

Mutator and Antimutator Effects of the Bacteriophage P1 *hot* Gene Product

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The Hot (homolog of theta) protein of bacteriophage P1 can substitute for the *Escherichia coli* DNA polymerase III θ subunit, as evidenced by its stabilizing effect on certain *dnaQ* mutants that carry an unstable polymerase III ϵ proofreading subunit (antimutator effect). Here, we show that Hot can also cause an increase in the mutability of various *E. coli* strains (mutator effect). The *hot* mutator effect differs from the one caused by the lack of θ . Experiments using chimeric θ /Hot proteins containing various domains of Hot and θ along with a series of point mutants show that both N- and C-terminal parts of each protein are important for stabilizing the ϵ subunit. In contrast, the N-terminal part of Hot appears uniquely responsible for its mutator activity.

The *Escherichia coli* chromosome is replicated with high efficiency and fidelity by DNA polymerase (Pol) III holoenzyme (HE), a large dimeric multisubunit enzyme complex that simultaneously copies the leading and lagging strands at the replication fork (for reviews, see references 15 and 24). HE contains two core polymerase subassemblies, each composed of three separate subunits, α , ϵ , and θ , which are tightly bound in the linear order α - ϵ - θ . The α subunit (*dnaE* gene product) (135 kDa) is the polymerase, while the ϵ subunit (*dnaQ* gene product) (28.5 kDa) is the 3'→5' exonucleolytic activity that functions as a proofreader for replication errors.

The precise function of the θ subunit (*holE* gene product) (8 kDa) within the Pol III core is less clear. θ binds tightly to the ϵ subunit and does not seem to interact with the α subunit (2, 32). Strains lacking θ (Δ *holE* strains) are viable, indicating that the subunit is not essential (31). On the other hand, our laboratory has shown that such deletion strains possess a mutator phenotype in mismatch repair-defective strains, suggesting that θ may have a positive effect on the accuracy of DNA replication, likely through its effect on the ϵ proofreading activity (33). This fidelity role is consistent with the increase in the ϵ exonuclease activity observed in an in vitro exonuclease assay in the presence of θ (32). In addition, large effects of the deletion of θ were observed in strains containing an impaired or unstable ϵ subunit (33). For example, the mutability of the temperature-sensitive *dnaQ49* mutator strain was increased nearly 1,000-fold upon the loss of θ (Δ *holE* strain). Based on these and other results, it was postulated (33) that the θ subunit fulfills a general stabilizing role for the intrinsically unstable ϵ subunit (7, 10, 12).

θ appears to be well preserved throughout the enterobacteria, suggestive of a meaningful role for the protein. Homologs of θ have also been found, surprisingly, to be encoded by two

conjugative plasmids as well as by bacteriophage P1 (3). In experiments with this P1 homolog produced by the P1 *hot* gene (homolog of theta) (22), we showed that the resulting Hot protein can substitute for θ in certain *dnaQ* mutators such as *dnaQ49*, as it was capable of reducing the high mutability of a *dnaQ49* Δ *holE* strain (3). In fact, for *dnaQ49*, which carries a V96G mutation in ϵ (33), Hot appeared to be significantly more efficient than θ in stabilizing this mutant. For other mutants, such as *dnaQ920* (R56W), *dnaQ923* (H66Y), and *dnaQ924* (L171F), Hot proved as efficient as θ . In contrast, Hot was not able to substitute for the θ function in *dnaQ928* (G17S), indicating that the precise interactions of θ and Hot with ϵ differ in certain details and that their effects may depend on the precise defect in ϵ (3).

The interaction between ϵ and θ has also been pursued by structural studies (5–7, 11, 13, 16). Nuclear magnetic resonance (NMR) solution structures of both θ and Hot have been obtained, revealing a largely superimposable three-part helical structure with unstructured N- and C-terminal segments (6, 26). Nevertheless, some small differences between the protein structures are apparent, as expected for two proteins that are approximately 50% identical (65% homologous). In addition, the biochemical behavior of Hot is slightly different from that of θ , as the purified protein appears to be more stable and better structured, allowing its solution structure to be determined in aqueous solution (6), whereas that of θ required mixed alcohol-water solvents (26).

In the present report, we describe and investigate certain instances where the substitution of θ by Hot produced, surprisingly, a mutator effect. This proved to be the case for the *dnaQ930* (H89Y) mutator strain as well as the *dnaQ*⁺ (wild-type [wt]) strain. In addition, we made use of the sequence and structural homologies of the two proteins to create a number of chimeric protein molecules, which might permit a more precise definition of the subdomains of either protein responsible for the antimutator and mutator effects. The results indicate that the N-terminal domain of Hot, which appears unstructured in NMR solution spectra, is specifically responsible for the Hot mutator effect. We also show

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TABLE 1. *E. coli* strains and plasmids used in this study

Strain or plasmid	Relevant genotype	Reference, source, or construction
<i>E. coli</i> strains		
CAG18486	<i>eda-51::Tn10</i>	30
CAG12068	<i>zeb-3190::Tn10</i>	30
CC101 to CC106	F'CC101 to F'CC106	4
KA796	<i>ara thi Δ(pro lac)</i>	29
MG1655	Wild type	30
NR9464	<i>mutL::Tn5</i>	27
NR9695	<i>dnaQ49 zae-502::Tn10</i>	28
NR11572	<i>dnaQ923 zae-502::Tn10</i>	33
NR11573	<i>dnaQ924 zae-502::Tn10</i>	33
NR11641	<i>dnaQ928 zae-502::Tn10</i>	33
NR11642	<i>dnaQ930 zae-502::Tn10</i>	33
NR13104	<i>ΔholE203</i>	3
NR16315	<i>ΔholE204::hot</i>	3
NR16319	<i>dnaQ923</i>	MG1655 × P1/NR11572
NR16320	<i>ΔholE203 dnaQ923</i>	NR13104 × P1/NR11572
NR16321	<i>ΔholE204::hot dnaQ923</i>	NR16315 × P1/NR11572
NR16322	<i>dnaQ924</i>	MG1655 × P1/NR11573
NR16323	<i>ΔholE203 dnaQ924</i>	NR13104 × P1/NR11573
NR16324	<i>ΔholE204::hot dnaQ924</i>	NR16315 × P1/NR11573
NR16325	<i>dnaQ928</i>	MG1655 × P1/NR11641
NR16326	<i>ΔholE203 dnaQ928</i>	NR13104 × P1/NR11641
NR16327	<i>ΔholE204::hot dnaQ928</i>	NR16315 × P1/NR11641
NR16328	<i>dnaQ930, zae-502::Tn10</i>	MG1655 × P1/NR11642
NR16329	<i>ΔholE203 dnaQ930</i>	NR13104 × P1/NR11642
NR16330	<i>ΔholE204::hot dnaQ930</i>	NR16315 × P1/NR11642
NR16785	<i>ΔholE204::hot eda-51::Tn10</i>	NR16315 × P1/CAG18486
NR16786	<i>ΔholE204::hot zeb-3190::Tn10</i>	NR16315 × P1/CAG12068
NR16787	<i>ara, thi, Δpro lac, ΔholE202::cat</i>	KA796 × P1/RM4193
NR17012	<i>ΔholE204::hot eda-51::Tn10</i>	KA796 × P1/NR16785
NR17013	<i>ΔholE204::hot zeb-3190::Tn10</i>	KA796 × P1/NR16786
NR17116	<i>dnaQ49</i>	MG1655 × P1/NR9695
NR17117	<i>ΔholE203 dnaQ49</i>	NR13104 × P1/NR9695
NR17118	<i>ΔholE204::hot dnaQ49</i>	NR16315 × P1/NR9695
NR17119	<i>mutL::Tn5</i>	MG1655 × P1/NR9464
NR17120	<i>ΔholE203 mutL::Tn5</i>	NR13104 × P1/NR9464
NR17121	<i>ΔholE204::hot mutL::Tn5</i>	NR16315 × P1/NR9464
RM4193	<i>ΔholE202::cat</i>	31
Plasmids		
pKO3		21
pKO3- <i>ΔholE</i>		3
pKO3- <i>holE</i>		3
pKO3- <i>hot</i>		3
pKO3- θ_{49} Hot		This work
pKO3-Hot θ_{50}		This work
pKO3- θ_{11} Hot		This work
pKO3-Hot θ_{12}		This work

that Hot and θ compete for their incorporation into the Pol III holoenzyme.

MATERIALS AND METHODS

Strains and media. The *E. coli* strains used, along with information on their source or construction, are listed in Table 1. P1 transductions were performed using P1*virA*. The various *dnaQ* alleles (Table 1) were transduced using linkage (~40%) with transposon *zae-502::Tn10* as described previously (33), followed by testing for the associated *dnaQ* mutator phenotype (scoring for rifampin-resistant mutants). The donor strains NR11569 (*dnaQ920*), NR11572 (*dnaQ923*), NR11573 (*dnaQ924*), NR11641 (*dnaQ928*), and NR11642 (*dnaQ930*) were *dnaQ* derivatives of NR9501 or NR9601 as described previously (33). Plasmid pKO3 (21) was obtained from G. Church (Harvard Medical School). Minimal media (MM) containing either glucose or lactose as a carbon source or LB broth were standard recipes (29). Antibiotics were added as follows: ampicillin, 100 μ g/ml; chloramphenicol, 20 μ g/ml; tetracycline, 15 μ g/ml; rifampin (Rif) 100 μ g/ml. Solid media contained 1.5% agar (Difco).

DNA isolation, PCR, and DNA sequencing. *E. coli* genomic DNA was prepared using the DNeasy tissue kit (QIAGEN Sciences). Plasmid DNAs were purified from 10-ml liquid cultures by using a Qiaprep Spin Miniprep kit (QIAGEN Sciences). All preparative PCRs were performed using the Expand

TABLE 2. Oligonucleotide primers

Primer	Sequence ^a
Pr1	5'-GAGAAATGCGGCCGCTGTAGTGTCTTTCGTTTATGCCCC-3'
Pr6	5'-CAAATCAGTCGACGCCAGCAGGTCGGGTTCTCC-3'
hotN-low ^b	5'-CAGCTGCGCAGATTCTCTGGTTG-3'
holEC-up ^b	5'-CAGAGAATCTGCGCAGCTGGTTTCG-3'
holEN-low ^c	5'-ATGAAATAGGTGCGCAAATGTTTCAGGCTG-3'
hotC-up ^c	5'-TGAACATTGCGCACCTATTTCATGGAAC-3'
HotNLow ^d	5'-CACTTTATCCATTCTCTCTGACTTTTAGCTGC-3'
HolECUp ^d	5'-GTCAGGAAGAAATGGATAAAGTGAATGTCG-3'
HolENLow ^e	5'-CTTATCCCGTTCTGTTTGTATCCAGTTTAGC-3'
HotCU ^e	5'-GATCAAACAGAACCGGGATAAAGGTTAACG-3'
SeqHotUp	5'-GGAATATTGACAGCTAAAAGTC-3'
SeqHotLow	5'-CTATTCTTTACGGCATCATC-3'
SeqholE Up	5'-CTGAAGAATCTGGCTAAAAGTC-3'
SeqholE Low	5'-GTTTTATTTAAGTTTGGGCTC-3'

^a Sequences in boldface type correspond to *holE* or *hot* coding sequences.

^b Primers with complementary 5' ends used to create pKO3-Hot θ_{50} .

^c Primers with complementary 5' ends used to create pKO3- θ_{49} Hot.

^d Primers with complementary 5' ends used to create pKO3-Hot θ_{12} .

^e Primers with complementary 5' ends used in creation of pKO3- θ_{11} Hot.

High Fidelity PCR system (Roche). Diagnostic PCRs were performed using *Taq* DNA polymerase (Invitrogen, Carlsbad, CA). Conditions for PCRs were recommended by the manufacturer. Oligo 6.8' software (Molecular Biology Insights, Inc., Cascade, CO) was used to design oligonucleotide primers (Table 2) and to determine annealing temperatures. DNA sequencing was performed using the Big Dye Terminator v1.1 cycle sequencing kit (Applied Biosystems, Foster City, CA) and Perkin-Elmer ABI models 377 and 3100 sequencers. Both DNA strands were sequenced.

Chimeric plasmids. The four chimeric plasmids pKO3- θ_{49} Hot, pKO3-Hot θ_{50} , pKO3- θ_{11} Hot, and pKO3-Hot θ_{12} (Table 1) were constructed by PCR methods in two-step procedures. In the first step, two separate PCR products containing the *holE* or *hot* gene sequence of interest were created, which were then fused in a second (overlap PCR) step to yield the desired final product. The starting materials were plasmids pKO3-*holE* and pKO3-*hot* (3), which contain the *holE* and *hot* coding sequences under the control of the *holE* promoter and are surrounded on either side by about 500 nucleotides of chromosomal *E. coli* DNA (3). The various primers used are listed in Table 2. Primers Pr1 and Pr6 have been described previously (3); they are "outside" primers, complementary to the terminal 5' and 3' sequences of the plasmid inserts, and contain NotI and SalI recognition sequences to mediate the insertion of the final product into plasmid pKO3 (21). Chimeras were designed with aid of the amino acid alignment of θ and Hot (see Fig. 3). (In the following text, note that due to the presence of an extra residue in the N terminus of Hot compared to θ , the numberings of the corresponding θ and Hot residues differ by 1.) For example, to create pKO3- θ_{49} Hot, we used (i) primers Pr1 and holEN-low to amplify the N-terminal part of *holE* (θ residues 1 to 49) from pKO3-*holE* and (ii) Pr6 and hotC-up to amplify the C-terminal part of *hot* (residues 51 to 83) from pKO3-*hot*. As "internal" primers, holEN-low and hotC-up contain a 24-base 5' overlap (Table 2); a second round of PCR on the two combined PCR products using outside primers Pr1 and Pr6 could be used to create a combined (~1,400-base) product encoding the chimeric θ_{49} Hot protein. Using the NotI and SalI restriction sites embedded in Pr1 and Pr6, the product was then inserted into the NotI/SalI sites of plasmid pKO3, yielding pKO3- θ_{49} Hot. The remaining chimeric plasmids (Table 1) were created in a similar fashion, using hotN-low and holEC-up for pKO3-Hot θ_{50} , HolENLow and HotCU^e for pKO3- θ_{11} Hot, and HotNLow and HolECUp for pKO3-Hot θ_{12} as internal primers. The resulting plasmids were analyzed by PCR and DNA sequencing. Sequencing primers were SeqHotUp and SeqHotLow (*hot*-specific primers) and SeqholEUp and SeqholELow (*holE*-specific primers). The sequencing also revealed, in addition to correct products, isolates containing one or more point mutations. Several of these mutants were included in some of the genetic experiments (see below).

Mutant frequency measurements. To measure mutant frequencies, the frequency of rifampin-resistant (Rif^r) mutants in cultures grown overnight was determined. In part of the experiments, we also determined the frequency of the Lac⁺ revertants using the series of *lac* alleles created previously by Cupples and Miller (4). Ten to 22 cultures for each strain started from individual colonies were grown overnight in 1 ml LB broth (containing chloramphenicol for experiments using plasmid pKO3 or its derivatives). Incubation temperatures are indicated in each of the tables or figures. For each culture, a 50- μ l aliquot of a

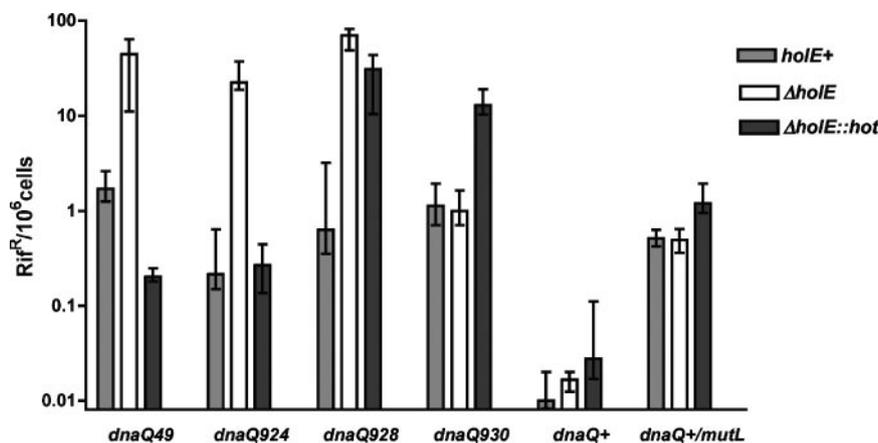


FIG. 1. Mutagenic and antimutagenic effects of the bacteriophage P1 Hot gene. Shown are the effects of the θ or Hot protein on several *dnaQ* mutator mutants and on *dnaQ*⁺ control strains. The strains are derivatives of MG1655 containing the *dnaQ49*, *dnaQ924*, *dnaQ928*, *dnaQ930*, or *dnaQ*⁺ allele in either the MG1655 (wt) (light gray), NR13104 (Δ *holE*) (white), or NR16315 (Δ *holE*::*hot*) (dark gray) background. All strains are mismatch repair proficient, except for the *dnaQ*⁺ *mutL*::Tn5 set (last entry). Cultures were grown and plated at 30°C (*dnaQ49*) or 37°C (others). The frequencies of rifampin-resistant mutants were calculated for 12 to 15 independent cultures for each strain, and the data were analyzed using Prism software (GraphPad). The graph shows the median values and interquartile ranges for the frequencies of rifampin-resistant mutants.

10^6 dilution was spread onto an LB or minimal glucose plate to determine the total number of viable cells. Fifty microliters of the undiluted or appropriately diluted culture was spread onto LB plates with Rif to determine the number of rifampin-resistant mutants or onto minimal lactose plates to determine the number of Lac⁺ revertants. The mutant frequency for each culture was calculated by dividing the total number of mutants by the total number of viable cells. The mutant frequency data were analyzed using Prism statistical analysis software (GraphPad). To validate the statistical significance of observed differences, the nonparametric Mann-Whitney test was used. An exact two-tailed *P* value was calculated for the tested data, and differences were determined to be statistically significant when the *P* value was <0.05.

RESULTS

Mutator and antimutator effects of P1 Hot. Our laboratory demonstrated previously that several *dnaQ* mutator strains carrying defects in the Pol III ϵ proofreading subunit are stabilized by θ (34). Their mutability, a reflection of their proofreading deficiency, was greatly enhanced in a Δ *holE* background. For example, the mutability of the *dnaQ49* (V96G) allele was enhanced more than 1,000-fold by the lack of θ . In general, we noted a correlation between the recessive nature of *dnaQ* mutants, an indication of some structural impairment, and their sensitivity to the absence of θ . Subsequently, we showed that the bacteriophage P1 Hot protein, a putative θ homolog, was able to substitute for θ in several of these mutants, including *dnaQ49* (3). Interestingly, for one other mutant, *dnaQ928* (G17S), Hot appeared to be ineffective, although it was stabilized significantly (>100-fold) by θ (3). It was suggested that the interactions of θ and Hot with ϵ , although presumably similar, were not precisely identical in all respects.

In the present study, we have extended the comparative analysis of θ and Hot to one more additional *dnaQ* mutator mutant, *dnaQ930* (H89Y) (33, 34). The H89Y defect in *dnaQ930* is genetically dominant, indicative of a mostly catalytic proofreading deficiency rather than a structural one (33). In such a case, stabilization by θ would not be expected and indeed was not observed (34). The effect of Hot on *dnaQ930* is shown in Fig. 1. In this experiment, we compared the effect

of several *dnaQ* mutator alleles in three different genetic backgrounds: wt (*holE*⁺), Δ *holE*, or Δ *holE*::*hot*. In the last case, the chromosomal *holE* gene encoding θ is replaced, precisely, by the P1 *hot* gene coding sequence so that Hot is expressed from the resident chromosomal *E. coli holE* promoter instead of θ (3).

The results for *dnaQ49*, *dnaQ924*, and *dnaQ928* confirm previous observations (3): *dnaQ49* (V96G) and *dnaQ924* (L171F) are stabilized by both θ and Hot (with *dnaQ49* more effectively stabilized by Hot than by θ), while the *dnaQ928* (G17S) mutator is stabilized by θ only. Interestingly, the *dnaQ930* (H89Y) strain displays a strong mutator phenotype when Hot is expressed. The Hot mutator effect is about 13-fold, as observed in several experiments, compared to the corresponding strains expressing or lacking θ . Thus, in addition to the established antimutator effects, P1 Hot is also capable of producing mutator effects.

To further investigate this mutator activity, we analyzed the effect of Hot in the corresponding *dnaQ*⁺ (proofreading-proficient) background. A modest (about twofold) but reproducible (and statistically significant) mutator activity was also observed in this case (Fig. 1). As effects on proofreading and DNA replication errors are often best evaluated in mismatch repair-deficient strains (due to the lack of correction of the replication errors), we also investigated the activity of Hot in a *dnaQ*⁺ *mutL* strain. Again, a consistent two- to threefold mutator effect was observed (Fig. 1). These results with the *dnaQ*⁺ and *dnaQ930* strains indicate that although Hot can substitute for θ in many *dnaQ* mutants and in fact is even more efficient in stabilizing the *dnaQ49* mutator, it is also capable of increasing the mutant frequency in *E. coli*.

Competition between θ and Hot. We made use of the *hot* mutator effect to investigate the possible competition between θ and Hot when they are present in the same cell. For this investigation, we used the mismatch repair-deficient *mutL* strains. High-level expression of θ or Hot is deleterious (3), but both proteins can be satisfactorily overproduced from the low-

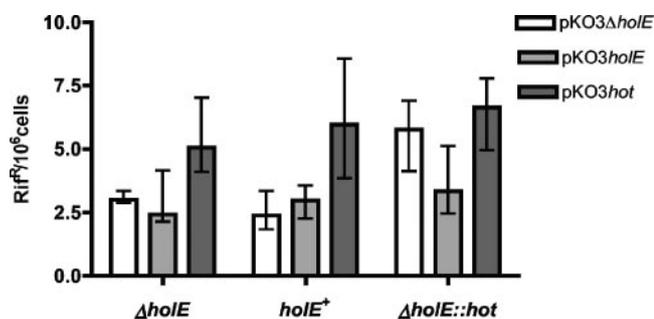


FIG. 2. Competition of θ and Hot for incorporation into the Pol III core. Shown are the effects of the overproduction of θ or Hot from low-copy-number plasmid pKO3 in a *dnaQ*⁺ strain containing the $\Delta holE$, *holE*⁺, or $\Delta holE::hot$ genetic configuration. The mismatch repair-defective strains used were NR17119 ($\Delta holE$), NR17120 (*holE*⁺), and NR17121 ($\Delta holE::hot$) (Table 1) containing the indicated pKO3 plasmids (Table 1). Cultures were grown at 30°C in LB plus chloramphenicol, and the plates were likewise incubated at 30°C. The frequencies of rifampin-resistant mutants were determined for 10 to 15 independent cultures for each strain, and the data were analyzed using Prism software (GraphPad). The graph shows the median values and interquartile ranges for the frequencies of rifampin-resistant mutants. The x axis indicates, for each of the three hosts, the three pKO3 plasmids containing $\Delta holE$ (white), *holE*⁺ (light gray), or $\Delta holE::hot$ (dark gray).

copy-number plasmid pKO3 (3). Three plasmids, pKO3-*holE* (containing the *holE* gene), pKO3-*hot* (containing the *hot* gene), and the control plasmid pKO3- $\Delta holE$ (3) (Table 1) were transformed, separately, into three *E. coli* strains, *holE*⁺, $\Delta holE$, and $\Delta holE::hot$, and the effect on the frequency of rifampin-resistant mutants was evaluated (Fig. 2). In the $\Delta holE$ strain (first set), we reproduced the *hot* mutator effect shown in Fig. 1. In the wild-type (*holE*⁺) strain (second set), expression of Hot from pKO3-*hot* also produced the mutator effect, despite the presence of θ in the cell. Finally, in the Hot-expressing ($\Delta holE::hot$) strain (third set), overexpression of θ from pKO3-*holE* clearly reduced the Hot-induced mutator effect. Thus, these results indicate that θ and Hot can compete for incorporation into the Pol III holoenzyme.

Mutator effects of $\Delta holE$. Previously, our laboratory reported that the loss of θ ($\Delta holE$ strain) causes a modest mutator effect in *dnaQ*⁺ strains (34). This was interpreted to indicate that θ exerts a positive effect on the ϵ proofreading activity even in the proofreading-proficient (*dnaQ*⁺) background (34). This result is confirmed in the results for the *dnaQ*⁺ *mutL*⁺ strain (Fig. 1), although in this case, no obvious effect was apparent in the *mutL* derivative. Nevertheless, a $\Delta holE$ mutator effect in the *mutL* background was clearly observed in a slightly different strain, KA796 (34). Here, we made use of the KA796 series to compare the mutator effect(s) of $\Delta holE$ and $\Delta holE::hot$. This background has the advantage that it permits the analysis of the specificities of mutations using the *lac* reversion system developed previously by Cupples and Miller (4). Specifically, KA796 was made $\Delta holE$ and $\Delta holE::hot$, which was followed by an introduction of the series of F'(pro *lacIZ*) episomes originally present in strains CC101 through CC106. The latter permit measurement, in parallel, of each of the six possible base pair substitutions at a specific site in the *lacZ* gene (4). The strains were also made *mutL*

(*mutL*::Tn5). The results for both Rif^r mutants and *lac* revertants are shown in Table 3. While both $\Delta holE$ and $\Delta holE::hot$ produce a similar 1.6-fold increase in the frequency of Rif^r mutants, their respective mutator effects are actually quite dissimilar, as viewed by the *lac* specificity data. For example, the lack of θ caused significant increases (indicated in boldface type in Table 3) for G · C → T · A, A · T → T · A, and A · T → G · C substitutions, consistent with our previous report (34). In contrast, the *hot* mutator did not increase the A · T → T · A transversions but instead increased the G · C → A · T transitions (1.8-fold) as well as the -1 frameshifts in strain FC40 (two- to threefold). Strain FC40 is routinely used for studying adaptive (or postplating) mutagenesis (9), but here, colonies were counted at 48 h. (We also observed a reproducible two- to threefold increase in postplating mutations in the case of the *hot*⁺ derivative but not in the $\Delta holE$ strain [data not shown].) Thus, while both Hot and the lack of θ produce a mutator effect, their specificities and, by implication, the precise mechanisms by which these effects are generated must be different.

θ -Hot chimeric proteins. The mutator effect exerted by the P1 Hot protein presumably reflects certain differences in the ϵ -Hot interaction compared to the ϵ - θ interaction. In the simplest model, Hot might bind more strongly to ϵ than θ , a possibility suggested by its stronger stabilization of *dnaQ49* (Fig. 1) (3). Such stronger binding, while greatly stabilizing *dnaQ49*, might lead to some structural or functional impairment of the exonuclease, observable as a mutator effect in some other *dnaQ* alleles. In an expanded version of this model, the mutator/antimutator effects might be ascribable to separate (sub)interactions within the ϵ -Hot and ϵ - θ complexes. We have investigated the latter possibility by creating several θ -Hot chi-

TABLE 3. Differential mutator effects of $\Delta holE$ and $\Delta holE::hot$: Lac⁺ or Rif^r mutant frequencies in mismatch repair-defective *mutL* strains^a

<i>lac</i> allele from strain	Mutation scored	No. of mutants (per 10 ⁶ cells)			Mutator effect (fold)	
		<i>holE</i> ⁺	$\Delta holE$	$\Delta holE::hot$	$\Delta holE/holE^+$	<i>hot</i> ⁺ / <i>holE</i> ⁺
All ^b	Rif ^r	0.44	0.71	0.7	1.6	1.6
CC101	A · T → C · G	0.0036	0.0027	0.0046	0.75	1.3
CC102	G · C → A · T	0.44	0.45	0.79	1	1.8
CC103	G · C → C · G	0	0	0		
CC104	G · C → T · A	0.009	0.038	0.017	4.2	1.9
CC105	A · T → T · A	0.0037	0.015	0.004	4	1
CC106	A · T → G · C	0.17	0.55	0.36	3.2	2.1
FC40 ^c	<i>lac</i> (-1) FS ^d	0.096	0.093	0.27	0.97	2.8

^a Strains are *mutL*::Tn5 derivatives of KA796 (*holE*⁺), NR16787 ($\Delta holE$), and NR17012 ($\Delta holE::hot$ *eda-51*::Tn10) or NR17013 ($\Delta holE::hot$ *zeb-3190*::Tn10) (Table 1) and contain the F'(pro *lac*) episome from strains CC101 through CC106 (Table 1), permitting measurement of the indicated, specific *lac* reversion frequencies. The *eda* or *zeb* transposon insertions did not affect the *hot* mutator effect (results not shown). For each strain with each F'(pro *lac*), 22 independent LB cultures were grown overnight at 37°C. The 22 cultures were derived from multiple isolates (transductants) for each strain. The frequencies of *lac*⁺ revertants and of Rif^r mutants were determined as described in Materials and Methods. The mutant frequencies were analyzed by Prism software, and statistically significant differences between *hot*⁺ and $\Delta holE$ or *hot*⁺ and $\Delta holE::hot$ strains (mutator effects) were assessed using nonparametric tests. Differences with *P* values of <0.05 are shown in boldface type.

^b The Rif^r data reflect the median value of eight independent experiments, each comprised of 22 cultures.

^c This strain was tested in the mismatch repair-proficient background.

^d FS, frameshift mutation.

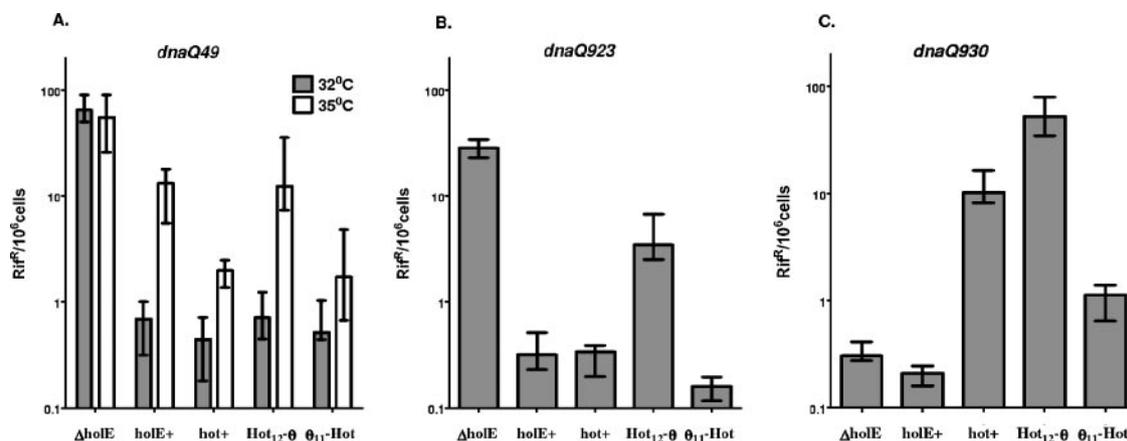


FIG. 5. Effects of the θ_{11} Hot and Hot $_{12}\theta$ chimeric proteins. The three panels show the effect of the θ_{11} Hot and Hot $_{12}\theta$ chimeric proteins along with the control proteins on the mutability of *dnaQ49* (A), *dnaQ923* (B), and *dnaQ930* (C). The strains used were NR17117 (*dnaQ49* Δ *holE*), NR16320 (*dnaQ923* Δ *holE*), and NR16329 (*dnaQ930* Δ *holE*) containing various plasmids as indicated along the x axis: pKO3- Δ *holE*, pKO3-*holE*, pKO3-*hot*, pKO3-Hot $_{12}\theta$, or pKO3- θ_{11} Hot. The cultures were grown in LB with chloramphenicol at 30°C and 35°C for the *dnaQ49* strains (A) and at 30°C for the *dnaQ923* and *dnaQ930* strains (B and C). Plates were incubated at the same temperature as the cultures. The frequencies of rifampin-resistant colonies were determined for 8 to 12 independent cultures for each strain, and the data were analyzed using Prism software (GraphPad). The graph shows the median values and interquartile ranges for the frequencies of rifampin-resistant mutants.

temperature dependent (14, 34), and previous studies have shown that while both Hot and θ can stabilize this allele, Hot is more effective than θ , a difference that becomes more pronounced at higher temperatures (3). The present data reproduce these findings. The results further indicate that the Hot $_{12}\theta$ protein behaves essentially like θ , while the θ_{11} Hot protein behaves like Hot. These results suggest that while the N-terminal 11 to 12 residues of θ or Hot may have a role in stabilizing the DnaQ49 protein, they are not the primary determinants for the greater efficiency of Hot in this respect. Apparently, these determinants lie in the remainder of the respective proteins.

A most informative result was obtained for the *dnaQ930* allele (Fig. 5C). Here, the mutator effect of Hot was strongly reproduced by the Hot $_{12}\theta$ protein, indicating that the N-terminal 12 residues of Hot are primarily responsible for the Hot mutator activity. A weaker mutator effect was also seen for the θ_{11} Hot protein, suggesting that some other structures within Hot might also be relevant for the Hot mutator effect for *dnaQ930*.

Further corroborative insights were obtained from the experiment with the *dnaQ923* mutant (Fig. 5B). While, ostensibly, θ and Hot are similarly active in stabilizing this *dnaQ* allele, the two chimeric proteins clearly reveal a split phenotype. The Hot $_{12}\theta$ chimera increased the *dnaQ923* mutant frequency up to threefold in comparison to either θ or Hot, while the θ_{11} Hot protein showed a three- to fivefold decrease. Although other explanations are possible, it appears that the N-terminal 11 to 12 residues of Hot are responsible for a mutator effect in both *dnaQ923* and *dnaQ930*, but in the *dnaQ923* mutant, this mutator effect is compensated for by a simultaneous antimutator effect of the remaining C-terminal part of Hot. As *dnaQ930* does not require any stabilization by either θ or Hot, this second effect is not observed for this mutant.

Effects of point mutants in chimeric proteins. During the creation of pKO3- θ_{11} Hot and pKO3-Hot $_{12}\theta$ plasmids, we also

obtained (presumably through PCR amplification errors) several mutants carrying amino acid substitutions. Several of these mutants were tested in parallel with the plasmids described above. The results are listed in Table 4.

For the Hot $_{12}\theta$ protein, two substitution mutants were obtained in the N-terminal Hot region. The Y2H and A7T mutations significantly increased the mutability of the *dnaQ49* strain, indicating that this N-terminal portion of Hot is important for the stabilization of the DnaQ49 protein. Y2H also increased the mutability of *dnaQ923*, consistent with the requirement of this allele for stabilization by Hot or θ . Y2H did not affect the Hot $_{12}\theta$ mutator effect on *dnaQ930*, consistent with the notion that *dnaQ930* does not require stabilization by

TABLE 4. Effect of point mutants in Hot $_{12}\theta$ and θ_{11} Hot chimeric proteins on the mutability of *dnaQ49*, *dnaQ923*, and *dnaQ930* mutants^a

Protein ^b	No. of Rif ^r mutants per 10 ⁶ cells			
	<i>dnaQ49</i>		<i>dnaQ923</i>	<i>dnaQ930</i>
	32°C	35°C		
None (Δ <i>holE</i>)	65	55	27	0.33
θ	0.69	13	0.33	0.29
Hot	0.4	2.0	0.30	13
Hot $_{12}\theta$	0.72	12	2.4	44
Hot $_{12}\theta$ (Y2H)	0.5	34	37	50
Hot $_{12}\theta$ (A7T)	12	55	0.42	0.59
Hot $_{12}\theta$ (N17H)	0.66	20	38	40
Hot $_{12}\theta$ (W51C)	71	27	28	3.2
θ_{11} Hot	0.52	1.7	0.11	1.4
θ_{11} Hot (D9Y)	0.78	8.8	0.17	0.96
θ_{11} Hot (V17I)	0.39		0.12	0.64
θ_{11} Hot (F53S)	63	92	7.5	0.87

^a Results are the averages of two or three determinations. The *dnaQ49* experiments were performed at 32°C and 35°C; the *dnaQ923* and *dnaQ930* experiments were performed at 30°C.

^b Amino acid numbers (in parentheses) refer to the original residue numbers in θ or Hot.

Hot or θ . Interestingly, the A7T mutation lowered the mutability of both *dnaQ923* and *dnaQ930* and in fact nearly completely abolished the mutator effect of Hot₁₂ θ on both alleles. These results are fully consistent with our proposal that the N-terminal 12 residues of Hot are responsible for the Hot mutator effect.

Two mutations, N17H and W51C, were obtained in the θ -specific part of Hot₁₂ θ . The N17H defect was generally deleterious for *dnaQ49* and, especially, *dnaQ923*, increasing the mutation frequency while not affecting the mutability of *dnaQ930*. These results are consistent with a stabilizing role of the C-terminal part. In contrast, W51C likely impairs the overall integrity of the protein, as the resulting hybrid lacked the ability to stabilize (*dnaQ49* and *dnaQ923*) and greatly reduced the mutator effect on *dnaQ930*. W51 in θ and the corresponding Y52 in Hot likely occupy a critical position within helix α_3 , as discussed previously (26).

The amino acid substitutions in the reciprocal θ_{11} Hot protein are less informative. The D9Y and V17I mutations are largely neutral, whereas F53S resembles the case of W51C discussed above, reducing both the stabilizing effect on *dnaQ49* and *dnaQ923* and the mutator effect on *dnaQ930*. Thus, F53S likely represents a structurally impaired protein that has lost most of its capacity to interact with ϵ .

DISCUSSION

The current results provide new evidence for the importance of the ϵ - θ interaction within the Pol III core in determining the fidelity of replication in *E. coli*. Previously, we demonstrated that the lack of the θ subunit caused a mutator effect in wild-type cells and in several proofreading-impaired *dnaQ* mutants (34). This strongly suggested a stabilizing role for the θ subunit, presumably keeping ϵ in a structural conformation favorable for proofreading. Currently, we show that Hot, the P1 homolog of θ that is capable of substituting for θ in this stabilizing role, can also generate a mutator effect, thus providing a further indication of the importance of the ϵ - θ (or ϵ -Hot) interaction in the optimal functioning of the proofreading activity. Specifically, the present results indicate that the extreme N-terminal residues (residues 1 to 12) of Hot are responsible for this mutator activity. In the published structure of Hot (or θ), this segment is not observed, presumably because it is poorly structured or conformationally active (6, 26). However, it is very likely that this segment plays an important role in the interaction with the ϵ subunit. The isolation of the A7T mutant in the Hot₁₂ θ chimera, showing abolishment of the mutator effect, further supports the importance of this segment for the ϵ -Hot interaction. Further structural studies on ϵ -Hot or ϵ - θ complexes may shed light on this important aspect.

Different mutational specificities for Δ *holE* and Δ *holE::hot*.

It is interesting that both the lack of θ and the substitution of θ by Hot lead to a very similar mutator phenotype when assayed by the frequency of rifampin-resistant mutants. However, more careful analysis using a series of defined *lac* reversions shows that the mutator effects are clearly distinct. If both effects result simply from the impairment of ϵ proofreading, one must assume that different base-base mismatches are differentially sensitive to the two modes of proofreading disturbance. As exonucleolytic proofreading involves multiple steps,

including the conformational changes associated with the transfer of the terminal mismatch from the polymerase active site to the exonuclease site, such differential effects should be considered. Alternatively, the differential specificities could reflect a more complicated mode of mutation production in Δ *holE* versus Δ *holE::hot* strains. For example, accessory DNA polymerases, such as Pol II or Pol IV, have been shown to be involved in the production of mutations, particularly when Pol III has difficulty extending certain terminal mispairs (1, 17). Possibly, HE without θ and HE containing Hot behave differently with respect to this phenomenon of polymerase trafficking. This will be an interesting area for further studies. Finally, a phylogenetic tree relating P1 Hot to other sequenced θ homologs clearly suggests that Hot is more closely related to homologs from more distant species such as *Klebsiella* than to θ itself (3). Thus, Hot is likely optimized for interaction with the ϵ subunit from other enterobacterial species, possibly explaining the observed mutator effect.

The role of *hot* for P1. A noteworthy aspect of the present work is the demonstration of direct competition between θ and Hot (Fig. 2). While we have not demonstrated a direct incorporation of Hot into the Pol III HE, the straightforward interpretation is that Pol III HE, when replicating the *E. coli* chromosome, contains Hot at least part of the time in the mixed *holE/hot* experiments and completely in the case of the Δ *holE* strain. This is, to our knowledge, the first demonstration of such a heterologous substitution in the Pol III HE. Such substitutions may also occur in P1-infected cells or in lysogens carrying the P1 prophage. These conclusions are relevant for the question as to why P1 carries, specifically, a gene for a θ homolog but no homolog for any other Pol III accessory subunit.

One possibility is that the increased mutation rate resulting from the incorporation of Hot in the HE is beneficial to the phage. As optimal mutation rates are proportional to genome size in DNA-based microbes (8), P1, based on its smaller genome, might tolerate and benefit from an increased mutation rate. Alternatively, the beneficial effect of Hot may be derived from an increased efficiency of replication. As the number of Pol III HE molecules per cell is very limited (23, 25), phage replication would probably benefit from an increase in their number. As ϵ is an intrinsically unstable protein (7, 10, 12) and θ and Hot stabilize ϵ , increased amounts of θ or Hot may result in increased amounts of the Pol III core and, ultimately, HE. The precise order of assembly of HE and its rate-limiting steps are unknown, and it might be worthwhile to study this under conditions of increased θ or Hot protein. Another interesting question is in which stage of the P1 life cycle the *hot* gene product is expressed or active. The gene has been classified as a "late" gene based on its predicted promoter structure (18, 19, 22), suggesting that it might be produced primarily late in the lytic cycle, when DNA replication has ceased and packaging ensues. It is hard to envision a role for a replication protein at this late stage, and this issue has to be investigated further, including expression of *hot* during lysogeny. Preliminary results from our laboratory (our unpublished data) have indicated that *hot* is expressed at least from the P1 prophage (lysogenic state).

In future studies, we will attempt to address the issue of the role of Hot in the P1 life cycle by investigating the properties

of a phage deleted for Hot as well as the important question of the timing of Hot expression as either a later or early gene.

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