THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 267, No. 13, Issue of May 5, pp. 8778-8784, 1992
Printed in U.S.A.
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Features of Replication Fork Blockage by the *Escherichia coli* Terminus-binding Protein*

(Received for publication, July 15, 1991)

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Blockage of the progress of a DNA replication fork in *Escherichia coli* can be ascribed to an inhibition of helicase action at the orientation-specific binding of a termination sequence (*ter*) by the *ter*-binding protein (Lee, E. H., Kornberg, A., Hidaka, M., Kobayashi, T., and Horiuchi, T. (1989) *Proc. Natl. Acad. Sci. U. S. A.* 86, 9104–9108). These observations have been extended to include the PriA helicase, thus confirming that blockage is general for helicases. The site of arrest of synthesis by a replication fork is at the very first nucleotide of the 22-base pair *E. coli-terB* sequence. Strand displacement by DNA polymerases is also inhibited, but is less profound and is orientation-specific. The *ter* sequences of plasmids R1-ter*R* and -ter*L* and of plasmids R6K and R100 have been compared with those of *E. coli-terA* and -*terB*.

In the replication of the circular *Escherichia coli* chromosome, two forks move bidirectionally from the unique origin (*oriC*) and terminate in a region that contains five terminus (*ter*) sites that are inverted repeats of a 22-bp[1] sequence (1, 2). Binding of *ter* by the *E. coli* terminus-binding protein (TBP), encoded by the *tau* (or *tus*) gene, is essential (3–5). One orientation of the bound sequences (*terB* and *terC*) blocks the clockwise (CW)-moving fork, whereas the other sequences (*terA, terD,* and *terE*) block the counterclockwise (CCW) fork (2).

Homologous functional and structural *ter* sites have also been demonstrated in *E. coli* plasmids R6K (6, 7), R1 (7, 8), and R100 (8) and in *Bacillus subtilis* (9, 10). Orientation-specific blockage of the replication forks observed *in vivo* (3, 4, 7, 8) has been observed with a reconstituted purified enzyme system for replication of the minichromosomal *oriC* plasmid (11). The component in the system most immediately affected is the DnaB helicase (12, 13). However, the obstruction to helicase action appears to be general inasmuch as two other helicases are also blocked in an orientation-specific manner (11).

This study was undertaken to explore other features of the TBP-*ter* complex (its capacity to block the action of the PriA helicase and strand displacement by DNA polymerases), to determine precisely the location of the blockage in or near the bound *ter* sequence, and to compare the relative blocking strengths of the various *ter* sequences.

* This work was supported by Grants GM07581 and AG02908 from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] The abbreviations used are: bp, base pair(s); TBP, terminus-binding protein; CW, clockwise; CCW, counterclockwise; SDS, sodium dodecyl sulfate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; kb, kilobase(s).

EXPERIMENTAL PROCEDURES

Strains, Enzymes, and Other Reagents—*E. coli* strains used were DH5α (*F⁻*, *ϕ80lacZΔ115, endA1, recA1, hrs⁻, hsm⁺, supE44, thi-1, gyrA*, λ-, Δ(*lacZYA-ArgF*), *U169*) and JM83 (*ara*, Δ(*lac-proAB*), *rpsL*, *ϕ80lacZΔ115*). Restriction endonucleases were *Acc*I, *Cla*I, *Dra*I, *Eco*RV, *Nae*I, *Pst*I, *Sma*I, *Sfi*I⁻, *Sac*I, *Sac*II, and *Sna*BI; and other enzymes used were T4 DNA ligase, T4 DNA polymerase, T4 polynucleotide kinase, and DNA polymerase I (large fragment), all from New England BioLabs, Inc. Calf intestine alkaline phosphatase was from Boehringer Mannheim. T7 DNA polymerase was from U. S. Biochemical Corp. T5 DNA polymerase was a gift from Dr. R. K. Fujimura (Oak Ridge National Laboratory). The enzymes used in the *oriC* reconstitution assay were as described (11, 12, 14). Other reagents were unlabeled deoxynucleoside and ribonucleoside triphosphates (Pharmacia LKB Biotechnology Inc.(, [γ-^{32}P]ATP (6000 Ci/mmol, 1 Ci = 38 GBq) and [α-^{32}P]dTTP (800 Ci/mmol) (Amersham Corp.), and bovine serum albumin (Pentex). TBP buffer contained 550 mM NaCl, 50 mM Tris-HCl (pH 7.5), 20% (v/v) glycerol, 1 mM EDTA, and 1 mM dithiothreitol.

Plasmids, Phage DNA, and Synthetic Oligonucleotides—Plasmids pTZ18R and pTZ19R were from Pharmacia. pTB101 contains a 678-bp *Hinc*II-*Pst*I fragment spanning *oriC* (positions −189 to +489) of pCM959 (15) cloned in the pBluescript vector (Stratagene). Plasmid pKHG300 (obtained from Dr. Horiuchi, Kyushu University, Fukuoka, Japan) is a pUC9-based vector carrying the *tau* gene and overproduces TBP >2000-fold (3). M13mp18 and M13mp19 replicative form DNAs were from New England BioLabs, Inc. M13mp18·*ter* CCW and M13mp19·*ter* CW DNAs were as described (11). Synthetic oligomers were synthesized by Alan Smith in the Protein and Nucleic Acid Facility at Stanford University.

In Vitro oriC Reconstitution Assay: Coupled and Staged—The *coupled* reconstituted *oriC* DNA replication reaction was essentially as described (11). In brief, the reaction mixture (40 μl) contained 30 mM Hepes/KOH (pH 7.6), 8 mM magnesium acetate, 2 mM ATP, template DNA (600 pmol as nucleotide), 0.3 mM each CTP, GTP, and UTP, 0.1 mM each dATP, dCTP, dGTP, and dTTP, 16 nM [α-^{32}P]TTP (100–300 cpm/pmol), 150 mM potassium glutamate, 80 ng of DnaA, 900 ng of *E. coli* single-stranded DNA-binding protein, 8 ng of DnaB, 27 ng of DnaC, 50 ng of gyrase A, 30 ng of gyrase B, 8 ng of HU protein, 17 ng of primase, 100 ng of DNA polymerase III holoenzyme, and 26 ng of the β-subunit of DNA polymerase III holoenzyme. After incubation at 30 °C for 30 min, reactions were stopped by adding 100 μl of 100 mM pyrophosphate containing 20 μg/ml carrier DNA (calf thymus). The extent of DNA synthesis was measured by liquid scintillation counting after precipitation with 10% (w/v) trichloroacetic acid and filtration on Whatman GF/C glass filters.

Progress of bidirectional DNA replication was assayed in a *staged reaction.* The prepriming complex was formed in 20 μl containing 40 mM Hepes/KOH (pH 7.6), 15% (w/v) glycerol, 200 μg/ml bovine serum albumin, 0.007% Brij 58, 0.25 mM EDTA, 200 mM potassium glutamate, 3.3 mM ATP, template DNA (600 pmol as nucleotide), 8 ng of HU protein, 8 ng of DnaB, 27 ng of DnaC, and 80 ng of DnaA. After 20 min at 37 °C followed by 2 min at 18 °C, 20 ng of TBP (or TBP buffer as a control) was added and incubated for 1 min. The omitted replication components (primase, gyrases A, and B, polymerase III holoenzyme, β-subunits, *E. coli* single-stranded DNA-binding protein, GTP, CTP, UTP, dATP, dCTP, dGTP, dTTP, and [α-^{32}P]dTTP), in a volume of 10 μl that also contained magnesium acetate at 10 mM and potassium glutamate at 200 mM, were added. Incubation was continued for 2, 4, and 6 min; and the reactions were stopped by adding EDTA to 20 mM. A small portion of each sample

was used to determine DNA synthesis as described above. The remainders of the samples were extracted twice with phenol and twice with chloroform and isoamyl alcohol, (24:1) and precipitated with ethanol. The DNA pellets were redissolved in buffer containing 10 mM Tris-HCl (pH 8.0), 0.2 mM EDTA and digested with the desired restriction endonucleases following the manufacturer's instructions. One unit of DNA replication activity promotes the incorporation of 1 pmol of nucleotide/min at 30 °C.

Purification of TBP—All operations were at 4 °C. *E. coli* strain JM83 (pKHG300) (3) was grown in 1.5 liters of L-broth to optical density ($A_{600\,nm}$) of 0.5, harvested by centrifugation in a Beckman JA-10 rotor for 10 min at 6000 rpm, and frozen in liquid nitrogen; frozen cells were resuspended in 20 ml of lysis buffer containing 50 mM Tris-HCl (pH 7.5), 10% sucrose, 5 mM dithiothreitol, 10 mM EDTA, 100 mM NaCl, 20 mM spermidine HCl (pH 7.5), $(NH_4)_2SO_4$ to 5% saturation, and 4 mg of lysozyme and were incubated on ice for 45 min. Cells were incubated at 37 °C for 5 min (inverting tubes once every minute), incubated on ice for 10 min, and centrifuged in a Sorvall SS-34 rotor for 1 h at 14,000 rpm (Fraction I, 20 ml). Ammonium sulfate (0.313 g/ml) was added during a 30-min period to Fraction I; stirring was continued for another 30 min. The precipitate was collected by centrifugation in a Sorvall SS-34 rotor for 20 min at 18,000 rpm and resuspended in 4 ml of buffer A (50 mM Tris-HCl (pH 7.5), 20 mM NaCl, 20% glycerol, 1 mM dithiothreitol, and 1 mM EDTA) (Fraction II, 4.2 ml). Fraction II was dialyzed against buffer A overnight and applied at 7.2 ml/h to a 13-ml column (2.6 cm high, 2.5-cm diameter) of DEAE-Sephacel equilibrated with buffer A. Flow-through fractions that contained TBP were collected (Fraction III, 21.8 ml). Fraction III was applied at 7.2 ml/h to a 15-ml heparin-agarose column (3.0 cm high, 2.5-cm diameter) equilibrated with buffer A. The column was washed with 30 ml of buffer A plus 280 mM NaCl prior to elution using a linear gradient of 280–980 mM NaCl in buffer A (45 ml). The peak of TBP activity collected in 12-ml fractions eluted between 500 and 700 mM NaCl. Protein concentrations were measured by the Bradford assay (28). One unit of TBP causes a 50% inhibition of replication of the *oriC-ter* CW·CCW plasmid (Fig. 1c) *in vivo* in 1 min at 30 °C. The purification data are shown in Table I.

Purified TBP is near homogeneity (>95%) as judged from Coomassie Blue staining of an SDS-polyacrylamide electrophoresis gel (16) and binds to the *ter* site in a 1:1 molar stoichiometry ratio when assayed by *oriC* reconstitution *in vitro*. Purified TBP does not inhibit DNA replication of *oriC* plasmids lacking *ter* sites even at a 30-fold molar excess.

Preparation of DNA Polymerase Substrates—A 64-mer oligonucleotide (40 ng) containing the complementary *ter* sequence was 5′-end-labeled with ^{32}P using T4 polynucleotide kinase (17) and mixed with M13mp18·*ter* CCW or M13mp19·*ter* CW single-stranded DNA (10 μg) in 100 μl of annealing buffer containing 10 mM Tris-HCl (pH 8.0), 100 mM NaCl, and 1 mM EDTA. The mixtures were heated at 100 °C for 2 min, cooled slowly to 50 °C, and incubated for 1 h and then cooled slowly to room temperature. The template, with the oligomer annealed to it, was purified by a Bio-Gel A-5 spun column (17), extracted with phenol and chloroform, and precipitated with ethanol. Annealing of the substrates was confirmed by electrophoresis on a 0.8% agarose gel followed by autoradiography. Similarly, a 30-mer (or 28-mer) oligonucleotide containing the *ter* sequence was annealed to M13mp18·*ter* CCW (or M13mp19·*ter* CW) DNA as described above.

Assay for Strand Displacement of DNA Polymerases—The reaction mixture (30 μl), containing 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 0.1 μg of DNA substrate (with annealed 5′-^{32}P-end-labeled *ter* oligomer, 6000 cpm), and 0.2 μg of M13 sequencing primer (17-mer), was incubated at 37 °C for 30 min and cooled to room temperature. The reaction volume was brought to 100 μl by adding 50 mM Tris-HCl (pH 7.5), 10 mM $MgSO_4$, 1 mM dithiothreitol, 50 μg/ml bovine serum albumin, and 250 μM each dATP, dCTP, dTTP, and dGTP. Samples were divided into two portions. To one was added 30 ng of TBP; to the other was added TBP buffer as a control. After incubation at 30 °C for 1 min, DNA polymerase was added; and at indicated time points, portions of each sample were transferred to an equal volume of "stop" solution containing 60 mM EDTA, 1% SDS, 20% glycerol, 0.02% bromphenol blue, and 0.02% xylene cyanol. Samples were electrophoresed on a nondenaturing 7% polyacrylamide gel, dried, and autoradiographed. Slices of the dried gel that contained undisplaced (substrate) and displaced (oligomer) bands were cut out; their radioactivity was measured in a liquid scintillation counter; and the percentage of displaced oligonucleotide was calculated. The DNA polymerases used were DNA polymerase I (large fragment), (5 units), T5 DNA polymerase (4.4 units), and T7 DNA polymerase (2 units).

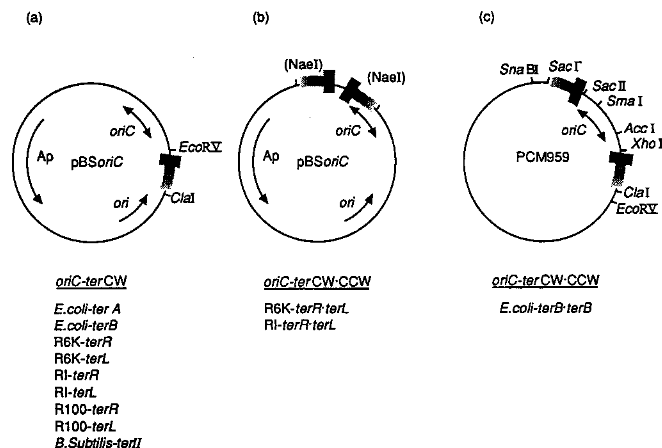

FIG. 1. **Construction of *oriC-ter* plasmids.** Pairs of complementary synthetic oligomers were annealed to generate *ter* duplexes with cohesive ends for *Cla*I and *Eco*RV restriction endonucleases. (*i*) *E. coli-terA*, 5′-ATCAATTAGTATGTTGTAACTAAAGTAT-3′ (28 mer) and 5′-CGATACTTTAGTTACAACATACTAATTGAT-3′ (30-mer); (*ii*) R100-*terR*, 5′-ATCATTATGAATGTTGTAACTACTTCAT-3′ (28-mer) and 5′-CGATGAAGTAGTTACAACATTCATAATGAT-3′ (30-mer); (*iii*) R100-*terL*, 5′-ATCTGTCTGAGTGTTGTAACTAAAGCAT-3′ (28-mer) and 5′-CGATGCTTTAGTTACAACACTCAGACAGAT-3′ (30-mer); and (*iv*) *B. subtilis-terII*, 5′-ATCATTGAATATTTAGTACATAGTGTAT-3′ (28-mer) and 5′-CGATACACTATGTACTAAATATTCAATGAT-3′ (30-mer). To generate a unique *Cla*I restriction site in pTB101 DNA (15), the additional *Cla*I site (at nucleotide 764) was inactivated by T4 DNA polymerase treatment after partial digestion of the plasmid DNA with *Cla*I. *a*, the *ter* sequences listed were inserted into a plasmid vector cleaved with *Cla*I and *Eco*RV restriction enzymes to generate the *oriC-ter* CW plasmids. Other *oriC-ter* plasmids were constructed by inserting the *ter* DNA fragments into pTB101 DNA; *E. coli-terB*, 204-bp *Acc*I-*Pst*I DNA fragment of the *oriC-ter* CW plasmid (11); R6K-*terR*, 74-bp *Alu*I-*Hae*III DNA fragment of pUC-R6K-*ter* (26); R6K-*terL*, 142-bp *Alu*I-*Hae*III DNA fragment of pUC-R6K-*ter*; R1-*terR*, 195-bp *Dra*I-*Sfi*I DNA fragment of pHM6050 (27); and R1-*terL*, 105-bp *Sfi*I DNA fragment of pHM6050 (27). The above DNA fragments containing *ter* sites were blunt-ended with T4 DNA polymerase and inserted into the *Eco*RV site of pTB101 DNA to generate *oriC-ter* CW plasmids. *b*, the R6K-*ter* sites (*terR* and *terL*) containing the 216-bp *Alu*I DNA fragment of the pUC-R6K-*ter* plasmid (26) and the R1-*ter* sites (*terR* and *terL*) containing the 554-bp *Dra*I-*Eco*RV DNA fragment of pHM6050 (27) were isolated, blunt-ended, and inserted into the *Nae*I site of pTB101 to generate *oriC-ter* CW·CCW plasmids. *c*, the *oriC-ter* CW·CCW (*E. coli-terB-terB*) plasmid was constructed by inserting the synthetic *ter* oligomers used for construction of *oriC-ter* CCW plasmids into the *oriC-ter* CW plasmid (11) that was cleaved with *Sac*I and *Sac*II restriction enzymes. The *T-like symbol* represents a *ter* sequence oriented such that the head of the *T* should block the progress of the replication fork.

TABLE I
Purification of TBP

Steps	Total protein	Total activity	Specific activity	Yield
	mg	*units* × 10^{-6}	*units* × 10^{-5}/*mg*	*%*
I. Lysate[a]	210			
II. Ammonium sulfate	113	14	1.23	(100)
III. DEAE-Sephacel	11.8	11	9.3	78
IV. Heparin-agarose	0.96	8	83	57

[a] The activity of this fraction could not be determined accurately.

Construction of PriA Helicase Substrates (See Fig. 8)—The 56-mer phage φX174 primosome assembly site (*pas*) recognized by priA protein (n′ protein) was synthesized with an extension of 4 residues at the 5′-end to generate the 60-mer: 5′-CCTTGAGGTTATTAACGCCGAAGCGGTAAAAATTTTAATTTTTGCCGCTGAGGGGTTGACC-3′. This 60-mer was annealed (by a 4-bp match) and

ligated to the pair of 36-mer complementary synthetic oligomers containing the 22-bp *E. coli-terB* sequence: oligomer 1, 5′-AAGGGGCACATAATAAGTATGTTGTAACTAAAGTGT-3′; and oligomer 2, 5′-TCACACACTTTAGTTACAACATACTTAT-TATGTGCC-3′. To verify the ligation, the 96-mer was isolated by gel electrophoresis in 7 M urea, 10% polyacrylamide and then annealed with a 5′-^{32}P-end-labeled complementary *ter* 36-mer (oligomer 1) to generate the CCW *ter* duplex, the sequence of which is oriented to block the PriA helicase movement from *pas*. In a similar way, the oppositely oriented *E. coli-terB* duplex substrate was prepared from the pair of synthetic *ter* oligomers: oligomer 3, 5′-AAGGGGCACAT-CATTTAGTTACAACATACTTATTGT-3′; and oligomer 4, 5′-TCACACAATAAGTATGTTGTAACTAAAGTATGTGCC-3′. The T-like symbol (see Fig. 8) enclosing the *ter* sequence is oriented such that the head of the T blocks the progress of the replication fork.

Two-dimensional Gel Electrophoresis—In vitro replicated DNA samples (3000–20,000 cpm) were cut with *Eco*RV and *Sna*BI restriction endonucleases and electrophoresed in two dimensions as described (18). The first dimension was in 0.4% agarose for 18 h at 1 V/cm at 4 °C in buffer containing 37 mM Tris borate (pH 8.3), 1 mM EDTA; DNA size markers (*Hin*dIII-digested λ DNA) were included. The second dimension was in 1% agarose for 4 h at 8 V/cm at 4 °C with 0.3 μg/ml ethidium bromide. Gels were dried, autoradiographed, and scanned by densitometry.

SDS, Agarose, and Polyacrylamide Gel Electrophoresis—SDS-polyacrylamide gel electrophoresis was as described (16). *Hae*III and *Eco*RV restriction endonuclease digests of replicated DNA and reaction samples from strand displacement of DNA polymerase assays were brought to 0.5% SDS, 10% glycerol, 0.01% bromphenol blue, and 0.1% xylene cyanol and were loaded on 7% polyacrylamide gel (acrylamide/*N,N*′-methylenebisacrylamide ratio of 30:1). Gels were electrophoresed in buffer containing 37 mM Tris borate (pH 8.3), 1 mM EDTA for 4 h at 10 V/cm, dried, autoradiographed, and scanned by densitometry. Also, slices of dried bands were measured for their radioactivity in a liquid scintillation counter. Agarose and polyacrylamide gel electrophoresis were essentially as described (17).

DNA Sequencing—DNA sequencing was by the dideoxynucleotide sequencing method (19) using the Sequenase system (U. S. Biochemical Corp.) according to the instructions of the manufacturer. In brief, the complementary *ter* oligomers (100-fold molar excess) and sequencing primer were annealed to template substrates by boiling for 2 min and cooling to room temperature slowly; a 20-fold molar excess of TBP was added; and the reaction mixture was incubated at room temperature for 1 min. The rest of the procedure was as described by the manufacturer.

RESULTS

Effectiveness of Several ter Sequences in Inhibition of DNA Replication—Among the 17 identified *ter* sequences (7–9), the capacity to block DNA activity *in vitro* has been reported for only the *E. coli-terB* (5, 11) and R6K-*terR* (20) sequences. *oriC* plasmids containing these or seven other *ter* sequences (Fig. 1*a*) were compared at an equimolar ratio of TBP to the *ter* sequence. Those of *E. coli* and R1 were more effective than those of R6K and R100 (Table II). The dissociation constant (K_D) for *E. coli-terB* is 10^{-12} M (5×10^9 M for R6K-*terR*) (1). Possibly the 2–3-fold stronger inhibition of DNA replication by *E. coli-terB* compared with R6K-*terR* (Table II) reflects differences in their affinity for TBP. However, K_D values for the other *ter* sequences have yet to be determined. The incomplete blockage of replication can be attributed to the bidirectional mode of *oriC* replication (11). Thus, a *ter* sequence oriented to block the CW fork fails to prevent movement of the CCW fork, and vice versa.

We tested *B. subtilis terII* sequence, mindful of the fact that the *E. coli* TBP is twice the size of that from *B. subtilis* (14.5 kDa) (21). An inhibition of only 10% was observed at a concentration 30-fold higher than that of the *E. coli* TBP (data not shown).

Limits of Progress of Replication Forks in oriC-ter Plasmids—The extent of the movement of a replication fork with an *oriC-ter* plasmid, with or without TBP, was analyzed by *Hae*III and *Eco*RV restriction enzyme cleavage as previously described (11). In the absence of TBP, the fragments generated by digestion of the *oriC-ter* plasmids indicated a bidirectional mode starting at or near *oriC* (Fig. 3), a pattern similar to the symmetrical bidirectional progress for the control *oriC* plasmid, whether or not TBP was present (11). With TBP, replication of an *oriC-ter* CW plasmid was unidirectional and blocked in the CW direction; with the *oriC-ter* CCW plasmid, movement was blocked in the CCW direction (Fig. 3). In these replications, full-length DNA appears within 2 min (11, 12). Thus, there was ample time for a replication fork to proceed through a CW *ter* site from the CCW direction, and vice versa. This problem was circumvented by constructing *oriC-ter* CW·CCW plasmids (Fig. 1, *b* and *c*), which blocked replication fork movements from *oriC* in both directions.

Replication of a plasmid with *ter* sequences oriented in opposite directions on *both* sides of *oriC* was almost completely inhibited (Fig. 2). The three constructions included a plasmid with two *E. coli-terB* sequences, one with R1-*terR* and -*terL*, and one with R6K-*terR* and -*terL*, each pair so oriented as to prevent both CW and CCW movements. Replication was sharply limited by the bound *ter* sequences to the *oriC* region (Fig. 3).

TABLE II
Comparative effectiveness of several ter sequences in the inhibition of DNA replication

Plasmid	DNA synthesis, ±TBP[a]
	%
pBS-*oriC*	97
E. coli-terA	35
E. coli-terB	23
R6K-*terR*	60
R6K-*terL*	64
R1-*terR*	33
R1-*terL*	34
R100-*terR*	58
R100-*terL*	57

[a] For each plasmid, the value for DNA synthesis with TBP is compared to that without it. Values, determined after incubations of 15 and 30 min, were similar; and the average is given. All the *ter* plasmids contained the *ter* sequence in pBS-*oriC* (Fig. 1*a*). The coupled reconstitution assay (see "Experimental Procedures") contained 3 ng of TBP and 75 fmol of DNA, as plasmid. The DNA synthesis of *oriC-ter* plasmids averaged 200 and 400 pmol for 15- and 30-min incubations, respectively, in the absence of TBP.

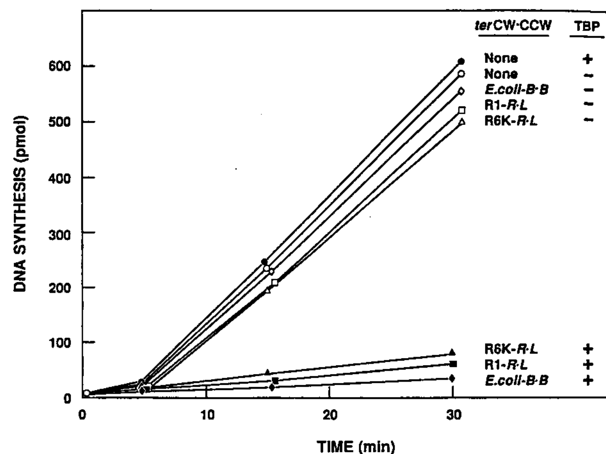

FIG. 2. **Inhibition of DNA synthesis of *oriC-ter* CW·CCW plasmids by TBP-*ter* complex.** The DNA substrate (75 fmol as circle) was used with or without TBP (30 ng). All plasmids were *oriC* with *ter* CW·CWW sequences, except where indicated (*None*). The *ter* sequences were a pair of *E. coli-terB*, a pair of R1-*terR* and -*terL*, and a pair of R6K-*terR* and -*terL*, as indicated.

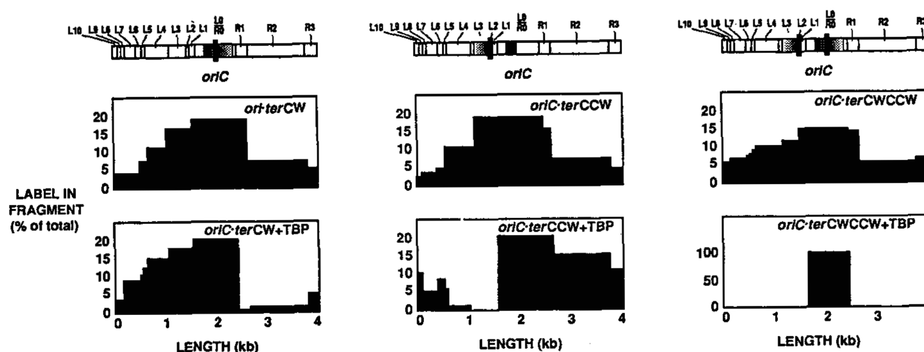

FIG. 3. **Progress of replication forks of *oriC* and *oriC-ter* plasmids.** The *oriC in vitro* reconstitution assay was staged, and the replication products were separated on 10% glycerol, 7% polyacrylamide gels and identified by autoradiography. DNA fragments were generated by *Hae*III and *Eco*RV cleavage of a 4-min DNA replication product; replication fragments *R0–R3* are in the CW order from *oriC;* fragments *L0–L10* are in the CCW order. TBP (30 ng) was in 10-fold molar excess over plasmid DNA (75 fmol as circle). The autoradiograph was scanned by densitometry, and the peaks were cut out and weighed. These intensity measurements were corrected for fragment size, and the percent of the total intensity of the lane in each fragment was calculated. The positions of minimal *oriC* and *ter* (*T-shaped area*) are shown. The fragments generated by *Hae*III and *Eco*RV digestion were as follows: *R0,* 785 bp; *R1,* 250 bp; *R2,* 926 bp; *R3,* 216 bp; *L0,* 785 bp; *L1,* 108 bp; *L2,* 9 bp; *L3,* 448 bp; *L4,* 484 bp; *L5,* 76 bp; *L6,* 85 bp; *L7,* 374 bp; *L8,* 22 bp; *L9,* 45 bp; and *L10,* 204 bp. The DNA size of *L3* is 262 bp for *oriC-ter* CCW and *oriC-ter* CW·CCW plasmids.

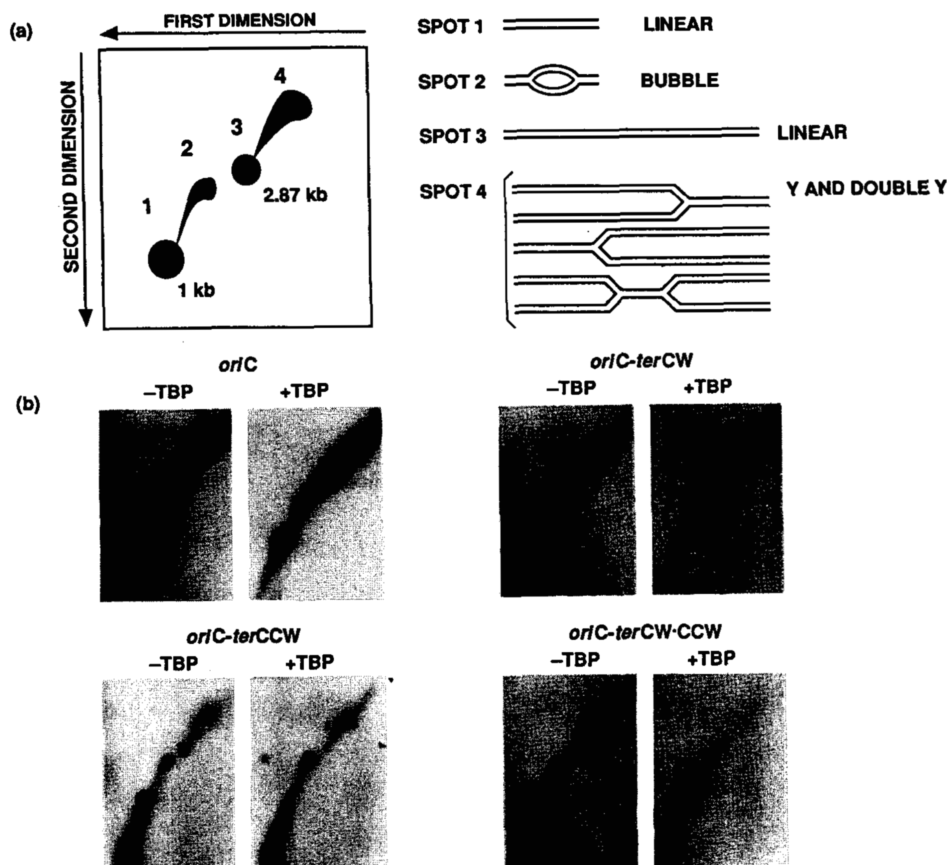

FIG. 4. **Analysis of replicated molecules by two-dimensional gel electrophoresis.** The *Eco*RV- and *Sna*BI-digested replication products were analyzed as described under "Experimental Procedures." *a,* the diagram indicates the sizes and migration patterns expected of the products (18). *Spot 1, oriC*-containing linear DNAs: 1.14 kb from *oriC,* 1.16 kb from *oriC-ter* CW, 0.92 kg from *oriC-ter* CCW, and 0.95 kb from *oriC-ter* CW·CCW; *spot 2,* replication intermediates of *spot 1* DNAs (bubble-shaped molecules); *spot 3,* 2.87-kb linear DNAs; *spot 4,* replication intermediates of *spot 3* DNAs (double-Y- and Y-shaped). *b,* two-dimensional gel profile of the replication products after 4 min of synthesis in a staged *oriC in vitro* assay. TPB, where indicated, was present in a 10-fold molar excess relative to *ter.*

Shapes of Replicated Molecules Analyzed by Two-dimensional Gel Electrophoresis—The first dimension separates molecules based on mass and the second, according to shape (18). In autoradiographs of the two-dimensional gels of *Sna*BI- and *Eco*RV-digested replication products (Fig. 4), two kinds of linear DNA molecules were observed from both the *oriC* and *oriC-ter* plasmids replicated in the absence of TBP: *oriC*-containing small linear molecules (*spot 1*) and large linear molecules (*spot 3*) (Fig. 4). In addition, *spot 2* contains bubble-shaped molecules, the replication intermediates of the small linear molecules; and *spot 4* contains the Y- and double Y-shaped molecules, the replication intermediates of the large linear molecules. In the presence of TBP, a similar pattern was obtained with the control (*oriC*) plasmid. However, with the *ter* CW and *ter* CCW plasmids, *spot 2* molecules were enriched 5–6-fold, and the *spot 1* molecules decreased drastically; with the *ter* CW·CCW (Fig. 1c) plasmid, only *spot 2* molecules were detected by autoradiography (Fig. 4). These results further demonstrate that the DNA replication forks were confined between the two *ter* sites and terminated their DNA synthesis at the *ter* sites.

Exact Location of Replication Block—Since replication forks

do not proceed beyond the *ter* site, the precise position of the blockage can be determined in a sequencing gel after cleavage with an appropriate restriction endonuclease. *Sma*I digestion of replication products of *oriC-ter* CW·CCW (Fig. 1*c*) generated a 120-bp DNA fragment (Fig. 5) that corresponds exactly to the distance from the *Sma*I site to the first nucleotide position of the *E. coli-terB* (CCW) site. A similar result was obtained from a digestion with *Acc*I (Fig. 5), which yielded a 137-bp fragment that also corresponds to the distance from the first nucleotide position of the *E. coli-terB* (CW) site to the *Acc*I site (Fig. 5). In addition to these 120- and 137-bp DNA fragments, others of 106, 463, and 476 bp were seen, presumably generated by synthesis from initiation sites in *oriC*. To confirm that the 120- and 137-bp DNA fragments are true replication-arrest bands, other restriction enzymes such as *Hin*dIII, *Bam*HI, *Aat*II, and *Bgl*II were used. For example, *Hin*dIII-*Sma*I double cleavage generated 120-bp (*Sma*I-*terB* (CCW)), 180-bp (*Hin*dIII-*terB* (CW)), 106-bp, and 290-bp fragments, all of which are readily interpreted as true DNA replication-arrest bands (data not shown). Also deduced from several restriction-enzyme analyses and comparisons of fragment sizes, two potential initiation sites were located at positions −70 and −100 downstream from *oriC* for the CW direction and 10 or more potential initiation sites within *oriC* for CCW synthesis (data not shown).

Effect of TBP-ter Complex on Strand Displacement by DNA Polymerase—Three DNA polymerases (T5, T7, and *E. coli* polymerase I (large fragment)), each lacking a 5′ → 3′ exonuclease but able to displace the strand annealed to the template (22), were examined for their ability to dissociate a *ter* sequence bound by TBP. The template-primers (Fig. 6*a*) possessed *ter* sequences oriented to block either the CCW or CW replication forks. Displacement of the annealed oligonucleotide was measured electrophoretically. In the absence of TBP, all three DNA polymerases displaced 60–85% of the annealed oligomers (Fig. 6*b*). In the presence or TBP, strand displacement by DNA polymerases was largely inhibited on both templates (CW and CCW). However, with M13mp18·*ter* CCW, in which *ter* is oriented to block fork movement, strand displacement was inhibited twice as much as with M13mp18 *ter* CW (Fig. 6*b*). The data also show the inhibition of strand displacement activity of DNA polymerases when the *ter* sequence is correctly oriented and TBP is present.

The location at which the DNA polymerase was blocked by the TBP-*ter* complex was readily determined with T7 DNA polymerase and chain-terminating dideoxynucleoside triphosphates. The blockage sites were at the fourth nucleotide from the 5′-end of *ter* CCW and at the fifth nucleotide from the 5′-end of *ter* CW (Fig. 7). Thus, this DNA polymerase penetrates several nucleotides into the complex, unlike the complete blockage of the forks observed in replication of the plasmids (Fig. 5). The greater effectiveness of a *ter* sequence against a replication fork is attributable to blockage of the DnaB helicase and the relative lack of strand-displacing activity possessed by the DNA polymerase III holoenzyme (23). Penetration of T7 DNA polymerase 3 bp from one end of the 22-bp *ter* sequence and 4 bp from the other leaves a 15-bp

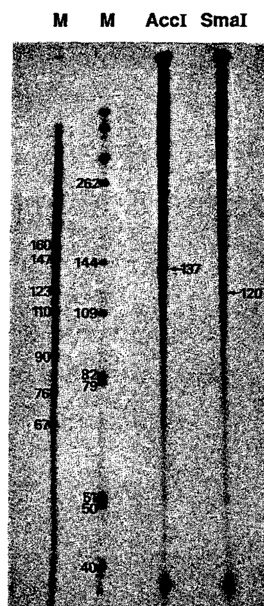

FIG. 5. **Site of arrest of DNA replication in *oriC-ter* CW·CCW plasmid.** The *oriC in vitro* reconstitution assay was staged after 6 min of DNA synthesis as described under "Experimental Procedures." The replication products of an *oriC-ter* CW·CCW plasmid (Fig. 1*c*) were digested with *Acc*I or *Sma*I and electrophoresed on a 7 M urea, 8% polyacrylamide sequencing gel (0.4 mm × 60 cm). The predominant bands observed with *Sma*I were at 120, 106, and 470 bp and with *Acc*I at 137, 453, and 465 bp. The 120-bp DNA band from *Sma*I and the 137-bp DNA band from *Acc*I were resistant to treatment with 0.05 M NaOH, but other bands were decreased slightly, suggesting that the 120- and 137-bp DNA bands are not RNA-primed DNA strands. The two lanes of size markers are indicated (*lanes M*). Essentially the same experiment was repeated six times with virtually identical results. The figure was from overexposed autoradiograph film to show all possible bands.

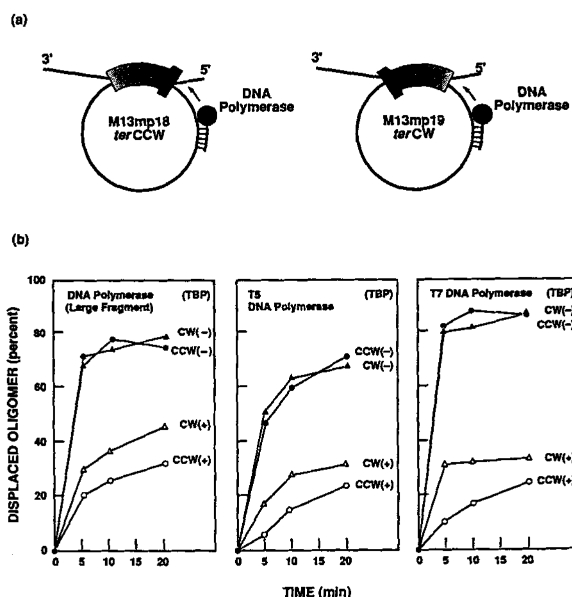

FIG. 6. **Effects of TBP-*ter* complexes on strand displacement by DNA polymerase.** *a*, the partial duplexes were constructed from M13mp18·*ter* CCW and M13mp19·*ter* CW circles as described (11) with annealed 64-mer oligomers containing the complementary *ter* sequence; a 7-nucleotide tail was present at the 5′-end of the partial duplexes and a 27-nucleotide tail was at the 3′-end. A 17-mer sequencing primer was annealed to each plasmid as described under "Experimental Procedures." *b*, The assay conditions were as described under "Experimental Procedures." The products of the DNA polymerase reactions with DNA polymerase I (large fragment, 5 units), T5 (4.4 units), and T7 (2 units) were separated by electrophoresis on a 10% glycerol, 7% polyacrylamide gel and detected by autoradiography. These amounts of the DNA polymerases were based on previous titrations of their strand displacement activity. TBP, where indicated, was present in a 20-fold molar excess over *ter*. Slices of the gel containing the displaced oligomers were measured for their radioactivity in a liquid scintillation counter. The percentage of displaced oligomer was based on the total radioactivity (displaced plus undisplaced oligomers).

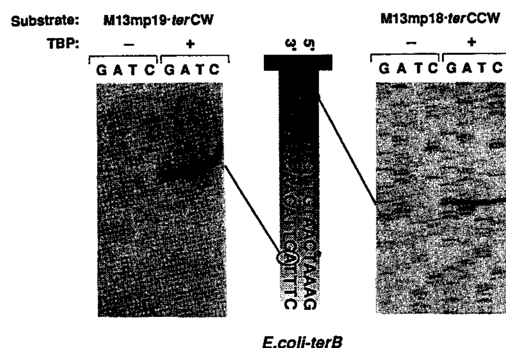

FIG. 7. **Site of arrest of replication by TBP-*ter* complex.** Using the partial duplexes with a sequencing primer (Fig. 6*a*) and T7 DNA polymerase, dideoxynucleotide sequencing (19) was performed according to the instructions of U. S. Biochemical Corp. Where indicated, TBP was present in a 20-fold excess over the *ter* sequence. The predominant termination sites are indicated. The *T-like symbol* representing the *ter* (*E. coli-terB*) sequence is oriented such that the head of the *T* should block the progress of a replication fork.

length between the two DNA polymerase termination sites protected by TBP.

Orientation-specific Blockage of PriA Helicase by TBP-ter Complex—The orientation-specific inhibition of helicases, including DnaB, Rep, and UvrD (helicase II), by a TBP-*ter* complex (11) was tested with PriA protein (24). The strand displacement activity of PriA protein was almost completely prevented by the TBP-*terB* complex and only in the orientation expected to block the $3' \rightarrow 5'$ movement characteristic of this helicase (Fig. 8). This finding makes an earlier suggestion that the blockage of DnaB helicase is due to a specific TBP-helicase interaction (20) even more unlikely.

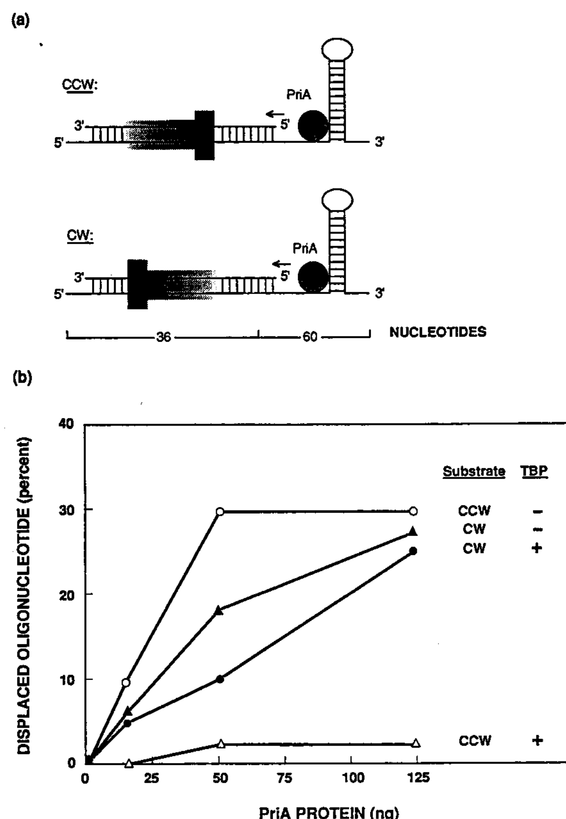

FIG. 8. **Blockage of PriA helicase by TBP-*ter* complex.** *a*, the helicase substrate, oriented to block the CCW movement of a replication fork, should prevent the action of the PriA helicase that moves in that direction; the CW substrate should offer no such impediment. *b*, assay of the PriA helicase. The reaction mixtures (20 μl) contained 20 mM Tris-HCl (pH 7.5), 9 mM $MgCl_2$, 4% (w/v) sucrose, 100 μg/ml bovine serum albumin, 1 mM ATP, 0.4 μg of *E. coli* single-stranded DNA-binding protein, 1.4 fmol of DNA substrate (linear oligomer), and 30 fmol of TBP. The amounts of PriA protein are indicated. The reaction was incubated at 30 °C for 10 min and terminated by adding 20 mM EDTA, 0.25% SDS. Samples were loaded on a nondenaturing 10% glycerol, 10% polyacrylamide gel; dried; and subjected to autoradiography. Slices of the dried gel that contained the undisplaced (substrate) and displaced (oligonucleotide) bands were cut out, and their radioactivity was measured in a liquid scintillation counter. The percentage of oligonucleotide displaced is recorded.

DISCUSSION

The termination of a replication fork by a protein-bound sequence has been observed for some circular genomes *in vivo* (3, 4, 7, 8) and with a reconstituted system of purified proteins (5, 11). The inverted repeats of a certain sequence (*ter*), when bound by TBP, block helicase actions in an orientation-specific manner (11, 20). In this study, we have further examined features of the capacity of a TBP-*ter* complex to prevent the separation of strands essential at a replication fork.

In a previous study (11), we used the *oriC* plasmid, which exploits the unique origin of the *E. coli* chromosome, and placed the *terB* sequence of *E. coli* on either side of it to block one or the other of the two replication forks. Inasmuch as the unblocked fork might come full circle, the inhibition of replication was incomplete. When oppositely oriented *ter* sequences were placed one on each side of *oriC*, both forks were stopped, and replication was confined to the minimal *oriC* region (Figs. 2 and 3). As discussed previously (11), the TBP-*ter* complex is especially effective in blocking the progress of the fork in the rolling-circle mode of replication that supervenes in the reaction. The precise mechanisms involved in this stage are unclear, as are the means by which the TBP-*ter* complex inhibits fork movement, presumably by preventing helicase action on this substrate.

The arrest of the replication fork by a TBP-*ter* complex was reflected in the migrations of replication intermediates analyzed by two-dimensional gel electrophoresis. A single bubble-shaped structure within *oriC* appeared when synthesis was blocked by complexes oriented in opposite directions on both sides of *oriC* (Fig. 4).

Blockage of the replication forks in the reconstituted *oriC* system could be attributed to inhibition of the DnaB helicase component of the system (11). However, the inhibition of two other helicases, Rep and UvrD (helicase II), by the same TBP-*ter* complex, also in an orientation-dependent way, indicated that the bound *ter* sequence (rather than specific TBP-helicase interactions) was responsible. The opposite conclusion was reached in another study (20), which reported that a TBP complex with a *ter* sequence from plasmid R6K blocked the actions of DnaB, but not those of Rep or helicase II. The suggestion that this discrepancy might be due to a tighter association of TBP with the *E. coli-terB* sequence compared with that of R6K (1) led us to examine the relative strengths of a variety of *ter* sequences. All were found to be rather similar in their capacity to inhibit a replication fork (Table II). The claim that the TBP-*ter* (R6K) complex fails to block helicase II has since been withdrawn (25). The generality of the TBP-*ter* complex inhibition has been extended to the helicase action of the T antigen of SV40 (25) and to that of the PriA helicase (Fig. 8).

The capacity of the TBP-*ter* complex to impede strand separation was demonstrable with DNA polymerases that have strand-displacing activity. The DNA polymerases of

phages T5 and T7 and the large fragment of *E. coli* polymerase I were all affected (Fig. 6), but the blockages were less severe and orientation-specific than those for the helicases.

The exact location at which a replication fork, advanced by DnaB helicase, is blocked by a TBP-*ter* complex was placed at the very first nucleotide on either side of the 22-bp *E. coli-terB* sequence (Fig. 5). In the replication of a ColE1-*ter* plasmid (5), arrest was observed, as in our studies (Fig. 5), at the very first nucleotide of the 22-bp *terB* sequence, but also at the second nucleotide and prematurely at sites 6 and 47 bp upstream. Separation of strands by a DNA polymerase was not only less orientation-dependent, but was also not as severely restricted; displacements appeared to be limited by a 15-bp region of the complex (Fig. 7). These effects need to be reconciled with a footprint of DNase I inhibition that showed that the entire 22-bp sequence was affected by TBP binding.[2] Clearly, more extensive and refined analyses of TBP binding are needed to account for the extent and particularly the orientation specificity of the binding of a *ter* sequence.

Acknowledgment—We are grateful to LeRoy L. Bertsch for his help in preparing the manuscript.

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