

## The C-Terminal Domain of DnaQ Contains the Polymerase Binding Site

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**The *Escherichia coli* *dnaQ* gene encodes the 3'→5' exonucleolytic proofreading ( $\epsilon$ ) subunit of DNA polymerase III (Pol III). Genetic analysis of *dnaQ* mutants has suggested that  $\epsilon$  might consist of two domains, an N-terminal domain containing the exonuclease and a C-terminal domain essential for binding the polymerase ( $\alpha$ ) subunit. We have created truncated forms of *dnaQ* resulting in  $\epsilon$  subunits that contain either the N-terminal or the C-terminal domain. Using the yeast two-hybrid system, we analyzed the interactions of the single-domain  $\epsilon$  subunits with the  $\alpha$  and  $\theta$  subunits of the Pol III core. The DnaQ991 protein, consisting of the N-terminal 186 amino acids, was defective in binding to the  $\alpha$  subunit while retaining normal binding to the  $\theta$  subunit. In contrast, the  $\Delta$ 186 protein, consisting of the C-terminal 57 amino acids, exhibited normal binding to the  $\alpha$  subunit but was defective in binding to the  $\theta$  subunit. A strain carrying the *dnaQ991* allele exhibited a strong, recessive mutator phenotype, as expected from a defective  $\alpha$  binding mutant. The data are consistent with the existence of two functional domains in  $\epsilon$ , with the C-terminal domain responsible for polymerase binding.**

The *Escherichia coli* DNA polymerase III (Pol III) holoenzyme (HE) is responsible for the efficient and accurate replication of the bacterial chromosome (for a review, see references 9, 10, and 16). This multisubunit enzyme contains a core polymerase with both polymerizing and exonucleolytic activities. Unlike those of most other proofreading polymerases, these two activities of Pol III are contained in two separate polypeptides. The polymerase activity is contained in the  $\alpha$  subunit (*dnaE* gene product), while the 3'→5' exonuclease (Exo) activity, which serves as a proofreader for replication errors, is contained in the  $\epsilon$  subunit (*dnaQ* gene product). The Pol III core also contains the  $\theta$  subunit (*holE* gene product), but the function of this subunit is unclear (4, 20, 21). The subunits are arranged in a linear fashion,  $\alpha$ - $\epsilon$ - $\theta$ , with  $\epsilon$  binding both  $\alpha$  and  $\theta$  (21). The precise interactions, both functionally and structurally, between the core subunits and of the core subunits with the additional accessory factors of the HE are the subject of active current interest (2, 7, 14).

Previously, in an effort to better understand the roles of the  $\epsilon$  subunit, we undertook a genetic analysis of a large collection of *dnaQ* mutator mutants (23). These studies confirmed and extended the importance of three conserved exonuclease motifs in the catalytic activity of the DnaQ enzyme (see Fig. 1) and also suggested that the C terminus of  $\epsilon$  might be required for interaction with the  $\alpha$  subunit. *dnaQ932*, a mutant containing a stop codon 3 amino acids from the C-terminal end of  $\epsilon$ , was recessive in the presence of a single copy of *dnaQ*<sup>+</sup>, suggesting that the DnaQ932 protein does not compete efficiently with the wild-type protein for binding to  $\alpha$ . Furthermore, the region between residues 190 and 212 has been identified as a so-called Q-linker (24). In certain proteins, Q-linkers act as a hinge to link domains that have different functions (22, 24). In  $\epsilon$ , this linker may tether the N-terminal exonuclease domain to the C-terminal polymerase-binding domain (Fig. 1).

The modular structure of  $\epsilon$  has been probed by limited proteolysis in vitro (17). This study revealed a stable 186-amino-acid N-terminal fragment, created by proteolytic cleavage on the N-terminal side of the proposed Q-linker (Fig. 1). The 186-amino-acid fragment, still comprising the three Exo motifs, has been investigated biochemically and shown to be fully proficient in exonuclease activity but to lack interaction with the  $\alpha$  subunit (17). Here, we present a genetic analysis of the function of the C terminus of  $\epsilon$ . We used the yeast two-hybrid assay to study the interactions of the proposed N- and C-terminal domains with the  $\alpha$  and  $\theta$  subunits. In addition, we analyzed the properties of an *E. coli* mutant carrying the truncated N-terminal 186-amino-acid  $\epsilon$  as the sole source of DnaQ protein.

The yeast two-hybrid system was used previously to probe the interactions of the  $\alpha$ ,  $\epsilon$ , and  $\theta$  proteins (8). For example, the  $\epsilon$  subunit specified by the strong mutator allele *mutD5*, carrying a Thr15Ile missense mutation in the Exo I motif, was shown to be fully proficient in binding to both the  $\alpha$  and  $\theta$  subunits, consistent with its specific catalytic defect and dominant mutator phenotype (5, 6, 18), while the  $\epsilon$  subunit specified by the recessive *dnaQ49* mutant (15) was defective in binding to  $\alpha$  (and, to various extents, also to  $\theta$ ).

Our first experiments used *dnaQ991*, a *dnaQ* allele generated by site-directed mutagenesis (Stratagene Quik Change kit) in the two-hybrid vector pGBT9-2-*dnaQ* (8), by converting codon 187 (Phe) into a TAA stop codon (TTT→TAA), resulting in a truncated  $\epsilon$  containing only the N-terminal 186 amino acids (Fig. 1). pGBT9-2-*dnaQ991* was combined in the same yeast cell with pGAD424 containing either the *dnaE*<sup>+</sup> gene or the *holE*<sup>+</sup> gene as described previously (8), and the relative strengths of the  $\alpha$ - $\epsilon$  and  $\epsilon$ - $\theta$  interactions were assayed by  $\beta$ -galactosidase activity. Table 1 shows that the  $\epsilon$  subunits specified by *dnaQ991* and *dnaQ49* have strongly reduced  $\alpha$ - $\epsilon$  interactions compared to those encoded by *dnaQ*<sup>+</sup> or *mutD5*, their interaction being essentially indistinguishable from the background level. Each protein was also assessed for its ability to interact with  $\theta$ . The  $\epsilon$ - $\theta$  interaction occurs more efficiently in the yeast two-hybrid system, presumably due to the smaller size

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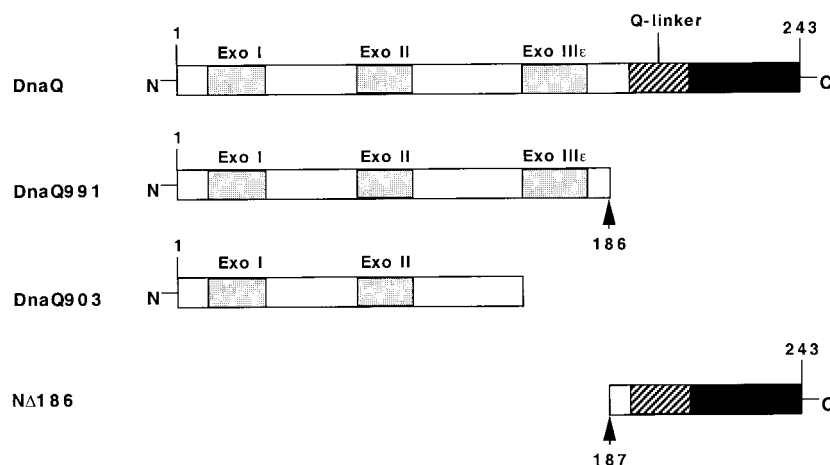


FIG. 1. Schematic drawing of *E. coli* DnaQ proteins. Highlighted are the conserved Exo I, II, and III $\epsilon$  motifs (1, 3), the proposed Q-linker (24), and the C-terminal domain. DnaQ991 is truncated at position 186, as indicated by the arrowhead. The N $\Delta$ 186 protein starts at position 187, as indicated by the arrowhead. Note that DnaQ903 lacks the Exo III $\epsilon$  motif in addition to the putative C-terminal  $\alpha$  binding site.

of  $\theta$  (8 kDa) compared to the  $\alpha$  subunit (130 kDa) (see also reference 8). The data show (Table 1) that the DnaQ991 protein is normal in the  $\epsilon$ - $\theta$  interaction, in contrast to the DnaQ49 protein, which is very defective in  $\theta$  binding under these conditions. The specific loss of binding of the DnaQ991 protein to the  $\alpha$  subunit is consistent with the proposed role of the C-terminal domain of  $\epsilon$  in binding to the polymerase subunit. The normal binding of DnaQ991 to the  $\theta$  subunit is consistent with the 186-amino-acid fragment being properly folded, as also suggested by the biochemical demonstration of exonucleolytic proficiency (17).

To more directly substantiate the role of the C terminus in the  $\alpha$ - $\epsilon$  interaction, we amplified the region of *dnaQ* encoding residues 187 to 243, the last 57 C-terminal amino acids of  $\epsilon$ , ligated the PCR product into pGBT9-2 (as described in reference 8) to form plasmid pGBT9-2-N $\Delta$ 186, and tested the resulting protein in the yeast two-hybrid system. Table 1 shows proficient binding of the N $\Delta$ 186 fusion protein to the  $\alpha$  subunit. This observation indicates that the C-terminal domain of

DnaQ is not only required, but is likely sufficient, for interaction with the polymerase.

The in vivo properties of the *dnaQ991* allele were also explored. As loss of the polymerase-binding domain would prevent  $\epsilon$  from being incorporated into the Pol III core, strains carrying *dnaQ991* are expected to mimic, in many respects, strains that lack  $\epsilon$  entirely. Deletion *dnaQ* strains have been reported previously in both *Salmonella typhimurium* and *E. coli* (11, 12, 20). The strains contain (extensive) deletions from the 3' end of *dnaQ*, resulting in truncated  $\epsilon$  proteins lacking not only the C terminus but also part or all of the exonuclease motifs (see DnaQ903 in Fig. 1). The strains exhibit poor growth as well as highly elevated spontaneous mutation rates. Cell health is improved considerably by spontaneously occurring suppressor mutations, termed *spq*, that arise in the *dnaE* gene. We inserted the 1.6-kb *SalI-SmaI dnaQ-rnh* fragment from pFF588 (20) into the *SalI-SmaI* linearized allele exchange vector pKO3 (13) and performed site-specific mutagenesis to create *dnaQ991*. The pKO3-*dnaQ991* plasmid was introduced into strain MG1655 (20), and a *dnaQ*<sup>+</sup>/*dnaQ991* heterodiploid was created by plasmid integration at the *dnaQ* locus at the nonpermissive temperature (13). Subsequent plating of the heterodiploids on Luria-Bertani (LB)-sucrose plates at 30°C yielded *dnaQ991* and *dnaQ*<sup>+</sup> haploid segregants. The *dnaQ991* segregants were readily distinguished pre-

TABLE 1. Interaction of mutant  $\epsilon$  subunits with Pol III core subunits  $\alpha$  and  $\theta$  as determined in the yeast two-hybrid assay<sup>a</sup>

<i>dnaQ</i> allele or deletion	$\beta$ -Gal activity <sup>b</sup> for the following interaction:	
	$\alpha$ - $\epsilon$	$\theta$ - $\epsilon$
Vectors only	0.001 $\pm$ 0.001	0.001 $\pm$ 0.001
<i>dnaQ</i> <sup>+</sup>	0.052 $\pm$ 0.032	37 $\pm$ 9
<i>mutD5</i>	0.030 $\pm$ 0.009	46 $\pm$ 18
<i>dnaQ49</i>	0.001 $\pm$ 0.001	0.015 $\pm$ 0.005
<i>dnaQ991</i>	0.001 $\pm$ 0.001	48 $\pm$ 5
N $\Delta$ 186	0.098 $\pm$ 0.047	0.001 $\pm$ 0.001

<sup>a</sup> Yeast transformants carrying pGBT9-2-*dnaQ* and either pGAD424-*holE* or pGAD424-*dnaE* were grown as described by Jonczyk et al. (8).  $\beta$ -Galactosidase ( $\beta$ -Gal) assays were performed with extracts at 30°C according to the method of Rose et al. (19), including correction for spontaneous *o*-nitrophenyl- $\beta$ -D-galactopyranoside (ONPG) hydrolysis under the given experimental conditions. Each value represents the average ( $\pm$  standard deviation) for three independent transformants, each analyzed in triplicate. Similar results were obtained in several repeated experiments.

<sup>b</sup> Expressed in units (amount of ONPG hydrolyzed per minute), calculated as  $(1,000 \times \text{OD}_{420}) / (\text{OD}_{600} \times \text{volume assayed} \times \text{time})$ , where  $\text{OD}_{420}$  and  $\text{OD}_{600}$  are the optical densities at 420 and 600 nm, respectively, volume is measured in milliliters, and time is measured in minutes.

TABLE 2. Mutability of the *dnaQ991* mutant and related strains<sup>a</sup>

Genotype	Total no. of cells (10 <sup>9</sup> /ml)	No. of Rif <sup>r</sup> mutants/ml	No. of mutants/10 <sup>6</sup> cells
<i>dnaQ</i> <sup>+</sup>	8.4	50	0.006
<i>dnaQ991</i>	0.49	55,200	111
<i>dnaQ991 spq-2</i>	3.0	244,000	82
<i>dnaQ903::tet</i>	0.15	14,640	99
<i>dnaQ903::tet spq-2</i>	1.8	199,600	109

<sup>a</sup> All strains are derivatives of MG1655. The *dnaQ991* strains were prepared as described in the text by using either MG1655 or its *spq-2* derivative. The *dnaQ903::tet* strains were prepared by transduction of the *dnaQ903::tet/dnaQ*<sup>+</sup> heterodiploid configuration (donor provided by R. Maurer) into MG1655 and its *spq-2* derivative, followed by segregation of *dnaQ903* haploids as described previously (20). Strains were grown at 37°C.

TABLE 3. Mutant frequencies in *dnaQ*<sup>+</sup>/*dnaQ* diploid strains<sup>a</sup>

Genotype	No. of Rif <sup>r</sup> mutants/10 <sup>6</sup> cells
Wild type .....	0.16
<i>dnaQ991/dnaQ</i> <sup>+</sup> .....	0.15
<i>dnaQ991/dnaQ</i> <sup>+</sup> <i>spq-2</i> .....	0.41
<i>dnaQ903::tet/dnaQ</i> <sup>+</sup> .....	0.10
<i>dnaQ903::tet/dnaQ</i> <sup>+</sup> <i>spq-2</i> .....	0.37

<sup>a</sup> Strains were constructed as described in the text and the legend to Table 2. Cells were grown overnight at 43°C in LB broth, plated on LB and LB Rif plates, and incubated at 43°C overnight. Plating at 37°C yielded similar results.

viously for *dnaQ* deletion derivatives (11, 20), and their strong mutator phenotype. Table 2 shows the mutant frequency for rifampin-resistant mutants of the *dnaQ991* haploids; *dnaQ* deletion (*dnaQ903::tet*) strains (20) are shown for comparison. The *dnaQ991* mutation resulted in a mutant frequency that was dramatically (>10,000-fold) increased over the background level, similar to that seen for *dnaQ903::tet*. A modest (~2-fold) but consistent increase in cell counts in stationary cultures of the *dnaQ991* haploid versus the *dnaQ903::tet* haploid was also observed (Table 2), suggesting that the presence of active free 3' exonuclease may be beneficial under the stressful conditions where the Pol III core consists of only the single  $\alpha$  subunit. Like that of the *dnaQ903::tet* strain, the health of *dnaQ991* haploids was significantly improved by the presence of the *spq-2* allele, a suppressor of the small-colony phenotype of *dnaQ903::tet* (20), as evidenced by a much healthier colony morphology and increased cell counts in the overnight cultures (Table 2). The *spq-2* allele did not affect the mutant frequency. Mutant frequencies of *dnaQ*<sup>+</sup>/*dnaQ991* and *dnaQ*<sup>+</sup>/*dnaQ903::tet* heterodiploids were also determined (Table 3). None of the heterodiploids exhibited mutant frequencies significantly different from those of the parental wild-type strain, indicating that *dnaQ991* is fully recessive. These mutational properties of *dnaQ991* are consistent with lack of binding of the DnaQ991 protein to the  $\alpha$  subunit and with the resulting absence of the  $\epsilon$  subunit from the HE.

In summary, our data support the contention that the  $\epsilon$  subunit is composed of two distinct domains, the N-terminal domain, which contains the exonuclease site (and also the  $\theta$ -binding site), and the C-terminal domain, which is necessary, and likely sufficient, for interaction with the polymerase. Our results do not rule out the existence of additional, secondary interactions between the polymerase and the N terminus of  $\epsilon$  that may be important for the proper coordination of the two proteins. The discovery of a separate domain within  $\epsilon$  for binding to the  $\alpha$  subunit will aid in further understanding the precise nature of the  $\alpha$ - $\epsilon$  interaction, which is likely important for efficient proofreading by the polymerase as well as for the structural integrity of the replication complex.

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