The θ Subunit of *Escherichia coli* DNA Polymerase III: a Role in Stabilizing the ϵ Proofreading Subunit

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The function of the θ subunit of *Escherichia coli* DNA polymerase III holoenzyme is not well established. θ is a tightly bound component of the DNA polymerase III core, which contains the α subunit (polymerase), the ϵ subunit (3'→5' exonuclease), and the θ subunit, in the linear order α - ϵ - θ . Previous studies have shown that the θ subunit is not essential, as strains carrying a deletion of the *holE* gene (which encodes θ) proved fully viable. No significant phenotypic effects of the *holE* deletion could be detected, as the strain displayed normal cell health, morphology, and mutation rates. On the other hand, *in vitro* experiments have indicated the efficiency of the 3'-exonuclease activity of ϵ to be modestly enhanced by the presence of θ . Here, we report a series of genetic experiments that suggest that θ has a stabilizing role for the ϵ proofreading subunit. The observations include (i) defined Δ *holE* mutator effects in mismatch-repair-defective *mutL* backgrounds, (ii) strong Δ *holE* mutator effects in certain proofreading-impaired *dnaQ* strains, and (iii) yeast two- and three-hybrid experiments demonstrating enhancement of α - ϵ interactions by the presence of θ . θ appears conserved among gram-negative organisms which have an exonuclease subunit that exists as a separate protein (i.e., not part of the polymerase polypeptide), and the presence of θ might be uniquely beneficial in those instances where the proofreading 3'-exonuclease is not part of the polymerase polypeptide.

The DNA polymerase III (Pol III) holoenzyme (HE) is the major chromosomal replication enzyme in *Escherichia coli* (19, 22, 30, 31). The enzyme is composed of 17 subunits, 10 of which are distinct (19, 31). HE contains two polymerase core molecules, each consisting of an α , ϵ , and θ subunit arranged in the linear order α - ϵ - θ . The two cores are connected through a dimer of the τ subunit, establishing the basic arrangement of a dimeric polymerase that simultaneously replicates the leading and lagging strands (14). In addition, the HE contains, for each core, a β -clamp (β_2) tethering the polymerase to the DNA to ensure high processivity and the five-subunit γ -complex ($\gamma\delta\delta'\chi\psi$) responsible for loading and unloading the β -clamp. The precise functioning of the HE complex in chromosomal replication is under active investigation (5, 24, 26, 31).

Within the Pol III core, α (the *dnaE* gene product) is the DNA polymerase, while ϵ (the *dnaQ* gene product) is the 3'→5' proofreading exonuclease. The function of θ (the *holE* gene product), which is tightly bound to ϵ , is unclear. Genetic analysis of the Pol III core constituents has provided insight into the role of its constituents. For example, *dnaE*(Ts) mutants, encoding temperature-sensitive polymerase subunits, are conditionally lethal, as expected in view of the essential nature of the Pol III replication function. Several *dnaE* mutants display mutator or antimutator effects (9, 28), indicating the important fidelity role of this enzyme. Many *dnaQ* mutants exhibit strong mutator phenotypes, indicating the importance of the 3'-exonuclease activity for replication fidelity (45). Dele-

tion mutants of *dnaQ* (23, 27) or mutants lacking the domain necessary for interaction with the polymerase (46) have been generated, but these mutants proved essentially inviable unless accompanied by a suppressing mutation in *dnaE*. Based on these studies, ϵ is assigned at least two functions: a fidelity function through its 3'-exonuclease activity and a structural function based on its tight, and presumably stabilizing, interaction with the polymerase (23, 27, 45, 46).

In contrast, the role of the θ subunit of the Pol III core is unknown. Loss of θ (Δ *holE*) results in healthy cells with no morphology changes and little or no change in mutant frequencies (42). Based on these studies, it was suggested that θ is not necessary or important for proper functioning of the Pol III core. θ does not affect DNA synthesis by α or α - ϵ (43); however, gel filtration (44), coexpression (1), and yeast two-hybrid experiments (18) have demonstrated a tight interaction between θ and ϵ , but none between θ and α . Interestingly, θ was shown to moderately affect the 3'→5' proofreading exonuclease activity, as addition of θ to an exonuclease assay measuring the removal of a G·T mispair increased ϵ -mediated excision of the terminal T residue by about 2.5-fold (44).

The above findings suggest that θ , while not essential, could play a role in DNA replication and its fidelity, presumably indirectly through its interaction with the ϵ subunit. The precise nature of this interaction is being pursued structurally by both nuclear magnetic resonance (NMR) and crystallography studies. Structures of both ϵ and θ have been reported (6, 15, 20), as well as the ϵ - θ interaction surface on ϵ (7). Here, we report on a series of genetic experiments on the ϵ - θ interaction. Specifically, we have studied (i) in greater detail, the possible mutator effect resulting from a *holE* deletion, (ii) the effect of the *holE* deletion on *dnaQ* mutator mutants, and (iii) the effect of θ on the α - ϵ interaction as measured by yeast two- and

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TABLE 1. List of *E. coli* strains used

Strain	Relevant genotype	Source or reference
MG1655	Wild type	41
ES1293	<i>mutL211::Tn5</i>	40
RM4030	<i>holE201::cat</i>	42
KA796	<i>ara thi Δ(pro-lac)</i>	38
NR9559	<i>mutL211::Tn5</i>	KA796 $\times$ P1/ES1293
CC101...CC111	<i>ara thi Δprolac F'(pro-lacIZ)</i>	4, 5
NR11852	<i>holE201::cat</i>	KA796 $\times$ P1/RM4030
NR11853	<i>mutL211::Tn5 holE201::cat</i>	NR9559 $\times$ P1/RM4030
NR9695	<i>dnaQ49 zae-502::Tn10</i>	37
NR9458	<i>mutD5 zaf-13::Tn10</i>	37
NR9501	<i>mutL211::Tn5</i>	45
NR9606	<i>mutL211::Tn5</i>	9

three-hybrid assays. The results suggest that θ may be a stabilizing factor for the ϵ subunit, which has been shown to be intrinsically unstable (11).

MATERIALS AND METHODS

Media. Luria-Bertani (LB) medium and minimal glucose medium (MM) were standard recipes (38). MMLac plates for determination of the *lacZ* reversion mutant frequency contained 0.2% lactose instead of glucose. Antibiotics were tetracycline (15 μ g/ml), rifampin (100 μ g/ml), kanamycin (50 μ g/ml), ampicillin (100 μ g/ml), and chloramphenicol (20 μ g/ml). For the yeast two-hybrid assay, yeast (*Saccharomyces cerevisiae*) strain Y187 (16) was grown in yeast-peptone-dextrose medium (standard recipe). Yeast strain Y190 (Y153, but *cyh*^r) (American Type Culture Collection, Manassas, Va.) was grown in yeast-peptone-dextrose for use in the yeast three-hybrid assay. For yeast transformation, yeast synthetic minimal medium was prepared as described previously (34).

Strains. The *E. coli* strains used are listed in Table 1. Constructions were generally performed by P1 transductions using P1virA. The *holE201::cat* allele was introduced from RM4030 (42) using chloramphenicol resistance as the selectable marker. The *mutL211::Tn5* allele was transferred from ES1293 (40) using kanamycin resistance. The *dnaQ49* allele was transferred from NR9695 (37) with the tetracycline resistance conferred by the *zae-502::Tn10* transposon, followed by testing for mutator phenotype ($\sim$ 40% linkage). The *mutD5* mutator allele was transferred from NR9458 (37) with the tetracycline resistance conferred by *zaf-13::Tn10*, followed by testing for mutator phenotype (90% linkage). The mutagenesis experiments shown below in Table 2 were performed with strains NR9559 (*mutL*) and NR11853 (*mutL holE*) into which the F'(*pro-lacIZ*) from strains CC101 through CC111 (3, 4) had been transferred by conjugation (F' transfer). The experiment shown below in Table 3 was performed using *dnaQ* and *dnaQ holE* derivatives of the mismatch-repair-defective strains NR9606 or NR9501. The complete genotype of NR9606 is *hisG4*(Oc) *trp-3*(Oc) *metE46 thi-1 fluA1 ara-9 tsx-3 supE44 galK2 λ^- rfbD1⁺ rpsL8 or rpsL9 malA1(λ^-), mutL1 lacZ118(Oc) *mutL::Tn5*, as described by Oller et al. (32). NR9501 is identical to NR9606 except that it contains *lacZ75*(Fs) instead of *lacZ118*(Oc) (J.-Y. Mo and R. M. Schaaper, unpublished data). This distinction is not relevant to the experiments presented here. Into these strains, we first introduced the series of *dnaQ* mutator alleles described by Taft-Benz and Schaaper (45), followed by introduction of Δ *holE*. In the case of the very strong *mutD5* and *dnaQ49* alleles, Δ *holE* was inserted first, followed by *mutD5* or *dnaQ49*.*

Plasmids. The plasmids used in the yeast two-hybrid assay were described previously (18, 46). The *dnaE* and *holE* genes were contained in plasmid pGAD424, while the various *dnaQ* alleles were contained in plasmid pGBT9-2 (18). Insertion of the *dnaQ* gene from the various *dnaQ* mutator strains was performed as described by Jonczyk et al. (18).

For the three-hybrid assay, vector pBridge (Clontech) was restricted with EcoRI and PstI and treated with calf intestinal alkaline phosphatase (CIAP). The *dnaQ* and *dnaQ49* genes from plasmids pGBT9-2*dnaQ* or pGBT9-2*dnaQ49* (18) were excised using EcoRI and PstI, and the *dnaQ* fragments were ligated into the EcoRI-PstI-CIAP-treated pBridge to create pBridge/*dnaQ* and pBridge/*dnaQ49*, respectively. The recombinant plasmids were sequenced to verify in-frame insertion and correctness of the inserted sequences. To insert the *holE* gene, pBridge/*dnaQ* and pBridge/*dnaQ49* were restricted with NotI and BglII and treated with CIAP. The entire *holE* gene (start to stop codon) was

PCR amplified from genomic DNA prepared from strain MG1655 (41) using primers containing NotI (forward primer) or BglII (reverse primer) restriction sites. The forward primer was 5'-AGTGTGGCGGCCGAATGCTGAAGAA TCTGGCTAAA-3' (the ATG start codon is underlined), and the reverse primer was 5'-GCGTGTGCGAGATCTCAGGCGTTATGTAAGAAAGTG-3'. The PCR product was restricted with NotI and BglII, gel purified, and ligated into NotI-BglII-CIAP-treated pBridge/*dnaQ* and pBridge/*dnaQ49*. The new plasmids, sequenced to verify correct in-frame sequence of the *holE* gene, were designated pBridge/*dnaQholE* and pBridge/*dnaQ49holE*, respectively. The *dnaE* plasmid used in this assay was pGAD42*dnaE*, as in the two-hybrid assay.

Mutant frequency determinations. In each experiment, mutant frequencies were determined from 8 to 15 independent cultures from each strain grown overnight in 1 ml of LB medium with agitation at the indicated temperature. Aliquots of appropriate dilutions were plated on LB-rifampin or MMLac plates to determine the number of Rif^r or Lac⁺ mutants, respectively. The total number of cells was determined by plating aliquots of appropriate dilutions on LB or MM plates, respectively. Mutant frequencies were calculated by dividing the median number of mutant colonies by the total number of cells.

Yeast two- and three-hybrid assays. Yeast strains were grown as previously described (18). For the two-hybrid assays, yeast strain Y187 was transformed (2) simultaneously with pGBT9-2*dnaQ* and pGAD42*dnaE* to permit assaying of the α - ϵ interaction, or simultaneously with pGBT9-2*dnaQ* and pGAD42*holE* to permit assaying of the ϵ - θ interaction. The *dnaQ* gene was either the wild type or any of the mutant *dnaQ* alleles listed below in Table 4. The assays were performed at 30°C as described before (46). For the three-hybrid assay, strain Y190 was transformed simultaneously with pGAD42*dnaE* and either pBridge/*dnaQholE* or pBridge/*dnaQ49holE*. The assay was performed as for the two-hybrid assay, except that 1 mM L-methionine was included in half of the cultures. β -Galactosidase activity was determined as described by Taft-Benz and Schaaper (46).

RESULTS

Mutator effect of *holE* deletion. Slater et al. (42) measured the effect of a *holE* deletion on spontaneous mutant frequencies and observed no significant effect on the rate of nalidixic acid-resistant (Nal^r) mutants and only a slight increase in the rate of rifampin-resistant (Rif^r) mutants. On this basis, it was concluded that θ plays no significant role in the fidelity of DNA replication. In order to reevaluate this question, we again measured mutant frequencies in Δ *holE* strains, using two modifications. First, we used mismatch-repair-defective (*mutL*) strains. In these strains, replication errors are not corrected and, hence, such strains provide a more direct view of replication errors. Second, we used a series of *lacZ* alleles that revert via defined base-pair substitution or frameshift pathways (3, 4). Such reversion systems might demonstrate effects on replication fidelity that would be obscured in forward systems such as Rif^r or Nal^r, in which multiple sites are scored simultaneously. We measured the number of *lac*⁺ revertants in *mutL* and *mutL Δ holE* strains carrying the *lacZ* alleles from strains CC101 through -111 (Table 2). We noted that mutant frequencies were consistently increased for several, although not all, of the *lac* alleles. The CC101 allele (A $\cdot$ T $\rightarrow$ C $\cdot$ G) proved quite variable in three independent experiments. We have observed significant variability with this allele in related experiments, presumably due to the variable production of 8-oxo-dGTP, which is believed responsible for the measured A $\cdot$ T $\rightarrow$ C $\cdot$ G transversion (12). More consistent results were obtained for the CC104 (G $\cdot$ C $\rightarrow$ T $\cdot$ A) allele (3.8-fold average increase), the CC105 (A $\cdot$ T $\rightarrow$ T $\cdot$ A) allele (3.5-fold average increase), and the CC110 (+A frameshift) allele (4.5-fold increase). Smaller increases were observed for strains containing the CC106 (A $\cdot$ T $\rightarrow$ G $\cdot$ C) (2.7-fold increase) and CC111 (−A

TABLE 2. *lac* reversion frequencies in mismatch-repair-defective *hol*⁺ and Δ *holE* strains^a

<i>lac</i> allele from strain:	Reversion event	Reversion frequency (per 10 ⁸ cells)		Δ <i>holE</i> / <i>hol</i> ⁺
		<i>hol</i> ⁺	Δ <i>holE</i>	
CC101	A · T → C · G	0.2	1.8	9
		0.6	1.3	2
		0.68	0.62	1.0
CC102	G · C → A · T	64	67	1.0
		100	58	0.6
		72	65	0.9
CC103	G · C → C · G	ND	ND	
		ND	ND	
		ND	ND	
CC104	G · C → T · A	2.9	22	7.6
		3.4	5.4	1.6
		3.4	8.1	2.4
CC105	A · T → T · A	1.2	5.0	4.2
		0.7	2.3	3.3
		0.68	2.1	3.1
CC106	A · T → G · C	17	82	4.8
		37	53	1.4
		29	55	1.9
CC107	+G	13,400	13,000	1.0
		19,800	19,600	1.0
CC108	-G	4,700	4,700	1.0
		3,700	4,900	1.3
CC109	-GC	5,100	5,700	1.1
		1,600	2,200	1.4
CC110	+A	96	430	4.5
		110	500	4.5
CC111	-A	1,900	4,200	2.2
		1,300	1,800	1.4

^a These experiments were performed in the mismatch-repair-defective *mutL* background. The strains used were NR9559 (*mutL*) and NR11853 (*mutL* Δ *holE*) (Table 1) into which the F'(*pro-lacIZ*) from the series CC101 to CC111 (4, 5) had been introduced by conjugation. The base-substitution or frameshift mutation measured by each *lac* allele is indicated in the second column. The multiple entries for each allele represent results of two or three independent experiments, each comprising 12 or 15 independent cultures for each strain. Entries in bold are alleles for which consistent *holE* mutator effects were observed. ND, no mutants detected.

frameshift) (1.8-fold increase) alleles. These increases in both base-substitution and frameshift mutant frequencies in Δ *holE* strains suggest that θ does play a role in DNA replication fidelity.

The *holE201::cat* construct contains a partial deletion of *holE*, resulting in deletion of the C-terminal 53 amino acids of θ (42). Although it is unlikely that the remaining 23 N-terminal amino acids have any residual activity, we also constructed a complete deletion of *holE* (residues 1 to 76) and again measured the above reversion frequencies. The data showed, likewise, modest but consistent mutator effects similar to those for strains carrying the *holE201::cat* construct (data not shown).

Effect of the *holE* deletion on the *dnaQ49*(Ts) allele. The effect of θ on *lac* mutant frequencies, as described above, is presumably due to its effect on the proofreading ϵ subunit with which it tightly interacts (1, 33, 44). To further investigate this

FIG. 1. Effect of *holE* on the Rif^r mutant frequencies of *dnaQ49* strains. Mutant frequencies were determined as described in Materials and Methods from 15 independent cultures grown at the indicated temperatures. The strains used were KA796 (wild type) and NR11852 (Δ *holE*) and their respective *dnaQ49* transductants, constructed as indicated in Materials and Methods. Note the log scale on the y axis. The frequencies for the two *dnaQ*⁺ strains (*hol*⁺ and Δ *holE*) were similar in this experiment, and the data points overlap (line indicated by *dnaQ*⁺).

possibility, we studied the effect of the θ deletion on a recessive *dnaQ*(Ts) allele, *dnaQ49*. The DnaQ49 protein contains an amino acid substitution (V96G) (47) that has been proposed to result in temperature-dependent destabilization of the protein. Based on the recessive nature of this allele (29), it was suggested that this destabilization leads to progressive loss of binding to the α subunit, resulting in the observed temperature-dependent mutator phenotype (17, 29). Indeed, Jonczyk et al. (18), using a yeast two-hybrid assay, showed that the DnaQ49 protein had decreased binding to the α subunit. Based on these findings, we reasoned that the unstable DnaQ49 protein might display increased sensitivity to the loss of the θ subunit.

We constructed *dnaQ49 holE*⁺ and *dnaQ49* Δ *holE201::cat* strains and measured their respective Rif^r mutant frequencies at various temperatures. The control *dnaQ*⁺ strains showed slightly elevated mutant frequencies at 37°C compared to 30°C (Fig. 1), as also noted by others (13). The *dnaQ49* strain, as expected, exhibited a large temperature-dependent increase in mutant frequency: at 25°C there was a modest 10-fold mutator effect and at 30°C the effect was about 100-fold, while at 37°C the effect was greater than 1,000-fold. Interestingly, the mutant frequency of the *dnaQ49* Δ *holE* strain was consistently elevated to the highest level, regardless of temperature. At 25°C, the strain showed a 10,000-fold mutator activity, more than 1,000-fold higher than the corresponding *dnaQ49 hol*⁺ strain. The mutant frequency reached, greater than 100×10^{-6} , is essentially at the level observed in strains completely defective in proofreading (10). The *dnaQ49* Δ *holE* strain also suffered from viability problems, as evidenced by lower viable titers in

TABLE 3. Rif^r mutant frequencies in *dnaQ* mutators with or without the θ subunit^a

<i>dnaQ</i> allele	Amino acid substitution	No. of mutants/10 ⁶ cells		$\Delta holE/hol^+$
		<i>hol</i> ⁺	$\Delta holE$	
<i>dnaQ</i> ⁺		3.4	2.5	
<i>dnaQ49</i>	V96G	105	440	4.1
<i>mutD5</i>	T15I	257	227	
<i>dnaQ920</i>	R56W	36	178	4.9
<i>dnaQ921</i>	G180D	28	30	
<i>dnaQ923</i>	H66Y	32	161	5.0
<i>dnaQ924</i>	L171F	11	144	13
<i>dnaQ928</i>	G17S	44	141	3.2
<i>dnaQ930</i>	H98Y	26	21	
<i>dnaQ932</i>	W241Ochre	29	30	
<i>dnaQ933</i>	G180N	45	33	

^a All strains in this experiment were also mismatch repair defective (*mutL::Tn5*). The strains were NR9501 or NR9606 (Table 1), into which the respective *dnaQ* alleles and the $\Delta holE$ allele were introduced as indicated in Materials and Methods. The amino acid substitutions for each of the *dnaQ* mutator mutants were as described previously (45). The experiments were performed at 37°C. The results represent the average mutant frequency for two to five experiments for each strain, with each experiment comprising eight independent cultures. The results indicated for the *dnaQ921* allele are the combined results for the *dnaQ921*, *dnaQ922*, and *dnaQ927* alleles, which all contained the same mutation, G180D (45), and which yielded indistinguishable results in this test. The entries in bold highlight the *dnaQ* alleles whose mutability was significantly enhanced by the absence of θ .

overnight cultures and the production of significantly smaller and more heterogeneous colonies on plates, as seen previously for near-completely proofreading-defective strains (10). These data suggest that θ plays an important role in stabilizing the intrinsically unstable DnaQ49 protein.

Effect of $\Delta holE$ on other *dnaQ* alleles. Since the DnaQ49 (V96G) protein is thought to be structurally compromised, we were interested to see if θ would exert a similar stabilizing effect on other, potentially less compromised mutant DnaQ proteins. Previously, we characterized in some detail a large number of new *dnaQ* mutator mutants (45). Ten of these displayed relatively moderate increases in mutant frequencies at 37°C compared to the *dnaQ49* strain, suggesting that these mutant *dnaQ* alleles encode proofreading subunits with less severe defects (45). For these 10 *dnaQ* alleles, we determined the Rif^r mutant frequency in both *holE*⁺ and $\Delta holE$ backgrounds at 37°C. We also included the *dnaQ49* and *mutD5* alleles. *mutD5* (T15I) is a strong, dominant, and temperature-independent mutator allele whose defect is thought to be catalytic with little effect on the protein structure (10, 29, 45). The strains used in this experiment were also mismatch repair defective (*mutL*). The use of the *mutL* background avoids complications brought about by saturation of mismatch repair that can occur in proofreading-defective strains (10, 35, 36, 39), and the effect of θ on the proofreading ability can be more effectively evaluated in this background. The results described in Table 3 indicate that four of the *dnaQ* alleles showed θ -related changes in mutant frequency similar to those of the *dnaQ49* strain: *dnaQ920* (R56W), *dnaQ923* (H66Y), *dnaQ924* (L171F), and *dnaQ928* (G17S). A 3.2- to 13-fold increase in mutant frequency at 37°C was observed upon loss of θ . In contrast, no effect was observed for the *mutD5* mutant. These data suggest that θ is generally capable of stabilizing a certain

TABLE 4. Yeast two-hybrid analysis of α - ϵ and ϵ - θ interactions for *dnaQ* mutator alleles^a

<i>dnaQ</i> allele	% β -Gal activity	
	α - ϵ interaction	ϵ - θ interaction
<i>dnaQ</i> ⁺	100	100
<i>dnaQ49</i>	4	0.01
<i>mutD5</i>	96	78
<i>dnaQ920</i>	5	28
<i>dnaQ921</i>	87	73
<i>dnaQ922</i>	84	100
<i>dnaQ923</i>	10	49
<i>dnaQ924</i>	6	83
<i>dnaQ927</i>	21	80
<i>dnaQ928</i>	3	59
<i>dnaQ930</i>	3	45
<i>dnaQ932</i>	2	74
<i>dnaQ933</i>	92	70

^a The two-hybrid assay was performed at 30°C as described by Taft-Benz and Schaaper (46). β -Galactosidase (β -gal) activity was measured and expressed in units (amount of *o*-nitrophenyl- β -D-galactopyranoside hydrolyzed per minute) and presented as the percentage of the *dnaQ*⁺ control value. The 100% values were 0.173 for the α - ϵ interaction and 56 for the ϵ - θ interaction. The *dnaQ* alleles are as described in Table 3. The ones indicated in bold were impaired in α - ϵ interaction as measured by this assay; with the exception of *dnaQ932* (see text), they correspond to the alleles sensitive to loss of θ in the mutagenesis experiment shown in Table 3. The numbers represent the average values for three separate transformants, each assayed in triplicate.

class of impaired ϵ subunits. It is likely that these mutants, like *dnaQ49*, carry defects that bear a structural component.

α - ϵ interactions as measured by yeast two-hybrid assays. Whether the *dnaQ* alleles that proved sensitive to the absence of θ suffer, like *dnaQ49*, from impaired interactions with the polymerase subunit was investigated using the yeast two-hybrid system. This system was used previously to evaluate both α - ϵ and ϵ - θ interactions (18, 46). Consistent with the genetic data, the DnaQ49 protein, but not the MutD5 protein, was shown to be impaired in its binding to the α subunit (18). Here, we inserted the various mutant *dnaQ* genes in the pGBT9-2*dnaQ* fusion vector (see Materials and Methods) and measured the relative strength of the α - ϵ and ϵ - θ interactions. The data in Table 4 indicate that among the tested *dnaQ* alleles, in addition to *dnaQ49*, several alleles (*dnaQ920*, *dnaQ923*, *dnaQ924*, *dnaQ928*, *dnaQ930*, and *dnaQ932*) suffered from significantly reduced α - ϵ interaction. The interaction of ϵ with θ was not affected significantly for any of the alleles except for *dnaQ49*. The ϵ - θ deficiency of *dnaQ49* at 30°C (but not at 25°C) has been described elsewhere (18, 46).

The reduced α - ϵ interaction for the *dnaQ920*, *dnaQ923*, *dnaQ924*, *dnaQ928*, *dnaQ930*, and *dnaQ932* gene products suggests that for these alleles a reduced ability to bind the polymerase provides at least part of the explanation for their proofreading defect. The *dnaQ932* allele contains a termination codon in the C terminus (Table 3) that defines a polymerase-binding domain (residues 187 to 243) (33, 46), and impairment in this domain is a sufficient explanation for this two-hybrid result. The remaining six alleles carry missense mutations in the N-terminal catalytic domain (residues 1 to 186), and we therefore conclude that their reduced α - ϵ interaction most likely results from a structural impairment, as in the case of *dnaQ49*. Five of these are also ones described in

TABLE 5. Effect of θ on the α - ϵ interaction in a yeast three-hybrid assay

Genes in pBridge		β -Gal activity ^a	
<i>dnaQ</i>	<i>holE</i>	No methionine	1 mM methionine
+	—	5.9	7.6
+	+	1.3	1.2
<i>dnaQ49</i>	—	0.01	0.045
<i>dnaQ49</i>	+	1.1	0.53
—	—	0.003	0.005

^a β -Galactosidase (β -Gal) activity is expressed in units (amount of *o*-nitrophenyl- β -D-galactopyranoside hydrolyzed per minute), calculated as described by Taft-Benz and Schaaper (46). Each value represents the average for three to six independent yeast transformants, each analyzed in triplicate. The experiment was performed at 30°C. The bottom row indicates results for empty vectors.

Table 3 for which the mutator phenotype was significantly enhanced by loss of θ . Thus, a good correlation exists between presumed structural defects in ϵ (as measured in the two-hybrid assay) and sensitivity to loss of θ (as measured by mutant frequencies). This correlation strengthens our suggestion that θ is important in maintaining ϵ in its proper conformation.

Yeast three-hybrid analysis. To show more directly that θ affects the α - ϵ interaction, we utilized the yeast three-hybrid assay using plasmid pBridge. This assay is similar to the two-hybrid assay but involves additional expression of a third protein, whose effect on the interaction of the two fusion proteins is studied. In our version of the system, we again expressed α and ϵ as interacting fusion proteins and assayed their interaction in the presence or absence of θ . We constructed plasmids pBridge/*dnaQholE* and pBridge/*dnaQ49holE* as described in Materials and Methods. From these plasmids, *dnaQ*⁺ or *dnaQ49* is expressed as a fusion protein, while *holE* is expressed as a free protein from the Pmet₂₅ promoter, which is repressible by the presence of 1 mM methionine. Thus, this assay allows, in principle, controlled expression of θ by manipulating the medium formulation. In our hands, we found the Pmet₂₅ promoter to be leaky and not completely repressible even by the presence of 1.5 mM methionine, a concentration at which the yeast cells were exhibiting growth delays. The leakiness of the Pmet₂₅ promoter on a multicopy plasmid was also noted previously (21).

In Table 5, we compare the strength of the α - ϵ interaction for both the wild-type and *DnaQ49* ϵ proteins under conditions promoting or inhibiting the expression of *holE*. The interaction of α with wild-type ϵ was relatively unchanged by the production of θ , although we observed a two- to fourfold decrease in the interaction when θ was expressed. This decrease could have been caused by intrinsic expression differences between plasmids with or without the *holE* gene or by small changes in plasmid copy number. In contrast, the α -*DnaQ49* interaction was dramatically increased (up to 100-fold) when θ was expressed. In fact, the strength of this interaction in the presence of θ was near that of the wild-type α - ϵ interaction. These results are in strong support of the conclusion that θ is an important stabilizing factor for the ϵ subunit.

DISCUSSION

The data presented here indicate an important role for the θ subunit of DNA Pol III in stabilizing the ϵ proofreading subunit. This effect of θ was demonstrated both indirectly, through the effect of θ on bacterial mutation frequencies, and directly, using the yeast three-hybrid assay.

The beneficial effect of θ was noted in particular for *dnaQ* alleles that were impaired in their interaction with the polymerase subunit, as determined from the two-hybrid assay. As the underlying *dnaQ* mutations were located in the N-terminal catalytic domain of ϵ , we suggest that these ϵ proteins suffer from a structural impairment (see also reference 45). This structural defect in the catalytic domain likely affects the stability of the full-length protein and its ability to interact effectively with α . This is likely the case, even though the C-terminal domain of ϵ was shown to contain the primary (but perhaps not only) determinant for binding to α (46). In addition to impaired interaction with α , the structural impairment of the ϵ catalytic domain could also have direct consequences for the exonuclease reaction itself. Thus, the proofreading impairment of these alleles could also have a catalytic component, as also suggested previously by analysis of the NMR model structure of ϵ (6). We noted that among θ -sensitive *dnaQ* alleles, *dnaQ928* (G17S) contains a mutation that altered residues in or near the catalytically important exonuclease I motif (45). Regardless of the precise mechanism of the proofreading defect, θ appears capable of stabilizing the structure, leading to improved proofreading ability and reduced errors. This proposed role of θ in stabilizing ϵ is consistent with the in vitro observation of stimulated terminal mismatch removal by purified ϵ in the presence of θ (44).

While large effects of θ were observed in the case of mutant *DnaQ* proteins, smaller, but significant effects were also observed with the *dnaQ*⁺ wild-type gene. Here, we demonstrated that the loss of θ is correlated with increased mutant frequencies for several mutational markers, including transitions, transversions, and frameshifts. This antimutagenic effect of θ suggests that the stability of ϵ can also be rate limiting to proofreading during normal DNA replication. One could speculate that this is relevant as to why *E. coli* and other gram-negative bacteria contain the θ subunit as part of their replication complex. As mutation rates are under genetic control (8), the modest effect of θ in reducing error rates could be significant on an evolutionary time scale.

It is interesting that the replication complex of gram-negative bacteria is characterized by both a θ subunit and a separate proofreading subunit (i.e., not part of the polymerase polypeptide). In other organisms where the proofreading exonuclease is part of the same polypeptide as the polymerase, no θ subunit is found, nor is any θ -like domain in the polymerase or other replication protein. This correlation of θ with a free proofreading exonuclease is certainly consistent with the idea of θ as being a specific, protective (or even chaperone-like) subunit for the benefit of ϵ .

A BLAST search revealed a *holE* homolog in the following gram-negative bacteria: *E. coli*, *Shigella flexneri*, *Salmonella enterica*, *Yersinia pestis*, *Wigglesworthia brevipalpis*, and *Photobacterium luminescens*. Most interestingly, a *holE* homolog is also found on two plasmids (*Proteus vulgaris* Rts1 and *S. enterica*

serovar Typhimurium pSLT) and on one phage genome (bacteriophage P1 *hot* gene). The function of these extrachromosomal *holE* homologs remains to be determined, but their presence is a further indication that θ must play a meaningful role.

Foster and Marinus (11), using *dnaQ-lacZ* fusion proteins, found evidence that ϵ is a rather unstable protein, as its cellular level is reduced in strains lacking DnaK, DnaJ, or GrpE heat shock proteins. Apparently, the chaperone function of these proteins is important in maintaining ϵ in the proper conformation and helping it to avoid rapid proteolysis. Interestingly, in vitro experiments with purified ϵ have also indicated it to be a protein of limited stability, including facile proteolysis (or hydrolysis) (33) and its relatively unstructured behavior during NMR experiments (6).

In addition to stabilizing ϵ within the Pol III core, another potentially important role of θ that needs to be considered is sequestration of free ϵ in the cell by capturing it in the ϵ - θ complex. In this complex, ϵ is significantly more stable than the free protein (7, 25). The order in which the Pol III core is assembled from its constituents is not known, and the complexation of ϵ with θ could be a first step designed to ensure sufficient supply of the intrinsically unstable ϵ for interaction with α to form the Pol III core. In this model, θ performs essentially a chaperone function.

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