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TerF, the Sixth Identified Replication Arrest Site in *Escherichia coli*, Is Located within the *rcsC* Gene

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We report the existence of a sixth replication arrest site, *TerF*, that is located within the coding sequences of the *rcsC* gene, a negative regulator of capsule biosynthesis. The *TerF* site is oriented to allow transcription of the *rcsC* gene but prevent DNA replication in the terminus-to-origin direction. Our results demonstrate that the *TerF* site is functional in both chromosomal and plasmid environments and that the stability of the Tus-*TerF* protein-DNA complex more closely resembles the plasmid R6K *Ter* sites than the chromosomal *TerB* site.

The DNA replication cycle of *Escherichia coli* concludes when the two replication forks converge in the terminus region, located 180° around the chromosome from the replication origin, *oriC*. If one replication fork arrives at the terminus region well in advance of the other, it will be prevented from exiting the terminus region by a replication arrest, or *Ter*, site. The *Ter* sites of the *E. coli* chromosome are DNA sequences of approximately 20 bp in length (10, 18) that bind the Tus protein (19, 23, 33) with a very high affinity (8). The Tus-*Ter* protein-DNA complex halts replication forks in an orientation-dependent manner both in vivo (4, 10, 15) and in vitro (17, 24, 27), apparently through interactions between Tus and the replicative helicase, DnaB (22, 25). In addition to its activity against DnaB, the Tus-*Ter* complex also shows activity against a variety of helicases and polymerases (1, 9, 12, 24). *Ter* sites have also been identified in several plasmid replicons (18, 20, 33), and analogs to Tus and the *Ter* sites have been found in the *Bacillus subtilis* chromosome (26, 36). Although significant advances have been made in our understanding of the biochemical aspects of the Tus-*Ter* complex (reviewed recently in reference 14), the role of the replication arrest sites in the biology of *E. coli* is less well understood.

Initially, only two replication arrest sites, called *TerA* (min 28 on the *E. coli* map) and *TerB* (min 35), were identified in the *E. coli* chromosome. These two sites were located at either boundary of the terminus region and were oriented to allow unimpeded replication in the origin-to-terminus direction but would arrest replication forks that had passed through the terminus and begun to replicate in the terminus-to-origin direction (4, 15). Consequently, *TerA* and *TerB* acted as one-way gates into the terminus region, allowing replication forks to enter but not exit this region. Subsequently, additional replication arrest sites were identified in and around the terminus region at min 23 (*TerE*), min 27 (*TerD*), and min 33 (*TerC*) (6, 10, 11). In each instance, the *Ter* site was oriented to allow replication in the origin-to-terminus direction. Thus, *TerA*, *TerD*, and *TerE* halt replication forks moving in the counterclockwise direction on the chromosome, and *TerB* and *TerC* halt replication forks proceeding in the clockwise direction. None of the identified *Ter* sites are located within structural genes; however, the *TerB* site is located within the promoter region of the *tus* gene (13, 18) and autoregulates *tus* gene expression (28, 30, 31). The positioning of *Ter* sites outside of transcriptional units presumably serves two purposes. (i) A functional *Ter* site located in a structural gene could disrupt gene expression when replication forks are arrested at the *Ter* site or if the *Ter* site is oriented to impede RNA polymerase and thereby interfere with transcription of the gene. (ii) If the *Ter* site is oriented to allow unimpeded transcription of the gene, an actively transcribing RNA polymerase may displace the Tus protein and thereby inactivate the *Ter* site with respect to arrest of DNA replication. The observation that *rho*-independent terminators are located within 100 bp of the intergenic *TerA*, *TerC*, and *TerD* sites (10) is consistent with this latter supposition.

We report the identification of a sixth functional replication arrest site, *TerF*, located at min 48 on the *E. coli* chromosome. This site is located within the coding sequences of the *rcsC* gene, which is a regulator of colanic acid capsule synthesis in *E. coli* (3, 35). The *TerF* site, spanning codons 716 to 721 of 933 total, is oriented to allow unimpeded transcription of the *rcsC* gene but arrest DNA replication in the terminus-to-origin direction. Analysis of the equilibrium and kinetic constants of binding show that *TerF* binds Tus protein as well as the R6K *Ter* sites, but less tightly than the chromosomal *TerB* site. We also demonstrate that the minimal *TerF* sequence arrests DNA replication in a plasmid vector and functions as a replication arrest site within the context of the *rcsC* structural gene in the *E. coli* chromosome.

Equilibrium and kinetic constants for the Tus-*TerF* interaction. The *TerF* site was identified by searching the GenBank data base for bacterial sequences similar to the consensus *Ter* sequence (Fig. 1). *TerF* differs from the derived consensus sequence for the chromosomal *Ter* sites in that it contains a G substitution at position 18 in place of the consensus T residue. Because it has been shown that a T→A mutation in the R6K *Ter* sites did not destroy Tus binding activity or replication arrest activity (32), it seemed likely that the *TerF* sequence could function as a replication arrest site. To determine whether the *TerF* site bound the Tus protein, oligodeoxyribonucleotides containing the *TerF* sequence were synthesized, bound to purified Tus protein, and analyzed by using a filter binding assay. The sequences of the two 33-base oligomers used in these studies were 5′-GATCCATCGTAGTTACAACATACGAAGGGCAGG and 5′-AATTCCTGCCCTTCGTATGTTGTAACTACGATG.

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Chromosomal																								
TerA	A	A	T	T	A	G	T	A	T	G	T	T	G	T	A	A	C	T	A	A	A	G	T	
TerB	A	A	T	A	A	-	T	A	-	-	-	-	-	-	-	-	-	-	A	A	G	T		
TerC	A	T	A	T	A	-	G	A	-	-	-	-	-	-	-	-	-	-	A	T	A	T		
TerD	C	A	T	T	A	-	T	A	-	-	-	-	-	-	-	-	-	-	A	A	T	G		
TerE	T	T	A	A	A	-	T	A	-	-	-	-	-	-	-	-	-	-	A	G	N	N		
TerF	C	C	T	T	C	-	T	A	-	-	-	-	-	-	-	-	G	-	C	G	A	T		
Plasmid																								
R6K*terR1*	C	T	C	T	T	-	T	G	-	-	-	-	-	-	-	-	-	-	A	A	T	C		
R6K*terR2*	C	T	A	T	T	-	A	G	-	-	-	-	-	-	-	-	-	-	C	T	A	G		
R100*terR1*	A	T	T	A	T	-	A	A	-	-	-	-	-	-	-	-	-	-	C	T	T	C		
R100*terR2*	T	G	T	C	T	-	A	G	-	-	-	-	-	-	-	-	-	-	A	A	G	C		
R1*terR1*	A	T	T	A	T	-	A	A	-	-	-	-	-	-	-	-	-	-	C	A	T	C		
R1*terR2*	T	T	T	T	T	-	T	G	-	-	-	-	-	-	-	-	-	-	A	A	G	T		
P307	A	T	T	A	T	-	A	A	-	-	-	-	-	-	-	-	-	-	C	A	T	T		
Consensus	N	N	A/T	A/T	A/T	G	A/T	A/G	T	G	T	T	G	T	A	A	C	T	A	A/C	N	N	N		
Position					5					10					15					20					

FIG. 1. Comparison of known *Ter* sequences from the *E. coli* chromosome and other replicons. Nucleotides identical to the derived consensus sequence are indicated by dashes, and nonidentical nucleotides are replaced by the substituted nucleotide. As presented, the *Ter* sites will arrest replication forks approaching from the 5′ side but allow replication forks approaching from the 3′ side to pass.

When annealed, these oligomers produced a 29-bp *TerF* fragment with 4-base extensions at each end.

The equilibrium and kinetic constants for *TerF* binding to purified Tus protein were determined by using the filter binding assay and binding buffer described by Gottlieb et al. (8). Four independent measurements of the dissociation constant of the equilibrium (K_D) were obtained and averaged to produce a binding constant of $1.1 \pm 0.1 \times 10^{-12}$ M for the Tus-*TerF* interaction. The results from one of these experiments are presented in Fig. 2. The affinity of Tus for the *TerF* site is therefore less than its affinity for the *TerB* site (3.4×10^{-13} M) but greater than its affinity for the R6K *TerR2* site (1.1×10^{-11} M). The dissociation constant (k_d) of the Tus-*TerF* complex was determined from three independent

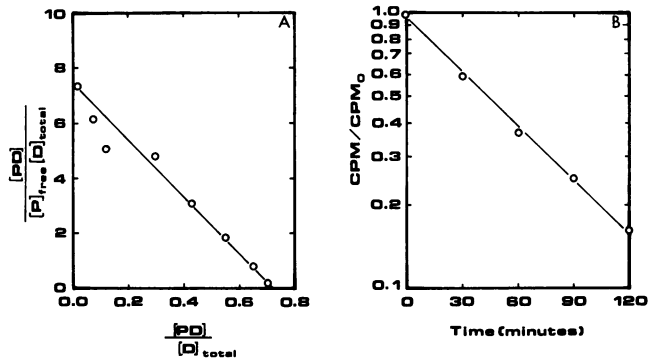

FIG. 2. Filter binding studies on the Tus-*TerF* interaction. (A) Scatchard plot of the equilibrium binding data. The concentration of *TerF* DNA in this experiment was 10^{-12} M, and data points represent the average of duplicate samples taken for each concentration of Tus. The values for the *y* axis are multiplied by 10^{-11}. The data from this particular experiment gave a K_D of 1.05×10^{-12} M. (B) Dissociation rate of the Tus-*TerF* complex. Tus protein (P) and *TerF* DNA (D) at 10^{-11} M each were incubated for 1 h, at which time unlabeled *TerR2* DNA was added to a final concentration of 2×10^{-10} M. Data points are the averages of duplicate samples taken at the indicated times. The fitted line for this experiment gave a half-life of 46 min and a calculated k_d of 2.5×10^{-4} s^{-1}.

experiments. The averaged values from these experiments indicated a half-life of 47 min and a dissociation rate constant of $2.5 \pm 0.1 \times 10^{-4}$ s^{-1}. The results from one of these experiments are also presented in Fig. 2. Thus, the Tus-*TerF* complex is roughly 10 times less stable than the Tus-*TerB* complex (half-life of 550 min) but shows stability similar to that of Tus-R6K *TerR2* complex (half-life of 43 min [8]). Two association rate experiments were performed, and the data were evaluated as described previously (8). Association of Tus and the *TerF* site occurred at the same rate (3.4×10^8 M^{-1} s^{-1}; data not shown) in the two experiments and was comparable to that of *TerB* (1.4×10^8 M^{-1} s^{-1}) but seven times faster than Tus-R6K *TerR2* complex formation (4.7×10^7 M^{-1} s^{-1}).

Replication arrest by the *TerF* site in plasmids. To demonstrate that the *TerF* sequence functioned as a replication arrest site in vivo, the *TerF* oligomer was cloned into a plasmid vector and tested for replication arrest activity. To insert the *TerF* site in an orientation that would halt plasmid replication, the annealed *TerF* oligomers, which contained *Bam*HI- and *Eco*RI-compatible ends, were cloned into the corresponding restriction sites in pUC18 and transformed into the *tus* mutant strain PK2607 (30). The resulting recombinant plasmid (pBS*TerF*) was sequenced to confirm the presence of the *TerF* site. A kanamycin resistance gene was then inserted into the *Eco*RI site of the pBS*TerF* polylinker to increase the spacing between the *TerF* site and the plasmid origin. The resulting plasmid, pBS*TerF-kan*, was transformed into isogenic *tus*$^+$ (PK457) and *tus* (PK2607) strains, and plasmid DNA was isolated by a neutral plasmid preparation procedure (21).

To determine whether the pBS*TerF-kan* plasmid contained an arrested replication fork, the plasmid DNA was digested with *Sma*I, electrophoresed on 0.6% agarose gels, transferred to nitrocellulose, and hybridized to a pUC-*kan* probe. *Sma*I cuts the pBS*TerF-kan* plasmid once in the kanamycin resistance gene and produces a double-Y structure if replication is arrested at the *TerF* site. The double-Y structure can be separated from the linear plasmid form by its slower migration during electrophoresis (36). As shown in Fig. 3, in addition to the 4.2-kb linear plasmid band produced by *Sma*I digestion, a slowly migrating band of apparent molecular size 6.5 kb was observed when pBS*TerF-kan* DNA was isolated from the wild-type PK457 strain but not when plasmid DNA was isolated from the *tus* strain PK2607. The *Sma*I digestion products from PK457/pBS*TerF-kan* were at the same position in the gel as the *Sma*I products from the well-characterized plasmid pTH101 (18), which is a pGEM vector containing a functional *TerB* site with a Kan^r gene inserted between the plasmid origin and the *TerB* site. Because the pTH101 and pBS*TerF-kan* plasmids are virtually identical in size and produced similar-size slowly migrating bands in *tus*$^+$ cells, we conclude from this experiment that plasmid replication was indeed halted at the *TerF* site in pBS*TerF-kan*. It should be noted that we did not test the *TerF* sequence in the opposite orientation and can therefore only assume that it demonstrates the functional polarity observed with all other identified *Ter* sites.

We also tested for replication arrest activity in a plasmid that contained the *TerF* site within the context of a functional *rcsC* gene. This plasmid, pJB201, contains a 4.0-kb fragment encoding the *rcsC* gene and complements strains containing the *rcsC137* allele (3), indicating that the gene is transcriptionally active. The *TerF* site in pJB201 is oriented to halt replication from the plasmid vector (pUC19) origin, allowing us to test for replication arrest by using the South-

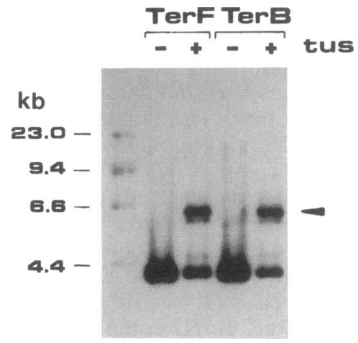

FIG. 3. Arrest of plasmid replication by the *TerF* sequence. DNA from plasmid pBS*TerF-kan* or plasmid pTH101, which contains the *TerB* site (17), was prepared from either *tus*$^+$ or *tus* strains under nondenaturating conditions. The plasmid DNAs were digested with *Sma*I, electrophoresed on a 0.6% agarose gel, transferred to nitrocellulose, and hybridized to a probe containing sequences from the plasmid vector. Digestion of both plasmids should yield a linear fragment of 4.2 kb if replication is not arrested at the *Ter* site but produce the linear fragment and a slower-migrating double-Y fragment if replication is arrested. A fragment of 6.5 kb in apparent molecular size (arrowhead) was observed only when the plasmids were isolated from cells containing a functional *tus* gene.

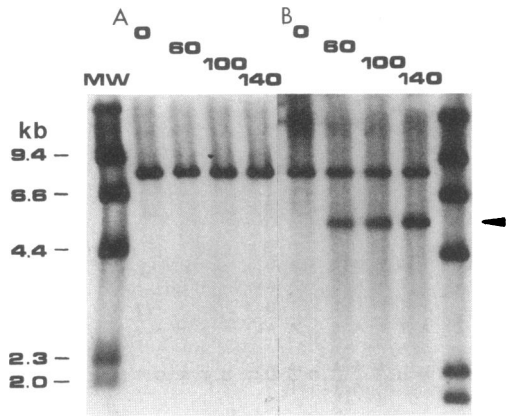

FIG. 4. Arrest of chromosome replication by the *TerF* sequence. Chromosomal DNA from strains PK2216 (A), which contains a P2*sig5* prophage inserted at min 16 and has a deletion removing *TerB*, *TerC*, and the *tus* gene, and PK2216/pPK1013 (B), which also contains a plasmid carrying a functional *tus* gene, was prepared at the indicated times following induction of the P2*sig5* prophage origin of replication. After digestion with *Eco*RI, the DNA was electrophoresed, blotted onto nitrocellulose, and probed with a fragment containing the *rcsB* gene. If replication is arrested at the *TerF* site, a fragment of 5.5 kb (arrowhead) will be detected in addition to the linear 8.5-kb *Eco*RI fragment.

ern blot assay described above. A slowly migrating band was observed in addition to the linear plasmid fragment only when pJB201 DNA was prepared from a *tus*$^+$ strain and not when plasmid DNA was prepared from a *tus* strain (data not shown), suggesting that *TerF* is functional even in the context of a transcriptionally active gene.

Replication arrest by the *TerF* site in the *E. coli* chromosome. We used PK998, which is a *dnaA*(Ts) strain containing a P2*sig5* phage origin at min 16 (15), to determine whether replication was halted at the chromosomal *TerF* site by using the modification by Pelletier et al. (29) of Weiss and Wake's Southern blot assay (36). Incubation of PK998 at 42°C suppresses replication initiation from *oriC* (min 84) by inactivating DnaA and induces replication from the P2*sig5* origin at min 16. The clockwise-traveling replication forks arising from the P2*sig5* origin arrive at the terminus region well ahead of counterclockwise-traveling replication forks, and replication arrest at the *Ter* sites can be easily detected by using a Southern blot assay.

In this assay, instead of probing for intact Y-shaped restriction fragments, the DNA is sheared by vortexing prior to restriction enzyme digestion to release one of the arms of the Y-shaped fragment. After restriction enzyme digestion, the sheared arm migrates in the gel faster than the full-length restriction fragment, to a position corresponding to the length between the *Ter* site and the restriction site. In the case of an arrested replication fork at the *TerF* site, an *Eco*RI digestion of chromosomal DNA will produce a Y-shaped *rcsC* restriction fragment of 8.5 kb in length with a 3-kb stem and 5.5-kb arms. If one of the arms is sheared off, a Southern blot of the *Eco*RI-digested DNA probed with an *rcsB* fragment should produce two bands on an autoradiograph: a band at 8.5-kb, representing the linear fragment, and a band at 5.5-kb, representing the released arm.

Attempts to demonstrate activity of the *TerF* site in strain PK998 were unsuccessful. We observed only the full-length 8.5-kb fragment and never detected the released arm when we probed *Eco*RI-digested chromosomal DNA with a 3.6-kb *Bam*HI-*Hin*dIII *rcsB* fragment (data not shown). We surmised that our inability to demonstrate *TerF* function was due to the presence of *TerC* and *TerB*, which are located between the P2*sig5* origin and *TerF* and probably prevent replication forks from reaching *TerF* during the time course of our experiments (140 min). To circumvent this problem, we used a derivative of PK998 called PK2216 (16), which contains a 160-kb deletion (Δ2038) that removes *TerC*, *TerB*, and *tus*. This strain was transformed with pPK1013, a plasmid containing an intact *tus* gene (19), and chromosomal DNA was prepared and tested for *TerF* activity. As shown in Fig. 4, in addition to the 8.5-kb linear fragment, a band of 5.5 kb was visible 60 min after induction of the P2*sig5* prophage, indicating that the *TerF* site was active in the chromosome.

The *TerF* site. The *TerF* replication arrest site displays characteristics that distinguish it from the five other replication arrest sites previously identified in the *E. coli* chromosome. First, it forms a complex with the Tus protein that is 10 times less stable than the Tus-*TerB* complex ($t_{1/2}$ of 47 min versus 550 min), indicating that it has binding characteristics that more closely resemble those of the R6K plasmid *Ter* sites than those of the chromosomal *Ter* sites. This difference in the stability of the Tus-*TerF* complex can most likely be attributed to the position 18 G substitution within the 11-bp core sequence (Fig. 1). The biochemical significance of this observation is that, as judged from recent comparisons between chromosomal and plasmid replication arrest sites (24), *TerF* should only be one-third to one-half as effective as *TerA* or *TerB* at halting replication. The biological significance of this observation is not currently known.

Second, of the six identified *Ter* sites, *TerF* is located furthest from min 31 (Fig. 5), the point in the terminus region where replication forks meet most often (2). The distance from *TerF* to the traditional terminus region and the placement of *TerC* and *TerB* between *oriC* and *TerF* suggests that *TerF* will only rarely, if ever, arrest replication forks arising from *oriC*. Our results support this contention, since *TerF*

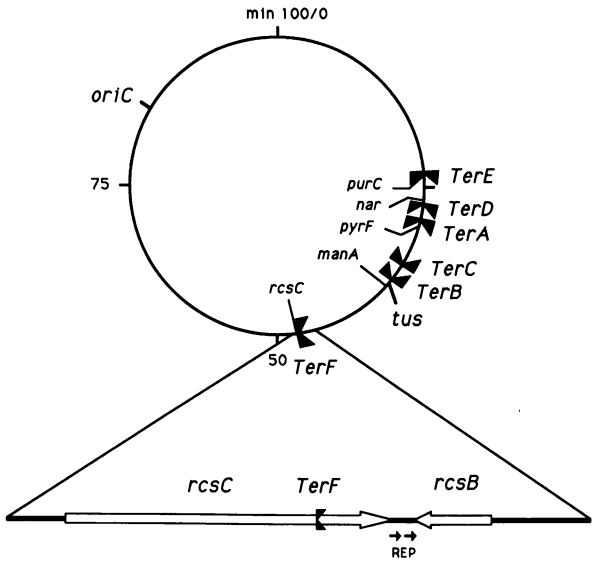

FIG. 5. Position of *TerF* in the *E. coli* chromosome and in the *rcsC* gene. The positions and orientations of all known *Ter* sites in the *E. coli* chromosome are shown in the upper diagram. The lower diagram shows the organization of the *rcsC-rcsB* region and the location of the *TerF* site, based on the sequence determined by Stout and Gottesman (35). The small arrows marked "REP" indicate sequence homology to the repetitive extragenic palindromic sequences found throughout the *E. coli* chromosome.

function could be observed only when *TerC* and *TerB* were removed. A similar postulation can also be made for *TerE*, since both *TerA* and *TerD* lie between it and *oriC*. The report that as many as 10 different *Ter* sites in the *E. coli* chromosome can be identified by hybridization to a consensus *Ter* sequence oligomer (5) indicates that additional *Ter* sites will ultimately be identified outside the terminus region. Taken together, these observations suggest that *Ter* sites perform a biological function in addition to their role as sites of arrest for replication forks initiated from *oriC*. Other possible functions might include arrest of DNA replication arising from other origins, such as integrated phage or plasmid origins or the *sdr* origins (5), a role in the higher-ordered structure of the *E. coli* chromosome, or, when positioned within a gene, a role as a regulator of gene expression.

Third, *TerF* is the first replication arrest site found within a structural gene. The only other *Ter* site located in a gene is the *TerB* site, which is located in the promoter region of the *tus* gene (13, 19) and autoregulates *tus* expression (28, 30, 31). In contrast, *TerF* is located in the last third of the coding sequences for *rcsC* (Fig. 5), which is one of the regulators for colanic acid capsular polysaccharide synthesis (3, 35). Although *TerF* is oriented to allow unimpeded transcription of *rcsC*, it is expected that an arrested replication fork at *TerF* would disrupt *rcsC* transcription and that *TerF* can, to a limited extent, affect *rcsC* expression. However, because *TerF* is not expected to be utilized in most replication cycles, the contribution of *TerF* to *rcsC* regulation is expected to be minimal.

Is there any significance to the placement of the *TerF* site in the *rcsC* gene? RcsC shares sequence similarities with the sensor proteins of other two component sensor-effector regulators, such as *ompR-envZ* and *phoB-phoR* (34), and is postulated to be a transmembrane protein (35). Mutations in *rcsC* increase the level of colanic acid synthesis, suggesting that the role of the RcsC sensor is to down-regulate the capsular synthesis genes (3). The effector protein that presumably receives the signal from RcsC is the product of the *rcsB* gene, which is located adjacent to and transcribed convergently with the *rcsC* gene (35) (Fig. 5). Interestingly, RcsB, in addition to stimulating capsular synthesis, upregulates the expression of *ftsZ* (7), a key intermediary in the pathway regulating cell division. We can only speculate at this time on the effect of an arrested replication fork at *TerF* on *rcsC* expression and any subsequent effect on bacterial physiology. However, in the future, it may be possible to assign significance to the positioning of *TerF* as the role of the *Ter* sites in the bacterial chromosome becomes clearer.

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