Escherichia coli Tus protein acts to arrest the progression of DNA replication forks *in vitro*

(DNA replication inhibition)

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ABSTRACT A polar DNA replication barrier is formed when the DNA-binding protein Tus forms a complex with any of the four 23-base-pair terminator (*ter*) sites found in the terminus region of the *Escherichia coli* chromosome. We have used a plasmid DNA replication system reconstituted with purified proteins *in vitro* to investigate the interaction of the Tus protein with the replication fork. Purified Tus protein alone is necessary and sufficient to arrest DNA replication on ColE1-type plasmid templates containing *ter* sites. Tus protein-catalyzed termination depends upon the orientation of the *ter* site in the plasmid DNA. Nucleotide resolution mapping of the terminated nascent DNA shows that leading-strand DNA synthesis arrests at the point of contact with the Tus protein, while the final lagging-strand primer sites are 50–70 nucleotides upstream. In addition, the distribution of leading-strand arrest sites changes when the composition of the proteins on the lagging-strand side of the replication fork is altered.

A cycle of DNA replication in *Escherichia coli* is initiated bidirectionally from the unique origin *oriC* and ends when the replication forks converge on the opposite side of the chromosome in a region called the terminus. Recent studies have demonstrated that the chromosomal terminus is bounded by polar terminator sites (generically called *ter* sites) that allow replication forks to enter the terminus but not exit (1, 2). The sequence of these chromosomal *ter* sites has been determined (3, 4), and similar sequences have been shown to be present in other replicons, such as the R6K and R100 plasmids (3, 5).

Function of the *ter* sites is entirely dependent on the presence of a trans-acting factor specified by the *tus* locus (6). The DNA sequence of the *tus* gene has been reported (7). This gene encodes a DNA-binding protein (Tus protein) of M_r 35,800 that forms a complex with the chromosomal *ter* sequences, causing inhibition of DNA replication fork progression (7). Arrest of DNA replication in the plasmid R6K is also dependent on the product of the *tus* gene (8–10).

Here, the ability of purified Tus protein to inhibit DNA replication fork progression has been examined. A DNA replication system reconstituted with purified proteins *in vitro* and capable of utilizing ColE1-type plasmid DNAs as templates was used to demonstrate that the Tus protein was sufficient to arrest the movement of DNA replication forks composed of the DNA polymerase III holoenzyme and the primosome. DNA replication fork inhibition in this system accurately mimicked termination *in vivo*. In addition, the point of DNA replication arrest has been mapped to nucleotide resolution, and the distribution of termination sites has been shown to vary when the composition of the proteins operating on the lagging-strand DNA template is altered.

MATERIALS AND METHODS

Bacterial Strains. Strain PK2691 is JM109 harboring the plasmid pTH311 that has the *tus* gene inserted behind the *tac* promoter of vector pKK223-33. Details on the construction of this Tus overexpressor plasmid will be published elsewhere (T.M.H., M. Tecklenburg, and P. Kuempel, unpublished data).

DNA Substrates. The *ter*-containing plasmid DNAs pTH101 and pTH201 have been described (3). Unamplified pBR322, pTH101, and pTH201 form I DNAs were prepared as described by Marians *et al.* (11).

Assay for Tus Protein Activity. Details of the filter-binding assay used to measure activity of the Tus protein fractions during purification (Table 1) will be published elsewhere (T.M.H., M. Tecklenburg, and P. Kuempel, unpublished data). One unit of activity is defined as the amount of Tus protein required to complex 1 fmol of an oligomer comprised of the T2 *ter* sequence.

Enzymes. Restriction enzymes were from New England Biolabs. *E. coli* DNA replication proteins were prepared as described by Minden and Marians (13).

DNA Replication Assays. DNA replication of plasmid templates (70 fmol) was performed essentially as described by Minden and Marians (12) except that the reaction mixture also included 200 mM potassium glutamate (pH 7.5) and 0.4 pmol of topoisomerase III (14). Newly synthesized DNA was labeled with either [^3H]dTTP or [α - ^{32}P]dATP. In experiments measuring arrest of DNA replication, purified Tus protein (fraction 6 of Table 1) was added to the substrate DNA 2 min prior to the addition of the replication proteins to allow formation of the Tus protein–terminator complex.

Determination of the Site of Replication Arrest. Reactions, in either the presence or the absence of primase, were terminated after a 20-min incubation at 30°C by treating with an equal volume of phenol. Tus protein was present at a 50-fold molar excess. DNA products were isolated by spin dialysis through columns of Sepharose 4B and then digested with the *Xho*I restriction endonuclease. After treatment with phenol, the DNA products were collected by precipitating with ethanol and dissolved in a denaturing gel-loading dye. The dideoxy sequence ladder was produced by using denatured pTH101 DNA as the template and the oligonucleotide 5'-TCGAGCAAGACGTTTCCCGTTGAAT-3' as a primer. This oligonucleotide is the same strand-sense as the nascent leading-strand DNA and has exactly the same 5'-end as the *Xho*I-cut material. Products were analyzed on 6% polyacrylamide (19:1) gels containing 50% urea.

RESULTS

Purification of the Tus Protein. To facilitate purification of the Tus protein, a DNA fragment containing the *tus* gene was inserted into the expression vector pKK223-3 (Pharmacia) and brought under the control of a *tac* promoter, yielding the

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Table 1. Purification of the Tus protein

Step	Protein, mg	Activity, units*	Specific activity, units/mg
1. Cell lysate†	1755	—	—
2. Ammonium sulfate†	873	—	—
3. Bio-Rex-70	50.2	5.82×10^8	1.16×10^7
4. CM-Sephadex	25.3	4.31×10^8	1.70×10^7
5. DE-52	7.5	1.59×10^8	2.13×10^7
6. Bio-Rex 70	3.8	8.39×10^7	2.22×10^7

The Tus protein was purified from 30 g of *E. coli* strain PK2691 that had been induced with 1 mM isopropyl β -D-thiogalactoside. Crude cell extracts were prepared by the procedure of McHenry and Crow (12). The lysate (fraction 1, 180 ml) was brought to 40% saturation with ammonium sulfate. The precipitate was then collected by centrifugation and discarded. The supernatant was then brought to 60% saturation with ammonium sulfate. After centrifugation, the protein pellet was resuspended in 10 ml of 50 mM sodium phosphate, pH 6.8/100 mM NaCl/0.1 mM EDTA/1 mM dithiothreitol/20% (vol/vol) glycerol (fraction 2), quick-frozen in liquid nitrogen, and stored at -80°C . Half of fraction 2 was thawed and dialyzed extensively against buffer A (50 mM imidazole hydrochloride, pH 6.8/100 mM NaCl/1 mM dithiothreitol/1 mM EDTA/and 20% glycerol), diluted to a protein concentration of 7 mg/ml, and loaded at 50 ml/hr onto a Bio-Rex (Bio-Rad) column (3.7 cm $\times$ 11 cm) equilibrated with buffer A. The column was washed with 300 ml of buffer A and eluted with a 1-liter gradient of NaCl (0.2–0.6 M) in buffer A. Active fractions (0.4 M NaCl) were pooled (fraction 3) and diluted with buffer A (without NaCl) to 0.1 M NaCl. Fraction 3 was then loaded at 50 ml/hr onto a CM-Sephadex (Pharmacia) column (2.6 cm $\times$ 5.5 cm) equilibrated with buffer A. The column was washed with 120 ml of buffer A and developed with a 300-ml gradient of NaCl (0.1–0.6 M) in buffer A. Active fractions (0.3 M NaCl) were pooled (fraction 4), dialyzed extensively against buffer B [50 mM Tris-HCl, pH 7.4 (at 4°C)/1 mM EDTA/1 mM dithiothreitol/20% glycerol], and loaded onto a DE-52 (Whatman) column (1.6 cm $\times$ 3 cm) equilibrated with buffer B. The column was washed with 20 ml of buffer B and the flow-through, which contained the Tus protein, was collected (fraction 5). Fraction 5 was dialyzed against buffer A, and the Tus protein was concentrated by loading onto a Bio-Rex column (0.9 cm $\times$ 2.7 cm). The protein was eluted from the column by washing with buffer A containing 0.6 M NaCl (fraction 6).

*One unit of activity is defined as the amount of Tus protein required to complex 1 fmol of an oligomer containing the T2 *ter* sequence in a filter-binding assay.

†The activity of these fractions could not be determined accurately because of the presence of proteins that interfered with the filter-binding assay.

plasmid pTH311. Bacterial cells carrying this plasmid grown in the presence of isopropyl β -D-thiogalactoside overexpress the Tus protein by 1600-fold. Soluble extracts prepared from these cells were used as a source to purify the Tus protein to >95% homogeneity (Fig. 1 and Table 1). The resulting preparation of Tus protein migrated in sodium dodecyl sulfate (SDS)-containing polyacrylamide gels with an apparent molecular mass of 36 kDa. Purified Tus protein was free of detectable nuclease activity (data not shown). Studies on the interaction between purified Tus protein and the T2 *ter* sequence have demonstrated that this reaction proceeded with a $K_d = 1 \times 10^{-12}$ M (T.M.H., M. Tecklenburg, and P. Kuempel, unpublished data).

Purified Tus Protein Inhibits DNA Replication from ColE1-Type Origins. The ColE1-type plasmids, such as pBR322 and its derivatives, replicate unidirectionally from a unique plasmid origin (15, 16). Initiation of DNA replication requires RNA polymerase, RNase H, and DNA polymerase I (17). Subsequent establishment of the replication fork requires the primosome [ref. 18; a multiprotein complex that moves in the overall 5'-to-3' direction along the lagging-strand DNA template and simultaneously unwinds the parental duplex DNA while it also primes Okazaki fragment synthesis (19)], the

FIG. 1. SDS/polyacrylamide gel electrophoresis of purified Tus protein. Samples were separated on a 15% polyacrylamide gel and stained with Coomassie brilliant blue. Lanes: 1, molecular mass markers; 2, 40 μg of fraction 1 (cell lysate); 3, 40 μg of fraction 2 (ammonium sulfate); 4, 4 μg of fraction 3 (Bio-Rex 70); 5, 4 μg of fraction 4 (CM-Sephadex); 6, 4 μg of fraction 5 (DE-52).

DNA polymerase III holoenzyme, and the single-stranded DNA binding protein. Propagation of this replication fork around the circular plasmid DNA requires DNA gyrase. Proper termination and segregation of the completed daughter DNA molecules requires topoisomerase III (20). Accurate and efficient replication of pBR322 DNA has been reproduced *in vitro* by using the purified replication proteins detailed above as well as DNA ligase (12, 21).

The effect of the Tus protein on the progression of a *bona fide* DNA replication fork was assessed by using the DNA replication system described above and two plasmid DNAs containing ColE1-type origins of DNA replication and also carrying *ter* sequences. In each case, the T2 *ter* sequence has been inserted 1658 nucleotides downstream of the origin of DNA replication; however, in plasmid pTH101 the orientation of the *ter* sequence is active for replication fork arrest, whereas in plasmid pTH201 it is inactive (Fig. 2 *Upper*; ref. 3). If the Tus protein inhibits DNA replication fork progression in the purified system with these plasmid DNAs as templates, only 40% of pTH101 will be replicated prior to DNA replication arrest, whereas DNA replication of pTH201 will proceed unimpeded.

The incorporation of [^3H]TMP into acid-insoluble product during pTH201 DNA replication (*ter* inactive) was unaffected by the addition of Tus protein, even at a molar excess of 50:1 (Fig. 2 *Lower*). However, the incorporation of radioactive label supported by pTH101 DNA (*ter* active) was significantly inhibited at a molar ratio of Tus protein to DNA of 5:1 and reached near maximal inhibition (45% of control) at a ratio of 10:1 (Fig. 2B). This indicated that the Tus protein was sufficient to inhibit plasmid DNA replication.

Tus Protein Acts by Terminating Progress of the DNA Replication Fork. The inhibition of DNA synthesis described in Fig. 2B could result either from a decreased rate of initiation or a premature termination of the nascent DNA. Electrophoresis of the reaction products through native agarose gels (Fig. 3) showed that when pTH101 DNA was used as a template in the presence of subsaturating concentrations of the Tus protein, a band having an electrophoretic mobility intermediate between that of form II (nicked circular) and form I (covalently closed circular) DNA accumulated as the major product. This band corresponds to partially replicated molecules (ref. 21; R. DiGate and K.J.M., unpublished data), suggesting, in fact, that the Tus protein was acting to arrest DNA replication fork movement.

Therefore, the size of the nascent DNA produced in reaction mixtures containing a saturating concentration of

FIG. 2. Tus protein arrests DNA replication in plasmids containing an active *ter* site. (A) Map of the plasmid DNA templates pTH101 and pTH201. The direction of plasmid DNA replication from the origin is indicated by the arrow. pTH101 contains a *ter* sequence (T2) oriented so that DNA replication forks will be arrested. pTH201 contains the *ter* sequence oriented in the nonfunctional direction. *amp^r* and *kan^r*, ampicillin and kanamycin resistance. (B) Effect of the Tus protein on pTH101- and pTH201-supported DNA replication *in vitro*. The indicated amounts of Tus protein (fraction 6) were added to standard DNA replication reaction mixtures containing [³H]TTP and either pTH101 (*ter* active) or pTH201 (*ter* inactive) as template DNAs (70 fmol). Incubations were at 30°C for 20 min. In the absence of Tus protein, 22.3 pmol and 21.2 pmol of [³H]TMP were incorporated into the acid-insoluble product with pTH101 and pTH201 DNAs, respectively, as templates. ○, pTH101 (*ter* active); ●, pTH201 (*ter* inactive).

Tus protein and either plasmid DNA template was examined. If the Tus protein were acting to arrest DNA synthesis at or

FIG. 3. Products of plasmid DNA replication in the presence of Tus protein. DNA replication assays with pBR322, pTH101 (*ter* active), or pTH201 (*ter* inactive) DNA as a template (70 fmol) were performed in the presence of [³²P]dATP and in the absence or presence of Tus protein (fraction 6) at the indicated protein/template ratio. DNA products were separated by electrophoresis through a 1% agarose gel in TAE buffer (40 mM Tris acetate/1mM EDTA) at 2 V/cm for 16 hr. Lanes: 1 and 2, products of pBR322 DNA replication in the absence or presence of Tus protein; 3–5, products of pTH101 DNA replication in the absence or presence of Tus protein; 6–8, products of pTH201 DNA replication in the absence or presence of Tus protein.

FIG. 4. Map of the origin of DNA replication-to-*ter* region of plasmid pTH101. The numbers above the line represent the distance in base pairs from the plasmid origin, and the direction of replication is indicated by the origin arrow. Restriction enzyme cleavage sites are indicated as follows: S, *Sma* I; C, *Cla* I; and X, *Xho* I. The map of pTH201 DNA (*ter* inactive) through this region is identical except that the *ter* site is oriented in the opposite direction. *kan^r*, Kanamycin resistance.

near the *ter* sequence, nascent DNA from reaction mixtures utilizing pTH101 as a template DNA should be about 1650 nucleotides in length, whereas the product from pTH201 templates should be full-length (roughly 4100 nucleotides) (Fig. 4).

Denaturing agarose gel electrophoresis of the reaction products (Fig. 5 Upper) indicated that this was the case. Essentially all of the nascent DNA produced with pTH101 DNA in the presence of the Tus protein was less than unit length, migrating as a sharp band with a size of about 1650

FIG. 5. Analysis of Tus protein-arrested nascent DNA. Standard DNA replication reaction mixtures containing [³²P]dATP, Tus protein (at a 50-fold excess over template), and either pTH101 (active *ter*) or pTH201 (inactive *ter*) DNA as a template (70 fmol) were incubated for 20 min at 30°C. DNA products were digested with the indicated restriction endonucleases (Res. Enz.). Portions of these samples were then electrophoresed either through a 1% alkaline agarose gel (A) at 2 V/cm for 16 hr with 30 mM NaOH/2 mM EDTA as the running buffer or through a 5% polyacrylamide (30:1) gel containing 50% urea (B) at 30 V/cm for 1 hr with 40 mM Tris/50 mM borate/1 mM EDTA, pH 8.3, as the running buffer. Size markers were 5'-end-labeled *Hae* III and *Hae* II restriction endonuclease digests of ØX174 replicative form DNA. nt, Nucleotides.

nucleotides. Digestion of the reaction products prior to gel electrophoresis with the *Sma* I, *Cla* I, or *Xho* I restriction endonuclease, all of which cleave the template DNA only once between the origin of DNA replication and the *ter* sequence (Fig. 4), resulted in a decrease in size of the nascent DNA consonant with a product spanning that region of the template. In contrast, the product formed with template DNA containing the inactive *ter* sequence was, even in the presence of this high concentration of Tus protein, full-length under all conditions examined. The faint bands that are visible represent origin-to-restriction site fragments produced from full-length DNA products that contain a nick at the origin.

Denaturing polyacrylamide gel electrophoresis was used to examine the region of nascent DNA from the restriction enzyme cleavage point to the actual site of termination (Fig. 5 Lower). The *Sma* I, *Cla* I, and *Xho* I cleavage sites are approximately 450, 270, and 180 nucleotides upstream of the *ter* sequence in pTH101 DNA. DNA fragments of the expected sizes were observed after denaturing polyacrylamide gel electrophoresis of the digested DNA products (Fig. 5 Lower), indicating that Tus protein-catalyzed inhibition of replication fork progression was occurring at or very near the *ter* sequence.

The smaller bands that are apparent in the arrested products are presumably the end products of lagging-strand DNA synthesis, since they are 50–100 bases shorter than the leading-strand DNA, and it is expected that the length of the lagging-strand DNA fragments will depend upon the placement of the last primer for Okazaki fragment synthesis.

Mapping the Arrest Site of Nascent DNA Synthesis. The exact site of replication arrest was determined. After replication of the *ter*-active plasmid DNA (pTH101) in the presence of a 50-fold excess of the Tus protein, the DNA was digested with the *Xho* I restriction endonuclease, and the products of the reaction were separated on a polyacrylamide/urea sequencing gel (Fig. 6 Upper). To determine the site where replication was arrested, a primer was synthesized that had the exact sequence of the 5' end of the *Xho* I-cut nascent leading strand. This primer was then elongated in the presence of dideoxynucleoside triphosphates to produce a sequencing ladder that had the identical composition and 5' end as the newly synthesized leading strand.

The products of two different DNA replication reactions were compared to distinguish between nascent leading and lagging strands. In the absence of added primase, only leading-strand DNA synthesis is observed, while in the presence of primase, both nascent strands are manifest (13). Thus, lagging-strand DNA products can be identified in Fig. 6 as those present only in the presence of primase, whereas leading-strand DNA products are present both in the absence and in the presence of primase.

The strongest leading-strand arrest site occurs in the presence of primase at the second A residue of the 23-base-pair consensus *ter* sequence (Fig. 6). Two other major leading-strand arrest sites occur one and seven nucleotides upstream of the strongest site. Thus, the DNA polymerase III holoenzyme is capable of approaching very close to the bound Tus protein before DNA synthesis is arrested.

Comparison of the DNA products formed in the presence and absence of primase allows the identification of the lagging-strand DNA products. It is likely that these DNA products represent the distance between the 5' end of the last primers made to initiate the synthesis of Okazaki fragments and the *Xho* I recognition site. These primers should be elongated by the DNA polymerase III holoenzyme back beyond the cleavage site even when DNA replication fork progression has been halted. If this were the case, then the synthesis of the last Okazaki fragments would be initiated between 50 and 100 nucleotides upstream of the termination

FIG. 6. Nucleotide resolution mapping of the site of Tus protein-catalyzed arrest of DNA replication. (A) Products of DNA replication with pTH101 (*ter* active) DNA as a template (70 fmol) in the presence of a 50-fold molar excess of Tus protein and in the presence or absence of primase were analyzed as described. (B) The sequence of the region around the *ter* site showing the positions of major DNA replication arrest sites. Arrowheads indicate the primary arrest sites of leading-strand DNA synthesis. Circles indicate the position of bands that are apparent only when primase is present in the reaction.

site of the leading-strand DNA. This presumably reflects the spatial organization of the proteins at the replication fork.

It is also noteworthy that the distribution of arrest sites of the nascent leading-strand DNA is altered in the absence of primase. The strong arrest point at the second A of the consensus *ter* sequence is diminished considerably, indicating that alteration of the composition and/or organization of the proteins on the lagging-strand DNA template affects the ability of the Tus protein to arrest moving replication forks.

Product analysis of the arrested nascent DNA showed that in this system the action of the Tus protein was absolute; all nascent DNA was arrested when the *ter* sequence was oriented in the proper direction, whereas no inhibition of DNA replication occurred when the *ter* sequence was in the opposite orientation. Presumably, the clarity of this effect was because of the use of a unidirectionally replicating DNA, such as those carrying ColE1-type origins of DNA replication.

Nucleotide resolution mapping of the sites of arrested nascent DNA showed that the replication fork synthetic machinery was capable of approaching very near to the bound Tus protein. This is interesting in view of the fact that the replication machinery on either strand is likely to be quite

large. The leading-strand side of the fork should at least be occupied by half of the dimeric DNA polymerase III holoenzyme (22, 23), which has a molecular mass in the range of 500 kDa. The nature of the protein components composing the primosome on the lagging-strand side is more problematical, although a current model (18) suggests that an almost equivalent mass would be present. The disposition of the lagging-strand half of the dimeric DNA polymerase III holoenzyme is even more uncertain.

The first protein component of the replication fork machinery to contact the bound Tus protein is most likely the DNA B protein, the replicative DNA helicase (24, 25). The polar nature of Tus protein-catalyzed termination is presumably reflected in the interaction of one face of the protein with one of the components of the DNA replication fork synthetic machinery. Since the Tus protein binds both strands of the DNA (10), it is likely that the protein itself is asymmetric and oriented directionally along the DNA so that when bound to the inactive *ter* sequence, it is displaced before it can interact productively with the DNA replication fork components.

DISCUSSION

Two different elements have been identified that are required for arrest of DNA replication in *E. coli*: the consensus *ter* sequence 5'-AATTATGTATGTTGTAAGTAAANT-3', which is located at several different sites in the *E. coli* chromosome and in plasmids such as R6K and R100 (3-5), and the trans-acting gene *tus* (6), which has been shown to encode a DNA-binding protein (7, 9, 10). It is not known whether other factors participate in the termination process.

Purification of Tus protein, with binding to a double-stranded oligomer comprised of the T2 *ter* sequence as an assay, resulted in a preparation that was >95% homogeneous for a 36-kDa polypeptide, in agreement with the predicted size of the *tus*-encoded protein (7). If 100% of the protein were active and bound to the DNA in a monomeric form, the theoretical maximum specific activity would be 2.8×10^7 units/mg. The purified Tus protein described here has a specific activity 80% of this value. The results presented here demonstrate that this preparation of the Tus protein was sufficient and necessary to arrest DNA replication in a DNA replication system *in vitro*.

The action of the Tus protein was examined in a DNA replication system reconstituted with purified proteins and capable of supporting the replication of plasmid DNAs carrying ColE1-type origins of DNA replication. Purified Tus protein acted to prohibit the orderly progression of the DNA replication fork. This observation mimicked those made *in vivo* in that arrest of DNA replication occurred at a *ter* sequence in an orientation-dependent manner. Tus protein-catalyzed termination could be observed at a molar ratio of Tus protein to template DNA as low as 1:1 and was essentially complete at a ratio of 10:1.

The variation in the presence and absence of the primase of the distribution of nascent leading-strand arrest sites demonstrated here is also interesting because the association of primase with the replication fork components occurs via the DNA B protein (26). Thus, the primase may be competing with the Tus protein for a site on the DNA B protein. Alternatively, the presence of the primase may cause some overall change in the organization of the DNA replication fork components, thereby altering the way in which one (or many) of these components interact with the Tus protein.

It seems likely that the position of the terminal Okazaki fragments reflects primers synthesized as the replication fork

approached the bound Tus protein. Presumably, once productive contact is made between the Tus protein and its active partner, the majority of the replication fork components dissociate. Under these conditions, the exposed single-stranded lagging-strand DNA template would be coated with the single-stranded DNA binding protein, preventing further access of either the primase or any other of the primosomal components. Thus, the location of the primers for the last Okazaki fragments synthesized probably reflects both the spatial organization of the synthetic machinery at the replication fork and the site specificity of the primase.

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