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# A host-encoded DNA-binding protein promotes termination of plasmid replication at a sequence-specific replication terminus

**(R6K replication termination/chemical footprinting)**

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**ABSTRACT** **We have purified ≈6600-fold an ≈40-kDa protein (Ter protein) encoded by *Escherichia coli* that specifically binds to two sites at the 216-base-pair replication terminus ($\tau$) of the plasmid R6K. Chemical footprinting experiments have shown that the Ter protein binds to two 14- to 16-base-pair sequences that exist as inverted repeats in the $\tau$ fragment. Site-directed mutagenesis of one of the terminus sequences ($\tau_R$) resulted in a mutant $\tau_R$ that failed to bind to the Ter protein. The same mutant terminus also failed to terminate DNA replication *in vivo*. These experiments strongly suggest that the interaction of the Ter protein with $\tau$ sequences plays an essential role in the termination of DNA replication, specifically at $\tau$.**

DNA replication can be divided into three steps—namely, initiation, ongoing replication, and termination. The ongoing replication step has been largely unraveled through analyses of several model replicons (for a review, see ref. 1). Progress has been made in the analyses of initiation of replication and its control (1). However, the termination step remains largely unclear.

The plasmid R6K initiates replication by interaction of Pi protein at one of three origins of replication (2, 3) and terminates DNA replication at a specific sequence called the terminus ($\tau$) that was revealed by *in vivo* experiments (2, 4–6). By using gap filling of newly terminated daughter molecules and molecular cloning, $\tau$ was localized in a 216-base-pair (bp) *Alu* I fragment (7). The nucleotide sequence of the 216-bp terminus region was also reported (8). Using an *in vitro* replication system, Germino and Bastia (9) reported that *Escherichia coli* contained soluble protein(s) that terminated DNA replication of pBR322 specifically at $\tau$ when the 216 bp was cloned in it. These experiments strongly suggested that a host-encoded protein(s) is likely to be involved in the termination process, probably by binding to the sequence. Previously, it was reported (6) that $\tau$ worked in either orientation.

In this report, we describe the identification and purification of a protein encoded by *E. coli* that specifically bound to a 14- to 16-bp sequence at two physically separable sites called $\tau_R$ and $\tau_L$ that are located as inverted repeats in the 216-bp $\tau$ fragment. We mutated 6 of 16 nucleotides of the Ter protein recognizing sequence $\tau_R$, and we observed that the mutations not only abolished binding of the site by Ter protein *in vitro* but also inactivated the ability of the mutated sequence to terminate DNA replication *in vivo*, thus assigning a key role to the Ter protein in the termination process.

## MATERIAL AND METHODS

**Plasmids.** pUC18-80 and pUC19-80 are plasmids containing the 80-bp $\tau_R$ sequence in opposite orientations with respect to the origin.

**Purification of Ter Protein.** Ter protein was purified from *E. coli* cells containing pUC19-2160 that were grown to midlogarithmic phase and then amplified for 3–4 hr with chloramphenicol (170 μg/ml). All subsequent steps were performed at 0°C–4°C. Step 1 involved enrichment of Ter protein by exploiting its *in vivo* affinity for the plasmid $\tau_R$ sequence (10). Wet packed cells (40 g) were suspended in 150 ml of buffer A [50 mM Tris·HCl, pH 7.5/2 mM EDTA/25% (wt/vol) sucrose/1 mM phenylmethylsulfonyl fluoride] to which 30 ml of lysozyme (10 mg/ml) in 250 mM Tris·HCl (pH 7.5) was added. After 10 min, 30 ml of 250 mM EDTA was added. After 10 more min, 210 ml of buffer B (50 mM Tris·HCl, pH 7.5/10 mM EDTA/0.1 mM dithiothreitol/0.4% sodium deoxycholate/1% Brij 58) was added and mixed gently for 15 min at 0°C. The lysate was cleared at 100,000 × *g* for 1 hr. The supernatant constituted fraction I. The plasmid DNA–protein complex was precipitated by adding polyethylene glycol 6000 (PEG) to 10%. After centrifugation, the pellet was dissolved in buffer C (50 mM Tris·HCl, pH 7.5/1 mM EDTA/200 mM NaCl/10 mM 2-mercaptoethanol). PEG was added to 5% and the pellet was redissolved in buffer C that contained 2 M NaCl. The plasmid DNA was precipitated away from stripped protein by adding PEG to 5%. The NaCl was removed by dialysis against Mono Q buffer, 20 mM Tris·HCl, pH 7.5/1 mM EDTA/10 mM 2-mercaptoethanol (fraction II), loaded onto a 10-ml heparin-agarose column (step 2). After washing with the same buffer, the column was eluted with 0–1 M NaCl gradient (60 ml) in Mono Q buffer. Fractions (1 ml) were collected. Ter activity, which eluted at 0.375 M NaCl, was measured by the gel shift assay using 20 fmol of labeled $\tau_R$ probe (11). As described later, the specificity of the assay was worked out using appropriate protein and competitor DNA concentrations. Peak fractions were pooled (recovery was ≈50% of fraction II) and was called fraction III. The fraction III was dialyzed against Mono Q buffer and loaded in five separate aliquots onto a 1-ml Mono Q column that was eluted with 30 ml of 0–0.3 M NaCl in Mono Q buffer (step 4). A typical elution profile and the assay of peak fractions are shown in Fig. 1. The Ter activity eluted at 0.09 M NaCl. The recovery was 90% of fraction III. One unit of Ter activity is arbitrarily defined as that amount able to fully shift 1 fmol of 80-bp $\tau_R$ DNA (specific activity, $10^6$ cpm/μg) in a gel shift assay in the presence of 100-fold excess of nonspecific sonicated calf thymus competitor DNA. Table 1 shows the degrees of purification from fractions I–IV. Although Ter activity in fraction I was difficult to measure with precision, we estimate that the *in vivo* affinity enrichment procedure yielded >100-fold purification of Ter at fraction II. The overall purification was at least ≈6600-fold over fraction I. The protein at this stage is free of endo- and exonuclease and nonspecific DNA binding activity (data not shown).

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**Preparation of Replicative Intermediates.** This was carried out as published (12).

**Copper-Phenanthroline Footprints.** This was performed as described (13).

**Photoaffinity Labeling of Ter Protein.** Using bromodeoxyuridine-substituted and $^{32}$P-labeled $\tau$ DNA, the labeling of protein was carried out as described (14).

**Site-Directed Mutagenesis.** Using the 19-mer described below, $\tau_R$ was mutated to mutant $\tau_R$ that has acquired a *Bgl* II site. The procedure is published (15).

## RESULTS

**Strategies for the Purification of Ter Protein.** Previous work (9) had shown that soluble protein fractions from *E. coli* were capable of terminating DNA replication *in vitro* at $\tau$. We therefore expected that at least a certain population of the putative Ter protein should exist in a soluble form in the host cytoplasm. We introduced the high copy number plasmid pUC19 containing a single copy of the 216-bp $\tau$ sequence, that was cloned between the *Eco*RI and *Hin*dIII sites of the vector, into *E. coli*, with the expectation that multiple copies of the $\tau$ sequence present in the cell milieu would sequester the putative Ter protein *in vivo* in the form of DNA–protein complexes.

The Ter protein sequestered by $\tau$ plasmid *in vivo* was purified >6600-fold through heparin agarose and Mono Q anion-exchange columns as described. The elution profile of the final column is shown in Fig. 1. Table 1 summarizes the purification steps. One unit of activity is defined as that amount of Ter protein able to fully complex 1 fmol of 80-bp $\tau_R$ DNA in the presence of 100-fold excess of competitor DNA.

The specificity of interaction of the protein with $\tau$ sequences was established by competitive gel shift assay (11). Gel shift assays in the presence of competitor DNA showed that the fraction III protein bound to the 216-bp $\tau$ fragment at

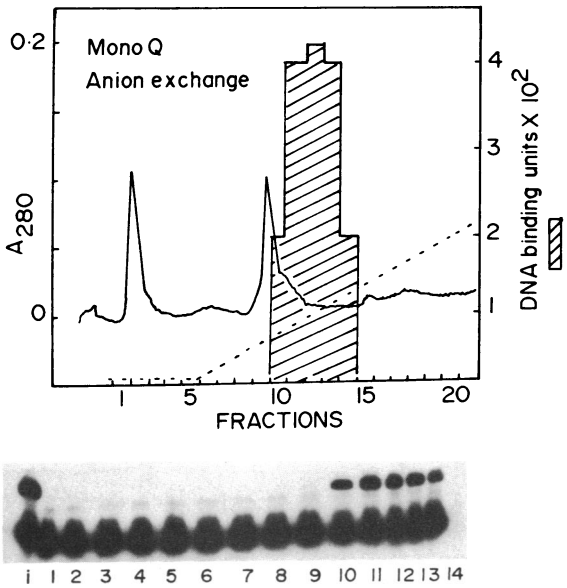


FIG. 1. The final step in the purification of Ter protein. (*Upper*) Elution profile of a 1-ml Mono Q column developed with a 30-ml 0–0.3 M NaCl gradient (dashed line) in Mono Q buffer (see text). The Ter activity eluted at 0.09 M NaCl. (*Lower*) The assay of fractions 1–14. One unit of Ter activity is defined as that amount able to fully complex 1 fmol of 80-bp $\tau_R$ fragment. Ten microliters of each 1-ml fraction was mixed with labeled $\tau_R$ DNA ($10^6$ cpm/$\mu$g), incubated, and loaded onto 5% polyacrylamide gels. The pooled peak fractions constituted fraction IV. Lane i, input fraction III protein from preceding heparin-agarose column.

Table 1. Purification of Ter protein

| | Fraction | Total protein, mg | Units of Ter | Specific activity | -fold purification over fraction II |
|---|---|---|---|---|---|
| I | Cell lysate | 3000 | — | — | — |
| II | *In vivo* affinity | 27.3 | 14,000 | 512 | 1 |
| III | Heparin agarose | 1.3 | 9,400 | 7,121 | 14 |
| IV | Mono Q | 0.23 | 8,000 | 34,040 | 66 |

Although it is difficult to make precise estimates of Ter activity in fraction I, our minimum estimate is that fraction II is 100-fold enriched over fraction I in specific activity. The overall minimum purification is, therefore, 6600-fold.

least at two sites (Fig. 2, lane F) as revealed by two discrete bands of DNA–protein complex that were resolved after polyacrylamide gel electrophoresis. When the $\tau$ fragment was cleaved with *Hae* III into an ≈140-bp and an ≈80-bp subfragment, each subfragment upon interaction with Ter protein revealed the formation of a single band of DNA–protein complex (Fig. 2, lanes B and D). Therefore, both the ≈140-bp and the ≈80-bp subfragments apparently contained at least one Ter binding site. These sites will be called $\tau_L$ and $\tau_R$ (Fig. 3 *A* and *B*). We monitored the sequence specificity of the DNA–protein interactions in two ways—first, under identical concentrations of the salt and competitor DNA, the protein bound specifically to the 216-bp $\tau$ fragment but not to the replication origin $\gamma$ (280 bp) fragment of R6K (Fig. 2, lane K) or to a fragment containing a piece of the $\beta$-lactamase gene of pBR322 (Fig. 2, lane H). Second, whereas the binding of Ter protein to labeled $\tau$ sequence could be blocked by competition by adding 10-fold excess of unlabeled homologous competitor DNA, it required, in contrast, a 500-fold excess of heterologous DNA (pUC19 DNA) to achieve the same level of competition (data not shown).

On the basis of the magnitude of the change in mobility of the $\tau$ DNA fragment when complexed with Ter protein, we estimated, using a recently published procedure (16), that the molecular mass of the protein binding to $\tau$ fragment was ≈80 kDa. If the Ter protein were a dimer, then the monomer molecular mass should be ≈40 kDa. We wished to identify

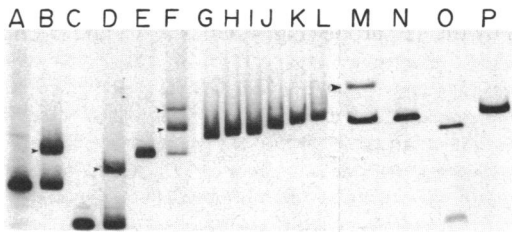


FIG. 2. Autoradiogram of a 5% polyacrylamide gel showing specific binding of Ter protein to the $\tau$ sites. Fraction III Ter protein was used in all experiments. Lanes: A, 143-bp $\tau_L$ fragment; B, $\tau_L$ complexed with 10 units of Ter protein (arrowhead shows DNA–protein complex); C, 80-bp $\tau_R$ fragment; D, $\tau_R$ complexed with 10 units of Ter protein (arrowhead indicates DNA–protein complex); E, 216-bp $\tau_L$-$\tau_R$ fragment; F, $\tau_L$-$\tau_R$ DNA–protein complexes with 10 units of Ter, two bands are shown (arrowheads); J, 280-bp ori $\gamma$ fragment; K and L, ori $\gamma$ fragment incubated with 100 and 200 units of Ter protein shows no significant gel shift; G, 265-bp ampicillin-resistance (Amp$^R$) fragment; H and I, Amp$^R$ fragment incubated with 100 and 200 units of Ter protein showing no significant gel shift; M, input wild-type $\tau_R$ DNA complexed with 10 units of Ter protein; N, input mutant $\tau_R$ DNA; O, input mutant $\tau_R$ digested with *Bgl* II; P, mutant $\tau_R$ incubated with 20 units of Ter protein. Note absence of gel shift showing failure to form DNA–protein complex. This figure is a composite of three gels. The slight change in mobility of lanes G, H and K, L is probably due to some nonspecific binding in the presence of excess protein.

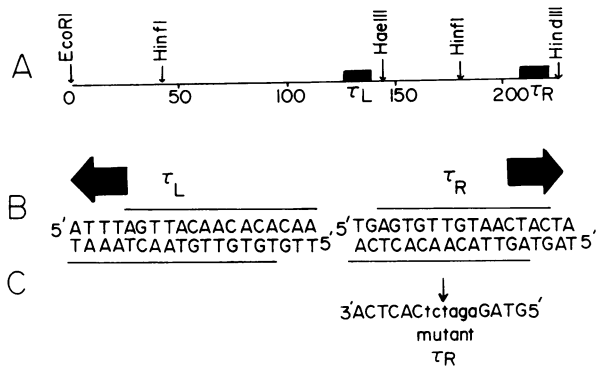


FIG. 3. Physical map and sequences of the $\tau$ sites. (*A*) Restriction map of the 216-bp *Alu* I fragment that was cloned between the *Hin*dIII and *Eco*RI sites of pUC19. The map is based on the results published by Bastia *et al.* (7, 8). (*B*) Summary of the interaction of the Ter protein with $\tau_R$ and $\tau_L$. The top and bottom lines show extent of protection from chemical cleavage (see Fig. 5). Arrows show the direction in which replication fork is blocked at $\tau_L$ and $\tau_R$. The sequence shown was previously published (7, 8). (*C*) Site-directed mutagenesis of $\tau_R$ that replaced 6 of 14 bases with a *Bgl* II site. The mutant $\tau_R$ is nonfunctional.

more definitively the size of the Ter protein in the partially purified protein fractions in the following manner.

**Cross-Linking of Ter Protein with Labeled Sites.** We cloned the 216-bp $\tau$ sequence into M13mp19 vector and, using a sequencing primer, synthesized a complementary strand, incorporating bromodeoxyuridine triphosphate, [$\alpha$-$^{32}$P]dATP, and [$\alpha$-$^{32}$P]dCTP into the newly synthesized DNA.

The bromodeoxyuridine-substituted and $^{32}$P-labeled $\tau$ fragment was cut out of the M13 DNA, and the labeled probe was mixed with Ter protein fractions and irradiated with short wavelength UV light (14). The following controls were performed. The Ter protein was also mixed with $^{32}$P-labeled and bromodeoxyuridine-substituted ori $\gamma$ DNA. The cross-linking of $\tau$ DNA to Ter protein was performed in the presence of specific and nonspecific competitor DNA. The results (Fig. 4) show that when excess DNA probe is digested away from the cross-linked DNA–protein complex and the products are analyzed in 15% SDS/polyacrylamide gels, an ≈14-kDa protein and an ≈40-kDa protein were specifically labeled by the Ter probe (Fig. 4, lanes A, C, and D). Specific

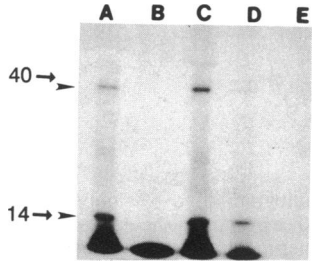


FIG. 4. Autoradiogram of 15% polyacrylamide gel of $^{32}$P and bromodeoxyuridine-labeled $\tau_R$ $\tau_L$ DNA cross-linked to fraction II protein. The cross-linking was performed as described (14). Lanes: A, protein cross-linked in the presence of 50 units of protein and 100-fold excess of heterologous competitor DNA; B, 10-fold excess of cold specific competitor; C, 200-fold excess of cold competitor; D, same as lane C but digested with 5-fold excess (over lanes A, B, and C) of micrococcal nuclease (60 units) and DNase I (70 units) at 37°C for 30 min. Note excess nuclease does not alter the 40- and 14-kDa bands but reduces their intensity. Note that specific competitor (lane B) abolishes the labeling of protein bands. Since a maximum of 8 kDa of $\tau$ sequence may be attached to the protein, their actual molecular masses should be less than 40 and 14 kDa. Coomassie blue staining of gel showed a prominent band near 14 kDa and a faint band at 36–37 kDa along with several other bands (data not shown).

unlabeled competitor, added in 10-fold excess, abolished the labeling of the two (40 and 14 kDa) protein bands (lane B). Excess of nonspecific competitor (100- to 200-fold) did not abolish this photoaffinity labeling (lanes A and B). Fivefold excess of nuclease did not change the mobility of the 40-kDa and 14-kDa bands (lane D). In the control bromodeoxyuridine-substituted $^{32}$P-labeled ori $\gamma$ probe, we observed that the ori $\gamma$ probe did not label the 40- or the 14-kDa protein (data not shown). These results strongly suggest that the 40-kDa protein is most likely the Ter monomer and the 14-kDa protein may be a degradation product or an accessory protein. However, with purer Ter protein (e.g., fraction IV), photoaffinity labeling only labeled the 40-kDa protein (data not shown). It should be kept in mind that mobility of the cross-linked protein reflects the bound DNA. Therefore, the real molecular mass of the Ter protein should be somewhat less than 40 kDa.

**Chemical Footprints of Ter Protein at $\tau$ Sequences.** To identify the sequences recognized by the Ter protein, we performed copper-phenanthroline footprinting of the Ter protein at the $\tau$ fragment by a recently described technique (13). The 216-bp fragment was cleaved by *Hae* III into ≈140-bp and ≈80-bp subfragments (Fig. 3*A*). These subfragments were 5′- and 3′-end-labeled with $^{32}$P separately at the *Hin*dIII, *Hae* III, and *Eco*RI ends. The asymmetrically end-labeled DNAs were complexed with protein. The DNA–protein complexes were resolved from free DNAs by gel electrophoresis. The gel slices were treated with copper-phenanthroline, and the cleaved DNA eluted and resolved in denaturing sequencing gels.

The results (Fig. 5, I and II) show that the Ter protein protects 14–16 bp of both the top and bottom strands of the $\tau_L$ from chemical cleavage. The footprint of the top strand is displaced to the right, with respect to the bottom strand footprint, by 3 nucleotides. Footprinting at $\tau_R$ showed similar

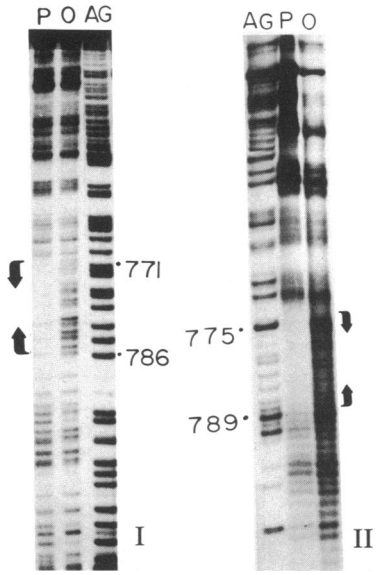


FIG. 5. Copper phenanthroline (Cu-Ph) footprints of Ter protein at $\tau_L$ sites. Fraction III protein was used in all experiments. Enough protein was added to complex 50% of input DNA. I, Footprint at $\tau_L$ 5′-labeled at the *Hae* III end. Lanes: AG, molecular mass markers provided by Maxam–Gilbert base-specific cleavage reactions; O, Cu-Ph cleavage of naked $\tau_L$ DNA; P, $\tau_L$–Ter complex. The coordinates marked by arrows are derived from the sequence of $\tau_R$ $\tau_L$ reported earlier (8). The bent arrows show the footprint. II, Footprint at $\tau_L$ 3′-end-labeled near the *Hae* III site (see Fig. 3). Lanes: AG, Maxam–Gilbert AG reaction; P, $\tau_L$–Ter complex, bent arrows show the protected region; O, naked $\tau_L$ DNA (control). The coordinates are as reported (8).

results. The footprint of the top strand was displaced to the right by two nucleotides with respect to the bottom strand (data not shown). Therefore, there is no preferential protection of one strand of $\tau_R$ or $\tau_L$ by the Ter protein.

**Site-Directed Mutagenesis of $\tau_R$.** We wished to examine whether the Ter protein plays a key role in the termination of DNA replication at $\tau_R$ (and $\tau_L$) in the following manner. Using the synthetic oligonucleotide 5′-CTAGTAGagatct-CACTCA-3′, we mutagenized 6 of 16 bases of $\tau_R$ that are protected by Ter protein. The mutants were selected by hybridization to 5′-end-labeled mutagenic oligonucleotide followed by washing at increasing temperatures as described (15). Furthermore, the mutant acquired a *Bgl* II site that was easily confirmed by digestion with *Bgl* II (Fig. 2, lane O) and by dideoxynucleotide sequencing (data not shown).

We examined the mutant $\tau_R$ (Fig. 3*C*), first for its ability to bind to Ter protein, and second for its ability to terminate DNA replication *in vivo*.

The 80-bp mutant $\tau_R$ failed to bind to 20 units of Ter protein under conditions under which wild-type $\tau_R$ readily bound to 10 units of Ter protein (fraction IV) as revealed by the gel shift assay (Fig. 2, lanes M and P).

**Termination of Replication *in Vivo*.** We cloned the wild-type $\tau_R$ and the mutant $\tau_R$ in both orientations with respect to ori, between the *Eco*RI and the *Hin*dIII sites of pUC18 and pUC19. The wild-type $\tau_R$ should block fork movement in pUC18 but not in pUC19 because of the polarized nature of the block (17). We prepared replication intermediates from both the wild-type and mutant $\tau_R$ clones and carried out the *in vivo* Ter assay described earlier (12). The assay relies on the fact that the majority of the replication intermediates have the structure shown in Fig. 6 (*Upper*). *Eco*RI/*Hin*dIII digestion should therefore release a large Y-shaped molecule and a small Y-shaped piece. The large Y-shaped DNA should be more retarded in an agarose gel than the corresponding *Eco*RI/*Hin*dIII-generated linear DNA from nonreplicating DNA. Furthermore, upon heat denaturation, the Y-shaped DNA should be disrupted by branch migration.

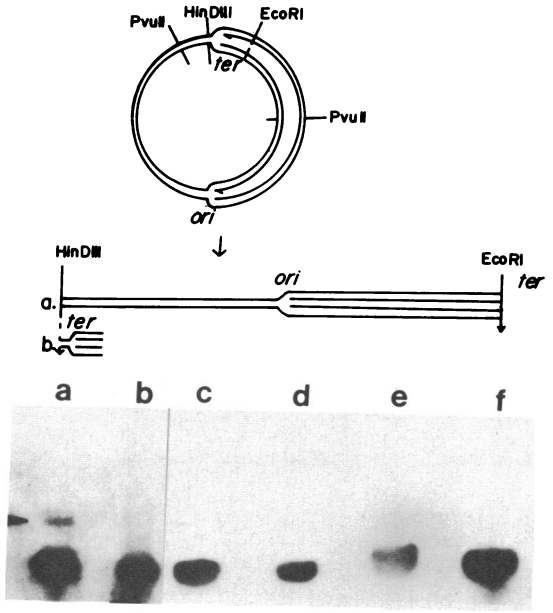


FIG. 6. Autoradiogram of a composite of a 1% and a 1.4% agarose gel blotted onto nitrocellulose showing termination intermediate. (*Upper*) Schematic representation of $\tau_R$ in pUC18 showing replication fork stalled at $\tau_R$. Probing with $^{32}$P-labeled pUC18 DNA probe would reveal the large Y-shaped termination intermediate (a) in addition to nonreplicated DNA. (*Lower*) *Eco*RI/*Hin*dIII-cut DNA samples from cells at logarithmic phase were electrophoresed in 1% (lanes a and b) or 1.4% agarose gels (lanes c–f) and blotted onto nitrocellulose. Approximately $10^6$ cpm of pUC18 DNA was used as a probe. Note termination intermediate (arrowhead) appears in pUC18-80 (lane a), but not in pUC18-80 mutant (lane b). No termination intermediate is visible in pUC19-80 (lane c) or pUC19-80 mutant (lane d). Upon heating (90°C, 5 min), the intermediate is destroyed in pUC18-80 (lane e). Heated pUC18-80 mutant DNA is shown in lane f.

The Southern blots of the relevant DNAs were probed with $^{32}$P-labeled pUC18 probe. The results from six independent experiments (Fig. 6 *Lower*) show that the pUC18 $\tau_R$ generated the characteristic larger termination intermediate (Y-shaped DNA), whereas the pUC18 mutant $\tau_R$ revealed no detectable amounts of the termination intermediate. Thus, the mutant $\tau_R$ neither binds to the Ter protein nor is capable of terminating DNA replication *in vivo*.

## DISCUSSION

The evidence presented in this paper supports the important conclusion that a sequence-specific replication terminus is specifically recognized by a host-encoded sequence-specific DNA-binding protein and that this interaction, most likely, is essential for blocking replication fork movement.

Is a specific replication terminus a universal feature of a replicon? A review of the available information shows that replicons such as phage λ at the early stage of replication (18) and simian virus 40 (19) do not have a specific replication terminus. Perhaps it is worthwhile to keep in mind that these are "runaway" replicons that are apparently not regulated. A specific terminus is more likely to be present in bidirectionally replicating circular replicons such as the chromosomes of *E. coli* (20, 21) and R6K (2, 4–9) that are regulated with respect to their copy number. Unidirectionally replicating plasmids such as ColE1 terminate replication at or near the origin (22).

What is the physiological role of a specific replication terminus? Clearly, the replication termini can be deleted from the chromosomes of R6K (5) and *E. coli* (23) without incurring any apparent lethality. However, *E. coli* strains with deleted termini grow more slowly (24).

Is the Ter protein that terminates replication at $\tau_L$ and $\tau_R$ of R6K also the same protein that terminates DNA replication at the terminus of *E. coli*? Hill *et al.* (24) reported that termination in *E. coli* requires an unidentified trans-acting factor. It is very likely that the Ter protein also terminates host replication and that the τ-like sequence may be a universal termination signal in *E. coli* and replicons that are similar to *E. coli* in other Gram-negative bacteria. The termini of R6K and *E. coli* show sequence homology (25, 26).

Unlike other DNA-binding proteins (e.g., *lac* repressor) that apparently do not terminate replication *in vivo*, Ter protein blocks replication *in vivo* and does so in a polarized fashion.

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