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Genetics

tus, the trans-acting gene required for termination of DNA replication in *Escherichia coli*, encodes a DNA-binding protein

(replication termination/*T1* and *T2* terminator signals)

THOMAS M. HILL*, MARIANNE L. TECKLENBURG, ANTHONY J. PELLETIER, AND PETER L. KUEMPEL

Department of Molecular, Cellular, and Developmental Biology, University of Colorado, Boulder, CO 80309-0347

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ABSTRACT **The components for termination of DNA replication in *Escherichia coli* include the terminator signals *T1* and *T2* and the trans-acting gene *tus*. We have shown previously that *tus* maps in a 4-kilobase region of the chromosomal terminus near *T2*. Through the use of deletion and insertion mutants, the location of the *tus* gene has now been precisely identified. We sequenced 2416 nucleotides in this region and identified a 927-base-pair open reading frame which encodes Tus. Insertion of a kanamycin-resistance gene in this open reading frame abolished *tus* activity. We also demonstrated that crude extracts of *tus*$^+$ cells contain a protein which binds to the *T2* terminator sequence.**

Two polar terminator signals which inhibit the progress of replication forks, designated *T1* and *T2*, have been identified in the terminus region the *Escherichia coli* chromosome (1, 2). *T1* is located at min 28.1 (3) or kilobase (kb) 90 on the Bouché terminus map (4), and it halts only counterclockwise-traveling replication forks, whereas *T2* maps at min 35.6 (3, 5) or kb 442, and it halts only clockwise-traveling replication forks. In addition to *T1* and *T2*, terminator signals have also been identified in the *E. coli* chromosome near min 34,† at min 27 (J.-M. Louarn, personal communication; T.M.H. and P.L.K., unpublished results), and in the plasmids R6K, R1, and R100 (6, 7).

In addition to the terminator signals, a trans-acting gene called *tus* (termination utilization substance) has been identified that maps near *T2* and that is required for function of the terminator signal *T1* (5). In this paper, we present further characterization of *tus*. Complementation of *tus* mutants was used to locate the *tus* gene and the nucleotide sequence of the *tus* coding region was determined,‡ identifying an open reading frame (ORF) which encodes the *tus* gene product. We demonstrate the physical location of the *tus* gene in relationship to the terminator signal *T2* and show that *tus* is also required for *T2* function. Finally, we show that the *tus* gene encodes a DNA-binding protein which associates with the terminator signal sequences.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. A list of strains used in these studies is presented in Table 1. Δ2474 was constructed *in vitro* by replacing the 0.35- and 1.3-kb *Eco*RV fragments (Fig. 1) in plasmid pPK1014 (see below) with a Kan^r gene cassette (Pharmacia) and crossing the plasmid into the *E. coli* chromosome by using previously described techniques (5). The *tus*::Kan insertion was constructed in the same manner. Strain PK2544 was constructed by transducing the *tus*::Kan insert into PK457 first and then transducing in the *recA56* allele.

Plasmids pPK1009 and pPK1007 (5) contain the 4.8-kb *Eco*RI fragment (kb 439–443.8) inserted into either pRI40 (8) or pUC19 (9), respectively. pPK1007 also contains a Kan^r gene and an additional 4 kb of terminus DNA from kb 428–432. Plasmid pPK1010 was constructed by digesting pPK1009 with *Hin*dIII and rejoining the fragment containing the vector and the 2.1-kb *Eco*RI/*Hin*dIII fragment. Plasmid pPK1013 was constructed by isolating the 2.7-kb *Hin*dIII/*Eco*RI fragment from pPK1007 and inserting it into pRI40. Plasmid pPK1014 was constructed by inserting the entire 4.8-kb *Eco*RI fragment into pUC19. The 1.3-kb *Eco*RV fragment (Fig. 1) was isolated from pPK1014 and inserted by blunt-end ligation into pRI40 to produce pPK1023. pPK1024 was constructed by partially digesting pPK1014 with *Eco*RV and inserting a Kan^r gene cassette into the resulting mix of partial digests. pPK1018, one isolate from the cloning, contained the Kan^r cassette inserted between the 1.3- and 1.15-kb *Eco*RV fragments. The 4.8-kb *Eco*RI fragment containing the Kan^r gene insert was then isolated from pPK1018 and reinserted into the *Eco*RI site of pRI40 to produce pPK1024.

Assay for *tus* Complementation. All plasmids were transformed into PK2340, which is a P2*sig*$_5$ (min 47) strain containing Δ2337 (Δ*tus*). Determination of *T1* activity by using a Southern blot assay has been described (3). Studies for determining if *tus* was required for *T2* activity were performed in derivatives of PK457. In this case, replication forks arrested at *T2* originated from *oriC*, rather than from a P2*sig*$_5$ prophage.

Sequencing Strategy and Technique. The 1.3- and 1.15-kb *Eco*RV fragments were inserted into the *Sma* I site of m13-mp18 (9), and deletions were generated by using the technique of Henikoff (10). The sequences were determined by using the dideoxynucleotide chain termination method of Sanger *et al.* (11). Sequenase (United States Biochemical) was used in place of the Klenow fragment of DNA polymerase. In places where overlapping deletions could not be obtained, synthetic oligomers were used as primers to determine the sequence.

DNA–Protein Binding Assay. Crude extracts of cultures were prepared by the following procedure: Exponentially growing cells in LB broth were harvested at an OD_{600} of 1.0 and centrifuged, and the cell pellet was washed once in binding buffer [10 mM Tris·HCl, pH 7.5/50 mM KCl/1 mM EDTA/5% (vol/vol) glycerol/1 mM dithiothreitol/50 μg of bovine serum albumin per ml/16 μg of phenylmethylsulfonyl fluoride per ml (12)]. After centrifugation, the cell pellet was resuspended in binding buffer in 1/50th of the original volume

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Abbreviations: Kan^r, kanamycin resistance; ORF, open reading frame.

*Present address: Department of Biosciences and Biotechnology, Drexel University, Philadelphia, PA 19104.

†Louarn, J. M., American Society for Microbiology Conference on the Organization of the Bacterial Chromosome, May 22–25, 1988, Pine Mountain, GA.

‡The sequence reported in this paper is being deposited in the EMBL/GenBank data base (accession no. J04507).

Table 1. List of strains

Strain	Genotype	Ref.
PK457	*trpR, trpA9605am, his-29am, ilv, pro-2, arg-427, thyA, deoB, tsx*	6
PK998	*dnaAts, rac, his, thr, leu, arg, thi-1, thyA, deoB* or *deoC, lac, xyl, mal, mtl, ara, rpsL, tonA*, P2*sig*$_5$ (min 16)	1
PK1012	*dnaAts, rac, his, thr, leu, arg, thi-1, thyA, deoB* or *deoC, lac, xyl, mal, mtl, ara, rpsL, tonA*, P2*sig*$_5$ (min 47)	1
PK2340	PK1012 plus Δ2337 (Δ*tus*), Kan^r	5
PK2359	*hsdS20, ara-14, leu, proA2, lacY1, galK2, rpsL20, xyl-5, mtl-1, supE44*, Δ2038 (Δ*tus*) *nth*::Kan plus pPK1007	This paper
PK2374	PK2340 plus pPK1009 (Spc^r)	5
PK2400	PK2340 plus pPK1010 (Spc^r)	This paper
PK2417	PK2340 plus pPK1013 (Spc^r)	This paper
PK2476	PK1012 plus Δ2474 (Kan^r)	This paper
PK2512	PK2340 plus pPK1023 (Spc^r)	This paper
PK2514	PK2340 plus pPK1024 (Kan^r)	This paper
PK2540	PK998 *tus*::Kan	This paper
PK2544	PK457 *recA56, tus*::Kan	This paper
PK2553	PK2544 plus pPK1013	This paper
PK2636	PK457 *recA56*	6

Kan^r, kanamycin resistance; Spc^r, spectinomycin resistance.

and sonicated. Cell debris was removed by a 20-min centrifugation in a Beckman Microfuge at 4°C. Protein concentrations were determined by using the assay of Bradford (13). A given amount of extract was incubated in binding buffer with 130 pg of the double-stranded synthetic oligomer (5′-AATTCATAAAATAAGTATGTTGTAACTAAAGTGGATC-3′ and its complementary strand) which had been end labeled with ^{32}P. In the experiments shown here, 155 ng of pBR322 DNA was added as a nonspecific competitor for DNA-binding proteins. Substitution of poly(dI·dC) (2 μg per reaction) in place of pBR322 DNA as the nonspecific competitor gave identical results. The incubation was carried out for 20 min at room temperature in a final volume of 20 μl. Electrophoresis in 5% polyacrylamide gels (20:1 crosslinking) was performed according to Fried and Crothers (14). The gel was then dried and exposed to Kodak X-Omat film.

RESULTS

We have previously demonstrated that a 4.8-kb *Eco*RI fragment from the *E. coli* terminus region contains the necessary information to complement *tus* deletion mutants (5). We isolated smaller fragments of the 4.8-kb fragment, inserted them into a low-copy-number vector, and tested the resulting plasmid constructions for complementation of *tus* mutants (Fig. 1). Plasmid pPK1010, which contains a 2.1-kb insert, and plasmid pPK1013, which contains a 2.7-kb insert, were used to transform strain PK2340. This strain is a derivative of PK1012 that contains Δ2337, which removes *tus* (5). Counterclockwise replication forks are not inhibited at *T1* in strains containing Δ2337 (Fig. 2) unless *tus* is supplied on a plasmid, such as with pPK1009 (5). When pPK1010 was tested in strain PK2340, it did not complement the Tus$^-$ phenotype (data not shown), whereas a similar experiment with pPK1013 clearly demonstrated reactivation of the *T1* terminator signal (Fig. 2). Consequently, the *tus* gene must be located on the 2.7-kb *Hin*dIII/*Eco*RI fragment. We then tested pPK1023, which contains the 1.3-kb *Eco*RV fragment. This plasmid was not capable of reactivating *T1* (data not shown), suggesting that the *tus* gene was either entirely contained in the region to the right of the 1.3-kb *Eco*RV fragment or spanned the junction between the 1.3- and 1.15-kb *Eco*RV fragments. That *tus* spans the junction was confirmed by testing Δ2474, which removes the 0.35- and 1.3-kb *Eco*RV fragments and eliminates *tus* function (data not shown).

We sequenced the 1.3- and the 1.15-kb *Eco*RV fragments. The sequence of 2416 nucleotides covering the two fragments is presented in Fig. 3. We find two ORFs of significant length within the sequenced region. A partial ORF, designated

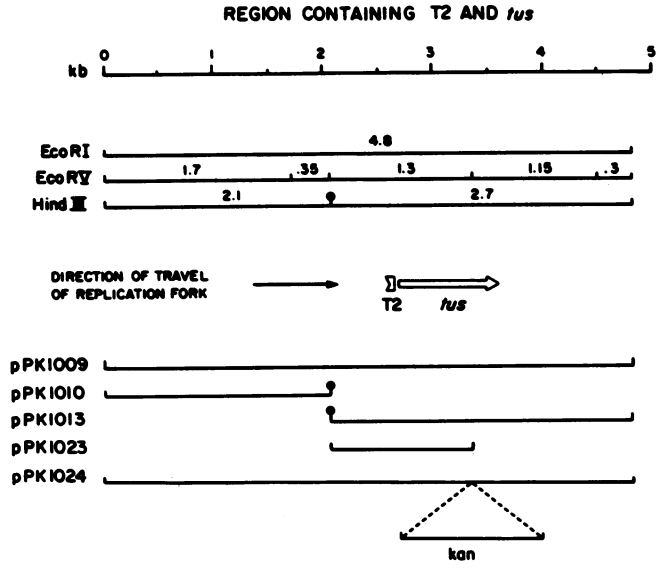

FIG. 1. Restriction map of the 4.8-kb *Eco*RI fragment containing *T2* and *tus* (kb 439–443.8). The locations of *Eco*RV and *Hin*dIII sites within the fragment are shown. The direction of replication forks inhibited at *T2* and the location of *T2* and *tus* are marked below the map. The DNA inserted into various plasmids is indicated along with the plasmid number. Complementation of *tus* strains by presence of the plasmid is also indicated.

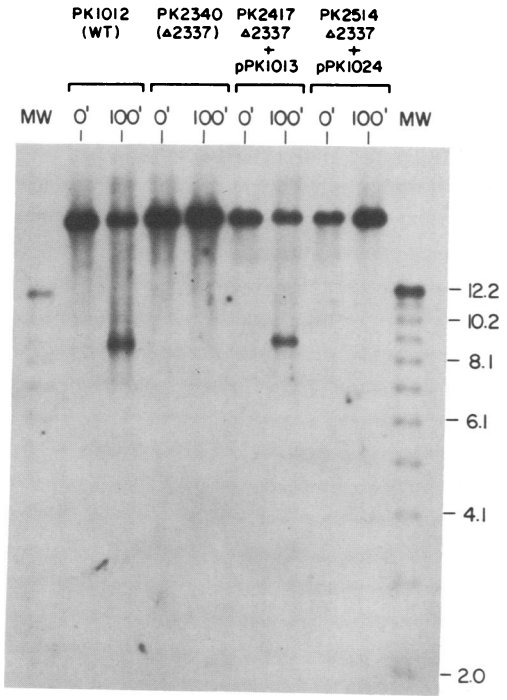

FIG. 2. Inactivation of *tus* by insertion of a Kan^r gene. Presence of *tus* was determined by *T1* activity. Strains containing a P2*sig*$_5$ prophage at min 47 were induced as described, and samples were taken at 0 and 100 min after induction. DNA from induced cells was digested with *Hin*dIII and blotted after electrophoresis. A linear band of 24 kb should be visible after probing with a fragment from pAP203 (3). If inhibition of replication at *T1* occurs, an additional band of 9 kb should also be visible (PK1012, 100-min sample). Δ2337, which removes *tus*, causes loss of *T1* function and, hence, loss of the 9-kb band. Providing pPK1013 restores *T1* function. However, insertion of a Kan^r gene between the 1.3- and 1.15-kb *Eco*RV fragments in pPK1024 prevents reactivation of *T1* function. MW, molecular weight markers, labeled in terms of kb.

ORF-1, starts upstream of the beginning of the sequence and ends at nucleotide 549, whereas a complete ORF of 927 base pairs, designated ORF-2, extends from nucleotide 628 through nucleotide 1554, spanning the junction of the two *Eco*RV fragments at nucleotide 1279. Expression of ORF-2 would produce a protein of 35,800 daltons with a calculated pI of 10.1. To test if ORF-2 encoded Tus, a plasmid (pPK1024) was constructed which contained a Kan^r gene inserted between the 1.3- and 1.15-kb fragments (Fig. 1). This plasmid was used to transform PK2340, and the resulting strain, PK2514, was tested for function of *T1*. In contrast to pPK1013, the Kan^r gene insert in pPK1024 prevented *T1* function, indicating that *tus* had been inactivated (Fig. 2).

Although it seemed likely, it had not been demonstrated that *T2* function in the chromosome required *tus*, nor had we been able to physically separate *T2* and *tus*. We therefore crossed the *tus*::Kan insert in pPK1024 into the chromosome of PK998 (P2*sig*$_5$ at min 16) and tested this strain (PK2540) for *T2* activity. The insert in *tus* eliminated *T2* activity (Fig. 4). However, it was still possible that the kanamycin gene had inserted into the *T2* sequence as well as interrupting the *tus* gene. *tus* was therefore provided in trans in a *tus*::Kan strain to determine if it reactivated *T2*. These experiments were performed in strains where replication forks were initiated from *oriC* rather than from a P2*sig*$_5$ prophage origin; consequently, the intensity of the hybridization signal is weaker (3). Fig. 4 demonstrates that the presence of pPK1013 caused replication inhibition at *T2* in a *tus*::Kan strain, indicating that only the *tus* gene had been inactivated. The recent identification of the sequence which acts as the terminator signal *T2* (6), which is located at nucleotides 595–617 in the sequence (Fig. 3), confirms our results that *T2* and *tus* are separate.

We have previously postulated that Tus may act as a DNA-binding protein that binds at the terminator signals *T1* and *T2* (5). To test this hypothesis, crude cellular extracts were prepared from strains containing the *tus*::Kan insert, a normal chromosomal copy of *tus*, or a plasmid carrying the *tus* gene. The extracts were incubated with a synthetic oligomer containing the 23-base-pair terminator signal from *T2* (6). When the products of the incubation were electrophoresed, a slower-migrating band was observed in the extract from the *tus*$^+$ strain but not in the *tus*::Kan extract (Fig. 5), indicating that the *tus* gene produced a protein which bound to the *T2* sequence. Increasing the amount of expression of the *tus* gene with either pPK1013 (low copy number) or pPK1007 (high copy number) gave increasing amounts of the retarded band. These results indicate that the *tus* gene product bound to the *T2* sequence and retarded the oligomer. A very faint band migrating at a position between the *T2* oligomer and the Tus/*T2* complex was also observed in lane B (*tus*::Kan) of Fig. 5. This band was repeatedly observed in extracts from all strains tested, suggesting that a cell protein other than Tus can bind to the terminator sequences with a low affinity.

DISCUSSION

The presently identified components for termination of DNA replication in *E. coli* consist of the terminator loci, which include *T1* and *T2*, and the trans-acting factor Tus. We mapped the *tus* gene to a specific region of a 4.8-kb *Eco*RI fragment, determined the nucleotide sequence of this region, and identified the ORF which encodes Tus. We also showed that crude extracts from *tus*$^+$ cells contained a protein that bound specifically to a small DNA fragment containing the *T2* terminator signal. We propose that the Tus protein prevents passage of replication forks by binding to the chromosomal terminator sequences.

Although the mechanism of replication termination is not known, we predict that the affinity of the Tus protein for different terminator sequences will determine the efficiency of replication fork inhibition at a particular termination site. Terminator sequences which bind Tus weakly will function poorly compared to sequences which bind Tus strongly. Determining the dissociation constant of Tus from various terminator sequences will test the validity of this hypothesis.

Analysis of the predicted amino acid sequence for Tus indicates that the mass of the protein is approximately 35,800 daltons. We confirmed the size of the *tus* gene product by constructing a plasmid which overproduces the Tus protein and identifying the overproduced product by electrophoresis on a sodium dodecyl sulfate/polyacrylamide gel. The size of the overproduced protein was approximately 36,000 daltons (T.M.H., M.L.T., and P.L.K., unpublished results). The predicted Tus protein also does not contain any of the consensus amino acid sequences for DNA-binding protein motifs such as the helix-turn-helix (15), "zinc finger" (16), or "leucine zipper" (17). Comparison of the protein sequence for Tus with the Protein Identification Resource data bank on Nov. 1, 1988, by using the Eugene sequence analysis programs (MBIR, Baylor College of Medicine) did not reveal other proteins with significant homology. Also, comparison with the Tus-like protein which has recently been identified for *Bacillus subtilis* (18, 19) did not show regions of homology.

Preliminary data indicate that the promoter for the *tus* gene is located at least 1200 base pairs upstream of the *tus* gene. We synthesized an oligomer complementary to nucleotides 640–657 and performed a primer extension to determine the location of the 5′ end of the *tus* message. A band of greater

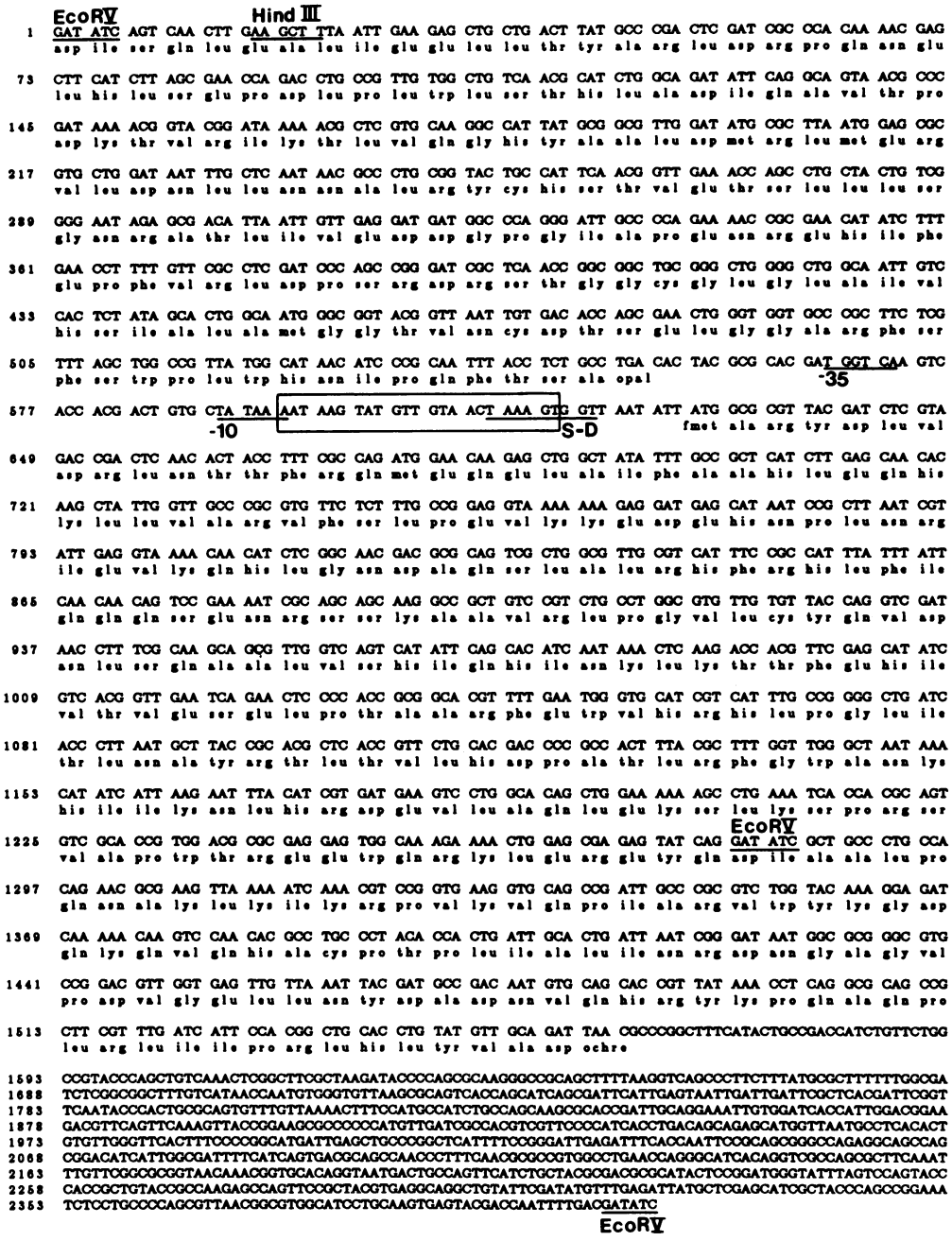

FIG. 3. Nucleotide sequence of the 1.3- and 1.15-kb *Eco*RV fragments containing *T2* and *tus*. The predicted amino acid sequence of open reading frame 1 (ORF-1) (nucleotides 1–549) and open reading frame 2 (ORF-2) or *tus* (nucleotides 628–1554) are indicated below the nucleotide sequence. The locations of a putative promoter (−35, −10) and the ribosomal binding site (S-D) are underlined and labeled. The location of certain restriction sites is also presented. The *tus*::Kan insert used in this study occurs at the *Eco*RV site that starts at nucleotide 1279. The location of the terminator signal *T2* (6) is indicated by the boxed sequence.

than 1200 nucleotides was consistently observed (A. M. Flower, C. S. McHenry, T.M.H., and P.L.K., unpublished results), indicating that the primary promoter for *tus* expression is probably located in front of ORF-1. In addition to the primary promoter, we identified a weak promoter immediately upstream of the *tus* gene sequences. A "-35 box" is located at nucleotides 567–572 and a "-10 box" at 590–595, just upstream of a Shine–Delgarno sequence (20) positioned between nucleotides 612 and 620. The presumed low activity of this promoter is based on a comparison of promoter sequences with known promoter activities (21).

The proximity of the *tus* gene to ORF-1 and the length of the *tus* mRNA suggests that *tus* is part of an operon. Since operons often contain genes of related function, we constructed a strain which contained an ORF-1::Kan insertion and tested for both *T1* and *T2* activity. The results indicated that loss of function of ORF-1 had no effect on replication inhibition (T.M.H., M.L.T., and P.L.K., unpublished results).

We have recently identified the DNA sequence which acts as the terminator signal at *T2* (6). A 23-base-pair sequence 5′-AATNAGTATGTTGTAACTAAAGT-3′ was found which is common to *T1* and *T2* and which causes replication forks to halt in a ColE1-derived plasmid. Function of the terminator signals was dependent upon the presence of *tus* and the orientation of the sequence with respect to the plasmid origin. The sequence for *T2* is located immediately upstream of the *tus* gene, at positions 595–617 in Fig. 3, suggesting that *T2* may act as a regulator of *tus* expression.

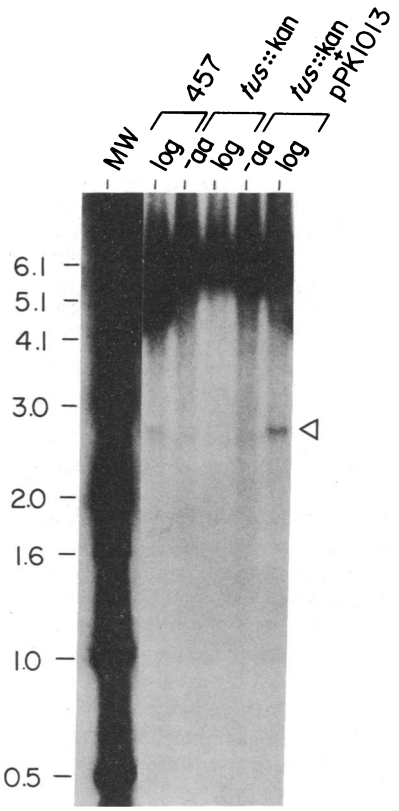

FIG. 4. Evidence that *tus* and *T2* are separate. Inhibition of replication forks originating from *oriC* was used to determine if *T2* could be reactivated in a *tus*::Kan strain when a *tus*$^+$ plasmid was provided. DNA was harvested from either amino-acid starved cultures (−aa), which should not show a termination band, or exponentially growing cultures (log). The DNA was then digested with *Eco*RI, electrophoresed and blotted, and hybridized to the 1.7-kb *Eco*RI/*Eco*RV fragment upstream of *T2* (see Fig. 1). The probe should hybridize to a 4.8-kb *Eco*RI fragment in wild-type DNA and a 6.1-kb fragment in DNA containing the *tus*::Kan insertion. If inhibition of replication at *T2* occurs, a band of 2.7 kb (open triangle) should also be visible in addition to the linear band. The termination band is present in PK457 (wild type), absent in PK2554 (which contains *tus*::Kan), and regained in PK2553 (pPK1013 + *tus*::Kan).

Binding of the Tus protein to the *T2* sequence would prevent function of the putative promoter just upstream of the *tus* gene, since the −10 box is directly in front of the *T2* sequence. Although this is not the primary promoter for *tus* expression, this promoter may be used when levels of Tus protein are diminished. Under these circumstances, the *T2* site may be unoccupied, allowing use of this promoter.

Horiuchi and Hikada (7) have recently reported unpublished results that suggest that a host protein binds to the R6K plasmid terminus. Since the R6K terminus contains two terminator sequences with high homology to the chromosomal terminator signals of *E. coli* (6, 7), we conclude that the *tus* gene encodes the host protein binding to the R6K terminus region.

Note Added in Proof. Hidaka *et al.* (22) have recently reported the nucleotide sequence for four termination sites.

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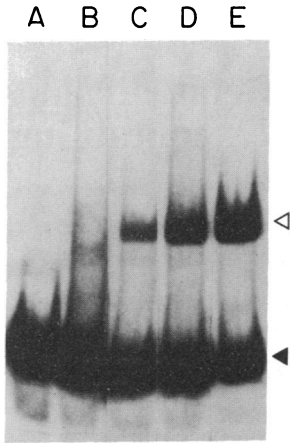

FIG. 5. Binding of Tus to a 37-base-pair oligomer containing the *T2* terminator signal. A double-stranded oligomer (37 base pairs) containing the 23-base-pair *T2* terminator sequence was end-labeled and mixed with 10 mg of total protein from crude cellular extracts. Incubation was carried out at room temperature for 20 min. Samples were then loaded onto a 5% polyacrylamide gel and electrophoresed for 1 hr at 180 V. Lane A, no protein added. Lane b, extract from PK2544, containing a *tus*::Kan insert. Lane C, extract from PK2636, containing a single chromosomal copy of the *tus* gene. Lane D, extract from PK2553, containing pPK1013, a *tus*$^+$ plasmid with a low-copy-number vector. Lane E, extract from PK2359, containing pPK1007, a *tus*$^+$ plasmid with a high-copy-number vector. ◀ indicates the position of the free oligomer, whereas ◁ indicates the position of the retarded oligomer.

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