proteins by measuring the rate of degradation of 3′ nucleotides from a 5′-³²P-labelled 23mer and from a partial duplex DNA 50mer:23mer, to determine if the mutations present in these proteins affected the catalytic activity (Figure 2, Table 2). The activities of the ϵ mutant enzymes encoded by the *dnaQ503*, *dnaQ509*, *dnaQ510* and *dnaQ515* alleles measured at two different enzyme concentrations using the

Table 1 *dnaQ* mutator alleles

Each *dnaQ* allele was sequenced completely to determine nucleotide changes (denoted in bold) and the deduced amino acid changes.

Allele	Nucleotide change(s)	Amino acid substitution(s)	No. of papillae	Fold increase
<i>dnaQ</i>	—	—	16	1.0
<i>mutD5</i>	ACC → ATC	T15I	96	6.0
<i>dnaQ501</i>	AGT → GGT	S238G	20	1.3
<i>dnaQ502</i>	CGC → TGC, CGA → TGG	R151C, R242W	37	2.3
<i>dnaQ503</i>	GGT → AGT, TCG → TTG	G180S, S184L	128	8.0
<i>dnaQ504</i>	GAA → AAA, GCA → GTA	E190K, A200V	39	2.5
<i>dnaQ505</i>	CTT → TTT	L171F	39	2.5
<i>dnaQ506</i>	CAT → CAA	H225Q	26	1.7
<i>dnaQ507</i>	ATT → AGT	I202S	53	3.3
<i>dnaQ508</i>	CAG → CTG	Q169L	20	1.3
<i>dnaQ509</i>	ACG → ATG	T183M	52	3.3
<i>dnaQ510</i>	GAA → AAA, GCG → GTG	E153K, A177V	106	6.7
<i>dnaQ511</i>	ATC → ACC, GTT → GCT	I170T, V215A	29	1.8
<i>dnaQ512</i>	GCC → GAC, GCG → GTG	A168D, A217V	22	1.4
<i>dnaQ513</i>	CAG → CAT, CAG → CGG	Q208H, Q233R	26	1.7
<i>dnaQ514</i>	AAC → AGC, GTT → GCT, CAA → CAT, GCC → ACC	N156S, V174A, Q197H, A227T	54	3.4
<i>dnaQ515</i>	GAA → GGA, ATT → GTT	E199G, I202V	43	2.7
<i>dnaQ516</i>	CAG → CTG	Q208L	20	1.3
<i>dnaQ517</i>	CAC → TAC, GCG → GTG, GCA → GTA, GCT → GTT, GCC → ACC	H162Y, A188V, A209V, A224V, A227T	76	4.8
<i>dnaQ518</i>	GTT → GAT, CTG → CAG, ATG → TTG, GAG → GGG	V174D, L176Q, M185L, E192G	113	7.1
<i>dnaQ519</i>	GGT → AAT, GAA → GGA, GCA → GTA	G180N, E199G, A200V	147	9.2
<i>dnaQ520</i>	CAG → CTG	Q195L	33	2.1
<i>dnaQ521</i>	CAG → CTG, TAT → TGT	Q169L, Y175C	43	2.7
<i>dnaQ522</i>	GTT → GCT, CAG → CTG, GTT → GCT, GAT → GGT	V174A, Q208L, V214A, D219G	21	1.3
<i>dnaQ523</i>	GCA → GTA	A200V	33	2.1

Figure 2 Exonuclease activities of the ϵ enzymes

Exonuclease reactions were prepared as described in the Experimental section containing wild-type ϵ or the indicated ϵ mutant. Enzyme dilutions were prepared at 10 $\times$ the indicated final concentration (C_f), and samples (1 μ l) were added to reactions. No enzyme was added to the reaction in the lane labelled 0. Reactions products were subjected to electrophoresis on a 23% (w/v) polyacrylamide denaturing gel. The position of migration of the 23mer is indicated.

23mer oligonucleotide are shown in Figure 2. Quantification of the activities shows that the purified ϵ mutant enzymes exhibited 3' $\rightarrow$ 5' exonuclease activities that ranged from 32% to 155% of the activity exhibited by the wild-type ϵ protein using the two different substrates (Table 2). These activities compare with that of the ϵ 186 fragment, which demonstrates $\sim$ 300% of the activity of the wild-type ϵ in this assay (Table 2), and of the MutD5 ϵ protein, which exhibits $\sim$ 2% activity relative to ϵ (Figure 2). The 50-fold reduction in exonuclease activity of the purified MutD5 ϵ protein is similar to that reported for the purified MutD5

holoenzyme [40]. The levels of exonuclease activity detected for the ϵ mutant proteins suggest that these mutations in ϵ have varying effects on the catalysis of nucleotides from single- and partial duplex DNA by this proofreading exonuclease, but none of the purified ϵ mutants exhibited the comparable 50-fold decrease in catalytic efficiency that was measured with the MutD5 ϵ protein.

Association of the ϵ mutants with α - and θ -subunits

Gel filtration chromatography was used to demonstrate a direct physical association between the α -subunit and eight of the 14 purified ϵ mutants. A tight physical association between the α -subunit and the ϵ 501, ϵ 502, ϵ 503, ϵ 509, ϵ 511, ϵ 513, ϵ 515 and ϵ 516 mutant proteins was apparent by co-fractionation of the α -subunit with these ϵ mutants (Table 2). The tight complex between the α -subunit and the ϵ 511 mutant is evident from the results presented in Figure 3(A) and is similar to the complexes generated between α and the eight ϵ mutants indicated above. Co-fractionation of the α - ϵ 511 complex during gel filtration was detected, with the peak of α - ϵ 511 eluting at fraction 6, consistent with the 154 kDa size of the complex. The excess ϵ 511 protein that was not in complex with α and some less-than-full-length ϵ 511 protein, presumably lacking the complete C-terminal region, fractionated as expected for the size of these proteins and were eluted from the column with the peak at fraction 24 (Figure 3A).

Six of the 14 ϵ mutants that were tested failed to associate with the α -subunit with sufficiently high affinity to co-fractionate during gel filtration chromatography (Table 2): the ϵ 504, ϵ 506, ϵ 507, ϵ 508, ϵ 510 and ϵ 512 mutants fractionated independently from the α -subunit when mixtures containing these proteins were subjected to gel filtration. As illustrated in Figure 3(B), the ϵ 504 peak at fraction 24 was well resolved from peak fraction 9 of the α -subunit. The positions of elution of the ϵ 504 mutant and the α -subunit are consistent with the 27 kDa and 127 kDa sizes of these proteins, and are representative of the results obtained

Table 2 Activities of ϵ mutants and interactions with α

Gel filtration chromatography was performed with mixtures of α and wild-type ϵ or ϵ mutant as described in the Experimental section. +, Co-elution of α and ϵ proteins; —, independent elution of the subunits. Activity values are means of three assays performed at two enzyme concentrations quantified as described in the Experimental section. ssDNA, single-stranded DNA; dsDNA, double-stranded DNA. Exonuclease stimulation assays were performed as described in the Experimental section. The stimulation of exonuclease activity of the ϵ mutants upon the addition of α was compared with the stimulation observed with wild-type ϵ .

ϵ protein	Co-fractionates with α	Activity (fmol of 3' nt excised)/min per 1 nM ϵ		Stimulation by α (fold)	Relative stimulation
		ssDNA	dsDNA		
ϵ	+	660	34	11	1.0
ϵ 186	—	2180	87	1.7	0.15
ϵ 501 (S238G)	+	356	16	5.5	0.50
ϵ 502 (R151C, R242W)	+	858	19	8.3	0.75
ϵ 503 (G180S, S184L)	+	264	11	7.5	0.68
ϵ 504 (E190K, A200V)	—	356	22	1.8	0.16
ϵ 506 (H225Q)	—	793	36	2.1	0.19
ϵ 507 (I202S)	—	218	13	2.0	0.18
ϵ 508 (Q169L)	—	211	15	1.5	0.14
ϵ 509 (T183M)	+	443	24	6.6	0.60
ϵ 510 (E153K, A177V)	—	528	38	1.2	0.11
ϵ 511 (I170T, V215A)	+	858	35	2.2	0.20
ϵ 512 (A168D, A217V)	—	1120	34	1.8	0.16
ϵ 513 (Q208H, Q233R)	+	958	35	5.3	0.48
ϵ 515 (E199G, I202V)	+	1020	21	18	1.6
ϵ 516 (Q208L)	+	614	17	5.0	0.45

Figure 3 Association of the ϵ 511 and ϵ 504 mutants with the α - and θ -subunits

Protein mixtures containing (A) α and ϵ 511, (B) α and ϵ 504, and (C) α , ϵ 504 and θ were prepared and subjected to gel filtration chromatography as described in the Experimental section. The indicated fractions (200 μ l) were concentrated and subjected to SDS/15%—PAGE. The gels were stained with Coomassie Brilliant Blue. The proteins present in peak fractions are indicated. Lane 1 contains molecular mass standards with sizes indicated in kDa.

with the six ϵ mutants that failed to associate with the α -subunit in this analysis (Table 2). The binding affinity (K_d) of the wild-type ϵ -subunit for the α -subunit has been estimated to be $<0.25 \mu$ M

[9]. It is possible that the ϵ 504, ϵ 506, ϵ 507, ϵ 508, ϵ 510 and ϵ 512 mutants interact with the α -subunit with an affinity too low to detect by gel filtration chromatography.

The six ϵ mutants that failed to associate with the α -subunit retained their tight association with the θ -subunit. Mixtures containing ϵ 504, ϵ 506, ϵ 507, ϵ 508, ϵ 510 or ϵ 512 with wild-type α and θ were subjected to gel filtration chromatography. Fractionation of the mixture of ϵ 504, α and θ is shown in Figure 3(C), and demonstrates formation of the ϵ 504– θ complex, with co-elution of ϵ 504 and the 8.8 kDa θ protein in fraction 24. The α -subunit eluted at fraction 9, indicating the independent fractionation of α in this mixture. Formation of the ϵ – θ complexes was detected in all mixtures containing ϵ 504, ϵ 506, ϵ 507, ϵ 508, ϵ 510 or ϵ 512 plus α and θ , with no evidence for formation of α – ϵ complexes. The independent fractionation of ϵ 504, ϵ 506, ϵ 507, ϵ 508, ϵ 510 and ϵ 512 from α indicates that the mutations present in these ϵ proteins result in reduced affinities for the α -subunit, with no detectable affect on affinities for the θ -subunit.

Stimulation of exonuclease activity of the ϵ mutants by the α -subunit

Stimulation of the exonuclease activity of ϵ by α has been interpreted to signify specific polymerase–exonuclease actions when the 3' end of the DNA is moved from the polymerase to the exonuclease active site, and the greatest stimulation of ϵ by α is observed using a correctly base-paired partial duplex DNA [8,9,28]. For all 14 of the ϵ mutants, some level of exonuclease stimulation was apparent upon addition of the α -subunit using a partial duplex DNA containing a 5'-³²P-labelled 23mer hybridized to a complementary 50mer template, suggesting interactions between these ϵ mutants and α (Table 2). The excision of 3' nucleotides from the radiolabelled 23mer by the ϵ 504, ϵ 506, ϵ 507, ϵ 511, ϵ 513 and ϵ 515 mutants upon addition of increased amounts of the α -subunit is shown in Figure 4. In control reactions, the activities of ϵ and the ϵ mutant enzymes in the absence of α -subunit resulted in 10–22 fmol of 3' nucleotides excised (Figure 4, lanes 2, 6, 10, 14, 18, 22 and 26). Addition of increased amounts of the α -subunit resulted in increased exonuclease

Figure 4 Stimulation of ϵ mutant exonuclease activities by the α -subunit

Exonuclease reactions containing partial duplex DNA were prepared as described in the Experimental section. The indicated ϵ protein (final concentration 0.5 nM) was mixed with the α -subunit at $10\times$ the indicated final concentration and incubated for 10 min at 24 °C. Samples (1 μ l) of the $\epsilon + \alpha$ (lanes 3–5) or ϵ mutant + α (lanes 7–9, 11–13, 15–17, 19–21, 23–25 and 27–29) mixtures were added to reactions. The reaction in lane 1 contains no α , ϵ or ϵ mutant. The reaction in lane 30 contains no ϵ or ϵ mutant protein. Reactions were incubated at 37 °C for 20 min and the products were subjected to electrophoresis on a 23% (w/v) polyacrylamide denaturing gel. The position of the 23mer is indicated.

activity by wild-type ϵ up to a maximal stimulation of 11-fold in the presence of excess α -subunit (Figure 4, lanes 3–5). Modest levels of exonuclease stimulation ranging from 1.8- to 2.2-fold were detected for the ϵ 504, ϵ 506, ϵ 507 and ϵ 511 mutants (Figure 4, lanes 7–9, 11–13, 15–17 and 19–21 respectively). Similar results were obtained using ϵ 186, ϵ 508, ϵ 510 and ϵ 512 (Table 2). Higher levels of exonuclease stimulation of 5.3- and 18-fold were detected for the ϵ 513 and ϵ 515 mutants (Figure 4, lanes 23–25 and 27–29 respectively). These higher levels of exonuclease stimulation were also detected for the ϵ 501, ϵ 502, ϵ 503, ϵ 509 and ϵ 516 mutants (Table 2). The variable levels of exonuclease stimulation exhibited by the ϵ mutants upon addition of the α -subunit probably reflect alterations in the α – ϵ interaction.

The ϵ 511 mutant was the only ϵ mutant of the 14 tested that demonstrated a tight physical association with α (Figure 3A) and a modest 2.2-fold level of exonuclease stimulation upon formation of the complex (Figure 4). The ϵ 504, ϵ 506, ϵ 507, ϵ 508, ϵ 510 and ϵ 512 mutants did not co-fractionate with the α -subunit during gel filtration chromatography and exhibited very modest 1.2–2.1-fold levels of exonuclease stimulation upon addition of the α -subunit (Table 2). These results are similar to those observed with the ϵ 186 mutant, which lacks the C-terminal region of ϵ and does not associate physically with the α -subunit during gel filtration [9]. In contrast, the ϵ 501, ϵ 502, ϵ 503, ϵ 509, ϵ 513, ϵ 515 and ϵ 516 mutants exhibited a tight physical association with the α -subunit, as shown by co-fractionation during gel filtration chromatography, and also exhibited higher 5.0–18-fold levels of exonuclease stimulation, more similar to the level of stimulation observed with wild-type ϵ upon addition of α (Table 2). Interestingly, only the ϵ 515 mutant exhibited a level of stimulation by the α -subunit as high as the level detected using the wild-type ϵ .

Stimulation of ϵ exonuclease activity by the θ -subunit in the DNA pol III core

The θ -subunit stimulates the exonuclease activity of ϵ slightly [5,8,9], and structural studies of the ϵ – θ complex suggest that θ provides stability in the DNA pol III core [10]. Exonuclease assays were designed to demonstrate stimulation of wild-type ϵ exonuclease activity by the θ -subunit in the DNA pol III core. The activity of ϵ alone was compared with its activity upon association with α , upon association with θ , and upon association with both α and θ in the α – ϵ – θ complex (Figure 5). In this experiment, the activity of ϵ alone resulted in 19 fmol of 3' nucleotides excised (Figure 5, lane 2). Addition of α to ϵ stimulated the ϵ exonuclease activity by 8.5-fold (Figure 5, compare lanes 2 and 3), whereas addition of θ to ϵ had little measurable effect on ϵ exonuclease

Figure 5 Stimulation of ϵ exonuclease activity by the θ -subunit in the DNA pol III core

Exonuclease reactions containing partial duplex DNA were prepared as described in the Experimental section. Wild-type ϵ (lanes 2–5) or mutant ϵ 511 (lanes 6–9), ϵ 513 (lanes 10–13) or ϵ 504 (lanes 14–17) protein was mixed with the α -subunit (lanes 3, 7, 11 and 15), the θ -subunit (lanes 4, 8, 12 and 16), or the α - and θ -subunits (lanes 5, 9, 13 and 17) at $10\times$ the final concentration (0.5 nM) and incubated for 10 min at 24 °C. Samples (1 μ l) of the mixtures were added to reactions. The reaction in lane 1 (NE) contained no enzyme. Reactions were incubated at 37 °C for 20 min and the products were subjected to electrophoresis on a 23% (w/v) polyacrylamide denaturing gel. The position of the 23mer is indicated.

activity (Figure 5, compare lanes 2 and 4). Addition of the θ -subunit to α – ϵ to constitute the α – ϵ – θ DNA pol III core resulted in a modest, but reproducible, further stimulation of exonuclease activity by approx. 15% relative to the activity detected in the α – ϵ complex (Figure 5, compare lanes 3 and 5). These results demonstrate that the θ -subunit directly affects the α – ϵ interaction, resulting in increased 3' exonuclease activity in the DNA pol III core, confirming previous results of others [5,8].

The θ -subunit partially restored stimulation of exonuclease activity of the ϵ 511 mutant in the DNA pol III core (Figure 5). The exonuclease activity of ϵ 511 alone resulted in 15 fmol of 3' nucleotides excised (Figure 5, lane 6), and addition of α or θ had no measurable effect on the activity of ϵ 511 (Figure 5, lanes 7 and 8). Addition of both the α - and θ -subunits to constitute the α – ϵ 511– θ DNA pol III core resulted in a 3.5-fold stimulation of the ϵ 511 exonuclease activity relative to addition of α alone (Figure 5, compare lanes 7 and 9). These results directly demonstrate the ability of θ to stabilize a mutant ϵ protein in the DNA pol III core complex to generate increased levels of exonuclease

activity, and provide further support for our recent structural studies on the θ binding site of ϵ [10]. This partial restoration of ϵ 511 exonuclease activity by addition of θ to constitute the α - ϵ 511- θ DNA pol III core is consistent with the specific contribution of θ to the proper positioning of the α 7 helix of ϵ that might be altered by the Ile $\rightarrow$ Thr change at residue 170 leading to incorrect orientation of the ϵ catalytic residues in the ϵ 511 double mutant (I170T/V215A) with respect to the α -subunit and diminished exonuclease activity in the α - ϵ complex. A mutation at the neighbouring residue, Leu¹⁷¹, has been shown to generate a mutator *dnaQ* allele [22].

The stimulation of ϵ 513 mutant exonuclease activity by θ more closely resembled that with wild-type ϵ . The activity of ϵ 513 alone resulted in 19 fmol of 3' nucleotides excised (Figure 5, lane 10). Addition of the α -subunit stimulated the ϵ exonuclease activity by 4-fold (Figure 5, compare lanes 10 and 11), and addition of the θ -subunit alone had no effect on the ϵ 513 activity (Figure 5, compare lanes 10 and 12). Similar to the result obtained using wild-type ϵ (Figure 5, lanes 2–5), addition of both the α - and θ -subunits to constitute the α - ϵ 513- θ DNA pol III core resulted in a modest further stimulation of exonuclease activity by approx. 20% relative to addition of α alone (Figure 5, compare lanes 11 and 13). However, neither of the exonuclease activities measured in the mutant α - ϵ 511- θ and α - ϵ 513- θ cores were as robust as the level of activity measured in the α - ϵ - θ DNA pol III core (Figure 5, compare lanes 5, 9 and 13). Stimulation of the ϵ 511 and ϵ 513 exonuclease activities contrasted sharply with the apparent lack of stimulation of the ϵ 504 exonuclease by the θ -subunit in the α - ϵ 504- θ core (Figure 5, lanes 14–17). For the other 11 ϵ mutants that were tested, the level of exonuclease stimulation upon addition of α alone was similar to that detected upon addition of α and θ together (results not shown). These results support the idea that the θ -subunit stabilizes the α - ϵ interaction in the DNA pol III core, and mutations in the C-terminal region of ϵ can affect the apparent stabilizing effect conferred in the DNA pol III core by the θ -subunit.

Polymerase/exonuclease ratios in the constituted DNA pol III cores

Efficient, high-fidelity DNA synthesis by the DNA pol III enzyme requires the appropriate balance between the forward polymerization reaction catalysed by the α -subunit and the reverse 3' excision reaction catalysed by the ϵ -subunit. A disruption in the balance between the forward chain elongation and proofreading reactions can result in a mutator DNA pol III with catalytically competent α - and ϵ -subunits [41,42]. The exonuclease assay was modified to measure in the same tube the competing polymerase and exonuclease activities of the α - and ϵ -subunits respectively in the DNA pol III core using a partial duplex DNA substrate. Protein mixtures were pre-incubated with the 5'-³²P-labelled partial duplex DNA in the presence of the next correct nucleotide for forward polymerization, dCTP, and reactions were subsequently initiated by the addition of MgCl₂. In these steady-state reactions, it is likely that the distributive nature of the DNA pol III core [43] results in multiple association/dissociation events from the partial duplex DNA substrate. Thus the ratio of forward polymerization catalysed by the α -subunit (as reflected in the amount of 24-nucleotide product) relative to reverse excision catalysed by the ϵ -subunit (reflected in the amount of oligonucleotide product <23 nucleotides) is indicative of the polymerase/exonuclease ratio in the DNA pol III core enzymes.

In control reactions, the activities of ϵ and the ϵ mutants gave values of 20–25 fmol of 3' nucleotides excised, as indicated by the appearance of oligonucleotide bands of <23 nucleotides in length (Figure 6, lanes 2, 6, 10 and 14). Addition of the α -subunit

Figure 6 Polymerase/exonuclease ratios in the constituted DNA pol III cores

Reactions containing partial duplex DNA were prepared as described in the Experimental section. Wild-type ϵ (lanes 3–6) or mutant ϵ 511 (lanes 7–10), ϵ 513 (lanes 11–14) or ϵ 504 (lanes 15–18) protein was mixed with the α -subunit (lanes 4, 8, 12 and 16), the θ -subunit (lanes 5, 9, 13 and 17) or the α - and θ -subunits (lanes 6, 10, 14 and 18) at 10 $\times$ the final concentration (1 nM) and incubated for 10 min at 24 °C. Samples (2 μ l) of the mixtures were added to reactions and incubated at 37 °C for 5 min prior to initiation by addition of MgCl₂. The reaction in lane 1 (NE) contained no enzyme. Reaction products were subjected to electrophoresis on a 23% (w/v) polyacrylamide denaturing gel. The positions of migration of the 23mer and 24mer are indicated.

to generate the α - ϵ complexes resulted in the appearance of a band of 24 nucleotides in length, corresponding to the addition of a single dCMP nucleotide to the 23mer primer by the α -subunit, and bands of <23 nucleotides in length, corresponding to excision of nucleotides by the ϵ -subunit (Figure 6, lanes 3, 7, 11 and 15). As expected, only bands 23 nucleotides in length or less were detected in reactions containing only ϵ (lanes 2, 6, 10 and 14) or ϵ plus θ (lanes 4, 8, 12 and 16). The reaction products were quantified in assays containing α - ϵ - θ (Figure 6, lane 5), α - ϵ 511- θ (lane 9), α - ϵ 513- θ (lane 13) and α - ϵ 504- θ (lane 17) to determine the polymerase/exonuclease ratios. For the reaction containing α - ϵ - θ , 13 fmol of 24mer and 130 fmol of oligomer product of <23 nucleotides in length were detected, indicating a calculated polymerase/exonuclease ratio of 0.1 for the wild-type DNA pol III core in this assay. For the α - ϵ 511- θ DNA pol III core, 22 fmol of polymerase product was detected, relative to the 62 fmol of exonuclease product. Thus the polymerase/exonuclease ratio for α - ϵ 511- θ was 3.5-fold higher than that for the wild-type core, indicating increased polymerization and reduced excision activity in the α - ϵ 511- θ core. To eliminate the possibility that the different polymerase/exonuclease ratios detected in the α - ϵ - θ and α - ϵ 511- θ mixtures might be attributed to excess ϵ or ϵ 511 not associated in the complexes, the DNA pol III cores were purified by gel filtration chromatography to remove possible excess ϵ or ϵ 511 and θ . Similar results were obtained using the gel filtration-purified DNA pol III core enzymes (results not shown). The polymerase/exonuclease ratios for α - ϵ 513- θ and α - ϵ 504- θ were 2.5- and 8-fold higher respectively than that detected for the wild-type core.

The calculated ratios of polymerase/exonuclease activities were determined for all 14 of the DNA pol III core enzymes, and the results are summarized in Table 3. The ϵ 504, ϵ 506, ϵ 507, ϵ 508, ϵ 510 and ϵ 512 mutants, which did not co-fractionate with the α -subunit during gel filtration chromatography, exhibited the highest polymerase/exonuclease ratios, which were 7.7–9-fold higher than that of the wild-type DNA pol III core. The ϵ 501, ϵ 502, ϵ 503, ϵ 509, ϵ 513 and ϵ 516 mutants, which exhibited a

Table 3 DNA pol III cores

DNA pol III cores were formed with wild-type α -subunit, wild type θ -subunit and the ε -subunit indicated. The mutation frequency (MF) is the number of colonies resistant to both chloramphenicol and 5-fluoro-2'-deoxyuridine divided by the total number of chloramphenicol-resistant colonies; values were averaged from two separate polymerization reactions (means $\pm$ S.D.). The total number of colonies resistant to both chloramphenicol and 5-fluoro-2'-deoxyuridine ranged from 49 to 425. The pol/exo (exonuclease) ratio is the total fmol of 3' termini extended divided by the total fmol of 3' termini excised. Pol activities ranged from 11 to 22 fmol, and exonuclease activities ranged from 14 to 180 fmol.

Polymerase	$10^4 \times$ MF	Increase in MF (fold)	Pol/exo ratio	Relative pol/exo ratio
None	0.3			
Wild-type core	1.6 ± 0.15	1.0	.1	1
α -Subunit	11.0 ± 1.3	7.0	–	–
ε 186 core	2.5	1.5	–	–
MutD5 core	9.2 ± 0.10	6.0	–	–
ε 501 core	1.7 ± 0.31	1.0	0.16	1.6
ε 502 core	1.7 ± 0.18	1.1	0.11	1.1
ε 503 core	3.8 ± 0.23	2.5	0.2	2.0
ε 504 core	4.4 ± 0.42	2.8	0.8	8.0
ε 506 core	4.3 ± 0.18	2.8	0.90	9.0
ε 507 core	4.1 ± 2.6	2.5	0.84	8.4
ε 508 core	5.6 ± 0.76	3.5	0.77	7.7
ε 509 core	2.0 ± 0.23	1.3	0.25	2.5
ε 510 core	4.2 ± 1.3	2.8	0.83	8.3
ε 511 core	3.8 ± 0.58	2.5	0.35	3.5
ε 512 core	3.9 ± 0.70	2.5	0.80	8.0
ε 513 core	3.8 ± 1.7	2.5	0.25	2.5
ε 515 core	3.1 ± 0.88	2.0	0.08	0.80
ε 516 core	3.0 ± 0.40	2.0	0.3	3.0

tight physical association with the α -subunit (as shown by co-fractionation during gel filtration chromatography), also exhibited polymerase/exonuclease ratios higher than that of the wild-type DNA pol III core, consistent with diminished proofreading activity in these enzymes. Only the α - ε 515- θ core exhibited a lower polymerase/exonuclease ratio than the wild-type DNA pol III core.

Mutant DNA pol III cores

A mutator phenotype exhibited *in vivo* by a *dnaQ* allele could result from perturbations in the α - ε (polymerase–exonuclease) interaction within the holoenzyme complex. To determine if we could measure an effect on the fidelity of synthesis by DNA pol III as a consequence of mutations in the ε proteins, DNA pol III core enzymes were constituted with purified α - and θ -subunits and the purified mutant ε proteins, and the accuracy of DNA synthesis by these enzymes was measured *in vitro*. The α -subunit DNA pol makes relatively few base substitution errors during *in vitro* DNA synthesis when measured in kinetic [44] and in forward mutation [45] assays. We selected a sensitive forward mutation assay designed by Eckert et al. [33] and used nucleotide pool imbalances during the *in vitro* DNA synthesis reactions to detect polymerization errors by the α -subunit DNA pol and the DNA pol III core enzyme (Table 3). In this assay, the DNA pol III enzymes synthesize DNA across a 203-nucleotide region of the HSV-*tk* gene. Polymerization errors generated during the *in vitro* DNA synthesis reactions inactivate the HSV-*tk* gene and are detected by resistance to 5-fluoro-2'-deoxyuridine conferred to cells containing a mutated copy of the HSV-*tk* gene [33,46]. Synthesis by DNA pol III core in the presence of equal nucleotide concentrations failed to generate detectable

mutations in the HSV-*tk* gene above the background mutation frequency (results not shown). When the nucleotide pools were biased 10-fold as described in the Experimental section, a 5-fold increase in mutation frequency over the background was detected during DNA synthesis *in vitro* by the DNA pol III core enzyme (Table 3). Under these conditions, synthesis by the α -subunit alone resulted in a 37-fold increase in the mutation frequency relative to background and a 7-fold increase in mutation frequency relative to the DNA pol III core-copied DNA (Table 3). These results establish the ability to detect polymerization errors in this assay by the core DNA pol III. The contribution of exonucleolytic proofreading *in vitro* by ε in the core DNA pol III in this assay is apparent from the 7-fold increase in mutation frequency detected in reactions containing only the α -subunit.

An increase in mutation frequency of at least 2-fold was apparent during DNA synthesis catalysed by 11 of the 14 DNA pol III cores constituted with the mutant ε proteins. The increases in mutation frequencies for these enzymes ranged from 2- to 3.5-fold above that observed for the wild-type DNA pol III core (Table 3). Increased mutation frequencies in these *in vitro* DNA synthesis reactions can be attributed either to deficiencies in the exonuclease catalytic activities or to dysfunctional exonuclease–polymerase interactions. To determine the effect of deficient ε exonuclease activity in the forward mutation assay, we constituted the DNA pol III core with the MutD5 ε protein (98 % reduction in activity) and measured the accuracy of DNA synthesis by this enzyme. The MutD5 core enzyme showed a 6-fold increase in mutation frequency relative to the wild-type DNA pol III core. The accuracy of the MutD5 DNA pol III core is similar to that of the α -subunit alone. To determine the effect of deficient exonuclease–polymerase interactions in the forward mutation assay, we prepared the DNA pol III core with ε 186 (> 400-fold reduction in binding to α -subunit [9]) and measured the accuracy of DNA synthesis by this enzyme. The ε 186 core enzyme showed a 1.5-fold increase in mutation frequency relative to the wild-type DNA pol III core. During *in vitro* DNA synthesis, the DNA pol III α -subunit extends misinserted bases poorly and is likely to dissociate upon nucleotide misinsertion [40,45]. The modest increase of only 1.5-fold in mutation frequency observed with the ε 186 core is consistent with dissociation of the α -subunit upon nucleotide misinsertion and subsequent excision of misinserted base by the independent action of ε 186. The relative contributions of catalytic deficiencies and exonuclease–polymerase dysfunction in the ε mutants were not revealed in these studies.

DISCUSSION

The identification of mutations in the *dnaQ* gene highlights the importance of the ε subunit for the accurate replication of the *E. coli* chromosome. Strains carrying mutations in *dnaQ* confer a mutator phenotype with increased mutation frequencies of as little as 2-fold and as much as 10^5 -fold [20,39,40]. More recent data on the structure of the ε -subunit polypeptide encoded by *dnaQ* requires that several mechanisms of ε dysfunction be considered in *dnaQ* alleles that generate the mutator phenotype. The stable complex of α , ε and θ implies an intimate co-ordination between these separate polypeptides in the DNA pol III core to facilitate the polymerization and proofreading steps that result in high-fidelity DNA synthesis in *E. coli* and other Gram-negative bacteria. Mutations in *dnaQ* affecting amino acids in ε that perform a critical function in the catalysis of 3' nucleotides are predicted to decrease the catalytic efficiency of the proofreading component of DNA pol III, increasing mutations during chromosomal replication. The proofreading exonuclease active site of

the ϵ -subunit resides within the N-terminal 186 amino acids [9]. Structural studies of this ϵ fragment by us [10,24] and others [25] provide support for the location of most of the previously identified mutator residues in ϵ at or near the region of the protein that is directly involved in interactions with the 3' end of a DNA substrate. Mutators at several acidic residues have been identified [22], and the ϵ 186 structure confirms the role of these residues in metal co-ordination critical for catalysis [24,25]. Mutator residues have been identified at positions in ϵ that make direct contacts with the DNA substrate and at positions that provide structural stability in the ϵ catalytic core through side chain and backbone contacts, providing rigidity in secondary structure elements in regions near the DNA binding pocket. Thus *dnaQ* mutator alleles that result in amino acid changes in the N-terminal 186 residues most probably reduce the catalytic efficiency of the DNA pol III proofreading enzyme to generate the mutator phenotype.

Mutations in *dnaQ* affecting the C-terminal region of ϵ generate mutator effects in *E. coli* through dysfunctional proofreading despite a catalytically active ϵ -subunit. The *dnaQ932* mutator allele [22] truncates the ϵ protein by three amino acids, and the *dnaQ-A* mutator allele [21] contains a single amino acid change (A223V) in the C-terminal region of ϵ . The exonuclease activities of these ϵ proteins have not been tested, but the positions of these changes are not predicted to affect the catalytic activities of these ϵ proteins. The identification of these mutator alleles suggested that complete catalytic function of ϵ is not sufficient *in vivo* to provide high-fidelity replication of the chromosome. The stable association between ϵ and α is established through the C-terminal region of ϵ [9,26], and elimination of the α -association domain of ϵ , as is the case in the *dnaQ991* allele, results in a dramatic mutator phenotype [22]. While complete elimination of the α - ϵ association results in a mutator phenotype, little is known about the effects of less obtrusive amino acid changes in the C-terminal region of ϵ on the α - ϵ association or interaction and contributions to a mutator phenotype.

In the present study, we developed a strategy to generate *dnaQ* mutants that retain ϵ catalytic activity and α - ϵ association, but exhibit a mutator phenotype due to a presumed proofreading defect related to the polymerase-exonuclease interaction. As predicted from the screening strategy, all of the 14 purified ϵ mutants tested had exonuclease activity (Table 2). Reduced catalytic activity was measured for some of the ϵ mutants, and this diminished catalytic activity in some of the ϵ mutants might contribute to the mutator phenotype. It is not possible at this time to assess the significance of the variable levels of exonuclease activity of the purified ϵ proteins with respect to *in vivo* mutagenesis. Surprisingly, we were able to demonstrate a stable α - ϵ interaction by gel filtration chromatography for only eight of the ϵ mutant proteins (ϵ 501, ϵ 502, ϵ 503, ϵ 509, ϵ 511, ϵ 513, ϵ 514 and ϵ 516). Eleven of the 14 DNA pol III core enzymes constituted with the ϵ mutants had diminished proofreading activity, as indicated by the increased mutation frequencies measured during *in vitro* DNA synthesis (Table 3).

The ϵ 511 protein exhibits properties indicative of dysfunctional proofreading when incorporated into the α - ϵ 511- θ DNA pol III core. The 3' exonuclease activity of the purified ϵ 511 protein is equal to that of the wild-type ϵ protein when measured using single-stranded and duplex DNA (Table 2). ϵ 511 co-fractionates with the α -subunit (Figure 3) and with the θ -subunit (results not shown) during gel filtration chromatography, indicating retention of the tight physical association between these three DNA pol III core subunits. Dysfunction in the α - ϵ 511- θ DNA pol III core is indicated by the diminished stimulation of ϵ 511 exonuclease activity using duplex DNA upon association with the α -subunit (Figure 4) and only partial restoration of this activity in the core

complex (Figure 5). The higher polymerase/exonuclease ratio of the α - ϵ 511- θ DNA pol III core relative to the wild-type enzyme (Figure 6) and decreased fidelity *in vitro* (Table 3) further substantiate the notion of dysfunctional proofreading in this complex. It seems likely that a channel or cleft exists between the α -subunit polymerase and the ϵ -subunit exonuclease active sites in DNA pol III. The *dnaQ511* mutator allele encodes an ϵ double-mutant protein with the two amino acid changes, I170T and V215A, suggesting that these positions in ϵ are involved in a pathway for DNA strand transfer from the α -subunit DNA pol active site to the ϵ -subunit exonuclease active site in the DNA pol III enzyme. Structural studies indicate that the α 7 helix of ϵ probably forms part of this channel at the entrance to the exonuclease active site [10,24,25]. Thus the position of the α 7 helix and the effect of the θ -subunit on the orientation of this structural component of ϵ in DNA pol III might enable the DNA to be shuttled into the exonuclease active site, and mutations in this region might impede the transfer of DNA between the polymerase and exonuclease sites.

The six *dnaQ* mutator alleles encoding proteins ϵ 504, ϵ 506, ϵ 507, ϵ 508, ϵ 510 and ϵ 512 that did not demonstrate stable interactions with the α -subunit *in vitro* by gel filtration chromatography raise additional possibilities for the expressed *in vivo* mutator phenotype. The ϵ proteins encoded by the *dnaQ504* (E190K, A200V), *dnaQ507* (I202S), *dnaQ508* (Q169L) and *dnaQ510* (E153K/A177V) alleles contain no amino acid changes in the C-terminal 40 amino acids, demonstrated previously to be sufficient for association of ϵ with α [9,26]. Thus no obvious explanation for the lack of stability in the α - ϵ mutant complexes *in vitro* can be proposed. Assuming that these ϵ mutants are incorporated into the holoenzyme complex *in vivo*, as required by the screening strategy used in the present study, perhaps these ϵ mutations affect more global protein-protein interactions within the replication apparatus, or affect specific interactions of ϵ with other proteins positioned at the replication fork to generate mutator effects *in vivo*. The *umuD* and *umuC* gene products encode the UmuD₂C (pol V) enzyme [47,48], and a relationship between UmuD₂C/pol V and pol III holoenzyme has long been suggested in genetic studies. More recent work indicates a direct interaction between the C-terminal domain of ϵ and the UmuD or UmuD' proteins of pol V [49,50]. Possible effects on this interaction of the ϵ proteins encoded by the mutator *dnaQ* alleles identified in the present study have not been tested. The possibility that error-prone DNA pol V participates in chromosomal replication under circumstances where DNA pol III is defective in the ϵ subunit due to *dnaQ* mutations that generate a mutator phenotype has been proposed [51], and must be considered for the *dnaQ* mutator alleles identified here.

The misinsertion of nucleotides by DNA pols causes a kinetic block to subsequent polymerization [52]. Proofreading exonucleases alleviate this kinetic block by removing the terminal mismatch. Studies using damaged DNA templates have led to the suggestion that when DNA pol III encounters a blocking lesion, it detaches from the 3' terminus and one of the lesion bypass polymerases pol II, pol IV or pol V are recruited [53]. It might be appropriate to extend this proposed mechanism to include a proofreading-deficient DNA pol III that is kinetically blocked at the replication fork after insertion of a terminal mismatch. Recruitment of a DNA pol with relaxed fidelity could provide a mechanism for continued replication of the chromosome, albeit at reduced fidelity.

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