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The Bacteriophage P1 *hot* Gene Product Can Substitute for the *Escherichia coli* DNA Polymerase III θ Subunit

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The θ subunit (*holE* gene product) of *Escherichia coli* DNA polymerase (Pol) III holoenzyme is a tightly bound component of the polymerase core. Within the core (α-ε-θ), the α and ε subunits carry the DNA polymerase and 3′ proofreading functions, respectively, while the precise function of θ is unclear. *holE* homologs are present in genomes of other enterobacteriae, suggestive of a conserved function. Putative homologs have also been found in the genomes of bacteriophage P1 and of certain conjugative plasmids. The presence of these homologs is of interest, because these genomes are fully dependent on the host replication machinery and contribute few, if any, replication factors themselves. To study the role of these θ homologs, we have constructed an *E. coli* strain in which *holE* is replaced by the P1 homolog, *hot*. We show that *hot* is capable of substituting for *holE* when it is assayed for its antimutagenic action on the proofreading-impaired *dnaQ49* mutator, which carries a temperature-sensitive ε subunit. The ability of *hot* to substitute for *holE* was also observed with other, although not all, *dnaQ* mutator alleles tested. The data suggest that the P1 *hot* gene product can substitute for the θ subunit and is likely incorporated in the Pol III complex. We also show that overexpression of either θ or Hot further suppresses the *dnaQ49* mutator phenotype. This suggests that the complexing of *dnaQ49*-ε with θ is rate limiting for its ability to proofread DNA replication errors. The possible role of *hot* for bacteriophage P1 is discussed.

Escherichia coli DNA polymerase III holoenzyme (HE) is the enzyme responsible for the faithful duplication of the bacterial chromosome. HE is a multisubunit, dimeric complex that replicates simultaneously the leading and lagging strands at the replication fork (for a review, see reference 31). Each half of the dimeric complex contains a polymerase (Pol) III core unit consisting of three subunits, α, ε, and θ, which are tightly bound in the linear order α-ε-θ. Of these, α (135 kDa) is the DNA polymerase (*dnaE* gene product), ε (28 kDa) is the exonucleotidic proofreader responsible for editing polymerase insertion errors (*dnaQ* gene product), and θ (8 kDa) is a small subunit whose function is not well defined (*holE* gene product). The HE (17 subunits total, 10 distinct) further consists of the two β-clamps (one for each core) that serve to tether the polymerase to the DNA and the DnaX complex ($\tau_2\gamma\delta\delta'\chi\psi$), which is responsible for connecting the two polymerases and for loading and unloading the β-clamp in the discontinuously synthesized lagging strand. The precise mechanisms by which HE is capable of replicating the bacterial chromosome with high speed and high accuracy are the subject of active investigation (21, 31, 33, 52).

Our laboratory has been interested in the roles and mechanisms of the ε and θ subunits within the Pol III core, particularly with respect to their contributions to the fidelity of the replication process (37, 48–50). ε is a critical subunit with at least dual functions. It is a fidelity subunit whose loss leads to strongly increased mutation rates (7, 13, 42, 48, 49). Second, it provides an important structural role within the core, most likely by its stabilizing effect on the α subunit (18, 23, 49). Consequently, a deletion mutant of the *dnaQ* gene is essentially nonviable unless compensated for by suppressor mutations in the polymerase subunit. ε has been investigated by genetic and physical methods. It consists of two separate domains, an N-terminal catalytic domain, which also contains the θ-binding site, and a C-terminal domain essential for binding to the α subunit. The structure of the N-terminal catalytic domain has been determined (5, 12), and the θ interaction domain on ε has been defined (3).

Much less is known about the θ subunit. θ binds to ε (but not to α) (2, 47), and this binding to ε was shown to increase the activity of the 3′ exonuclease of the ε subunit on a terminal G · T mismatch in vitro by about 2.5-fold (47), supporting the idea of a possible fidelity role for θ. In vivo experiments showed that a Δ*holE* strain is normally viable (45), indicating that the subunit is not essential. A possible role for θ in controlling the fidelity of DNA replication was further suggested by measurements of mutant frequencies in mismatch repair-defective strains, in which Δ*holE* derivatives showed two- to five-fold-higher mutator activities for selected mutational markers (50). Based on these results, it was suggested that θ exerts a positive effect on replication fidelity, presumably indirectly by promoting the proofreading ability of ε. These experiments also revealed θ to play a particularly important role in stabilizing the temperature-sensitive *dnaQ49* allele (50), which produces a temperature-sensitive ε subunit (7, 13, 41, 42). At low temperature (28°C), a *dnaQ49* strain displays only a modest mutator phenotype. However, a *dnaQ49* Δ*holE* strain shows, at this temperature, a very high mutator activity (1,000-fold enhanced over that of the single-*dnaQ49* level) (50). A

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similar effect of θ was observed with several other *dnaQ* mutator alleles, particularly those that appeared to carry a structurally impaired ε subunit (50). Thus, it was proposed that one function of θ is to stabilize ε. Such a stabilizing function is likely to be beneficial, as ε appears to be intrinsically unstable (8) or poorly folded (5, 10).

The studies reported here focus on the *holE* homolog, named *hot* (*h*omolog *o*f *t*heta), present in the genome of bacteriophage P1 (25). P1 phage relies entirely on the *E. coli* replication machinery for its replication during both the lytic and lysogenic stages (25). The phage encodes only a few other potential replication proteins, such as *ban* (a *dnaB* helicase homolog), *ssb* (an *ssb* homolog), and *humD* (a *umuD′* homolog), which have been shown to complement corresponding host defects, but it does not contain any other putative components of the Pol III HE (25). Thus, the presence of this putative homolog of the Pol III core is of interest. θ homologs have been found, in addition, in certain large conjugational plasmids of enterobacteriae, *Salmonella* pSLT (30) and *Proteus vulgaris* Rts1 (34), further supporting a meaningful function for this protein. Indirectly, study of these proteins may help elucidate the function of θ in *E. coli*. For example, the greater in vitro stability of Hot than that of θ has permitted determination of the solution structure of Hot by nuclear magnetic resonance methods (4).

Here, we have investigated whether Hot, the P1 θ homolog, can substitute for θ in stabilizing certain *dnaQ* alleles characterized by structurally impaired ε subunits. We show that the presence of the *hot* gene stabilizes *dnaQ49* and similar *dnaQ* alleles, suggesting that Hot protein is indeed capable of substituting for θ. We also observed that at least one *dnaQ* allele can be stabilized by θ but not by Hot, indicating that the interactions of the two proteins with ε are not completely identical. We discuss the possible reasons why the bacteriophage P1 may have a θ homolog.

MATERIALS AND METHODS

Strains and media. The *E. coli* strains used, along with information on their construction, are listed in Table 1. P1 transductions were performed using P1*virA*. P1 used for the cloning of the *hot* gene was from a P1 *c*1-100 Tn*9* lysogen (25) obtained from P. Schendel (Genetics Institute, Cambridge, MA). The construction of MG1655 derivatives NR13104 (Δ*holE203*) and NR16351 (Δ*holE204*::*hot*) is detailed below. The various *dnaQ* alleles (Table 1) were transduced using linkage (~40%) with transposon *zae-502*::Tn*10*, followed by testing for the associated *dnaQ* mutator phenotype (scoring for rifampin-resistant [Rif^r] mutants). The donor strains NR11569, NR11572, NR11573, and NR11641 were *dnaQ* derivatives of NR9501 or NR9601 as described previously (48). Plasmid pKO3 (24) was obtained from G. Church (Harvard Medical School). LB broth was the standard recipe (39). Unless indicated otherwise, antibiotics were added as follows: ampicillin at 100 μg/ml, chloramphenicol at 20 μg/ml, tetracycline at 15 μg/ml, and rifampin at 100 μg/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). Bacteriophage P1 DNA was purified from a P1 *c*1-100 Tn*9* lysogen using the QIAGEN large construction kit (QIAGEN Sciences). Plasmid DNAs were purified with a Qiaprep spin miniprep kit (QIAGEN Sciences). All preparative PCRs were performed using the Expand high-fidelity PCR system (Roche). Analytic PCRs were performed using *Taq* DNA polymerase (Invitrogen). Conditions for PCRs were as 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 of plasmids pUC18*hot* and pKO3*hot* or the PCR product containing the chromosomal Δ*holE*::*hot* insert (strain NR16315) (Table 1) was performed using the Big Dye Terminator v1.1 cycle sequencing kit (Applied Biosystems, Foster City, CA) and Perkin-Elmer ABI model 377 and model 3100 sequencers. Both DNA strands were sequenced.

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

Strain	Relevant genotype	Reference or construction
MG1655	Wild-type	44
NR13104	Δ*holE203*	This work
NR16315	Δ*holE204*::*hot*	This work
NR9695	*dnaQ49 zae-502*::Tn*10*	38
NR11569	*dnaQ920 zae-502*::Tn*10*	48
NR11572	*dnaQ923 zae-502*::Tn*10*	48
NR11573	*dnaQ924 zae-502*::Tn*10*	48
NR11641	*dnaQ928 zae-502*::Tn*10*	48
NR16316	*dnaQ920*	MG1655 × P1/NR11569
NR16317	Δ*holE203 dnaQ920*	NR13104 × P1/NR11569
NR16318	Δ*holE204*::*hot dnaQ920*	NR16315 × P1/NR11569
NR16319	*dnaQ923*	MG1655 × P1/NR11572
NR16320	Δ*holE203 dnaQ923*	NR13104 × P1/NR11572
NR16321	Δ*holE204*::*hot dnaQ923*	NR16315 × P1/NR11572
NR16322	*dnaQ924*	MG1655 × P1/NR11573
NR16323	Δ*holE203 dnaQ924*	NR13104 × P1/NR11573
NR16324	Δ*holE204*::*hot dnaQ924*	NR16315 × P1/NR11573
NR16325	*dnaQ928*	MG1655 × P1/NR11641
NR16326	Δ*holE203 dnaQ928*	NR13104 × P1/NR11641
NR16327	Δ*holE204*::*hot dnaQ928*	NR16315 × P1/NR11641

Construction of NR13104 containing a precise *holE* deletion (Δ*holE203*). Strains containing partial deletions of the *holE* coding sequence have been described previously (45). In the present study, we used a strain, NR13104, containing a complete deletion of the *holE* gene, created by the method of Link et al. (24), as described below. NR13104 contains a precise chromosomal deletion of the 226 bp between the *holE* start and stop codons, replaced by a 36-bp stuffer fragment encoding the 12-mer peptide MVINLVCEGYCV. The primers used are listed in Table 2. Outside primers were GR-2, positioned 496 bp upstream of the *holE* ATG codon (and containing a NotI restriction site in its 5′ tail), and GR-4, positioned 494 bp downstream of the *holE* stop codon (and containing a SalI restriction site). Inside primers were GR-1 and GR-3, which contain a 33-base overlapping sequence at their 5′ ends encoding the 12-residue stuffer fragment. Genomic DNA of strain MG1655 was prepared and amplified by PCR in two separate reactions using primer pairs GR-1–GR-2 and GR-3–GR-4. The resulting two products were then mixed together in a second round of PCR amplification using the outside primers GR-2 and GR-4. Due to the complementarity of the GR1 and GR3 sequences, this yielded an ~1,000-bp PCR product containing the *holE* deletion. The fragment was purified and inserted between the NotI and SalI sites of plasmid pKO3 (24), yielding plasmid pSTBΔ*holE*. This plasmid was used for integration into the MG1655 chromosome at the *holE* sequence, leading to replacement of the *holE*$^+$ gene by the Δ*holE* (Δ*holE203*) fragment by the method of Link et al. (24).

Cloning of the P1 *hot* gene under the control of the *holE* promoter. To clone the P1 *hot* gene under the control of the *holE* promoter, three steps of PCR were used as diagrammed in Fig. 1. The primers used are listed in Table 2. In the first step, we created three separate PCR products (A, B, and C in Fig. 1). In reaction A, a 499-bp fragment of DNA upstream of the *E. coli holE* gene was amplified using primers Pr1 and Pr2 on *E. coli* MG1655 chromosomal DNA, yielding a product that included the *holE* promoter but ended at the ATG start codon. In reaction B, primers Pr3 and Pr4 were used on bacteriophage P1 DNA to create a 313-bp fragment containing the entire P1 *hot* coding sequence starting at the ATG start codon and ending 27 nucleotides past the TAG stop codon. In reaction C, 564 bp of *E. coli* chromosomal DNA downstream of the *holE* gene was amplified using primers Pr5 and Pr6 on *E. coli* chromosomal DNA. The "outside" primers Pr1 and Pr6 contained tails carrying NotI and SalI restriction sequences, respectively, to facilitate the eventual cloning of the desired construct. "Inside" primer pairs Pr2-Pr3 and Pr4-Pr5 contained complementary tails and sequences (Table 2) that permitted assembly of these three fragments by overlap PCR into one large, 1,399-bp fragment, as described below. In the second step (Fig. 1D), fragments A and B were joined using Pr1 and Pr4 based on the overlap provided by Pr2 and Pr3. The resulting fragment contained the P1 *hot* coding sequence fused to the *holE* promoter, while at the same time *holE* was deleted starting from its ATG codon. In the final step (Fig. 1E), the fragment generated in step 2 was joined to fragment C using primers Pr1 and Pr6, facilitated by the

TABLE 2. Oligonucleotide primers used in PCRs

Name	Sequence[a]
GR-1[b]	5′-**CACGCAATAACCTTCACACTCCAAATTTATAAC**CATTCTTAATCTCCTCATCATTCGCGG-3′
GR-2	5′-AAGGAAAAAAGCGGCCGCTGTAGTGTCCTTTCGTTTTATGCCC-3′
GR-3[b]	5′-**GTTATAAATTTGGAGTGTGAAGGTTATTGCGTG**TAAAACTTATACAGAGTTACACTTTCTTACATAACG-3′
GR-4	5′-CGCACGCATGTCGACCGGTTGTTCATCGACCACC-3′
Pr1	5′-GAGAAATGCGGCCGCTGTAGTGTCCTTTCGTTTTATGCCC-3′
Pr2[c]	5′-**CATTCTTAATCTCCTCATCATTCGC**-3′
Pr3[c]	5′-**GCGAATGATGAGGAGATTAAGA***ATGTACGATTGGAATATTGCAGC*-3′
Pr4[d]	5′-**CTCAATAGGATC***CGAGAAGTTCAAAACGGTTAACTACC*-3′
Pr5[d]	5′-***ACTTCTCG*GATCCTATTGAG**CAGAGTTACACTTTCTTACATAACGC-3′
Pr6	5′-CAAATCAGTCGACGCCAGCAGGTCGGGTTCTCC-3′

[a] Underlines represent NotI (GR-2, Pr1) or SalI (GR-4, Pr6) sequences. Double underlines indicate *holE* or *hot* start (GR-1, Pr3) or stop (GR-3, Pr5) codons. P1 *hot* sequences (in Pr2, Pr4, and Pr5) are italicized.
[b] The first 33 bases of GR-1 and GR-3 are complementary (boldface).
[c] The first 25 bases of Pr2 and Pr3 are complementary (boldface) and include the *hot* ATG start codon.
[d] The first 20 bases of Pr4 and Pr5 are complementary (boldface).

overlap of primers Pr4 and Pr5. This joined the *hot* gene to the *E. coli* chromosomal sequence downstream of the *holE* gene. In the final product, the *hot* TAG stop codon was followed by 27 nucleotides of downstream *hot* sequence and then by 12 unrelated nucleotides containing a BamHI site (not relevant to the present study), which replaced 8 nucleotides that normally follow the *holE* TAA stop codon. The final 1,399-bp product contained the P1 *hot* open reading frame surrounded on either side by about 500 nucleotides of *E. coli* chromosomal DNA (Δ*holE*::*hot*). This product was blunt ended into the SmaI site of plasmid pUC18, yielding pUC18*hot*. The plasmid was sequenced to confirm the correctness of the construct.

Plasmid constructions. In addition to pUC18*hot* described above, two additional pUC18 plasmids were made, as well as corresponding derivatives of low-copy-number plasmid pKO3 (24). Primers Pr1 and Pr6 (Table 2) were used to obtain a PCR fragment from genomic DNA of strains MG1655 (*holE*$^+$) and NR13104 (Δ*holE*). These fragments contained the *holE* gene and the Δ*holE* deletion, respectively, flanked by approximately 500 base pairs of genomic sequence on either side of *holE*. The fragments were inserted directly into the SmaI restriction site of pUC18, yielding pUC18*holE* and pUC18Δ*holE*, respectively. Plasmid pKO3 is a low-copy-number (three to five copies) temperature-sensitive vector that can be used for creating gene deletions or genomic replacements (24). The PCR inserts from the three pUC18 plasmids were transferred into pKO3 using the NotI-SalI restriction sites contained in their ends, yielding pKO3*holE*, pKO3*hot*, and pKO3Δ*holE*, respectively.

Insertion of the P1 *hot* gene into the *E. coli* chromosome. The pKO3*hot* plasmid containing the P1 *hot* gene under the control of the *holE* promoter (see above) was used to insert the *hot* construct into the *E. coli* chromosome by the method of Link et al. (24). NR13104 (Δ*holE203*) cells were transformed by pKO3*hot*, and transformants were selected at 40°C on LB plates containing chloramphenicol. Several chloramphenicol-resistant colonies were diluted in LB broth and plated at 30°C on LB broth containing 5% sucrose. Single colonies were tested for loss of chloramphenicol resistance as well as the presence of the *hot* gene by PCR using primers Pr3 and Pr4 (Table 2). A PCR product containing the recombinant gene was sequenced to confirm the correctness of the construct. The new chromosomal allele was designated Δ*holE204*::*hot* (Table 1)

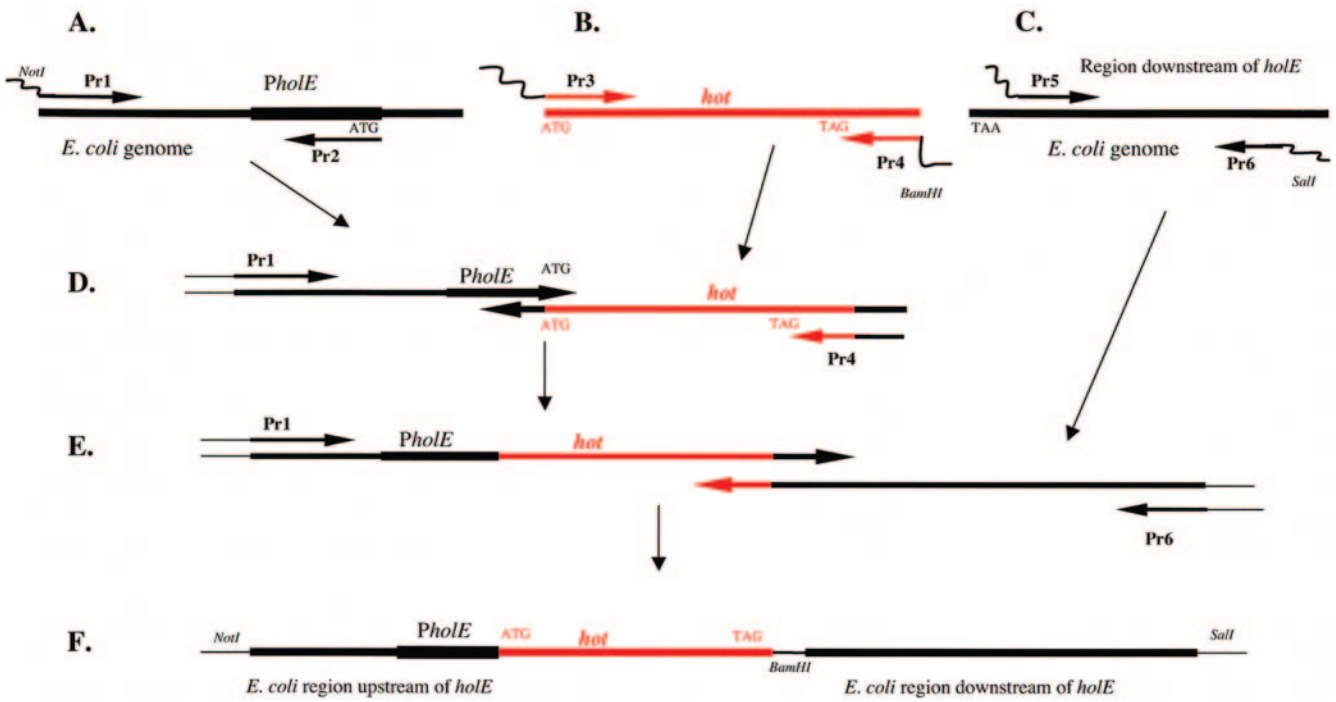

FIG. 1. Three steps of PCR to replace the *holE* coding sequence by the P1 *hot* open reading frame. Red lines indicate P1 genomic DNA, and the black lines indicate *E. coli* genomic DNA. P*holE* denotes the *holE* promoter. Arrows indicate the 5′→3′ direction of DNA primers. See Materials and Methods for further details and Table 2 for primer sequences.

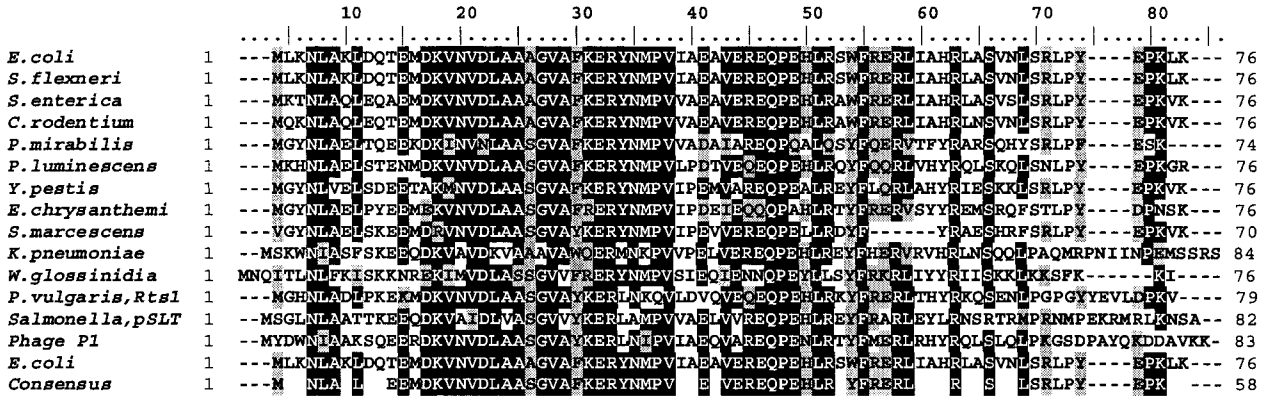

FIG. 2. Alignment of θ homologs. A ClustalW multiple-amino-acid sequence alignment was created using BioEdit software (11) and appears with the indicated consensus sequence. The sequences were obtained by BLAST searches of the *coli*BASE database (*P. mirabilis*, *C. rodentium*, *S. marcescens*) or the NCBI database (others) as described in Materials and Methods. Multiple, but identical, entries were found for most of the θ homologs, except for *Y. pestis* and *Y. pestis KIM*. The θ homolog from the latter contained several extra amino acids at its N terminus. These are not shown here. The *E. coli* θ sequence is presented on both the top and bottom to facilitate comparisons. Identical amino acids (70%) are shown with a black background, and similar residues (70%) are shown with a gray background. The indicated species are in the order *Escherichia coli*, *Shigella flexneri*, *Salmonella enterica*, *Citrobacter rodentium*, *Proteus mirabilis*, *Photobacter luminescens*, *Yersinia pestis*, *Erwinia chrysanthemi*, *Serratia marcescens*, *Klebsiella pneumoniae*, *Wigglesworthia glossinidia*, *Proteus vulgaris*, *Salmonella* sp., bacteriophage P1, and *Escherichia coli*.

Protein expression. Expression of θ and Hot was assayed by Western blotting using a rabbit anti-θ polyclonal antibody (prepared by Biocon, Inc., Rockville, MD). Strains containing various pUC18 plasmids (see above) were grown overnight at 37°C in 1 ml LB broth plus ampicillin (500 μg/ml). Control strains without plasmid were grown in LB broth. Cells were centrifuged and washed with Tris-buffered saline; the pellet was sonicated and lysed in 100 μl of Tricine-sodium dodecyl sulfate loading buffer (Invitrogen) containing 5% β-mercaptoethanol. Protein samples (8 μl) were electrophoresed through a 16% polyacrylamide-Tricine-sodium dodecyl sulfate gel (Invitrogen) and transferred to a nitrocellulose membrane using a MilliBlot graphite electroblotter apparatus (Millipore). To remove nonspecific binding activities, the anti-θ antibody was pretreated (3 h at room temperature) with a cell lysate of strain NR13104 (Δ*holE*) (prepared by sonication) in I-block solution (Applied Biosystems), and the precipitate was removed by centrifugation. Goat anti-rabbit antibody labeled with alkaline phosphatase (Bio-Rad) was used as secondary antibody. The Western-star kit (Applied Biosystems) was used for membrane development, as recommended by the manufacturer. Purified θ, used as a protein marker, was as described previously (22).

Mutant frequencies. To measure mutant frequencies for each strain, the frequency of Rif^r mutants in overnight cultures was determined. Ten to 15 cultures for each strain started from individual colonies were grown overnight in 1 ml LB broth at 37°C. For each culture, a 50- to 100-μl aliquot of a 10^{-6} dilution was spread on an LB plate to determine the total number of viable cells, and 100 μl of the undiluted or appropriately diluted culture was spread on LB broth-Rif plates to determine the number of rifampin-resistant mutants. The mutant frequency for each culture was calculated by dividing the total number of mutants by the total number of viable cells. The data were analyzed using the statistical analysis software Prism (GraphPad).

Database searches and protein sequences comparison. BLAST (BLASTP) searches for homologs of *E. coli* θ protein were performed with the GenBank (http://www.ncbi.nlm.nih.gov/) and *coli*BASE (http://colibase.bham.ac.uk) databases, using the sequence of *E. coli* θ as a query. The *Klebsiella pneumoniae* sequence was found by translated BLAST (TBLASTN) on the NCBI microbial genome database. A ClustalW multiple-amino-acid sequence alignment was created using BioEdit software with BLOSUM62 as the similarity matrix (11). This alignment was used in conjunction with Tree Top analysis tools of the GeneBee Molecular Biology server (http://www.genebee.msu.su/genebee.html) to create a phylogenetic tree of θ proteins (by topological algorithm).

RESULTS

Alignment of θ with its homologs. In Fig. 2, we present an amino acid alignment of *E. coli* θ with several homologs as found by BLAST searches in GenBank and *coli*BASE (see Materials and Methods). The bacteria in this group (*Shigella flexneri*, *Salmonella enterica*, *Citrobacter rodentium*, *Proteus mirabilis*, *Photorhabdus luminescens*, *Yersinia pestis*, *Erwinia chrysanthemi*, *Serratia marcescens*, *Klebsiella pneumoniae*, and *Wigglesworthia glossinidia*) are all gram-negative enterobacteriae with relatively close relatedness to *E. coli*. The homology with θ is generally good, as expected for these related organisms. The homology is strongest in the N-terminal part (residues 1 to 56 of θ) and is more diverse in the remaining C-terminal part (residues 57 to 76). The alignment also includes two homologs residing on large conjugative plasmids: plasmid Rts1, originally isolated from *Proteus vulgaris* (34), and plasmid pSLT from *Salmonella enterica* serovar Typhimurium LT2 (30). Rts1 is a large (2.1-Mb) conjugative plasmid, while pSLT (0.94 Mb) is the conjugative virulence plasmid of *S. enterica* serovar Typhimurium LT2. Their homology with θ is lower but still generally good (44 to 54% identical, 57 to 62% similar).

The alignment includes the bacteriophage P1 Hot protein, the product of the phage *hot* gene (the mnemonic *hot* derives from *h*omolog *o*f *t*heta) (25). Hot also has good homology to θ (47% identity, 61% similarity), particularly in the N-terminal part. In the following, we describe experiments aimed at finding out whether the P1 *hot* gene product can act as a functional replacement for θ in its role of Pol III accessory factor.

***hot* expression in *E. coli*.** The *hot* gene on P1 has been reported to be expressed from a "late" promoter (19, 20, 25). Late P1 promoters regulate the expression of proteins needed at the end of the phage cycle, including the coat proteins needed for phage packaging (25). The late promoters require the P1 gene *10* (*lpa*) product, a transcription factor specific for late phage gene expression (19, 20, 25). Expression of this transcription factor is reportedly toxic to *E. coli* (19), and therefore we decided to not use the P1 native expression system. Instead, we used PCR methods to create a construct in which the *hot* gene is expressed from the *E. coli holE* promoter. Specifically, we amplified the *hot* coding sequence and inserted it in front of the *holE* promoter with its ATG start codon

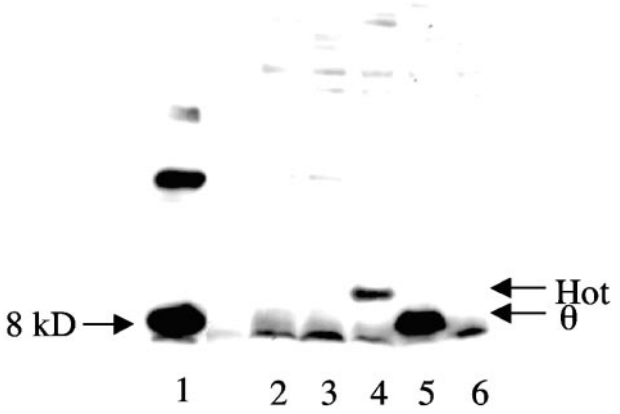

FIG. 3. Expression of θ and Hot from pUC18 plasmids detected by Western blot analysis using anti-θ antibody. Strain NR13104 (Δ*holE*) containing various pUC plasmids was grown overnight at 37°C in LB broth plus ampicillin (500 μg/ml). The control strains NR13104 (Δ*holE*) and MG1655 (*hol*$^+$) were grown in LB broth. See Materials and Methods for details on lysate preparation, gel electrophoresis, and the Western blot procedure. Lane 1, purified θ protein; lane 2, Δ*holE* strain, no plasmid; lane 3, Δ*holE* strain containing pUC18; lane 4, Δ*holE* strain containing pUC18*hot*; lane 5, Δ*holE* strain containing pUC18*holE*; lane 6, MG1655 (wild type). The higher-molecular-mass band in lane 1 may represent a θ dimer, but this was not further investigated.

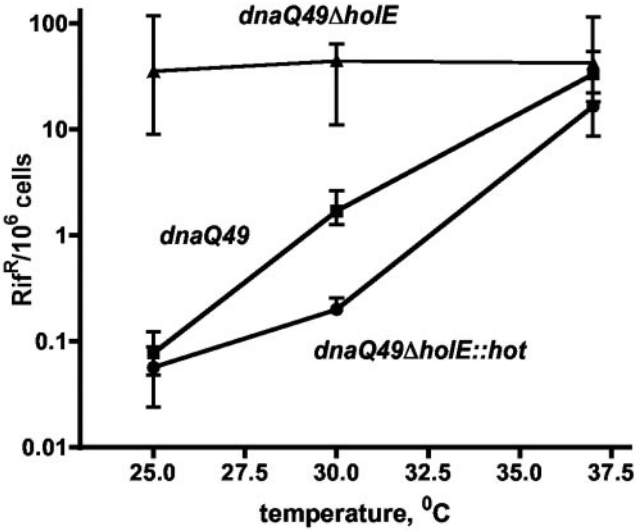

FIG. 4. Mutability of *dnaQ49* strains containing a chromosomal *holE*$^+$, Δ*holE*, or Δ*holE*::*hot* allele. Twelve to 18 independent cultures for each strain were incubated overnight at the indicated temperatures; plates were incubated at 30°C. The median mutant frequencies along with the indicated interquartile ranges were calculated using Prism (GraphPad) (see Materials and Methods). Note the log scale on the *y* axis.

precisely in place of the *holE* ATG codon, while *holE* itself was deleted in the process. The procedure is diagrammed in Fig. 1 and described in more detail in the Materials and Methods. Initially the new construct was obtained in multicopy plasmid pUC18, yielding pUC18*hot*. Western blots on extracts from a Δ*holE* strain containing pUC18*hot* showed that the recombinant cells produced a significant amount of Hot protein (Fig. 3, lane 4). The size of Hot is consistent with its molecular size (83 amino acids). It is clearly distinct from θ (76 amino acids), either as purified θ or in extracts of pUC18*holE* containing strains (lanes 1 and 5). pUC18*holE* is identical to pUC18*hot* except that it contains *holE* instead of *hot* (see Materials and Methods). The antibody used was a polyclonal antibody generated against θ, and the recognition of Hot by this antibody is consistent with presumed similarities between θ and Hot. The antibody was not capable of detecting either θ (Fig. 3, lane 6) or Hot (not shown) in extracts when expressed from a single chromosomal gene copy.

Single-copy replacement of *holE* by *hot*. Cells containing pUC18*hot* and pUC18*holE* produced small and heterogeneously sized colonies. Also, the plasmids were rapidly lost unless high levels of ampicillin were included in the medium. These results suggested that excessive amounts of θ of Hot are deleterious. When the inserts were transferred to the low-copy-number vector pKO3 (three to five copies) (see Materials and Methods), colonies were of normal size and were capable of stable plasmid maintenance. However, for our study to investigate possible replacement of θ by Hot under normal single-copy conditions, we decided to generate a strain in which *hot* is integrated, as a single copy, in the *E. coli* chromosome, replacing *holE*. This replacement was readily possible using the pKO3*hot* plasmid by the method of Link et al. (24). This yielded strain NR16315 (Δ*holE204*::*hot*) (Table 1 and Materials and Methods), in which *hot* is expressed chromosomally from the native *holE* promoter.

One conveniently assayable phenotype associated with the lack of θ (Δ*holE* strain) is reduced stability of the *dnaQ49* mutator mutant. The *dnaQ49* mutant (containing the V96G mutation) carries a defective polymerase III ε subunit whose stability is greatly dependent on the θ subunit (50). The *dnaQ49* mutant is only a modest mutator at low temperatures (25°C), but it becomes a very strong mutator (up to 1,000-fold enhanced) at 37°C due to collapse of its proofreading ability (13, 17, 36, 40). In contrast, the *dnaQ49* Δ*holE* strain is an exceptionally strong mutator even at the lowest temperature (50).

To assay the effect of *hot* on the *dnaQ49* mutator, we compared the mutabilities of three *dnaQ49* strains: the *dnaQ49* *holE*$^+$, *dnaQ49* Δ*holE*, and *dnaQ49* Δ*holE*::*hot* strains. The results in Fig. 4 show that, as before (50), the *dnaQ49* Δ*holE* strain is an extremely strong mutator regardless of the temperature compared to the *dnaQ49* *holE*$^+$ strain, which shows greatly reduced mutator activity at the lower temperature and reaches high levels of mutagenesis only at 37°C. Quantitatively, the presence of θ reduces the mutant frequency at 25°C by about 1,000-fold. Importantly, the results also show that the *dnaQ49* Δ*holE*::*hot* strain—containing *hot* instead of *holE*—behaves like the *dnaQ49* strain; i.e., it displays low mutability at low temperature. This indicates that Hot, like θ, can stabilize the *dnaQ49* ε subunit. Interestingly, the stabilizing effect of Hot appears actually greater than that of θ. In this experiment, the mutant frequency of the *dnaQ49* Δ*holE*::*hot* strain is 3- to 10-fold lower at the three temperatures than that of the *dnaQ49* *holE*$^+$ strain. In four independent experiments, the

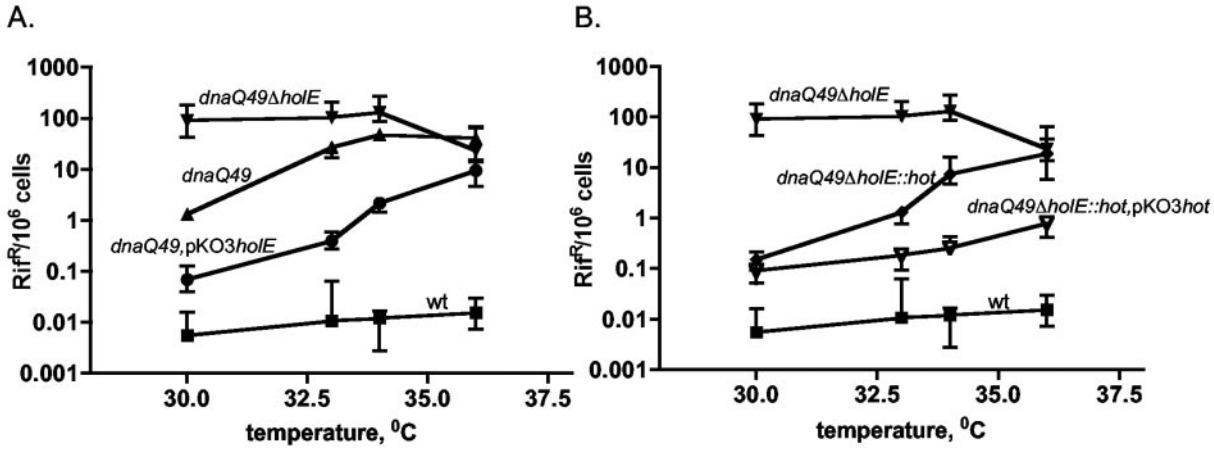

FIG. 5. Reduction of the *dnaQ49* mutator activity by multiple copies of the *holE* (A) or *hot* (B) gene. In panel A, the mutant frequency of the *dnaQ49* Δ*holE* strain is compared to those of its counterparts containing either one copy (*dnaQ49*) or multiple copies (*dnaQ49* pKO3*holE*) of the *holE* gene. The MG1655 (wild-type [wt]) strain is shown as a control. In panel B, the *dnaQ49* Δ*holE* strain is compared to its counterparts containing either one copy (*dnaQ49* Δ*holE*::*hot*) or multiple copies (*dnaQ49* Δ*holE*::*hot* pKO3*hot*) of the P1 *hot* gene. All strains not containing either pKO3*holE* or pKO3*hot* carried pKO3Δ*holE*. Cultures were grown overnight at 30, 33, 34, or 36°C in LB broth plus chloramphenicol; plates were incubated at 30°C. Mutant frequencies are based on 12 independent cultures for each strain (see Materials and Methods). The median values and interquartile ranges (indicated) were calculated using Prism software (GraphPad).

average reductive effect of Hot was 2.8-, 15-, and 5.7-fold greater than the reductive effect of θ at 25, 30, and 37°C, respectively. Based on these experiments, we conclude that P1 Hot can replace the θ subunit and must be considered a functional homolog of θ.

Multiple copies of *holE* or *hot* further suppress the *dnaQ49* mutator effect. The above-described experiments were also performed with the *holE* and *hot* genes on the low-copy-number plasmid pKO3, as shown in Fig. 5. Panel A shows that expression of θ from pKO3*holE* at temperatures between 30 and 34°C reduces the mutant frequency of the *dnaQ49* strain by 1 order of magnitude compared to that of a strain with a single chromosomal *holE* copy (note that pKO3 has a temperature-sensitive replication origin and cannot be grown above 36°C). A similar effect is seen in the case of Hot protein expressed from pKO3*hot* (Fig. 5B). At 30°C, a single copy of *hot* exerts a maximal effect, although at a higher temperature, multiple copies are clearly more effective than the single copy. Overall, the mutability of the *dnaQ49* strain with multiple *hot* copies shows only weak temperature dependence. These results, showing that multiple copies of *holE* and *hot* further stabilize the *dnaQ49* mutant, have implications for the precise mechanism underlying the *dnaQ49* mutator effect. In addition, they reinforce the greater efficacy of Hot in suppressing the *dnaQ49* mutator effect.

Effect of *hot* on other *dnaQ* mutator alleles. In the previous study on the θ subunit (50), we also investigated a series of *dnaQ* alleles other than *dnaQ49* (V96G). We found several additional alleles whose mutability was further elevated in the Δ*holE* background. We concluded that these other *dnaQ* alleles, like *dnaQ49*, contain certain structural defects that are exacerbated by the lack of a supporting θ subunit. In the experiment whose results are shown in Fig. 6, we tested the relative efficiency of θ and Hot in stabilizing these other *dnaQ* mutators. Hot, like θ, was capable of restoring low mutability to the Δ*holE dnaQ920* (R56W), Δ*holE dnaQ923* (H66Y), and Δ*holE dnaQ924* (L171F) strains, further corroborating the ability of Hot to substitute for θ. Interestingly, little or no effect of Hot was observed for the *dnaQ928* (G17S) allele, although this allele is readily stabilized by θ (Fig. 6). These results suggest that while both θ and Hot interact with and presumably stabilize ε, there are additional aspects to this interaction that are not identical for θ and Hot.

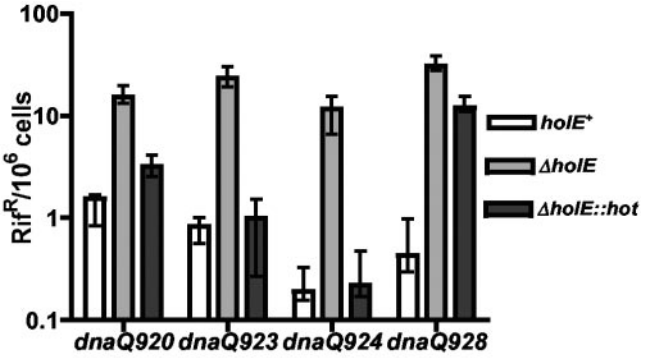

FIG. 6. Effect of θ and Hot on *dnaQ920*, *dnaQ923*, *dnaQ924*, and *dnaQ928* mutants. Mutant frequencies were calculated for 12 independent cultures for each strain (see Materials and Methods). Cultures were grown overnight at 37°C in LB broth; plates were also incubated at 37°C. (Note that the mutant frequencies for the mutator strains are not comparable to those reported previously [50] because the latter were determined in the mismatch repair-deficient *mutL* background). Data were analyzed using Prism software (GraphPad). The graph shows median values and the interquartile ranges for the frequency of rifampin-resistant mutants. The *x* axis indicates, for each *dnaQ* allele, the three strains containing the *holE*$^+$, Δ*holE*, or Δ*holE*::*hot* chromosomal configuration.

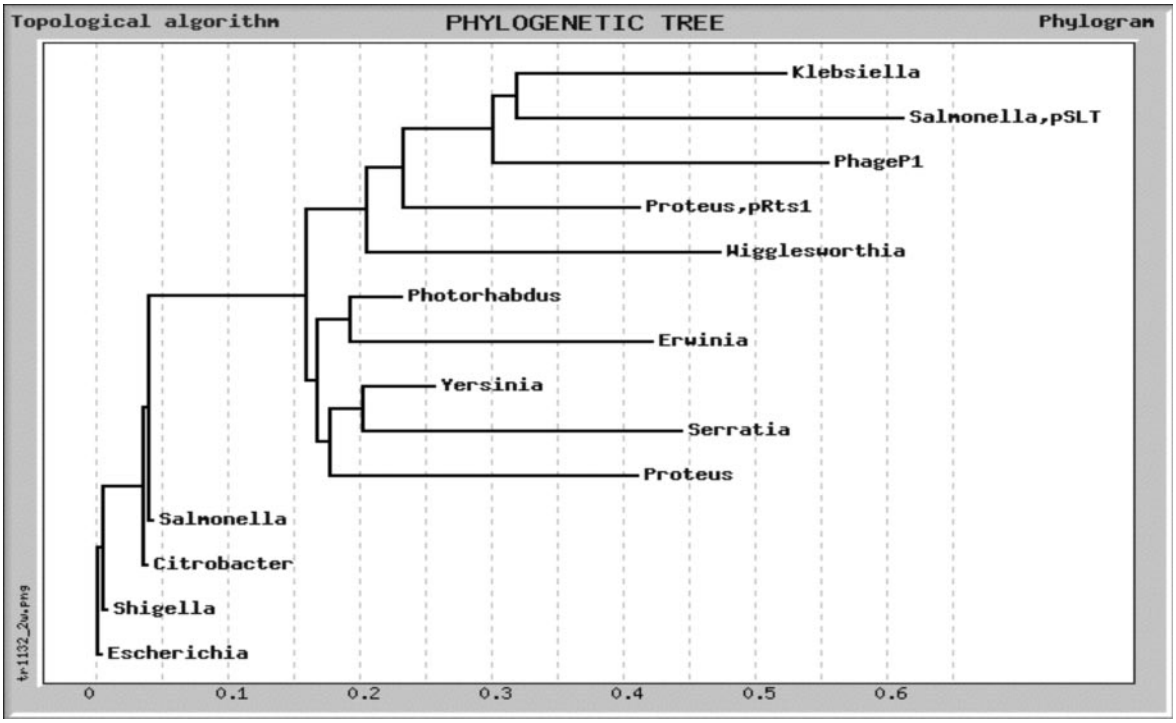

FIG. 7. Phylogenetic tree of θ protein homologs. The θ homologs are as shown in the alignment of Fig. 2. The tree was obtained using Tree Top sequence analysis tools on the GeneBee Molecular Biology Server (http://www.genebee.msu.su/genebee.html) and a topological algorithm.

DISCUSSION

The present data indicate that the phage P1 Hot protein, a homolog of the *E. coli* Pol III θ subunit, can compensate for the lack of θ, as observed through the stabilizing effect of the *hot* gene in several *dnaQ* strains. This compensation most logically involves a direct substitution for θ in its interaction with the ε subunit and, likely, the incorporation of Hot into the Pol III core. The presence of this functional homolog of θ in the bacteriophage raises the question of why the phage contains this homolog. The answer is likely intertwined with the precise function of θ in *E. coli*.

The role of θ. We have previously suggested (50) that θ might act as a chaperonin for the ε subunit. This suggestion was based on the stabilizing effect of θ on several impaired *dnaQ* alleles, as well as on *Saccharomyces cerevisiae* tri-hybrid assays showing a strongly improved α-ε interaction in the presence of θ (50). The proposal is consistent with other observations of θ, such as the tight interaction between ε and θ (47), the intrinsic instability of ε in vivo (8), and the improved in vitro behavior of purified ε in the presence of θ (5, 10, 12). We also noted that θ is found among organisms with a 3′ proofreading activity that is present on a separate subunit (rather than being part of the polymerase polypeptide), suggesting that the need for θ may be related to the peculiarities of a free ε subunit. On the other hand, based on recent GenBank searches, we note that θ is not found among several other gram-negative bacteria that also contain an isolated proofreading activity, such as *Vibrio cholerae*, *Haemophilus influenzae*, *Pseudomonas aeruginosa*, or *Photobacterium profundum*. Therefore, the relevance of this connection remains unclear. We cannot exclude the possibility that in those organisms, θ orthologs exist but are not detected by BLAST search due to sequence diversion. Whether the stability of ε will be a limiting factor for efficient or faithful replication in a given organism will likely depend on multiple factors, such as the intrinsic stability of the particular ε, the nature of its interactions with the α subunit, and the levels of chaperone/heat shock proteins (8), which may all differ in different taxa of gram-negative bacteria.

The relatedness of θ proteins. The homology of the various θ proteins as presented in Fig. 2 is extensive, particularly in the N-terminal part. Additional insight into the relatedness of the proteins may be gleaned from Fig. 7, where we show a phylogenetic tree constructed based on the relatedness to θ. It is clear that, within this scheme, *E. coli* θ, along with the proteins from the closely related *Salmonella*, *Shigella*, and *Citrobacter* species, is more distantly related to P1 Hot. Hot is part of a separate grouping that contains the two plasmid-encoded homologs (*Salmonella* pSLT and *Proteus* Rst1) as well as the proteins from *Klebsiella* and *Wigglesworthia*. Thus, it is unlikely that *hot* is a recent acquisition from its current host, *E. coli*. Instead, it may have its origin in a different species. The same argument applies to the homologs encoded by the *Salmonella* pSLT and *Proteus* Rts1 plasmids, which also differ significantly from their host homologs. Thus, P1 Hot may be optimized for interaction with a phylogenetically different ε subunit. We note that *Wigglesworthia glossinidia* is an endosymbiont (of tsetse flies) with a greatly reduced genome (698 kb, ~15% of the *E. coli* chromosome) (1). That the organism has retained the θ homolog despite the severe size reduction of its genome is a

further suggestion that the θ homolog fulfills a relevant function. On the other hand, two other symbionts from the enterobacterial group whose genomes have been sequenced and which also have a reduced genome, *Buchnera aphidicola* (43, 51) and *Blochmannia floridanus* (9), do not possess a θ homolog.

Relative effects of θ and Hot in suppressing *dnaQ* mutator alleles. Our results with the *dnaQ49* mutant show that Hot protein is more effective than θ in stabilizing this allele (Fig. 4). This observation may indicate that Hot interacts more effectively with ε, leading to additional stabilization of this subunit. Alternatively, Hot protein may be intrinsically more stable, leading to a higher effective protein concentration available for interaction with ε. As seen from Fig. 5, increased θ or Hot lead to additional reduction in the *dnaQ49* mutator activity. Further support for this hypothesis comes from structural studies performed on θ and Hot. Nuclear magnetic resonance analysis of the θ solution structure showed it to be a rather unstructured protein (15, 22). Hot behaved significantly better in these experiments, permitting determination of its solution structure (4). Circular dichroism studies also indicated that the stability of Hot was greater than that of θ (4). On the other hand, experiments with the *dnaQ920*, *dnaQ923*, and *dnaQ924* alleles showed θ and Hot to be similarly effective in stabilizing these alleles, while *dnaQ928* could be stabilized only by θ (Fig. 6). These results are suggestive of differences in the precise ε-θ and ε-Hot interactions. In a subsequent report (A. K. Chikova and R. M. Schaaper, unpublished data), we describe certain circumstances in which the substitution of Hot for θ actually increases mutagenesis. Analysis of the ε-θ and ε-Hot complexes by structural methods may shed further light on these questions.

Effects of multicopy *holE* or *hot* on the *dnaQ49* mutant. The data of Fig. 5 indicate that the *dnaQ49* mutator effect can be further reduced when these proteins are expressed from plasmid pKO3, when protein levels may be expected to be increased three- to fivefold based on the plasmid copy number. This observation has implications for the nature of the *dnaQ49* defect. The *dnaQ49* mutation is genetically recessive (13, 29). This has been interpreted to indicate that the encoded ε subunit is defective in its binding to the Pol III α subunit. Indeed, yeast two-hybrid analyses have shown the α-ε interaction to be severely diminished for this mutant (14, 49, 50). On the other hand, it is likely that part of the *dnaQ49* defect reflects a catalytic deficiency. The amino acid substitution responsible for the *dnaQ49* defect, V96G, resides in the N-terminal catalytic domain, while the primary determinant for interaction with the α subunit resides in the C-terminal domain (residues 187 to 243) (35, 49). Also, the V96G mutation resides near the exonuclease II motif (48), which provides a component of the ε catalytic site.

The observation that θ and Hot overexpression further reduces the *dnaQ49* mutant frequency suggests that in *dnaQ49* strains the chromosome is replicated, at least part of the time, by a form of HE that lacks θ. In fact, this form of HE may also lack ε, as yeast two- and three-hybrid assays have shown the DnaQ49-α interaction to be dramatically reduced in the absence of θ (14, 49, 50). HE containing a core consisting of only the α subunit has been prepared in vitro and shown to synthesize DNA effectively, albeit with reduced processivity (16, 28, 46). Chromosomal DNA synthesis by this kind of HE would naturally be highly mutagenic due to the lack of any proofreading. The existence of such a proofreading-defective form of HE in vivo has been speculated upon (6), but our current results may provide a first experimental indication of their existence.

What is the function of Hot in P1? It seems most logical to analyze this question in the context of the function of θ. Assuming that the function of θ is to support the intrinsically unstable ε subunit, one possibility is that the function of Hot may be to help sequester and stabilize ε for the benefit of the phage. As P1 is dependent on Pol III HE for its replication (25), establishing sufficient amounts of ε for incorporation in the Pol III core may be one way to ensure the availability of a sufficient number of Pol III HE molecules for phage replication. There are only limited numbers of Pol III core and Pol III HE molecules per cell (26, 27, 32), making this an issue of relevance to the phage. The need for additional HE would be most acute in the lytic cycle but could also be felt during P1 plasmid maintenance. One interesting concern with this model is that *hot* has been classified as a late gene (19, 20, 25), and Hot protein would be expected to be produced primarily during the phage packaging stage. One (speculative) possibility is that Hot, after being produced late in infection, is encapsulated in the virion and released upon infection (25). On the other hand, the *hot* promoter does not conform exactly to the late promoter consensus, because its −10 and −22 sequences, characteristic for the P1 late genes, are spaced 9 nucleotides apart instead of the canonical 4 (25). Thus, it will be important to investigate the precise timing of Hot expression in the phage life cycle, including the prophage stage, to address these questions. It will also be of interest to investigate the viability or properties of a P1 mutant lacking *hot*.

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