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Escherichia coli replication termination protein impedes the action of helicases

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ABSTRACT **Identification of the consensus sequence for termination of replication (*ter*) in *Escherichia coli* and the isolation of the *ter*-binding protein (TBP) allowed us to test their effects on replication forks initiated at the unique origin of the *E. coli* chromosome (*oriC*) in a purified enzyme system. Replication was severely impeded by *ter* in a unique orientation when purified TBP was supplied to bind it. The target for blockage within the replication complex can now be ascribed to the inability of dnaB helicase to separate the duplex strands when it encounters *ter* bound by TBP. Other helicases, such as rep and uvrD proteins, that translocate on DNA and displace strands in the direction opposite to that of dnaB protein are also blocked, but only when the TBP-bound *ter* is oriented in the other direction. From these results, we infer that the orientation of *ter* confers a particular polarity on the TBP seated on it, such that a helicase is blocked when it confronts TBP from one side, but can act, presumably by displacing TBP, when facing its other side. Thus, the intrinsic nature of the oriented TBP–*ter* complex is responsible for impeding the helicases, rather than any protein–protein interactions.**

Highly processive DNA polymerases, unlike RNA polymerases, continue synthesis on a template strand to the extent that it is available. Template signals comparable to those for termination of transcription that decelerate or block the progress of a replication fork are generally lacking or unidentified. Notable exceptions are the termination regions identified in plasmid R6K (1, 2) and the genomes of *Escherichia coli* (3–5) and *Bacillus subtilis* (6, 7). Replication forks moving bidirectionally from the unique origin of each of these genomes are impeded by sequences distantly located around the circular chromosome. Blockage depends on termination (*ter*) sequences of ≈22 base pairs (bp), present as inverted repeats (8), that are nearly identical in R6K and *E. coli* (5). A dramatic property of the inverted repeats is that one orientation blocks fork progress only in the clockwise (CW) direction and the other does so only in the counterclockwise (CCW) direction. An *E. coli* protein that specifically binds the *ter* sequences, encoded by the *tau* (*tus*) gene, is essential for impeding replication at *ter in vivo* (9–12). The purified *ter*-binding protein (TBP; ref. 13 and M.H., T.K., S. Takenaka, and T.H., unpublished results) shows the anticipated sequence-specific binding activity.

With the *ter* sequence and purified TBP available, we wished to determine how they operate in combination to impede the progress of a replication fork. In the present study, *ter* sequences placed in plasmids on either side of *oriC* (the unique origin of the *E. coli* chromosome) and bound by TBP exert their orientation-specific blockage of replication by impeding the actions of helicases.

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MATERIALS AND METHODS

Reagents. Sources were: unlabeled deoxynucleoside triphosphates and ribonucleoside triphosphates (Pharmacia LKB Biotechnology); [γ-^{32}P]ATP (6000 Ci/mmol; 1 Ci = 37 GBq) (DuPont/New England Nuclear); [α-^{32}P]dTTP (800 Ci/mmol) (Amersham); bovine serum albumin (Pentex).

Plasmids, Phage DNA, and Synthetic Oligonucleotides. pCM959 (14) is a minichromosome (4012 bp) consisting solely of *E. coli* DNA encompassing *oriC* and its flanking genes. M13mp18RF and M13mp19RF DNA were from New England Biolabs. Synthetic oligomers were made by K. Relloma (Stanford University) using a DNA synthesizer (Coder 300, DuPont).

Enzymes and Proteins. T4 polynucleotide kinase, T4 DNA ligase, and *Hae* III, *Hin*dIII, *Eco*RV, *Pst* I, *Sac* I, *Sac* II, and *Xho* I were purchased from New England Biolabs. UvrD protein was a gift from Paul Modrich (Duke University, Durham, NC). Rep protein and highly purified replication proteins were prepared as described (15, 16). TBP was overproduced 2000-fold in cells carrying a plasmid with mutations at the promoter site of the *tau* gene. The protein was purified from cell extracts to apparent homogeneity by DEAE-Sephacel and Heparin-Sepharose CL-6B chromatography (M.H. *et al.*, unpublished results).

Reconstituted *oriC* DNA Replication. The reaction mixture (40 μl) contained 30 mM Hepes·KOH (pH 7.6), 8 mM magnesium acetate, 2 mM ATP, template DNA (600 pmol of nucleotide), 0.3 mM CTP, 0.3 mM UTP, 0.3 mM GTP, 0.1 mM dGTP, 0.1 mM dCTP, 0.1 mM dATP, 0.01 mM [α-^{32}P]TTP (100–200 cpm/pmol), 150 mM potassium glutamate, 100 ng of dnaA, 700 ng of SSB, 60 ng of dnaB, 20 ng of dnaC, 50 ng of gyrase A, 10 ng of gyrase B, 8 ng of HU, 10 ng of primase, 70 ng of DNA polymerase III holoenzyme, and 75 ng of β subunit of DNA polymerase holoenzyme. After incubation at 30°C, reactions were stopped by adding EDTA to 20 mM. The extent of DNA synthesis was measured by liquid scintillation counting after precipitation with 10% (wt/vol) trichloroacetic acid and filtration onto Whatman GF/C glass-fiber filters. The progress of bidirectional replication was assayed in stages. The prepriming complex was formed in 20 μl containing 40 mM Hepes·KOH (pH 7.6), 15% (vol/vol) glycerol, bovine serum albumin (200 μg/ml), 0.007% Brij 58, 0.25 mM EDTA, 200 mM potassium glutamate, 3.3 mM ATP, template DNA (600 pmol of nucleotide), 8 ng of HU, 60 ng of dnaB, 20 ng of dnaC, and 100 ng of dnaA. After 15 min at 37°C, the temperature was shifted to 18°C for 2 min, followed by addition of the missing replication components (see above) in a final volume of 30 μl that also contained magnesium acetate at 10 mM. Incubation was continued for 2, 4, or 6 min; the reactions were stopped by adding EDTA to 20 mM. A small portion of each sample was used to determine DNA incorporation as described above.

Abbreviations: TBP, *ter*-binding protein; CW, clockwise; CCW, counterclockwise.

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Samples were extracted twice with phenol, once with phenol/chloroform (1:1), twice with chloroform, and then precipitated with ethanol. The DNA pellets were redissolved in buffer containing 10 mM Tris·HCl (pH 8.0) and 0.5 mM EDTA and digested with *Hae* III and *Eco*RV as directed by the supplier.

Agarose and Polyacrylamide Gel Electrophoresis. Essentially as described in ref. 17. Samples (10–20 μl) of the replication reaction were made up to 0.5% SDS and loaded on a 0.8% alkaline agarose gel (in 50 mM NaOH/1 mM EDTA). The gels, electrophoresed for 16 hr at 40 V, were neutralized by soaking in 1.5 M Tris·HCl (pH 7.5), dried, and subjected to autoradiography. Restriction endonuclease (*Hae* III and *Eco*RV) digests of replicated DNA were brought to 0.5% SDS and loaded on a 7% polyacrylamide gel (acrylamide/*N,N'*-methylenebisacrylamide ratio 30:1) containing 10% glycerol, 37 mM Tris borate (pH 8.3), and 1 mM EDTA. The gels, electrophoresed for 4 hr at 10 V/cm, were dried, autoradiographed, and scanned by densitometry.

Preparation of the DNA Helicase Substrates. The 244-bp *Hin*dIII–*Pst* I fragment containing the *ter* sequence was excised from *oriC ter* CW and inserted into the *Hin*dIII–*Pst* I cleaved site of M13mp18 DNA (duplex replication form) from which the viral, single-stranded circular M13mp18 *ter* CCW was generated (18). A 10-fold excess (molar ratio) of a 30-mer oligonucleotide containing the *ter* sequence [5'-end labeled with T4 polynucleotide kinase (17)] was annealed to M13mp18 *ter* CCW in 10 mM Tris·HCl (pH 8.0), 100 mM NaCl, and 1 mM EDTA at 100°C for 2 min and cooled slowly to 50°C. Incubation was continued for 1 hr at 50°C, and then samples were cooled to room temperature. The template, with the 30-mer oligonucleotide annealed to it, was purified by a Bio-Gel A-5m spun column (17), extracted with phenol and chloroform and then precipitated with ethanol. Similarly, a *Hin*dIII–*Pst* I-cleaved 244-bp fragment of *oriC ter* CW was inserted into the *Hin*dIII–*Pst* I site of M13mp19 to generate a 28-bp duplex with M13mp19 *ter* CW. The 5'-end labeling of the 28-mer oligonucleotide, annealing, and purification procedures were as above.

Assays of Helicases: rep, uvrD, and dnaB. Assays of rep and uvrD were as described (19, 20). In brief, the reaction mixture (20 μl) contained 40 mM Tris·HCl (pH 7.6), 5 mM dithiothreitol, 2.5 mM $MgCl_2$, bovine serum albumin (50 μg/ml), 2.5 mM ATP, and 0.12 pmol (as circles) of the DNA substrate. The amounts of rep, uvrD, and TBP were as indicated. The reaction was incubated at 37°C for 10 min and terminated by adding an equal volume of a solution containing 80 mM EDTA, 1% SDS, 20% glycerol, 0.02% bromophenol blue, and 0.02% xylene cyanol. Samples were loaded on a nondenaturing 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; from the radioactivity measured in a liquid scintillation counter, the percentage of displaced oligonucleotides was calculated. The dnaB helicase assay was as described (21). The mixture (20 μl) contained 40 mM Hepes·KOH (pH 7.6), 11 mM magnesium acetate, 3.4 mM ATP, bovine serum albumin (50 μg/ml), and 0.12 pmol of DNA circles. The amounts of TBP and dnaB were as indicated. The procedure was the same as for rep and uvrD.

Preparation of Fraction II. *E. coli* W3110 cell paste was lysed as described (22). The supernatant (fraction I) was precipitated with ammonium sulfate (0.26 g/ml). The centrifuged pellet was resuspended in buffer containing 20% glycerol, 50 mM Hepes·KOH (pH 7.6), 100 mM NaCl, 1 mM dithiothreitol, and 1 mM EDTA.

RESULTS

Construction of *oriC ter* Plasmids. Segments containing the 22-bp *ter* sequence were inserted in the 4-kilobase plasmid (pCM959) that contains *oriC*, the minimal 245-bp replication origin of the *E. coli* chromosome (Fig. 1). In one plasmid (*oriC ter* CW), *ter* was placed to the right of *oriC* in an orientation expected to block the replication fork progressing CW from *oriC*. In another plasmid (*oriC ter* CCW), *ter* was placed to the left of *oriC* in an orientation that should block CCW replication. The orientations were based on the polarity of the sequences observed *in vivo* (2, 5, 11). The copy numbers of the *ter* CW and *ter* CCW plasmids appeared to be ≈60% that of the *oriC* plasmid. On the other hand, plasmids in which *ter* sequences were placed on both sides of *oriC* (oriented to prevent both CW and CCW replication) were present at only 5–10% the copy number of the *oriC* plasmids. Thus the capacity for autonomous replication of an *oriC* plasmid is severely reduced when the progress of replication forks is impeded in both directions.

Replication of *oriC ter* Plasmids Is Inhibited. With the purified enzyme system, replication of the *oriC ter* plasmids, compared to that of *oriC* was only slightly less active (Fig. 2). However, upon the addition of a crude *E. coli* enzyme fraction (fraction II), the inhibition of replication reached 40% (data not shown). The factor in the crude fraction that elicits the *ter* activity could be replaced by purified TBP. Saturation for the inhibition of replication was achieved at about an equimolar ratio of TBP to *ter*.

In the replication of *oriC* plasmids, the initial products are genome-length strands; in an ensuing mode of replication, the

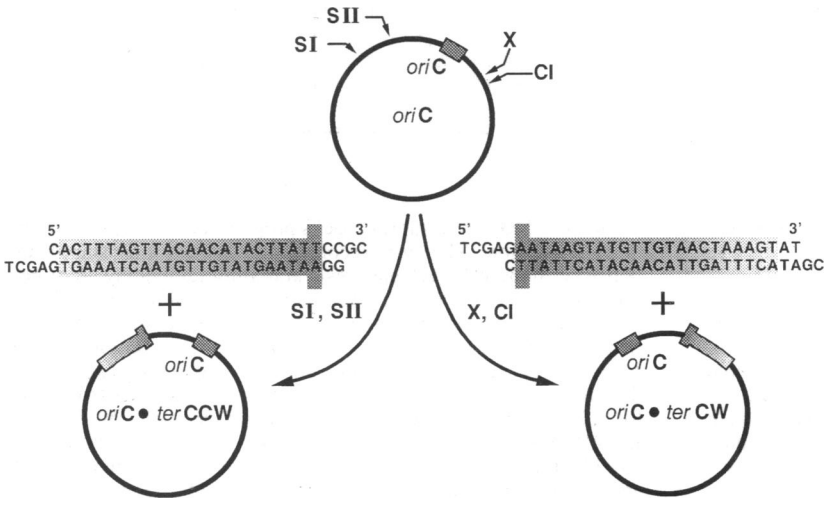

FIG. 1. Construction of *oriC ter* CCW and *oriC ter* CW plasmids. Two complementary synthetic oligomers of 30 and 28 nucleotides were annealed to generate a 26-bp duplex containing the 23-bp *ter* consensus sequence and restriction nuclease *Sac* I (SI) and *Sac* II (SII) cohesive ends. *oriC* plasmid DNA (pCM959, 4012 bp) was cleaved by *Sac* I and *Sac* II to generate a site for insertion of the synthetic duplex. In the resultant *oriC ter* CCW plasmid circle (3812 bp), the *ter* sequence is oriented to block CCW DNA replication. In a similar way, *ter* in a 26-bp sequence with cohesive ends for *Xho* I (X) and *Cla* I (Cl) was inserted into an *oriC* plasmid cleaved by the same enzymes. In the resultant *oriC ter* CW plasmid (4028 bp), the *ter* sequence is oriented to block the CW DNA replication fork. The T-like symbol representing *ter* is oriented such that the head of the T blocks the progress of the replication fork, based on the polarity observed *in vivo* (2, 5, 11). The cleavage sites (in bp) in *oriC* are: *Cla* I, 426; *Sac* I, 3633; *Sac* II, 3849; and *Xho* I, 417.

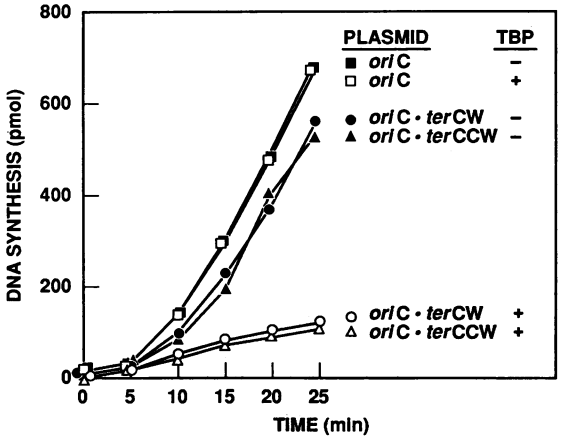

FIG. 2. TBP inhibits replication of *oriC ter* plasmids. TBP (72 ng) was added where indicated (+). −, TBP not added.

products are strands of multimer length. Analysis of the distribution of monomer- and multimer-length products by alkaline agarose gel electrophoresis showed that the inhibition of replication observed with the *ter* plasmids was largely at the presumed rolling-circle stage of product amplification (Fig. 3). The result is explicable in view of the bidirectional mode of *oriC* plasmid replication (23, 24). When the CW direction is blocked by *oriC ter* CW, a unit-length strand can still be produced in the CCW direction; similarly, blockage of the CCW direction permits a unit-length strand to be generated in the CW direction. Multimer-length strand synthesis, requiring repeated traversals of the entire genome, is the stage most affected by the TBP–*ter* complex as manifested in the total DNA synthesis.

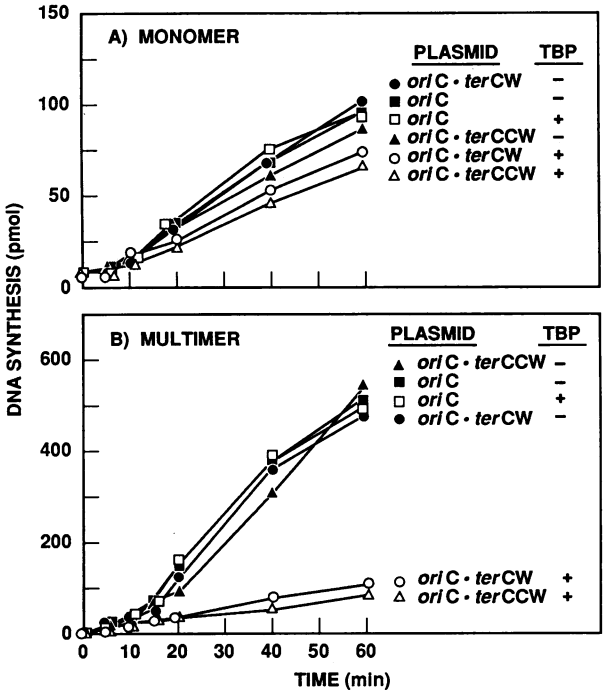

FIG. 3. Stage in *oriC ter* plasmid replication inhibited by the TBP–*ter* complex. Products of replication (reconstituted system), containing about 10 ng of TBP where indicated (+), were subjected to alkaline agarose gel electrophoresis and autoradiography. Radioactivity in slices of the dried gel that contained the monomer-length (*A*) and multimer-length (*B*) DNA bands was measured in a liquid scintillation counter. Each value represents the average of two experiments. −, TBP not added.

Replication Block Is Located at or Near the *ter* Site. The progress of replication of *oriC* and *oriC ter* CW plasmids, with or without TBP, was monitored by the sequential appearance of radioactive nucleotides in fragments of the plasmid generated by *Hae* III and *Eco*RV cleavage (Fig. 4). Based on the amount of label in each fragment (corrected for its size), as a percentage of the label in all fragments, replication of the *oriC* plasmid proceeded bidirectionally, starting at or near *oriC* as reported (23, 24); the presence of TBP had no effect on the pattern. However, the pattern of *oriC ter* CW plasmid replication differed strikingly when TBP was present. Replication was unidirectional, moving leftward (CCW) because the TBP–*ter* CW complex blocked its CW movement. The same experiment, repeated with *oriC ter* CCW, produced a pattern that was virtually the mirror image of that observed with *oriC ter* CW; replication proceeded only CW from *oriC* (data not shown).

TBP Impedes the Actions of Helicases. Progress of replication of duplex DNA requires a separation of the strands to provide the template and depends on the action of a helicase as the leading element. In the case of an *oriC* plasmid, once it is primed, replication by DNA polymerase III holoenzyme depends on the actions of dnaB protein and gyrase (24, 25); the rate of fork movement is determined by the rate of unwinding. To assess how dnaB protein and the helicases are affected by *ter*, substrates were constructed in which 30-bp

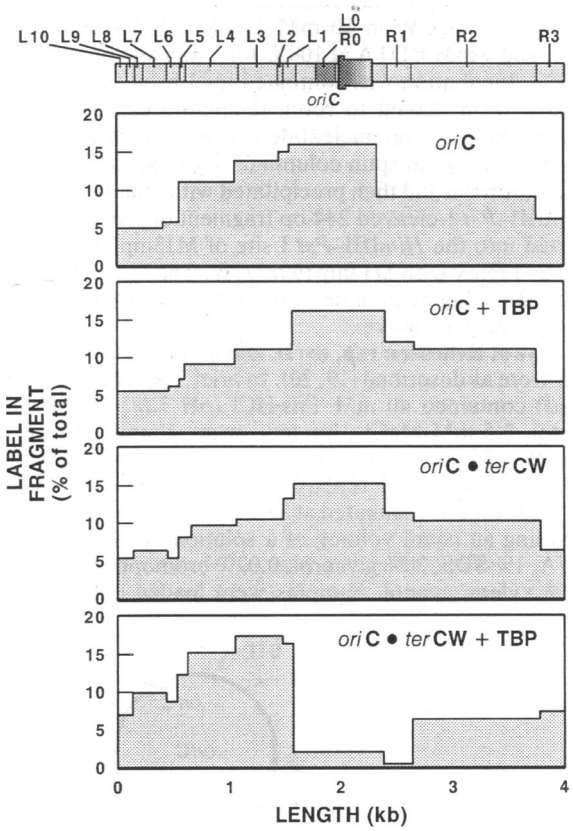

FIG. 4. TBP–*ter* complex blocks progress of the replication fork in one direction. The *oriC* reconstituted system was staged and polyacrylamide gel electrophoresis and autoradiography were performed. The autoradiogram was scanned by densitometry and each peak on the graph was cut out and weighed. The density values were corrected for fragment size. The values shown are for a 6-min replication. DNA fragments were generated by *Eco*RV and *Hae* III digestion; L0 to L10 represent fragments in CCW order from *oriC*; R0 to R3 are in CW order. Sizes (in bp): R0, 785; R1, 250; R2, 926; R3, 216; L0, 785; L1, 108; L2, 9; L3, 448; L4, 484; L5, 76; L6, 85; L7, 374; L8, 22; L9, 45; L10, 204. The positions of the minimal *oriC* and *ter* (T-shaped area in L0/R0) are shown. kb, Kilobases.

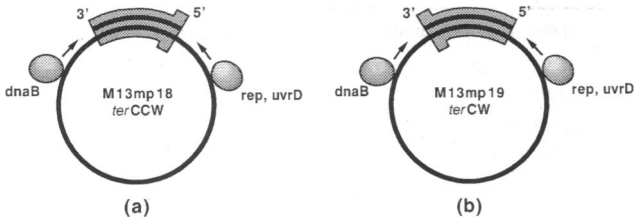

FIG. 5. Substrates for the helicases. Two substrates were constructed. (*a*) In M13mp18 *ter* CCW, the *ter* sequence (T-shaped area) is oriented to block CCW replication. (*b*) In M13mp19 *ter* CW, the *ter* sequence (T-shaped area) is oriented to block CW replication. The orientations of *ter* sequences were confirmed in each case by use of the M13 dideoxynucleotide sequencing method (26).

oligonucleotides were annealed to single-stranded circles to generate the duplex *ter* sites in either orientation (Fig. 5). Upon exposure to a helicase, displacement of the oligonucleotide was measured electrophoretically.

Three helicases were examined: dnaB protein, which translocates on a DNA strand in the 5′→3′ direction (CW) (21), and rep (19) and uvrD (20) proteins, which translocate in the 3′→5′ direction (CCW) (Fig. 5). Strand displacement by each of the helicases was observed in the absence of TBP, showing that the *ter* sequence as such offers no impediment (Figs. 6 and 7). The dnaB protein that prefers to displace a strand with an unannealed 3′ end (e.g., a "tail") (21) was still sufficiently active on this fully annealed ("tailless") oligonucleotide. In contrast, in the presence of TBP, dnaB protein failed to displace the strand specifically when *ter* was oriented to block CW replication, whereas rep and uvrD proteins were impeded in their actions by *ter* only in the orientation that blocks CCW movement (Figs. 5–7). Thus, TBP binds *ter* in both orientations, but blocks the action of a helicase only in one direction, the same polarity as that observed for blockage of a replication fork *in vivo*.

The appearance of the displaced oligonucleotide as two bands in the native gel electrophoresis (Fig. 6) in all the samples (including the heat-treated substrate) is likely due to the formation of some secondary structure or to dimerization, possibly through annealing at a potential hairpin loop in the middle of *ter*. Electrophoresis in a denaturing urea gel revealed the oligonucleotide in all samples as a single band in the position expected for its size.

DISCUSSION

In recent years, knowledge regarding the termination of replication has been enlarged by two major advances: (*i*) recognition that a defined sequence (*ter*), present as inverted repeats (opposite orientations), is responsible for termination by impeding the bidirectional replication of plasmid (1, 2), *E. coli* (5, 11), and *B. subtilis* (6) genomes; and (*ii*) isolation of TBP, the product of the *tau* or *tus* gene, which in a complex with *ter* is essential for the termination event (7, 10, 12, 13).

With a system of purified proteins that carries out the bidirectional replication of plasmids initiated at *oriC* (the unique origin of the *E. coli* chromosome) (16, 24), we have been able to show that both *ter* and TBP are necessary and sufficient for reconstitution of termination *in vitro* (Figs. 2 and 3). As expected, one orientation of *ter* impedes replication fork movement only in a CW direction (Fig. 4), whereas the other orientation inhibits it only in the CCW direction. As further confirmation, replication of plasmids with the *ter* sequence on both sides of *oriC* is nearly completely blocked (E.H.L. and A.K., unpublished results).

Inasmuch as fork movement in the reconstituted *oriC* plasmid replication system depends on the action of a helicase to separate the strands of the duplex, the behavior of

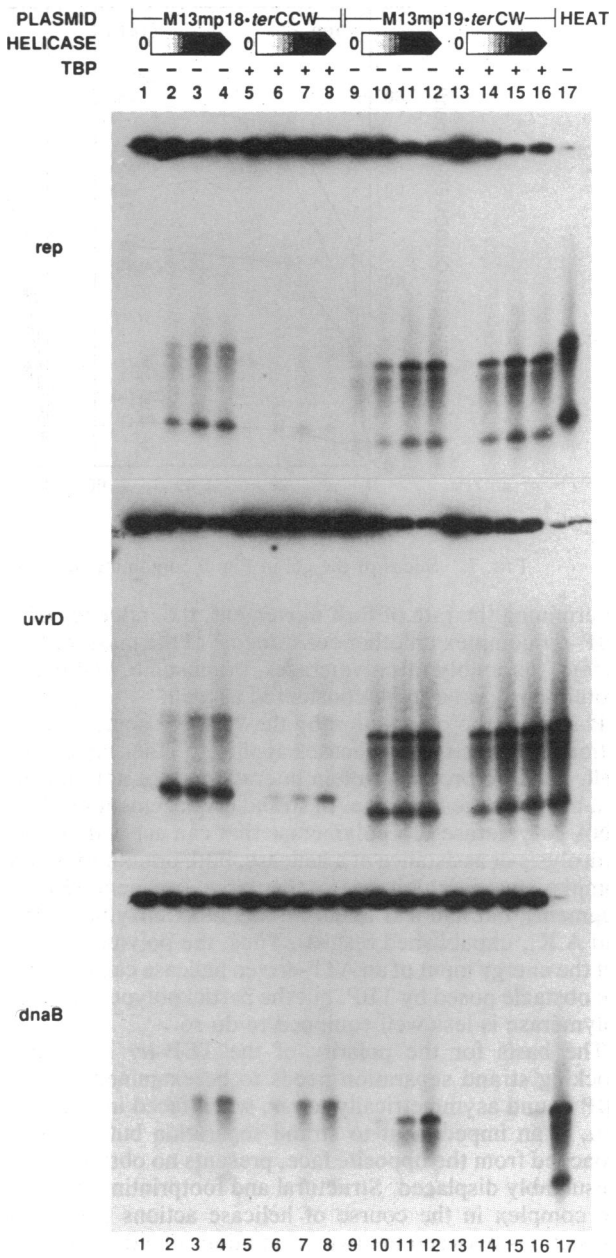

FIG. 6. TBP–*ter* complex inhibits helicases in an orientation-specific manner. The products of the reactions were separated by 10% polyacrylamide gel electrophoresis and detected by autoradiography. In lane 17 the substrate was heated at 100°C for 2 min. Helicases tested were rep (*Top*), uvrD (*Middle*), and dnaB (*Bottom*). As indicated, the helicase protein was omitted in lanes 1, 5, 9, and 13 and present in increasing amounts in lanes 2–4, 6–8, 10–12, and 14–16. The increasing amounts of protein in the three-lane groups above were 4, 17, and 53 ng for rep protein; 14, 41, and 125 ng for uvrD helicase; and 26, 150, and 272 ng for dnaB helicase. The corresponding molar ratios of helicase to substrate were 0.5, 2, and 7 for rep; 2, 5, and 14 for uvrD; and 0.7, 4, and 7 for dnaB.

helicases encountering a TBP–*ter* complex was examined (Fig. 5). Each of three helicases was strikingly impeded in the separation of strands at a *ter* site bound by TBP. The dnaB protein, which translocates on DNA in a 5′→3′ direction, was blocked by the TBP–*ter* complex only when oriented to impede CW fork movement, whereas the actions of rep and uvrD proteins, which translocate on DNA in the 3′→5′ direction, were inhibited by the TBP–*ter* complex only in the orientation that blocks CCW movement (Figs. 6 and 7). Even though one or another helicase may be a major element in

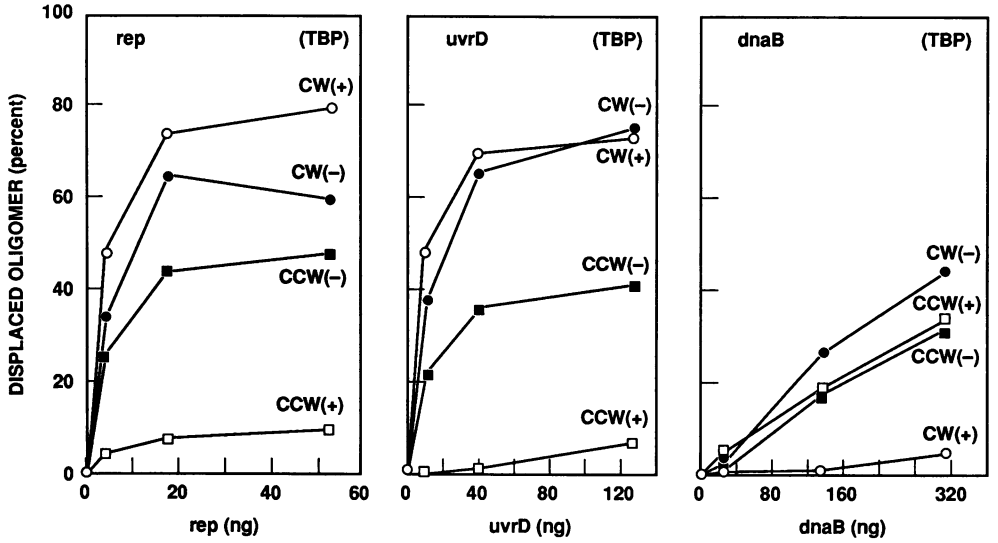

FIG. 7. Slices of the gel in Fig. 6 containing the displaced and undisplaced oligomers were analyzed for radioactivity.

determining the rate of fork movement, the influence of the TBP–*ter* complex on other constituents of the massive "replisome" assembly of polymerases, primosome, and binding proteins will have to be considered as well.

Plausibly, the obstruction by the TBP–*ter* complex to the actions of these three helicases applies to other helicases as well. Specific protein–protein interactions are not indicated as a basis for the blockage. In strand displacement by *E. coli* DNA polymerase I, a polymerase that can act without ATP hydrolysis or assistance of a helicase, both orientations of the complex were inhibitory to the large fragment (Klenow fragment), but had less effect on the intact enzyme (E.H.L. and A.K., unpublished results). Thus, the polymerase without the energy input of an ATP-driven helicase can overcome the obstacle posed by TBP, but the partial polypeptide of the polymerase is less well equipped to do so.

The basis for the polarity of the TBP–*ter* complex in blocking strand separation needs to be examined. Clearly, TBP bound asymmetrically to *ter*, when faced in one direction, is an impediment to strand separation but, when approached from the opposite face, presents no obstacle and is presumably displaced. Structural and footprinting studies of the complex in the course of helicase actions may reveal distinctions between the two orientations of TBP bound at a *ter* site. Further work is also needed to determine the degree to which the TBP–*ter* complex promotes pausing or even the dissociation of otherwise processive helicase and replication proteins.

The value of a termination mechanism to a replicon is uncertain. Most genomes (e.g., ColE1 plasmid, phage λ, and simian virus 40) lack such a mechanism and those that possess it suffer no serious disadvantage when *ter* sites are removed or the TBP is inactivated by mutation. Perhaps the topological states generated by replication forks as they approach each other or approach the origin of replication may offer difficulties, such as a susceptibility to a rolling-circle mode of replication, that are resolved by slowing the pace of strand separation at the advancing fork. The temporally regulated replication of large chromosomes may be another instance in which a termination mechanism is employed.

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