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The replication terminator protein of the Gram-positive bacterium *Bacillus subtilis* functions as a polar contrahelicase in Gram-negative *Escherichia coli*

(termination of replication/DNA–protein interaction)

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ABSTRACT **The replication terminator protein (RTP) of *Bacillus subtilis* is a dimer with a monomeric molecular mass of 14.5 kDa. The protein terminates DNA replication at a specific binding site. Although the protein has been crystallized and its crystal structure has been solved, the lack of an *in vitro* replication system in *B. subtilis* has been a serious impediment to the analysis of the mechanism of action of this protein. We have discovered that the protein is functional in the Gram-negative bacterium *Escherichia coli in vivo* and *in vitro*. RTP blocked replication forks initiated from a ColE1 replication origin at the cognate DNA-binding site (BS3) in a polar mode. The protein did not block rolling circle replication initiated from the pT181 origin in cell extracts of *Staphylococcus aureus*. RTP antagonized the helicase activity of DnaB but not that of helicase II of *E. coli*. Thus, RTP functioned as a polar contrahelicase blocking a helicase that participates in symmetric DNA replication but it did not impede rolling circle replication nor the action of a helicase involved in DNA repair.**

The initiation of DNA replication in the Gram-positive bacterium *Bacillus subtilis* occurs at a unique replication origin (1–4), and the forks, under normal physiological conditions, travel bidirectionally until meeting each other at sequence-specific replication termini located at a site diametrically opposite to that of ori (5–8). During stringent response the replication forks are transitorily arrested at two sites located ≈200 kb from and on each side of the ori (9, 10). These sites, which are responsive to stringent control, release the arrested forks under relaxed conditions to progress and terminate at the normal terminator sites. The process of termination is controlled by the binding of a homodimeric protein, of subunit molecular mass of 14.5 kDa, to two terminator sites that are present as inverted repeats (IR's) (5–7). One set of the sequence motifs constituting each IR, by itself, impedes fork movement in *B. subtilis* in a polar fashion (8). Polarity means that each IR, in one orientation with respect to a specific direction of fork movement, is able to arrest the fork whereas when located in the opposite orientation, is unable to do so (see Fig. 1). The polar block to fork movement has also been observed in *Escherichia coli*, where a 36-kDa monomeric protein interacts with specific τ sequences and the protein acts as a polar contrahelicase (11, 12) impeding DNA unwinding by DnaB helicase (13).

Although the topology of DNA replication of *B. subtilis* has been elegantly analyzed by Sueoka and coworkers (1–3) and many mutants that identify the replication genes of *B. subtilis* have been isolated and mapped (14, 15), the enzymology of DNA replication in this system has not been worked out. Hitherto, there has been no evidence for the existence of an *in vitro* replication system that faithfully initiates replication from the chromosomal ori in a cell-free environment.

Although the primary sequence of the DnaB initiator protein has been reported (16), the initiator (16) and the terminator proteins have been purified (6, 17) and the terminator has been crystallized and analyzed by x-ray diffraction and NMR spectroscopy (17), little or nothing is known about the replicative helicase, primase, and polymerases of *B. subtilis* (18).

The primary structure of the replication terminator protein (RTP) of *B. subtilis* has no detectable homology with the ter protein of *E. coli*. Two dimers of RTP interact with each cognate binding site and the cooperative interaction between the two dimers is apparently needed for termination (7, 8). In contrast, a single monomer of *E. coli* ter protein binding to a single τ sequence motif effects polarized termination of replication (19–22). The identification of the *tus* gene of *E. coli* encoding the ter protein (19) and the interaction of ter with the terminator sites (τ) has been reported (22).

The availability of crystals of RTP that diffract to high resolution (17) has resulted in the elucidation of the three-dimensional (3D) structure of the protein (D. Bussiere, D.B., and S. White, unpublished data). The availability of the 3D structure promises to help unravel the molecular mechanism of replication termination provided that (*i*) a defined *in vitro* system consisting of pure proteins is established to study the biochemistry of normal RTP and (*ii*) guided by the crystal structure, appropriate mutants of RTP are isolated that alter the biochemical properties of the protein in a predictable or revealing fashion, thus correlating structure to function.

In view of these important goals, we wanted to investigate whether RTP would terminate replication initiated from the origin of replication of ColE1 *in vitro* using cell extracts and purified enzymes of the well-studied *E. coli* system. In this paper, we report that RTP blocks replication initiated from ColE1 ori *in vitro* at the cognate BS3 sites. We also report physiological observations that are consistent with polarized termination occurring *in vivo*. We have shown that despite the lack of homology in the primary sequence of RTP and ter, RTP blocked the activity of DnaB helicase in a polar fashion. Interestingly, rolling circle replication initiated from the ori of the plasmid pT181 was not impeded by RTP. The activity of helicase II of *E. coli* that is involved in DNA repair (23) was also not impeded by RTP. Thus the ability of RTP to function as a polar replication terminator in the well-defined *E. coli* system along with its 3D structure promise to help unravel the molecular basis of its contrahelicase activity.

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Abbreviations: RTP, replication terminator protein; IR, inverted repeat; 3D, three-dimensional; 2D, two-dimensional.

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MATERIAL AND METHODS

Bacterial Strains and Plasmids. The wild-type *E. coli* strain W3110 was used in most transformation experiments; the strain JS117 (Tus Δ) was used as the source of cell extracts for *in vitro* replication experiments. Wild-type *Staphylococcus aureus* RN4220 cells were used for cell extract preparation. The plasmid pWS46 that overproduces RTP was a gift from R. G. Wake (6). The plasmids pUC18-BS3 and pUC19-BS3 contained the replication terminator site (BS3) of *B. subtilis* in the nonfunctional and functional orientations, respectively. The BS3 site was cloned between the *Eco*RI and *Hin*dIII sites of the plasmid vectors. The plasmids pSK101 and pSK112 contained the origin of replication of pT181 in addition to the terminator site (BS3) and the ColE1 ori (as shown in Fig. 3 *Upper*).

***In Vitro* DNA Replication and Analysis of Replication Intermediates by Brewer–Fangman Gels.** Cell extracts from *E. coli* and *S. aureus* were prepared as described (11, 24, 25). The *E. coli* extracts and *S. aureus* extracts (along with the RepC protein of pT181) were used to initiate replication from the ColE1 ori and the pT181 ori, respectively, as described (11, 24, 26). Various quantities of purified RTP were added to the *in vitro* system to investigate the effect on fork movement.

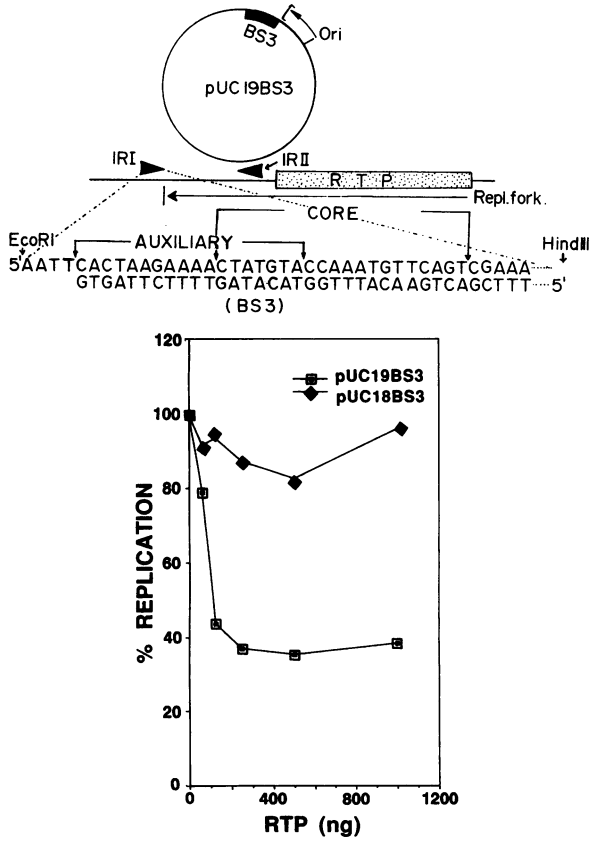

FIG. 1. Templates used in the *in vitro* replication reactions and the effect of RTP on the extent of replication. (*Upper*) The sequence of BS3 that was synthesized as an *Eco*RI–*Hin*dIII fragment was cloned in opposite orientation with respect to the ori in pUC18 and pUC19 vectors. IRI and IRII refer to the IRs with BS3 being the same as IRI. RTP, coding region of RTP. The fork moving right to left progresses past the IRII sequence and is arrested at IRI. (*Lower*) The extent of replication of the pUC18-BS3 and pUC19-BS3 templates is shown as a function of amounts of exogenously added RTP. Note that the replication of pUC19-BS3 and pUC18-BS3, which have the terminator site in the functional and nonfunctional orientation with respect to the replication origin, respectively, are inhibited by 60% and ≈10% by 100–200 ng of RTP, thus, showing polarity of the termination process *in vitro*.

The replication intermediates were purified and analyzed by Brewer–Fangman two-dimensional (2D) gels as described (11, 27).

Purification of Proteins. RTP was purified to homogeneity as described (17) from the overproducer clone pWS46 (5). Purification of DnaB of *E. coli* has been published (11). Helicase II of *E. coli* was a gift from S. Matson (University of North Carolina, Chapel Hill). RepC protein of pT181 was purified as described (26).

Helicase Assays. The binding site BS3 of RTP was cloned in two orientations at the *Eco*RI–*Hin*dIII sites of M13mp18 and M13mp19. Complementary synthetic oligonucleotides were 5′ end-labeled with [γ-^{32}P]ATP and hybridized to the single-stranded circular DNA to prepare substrates for helicase assays. The assays were performed as described (11).

RESULTS

RTP Blocks Cairns-Type Replication *in Vitro* and *in Vivo* in the *E. coli* System. We constructed hybrid replicons containing the origin of replication of ColE1 (pUC18 or pUC19 vector) and the replicator terminator site (BS3) of *B. subtilis*, which was cloned into the *Eco*RI–*Hin*dIII sites in the functional and the nonfunctional orientations (see Fig. 1 *Upper*). When these hybrid replicons were introduced into an *E. coli* strain that contained a resident plasmid expressing low levels of the RTP, we consistently observed that the pUC18-BS3 recombinant (nonfunctional orientation of the terminator site BS3) consistently transformed the bacterial cells into ampicillin resistance, and the cells produced colonies of normal size on ampicillin (100 μg/ml)-containing rich medium plates after overnight growth at 37°C. In contrast, pUC19-BS3 DNA (with the terminator site present in the functional orientation

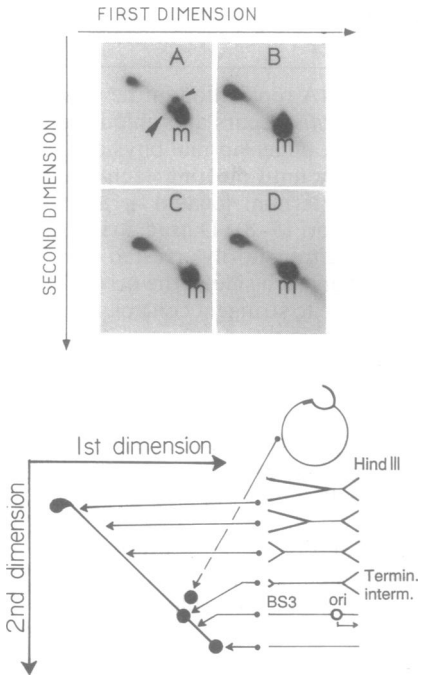

FIG. 2. Analysis of the products of *in vitro* replication shown in Fig. 1, by 20 Brewer–Fangman gel electrophoresis. (*Upper*) pUC19-BS3 replicated in the presence of a 20-fold molar excess of RTP over DNA template. A, m, monomer; longer arrowhead, termination intermediate; shorter arrowhead, "off the arc" σ-shaped DNA (see *Lower*); B, pUC19-BS3 without RTP; C, pUC18-BS3 plus 20-fold molar excess of RTP; and D, pUC18-BS3 without RTP. Note that, as expected, the twin termination spots are absent in B–D. (*Lower*) Interpretive diagram showing the expected pattern of the 2D gel analysis of an unidirectionally moving fork transiently arrested at a terminator site.

with respect to the origin) yielded a 100- to 1000-fold less number of colonies after overnight growth under identical conditions. Furthermore, most of the colonies that appeared were very small in diameter, indicative of severe retardation of growth under ampicillin selection. Extraction of plasmid DNA from equal number of cells containing pUC18-BS3 and pUC19-BS3 revealed an ≈10-fold reduction in copy number in pUC19-BS3 as compared with pUC18-BS3 (data not shown). These observations were consistent with the interpretation that RTP was functional *in vivo* in *E. coli* and retarded replication in a polar fashion in pUC19-BS3 but not in pUC18-BS3.

We wished to confirm the *in vivo* observation by replicating pUC18-BS3 and pUC19-BS3 (Fig. 1 *Upper*) templates *in vitro* in cell extracts of *E. coli* and various concentrations of exogenously added purified RTP. A typical set of results shown in Fig. 1 confirms that 200 ng of RTP inhibited the extent of replication of pUC19-BS3 by 60%, whereas that of pUC18-BS3 was inhibited by <10%. Note that the orientation of BS3 that was present in pUC19 was the one that is identical to the orientation that is also functional in *B. subtilis in vivo* (7, 8).

We sought to confirm further the polar inhibition of fork movement in *E. coli* elicited by RTP *in vitro* by analyzing the replication intermediates in Brewer–Fangman 2D gels. The pUC18-BS3 and pUC19-BS3 DNAs were replicated *in vitro* in the presence and absence of RTP and with [α-^{32}P]dATP as the labeled substrate. The pUC18-BS3 and pUC19-BS3 products were purified and cleaved with *Eco*RI and *Hin*dIII, respectively, and electrophoresed and separated by molecular mass in the first dimension and by molecular shape, in the presence of ethidium bromide, in the second dimension. The pUC19-BS3 template yielded a hook-shaped pattern with twin termination spots (Fig. 2 *Upper*, arrowheads in A) above the monomer spot (Fig. 2 *Upper*, A, m) in the presence of RTP (see interpretive diagram in Fig. 2 *Lower*). The twin termination spots were missing when RTP was withheld (Fig. 2 *Upper*, B). The "on the arc" spot (Fig. 2 *Upper*, longer arrowhead in A) represents double Y-shaped intermediates generated by arrest of the replication fork at BS3 followed by cleavage of both arms of the replication bubble at the *Hin*dIII site located just beyond the terminator site. The off the arc spot (Fig. 2 *Upper*, shorter arrowhead in A) was, we believe, generated by incomplete cleavage of the replication bubble (see Fig. 2 *Lower*). Cleavage of one of the two arms of the bubble would generate a o-shaped molecule that would put the spot off the arc (28).

These twin termination spots were missing in pUC19-BS3 (Fig. 2 *Upper*, B) and pUC18-BS3 (Fig. 2 *Upper*, D) samples in the absence of exogenously added RTP. When RTP was added to an *in vitro* reaction that included the pUC18-BS3 template, having BS3 in the nonfunctional orientation, consistent with the polarity of replication blockage by RTP, no termination spots were visible (Fig. 2 *Upper*, C). Thus, the *in vitro* replication analysis confirmed the *in vivo* observation that RTP of *B. subtilis* functions in *E. coli* and retains its polarity of action.

RTP Does Not Impede Rolling Circle Replication in Cell Extracts of *S. aureus*. The rationale behind these experiments was to examine whether RTP specifically blocked only Cairns-type replication but not rolling circle replication that occurs in certain bacterial plasmids such as pT181 of *S. aureus* (25, 26) and, presumably, also during conjugation transfer of plasmids from a donor bacterium to a recipient. We have previously shown that the rep helicase involved in ϕX174 replication is not blocked by ter of *E. coli* (11). We have recently found that helicase I, which is involved in conjugational transfer is blocked neither by ter nor by RTP (B.K.M. and D.B., unpublished data). Note that RTP of *B.*

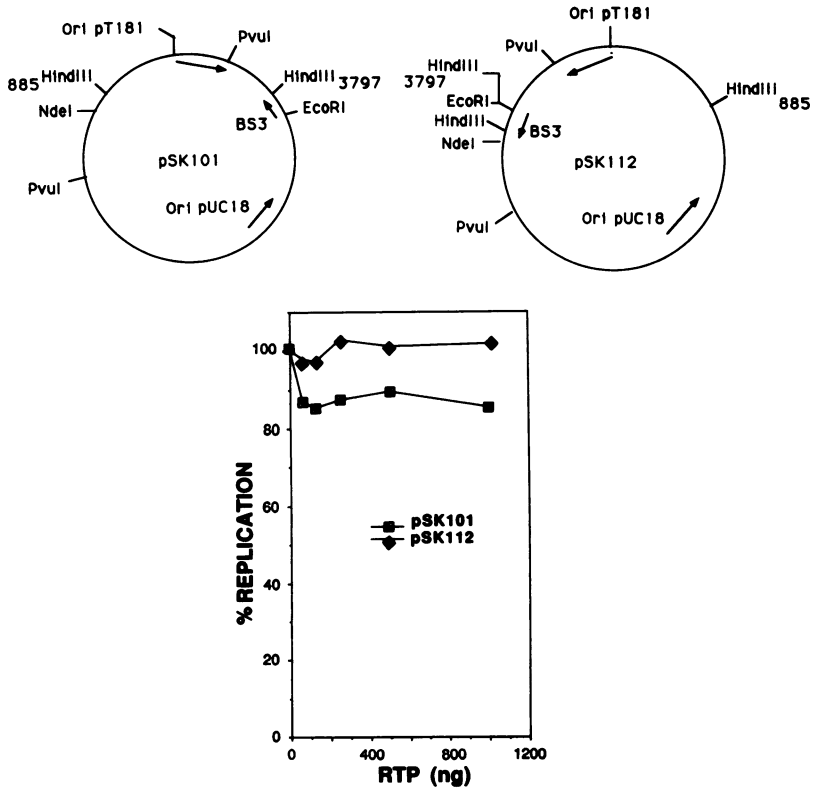

FIG. 3. Effect of RTP on rolling circle replication from pT181 origin in cell extracts of *S. aureus*, initiated by RepC protein of the plasmid pT181. (*Upper*) DNA templates used in the reactions. (*Lower*) Quantitation of the *in vitro* reaction showing no significant blockage of rolling circle replication from either of the templates pSK101 and pSK112. The pSK101 and pSK112 contained the BS3 terminus in opposite orientation with respect to the pT181 ori.

subtilis is known to function *in vivo* and blocks heterologous Cairns-type Gram-positive replicons at the BS3 site (29).

We investigated whether RTP would block rolling circle replication *in vitro* of hybrid replicons pSK101 and pSK112 (Fig. 3 *Upper*) that contained pT181 origin of replication and BS3 in either orientation with respect to the origin and the unidirectionally moving replication fork. In cell extracts of *S. aureus* that were supplemented by exogenously added RepC initiator protein, rolling circle replication of pSK101 and pSK112 was initiated in the presence of increasing levels of RTP. The replication of pSK101 (BS3 in functional orientation) was inhibited by <10% and that of pSK112 (BS3 in nonfunctional orientation) was inhibited to 20% of maximal replication (Fig. 3 *Lower*). Analysis of the replication intermediates by 2D gel electrophoresis revealed no stalled termination intermediates (data not shown).

Thus, the results lead us to conclude that RTP did not appreciably block rolling circle replication *in vitro* in cell extracts of the Gram-positive *S. aureus*. Although the helicase involved in pT181 replication has not been identified to date, it is probably a reasonable expectation that RTP would not impede the DNA unwinding action of such a helicase at a BS3 site.

Polar Contrahelicase Activity of RTP on DnaB Helicase. The terminator protein (ter) of *E. coli* is a polar contrahelicase to the replicative helicase DnaB (13). Although ter and RTP show no significant primary sequence homology (6, 19), the observation that RTP blocks DNA replication of a plasmid in Gram-negative *E. coli* suggested that the protein might also block the DNA unwinding activity of *E. coli*-encoded DnaB helicase in a polar fashion.

We investigated the effect of RTP on the catalytic activity of DnaB helicase by constructing M13mp18-BS3 and M13mp19-BS3 recombinant single-stranded DNA circles that contained the BS3 site in opposite orientations. Two mutually complementary synthetic 57-mer oligonucleotides comprising the BS3 sequence were synthesized, 5′ end-labeled, and hybridized to the mp18 and mp19 single-stranded circles and, using the labeled heteroduplex circular DNA substrate, helicase assays were performed. A typical set of results is shown in Fig. 4. The results show that in the M13mp18-BS3 substrate, which contained the BS3 site in the nonfunctional orientation with respect to the 5′ → 3′ direction of propagation of DnaB helicase, the RTP had no significant influence on the ATP-dependent DNA unwinding activity. In contrast, in the M13mp19-BS3 substrate, addition of a 20-fold molar excess of RTP over the helicase substrate caused an 80% inhibition of DnaB helicase activity. Thus, despite the lack of any significant primary sequence homology between ter and RTP, the Gram-positive terminator protein (RTP) acted as a polar contrahelicase of the Gram-negative replicative helicase DnaB.

RTP Does Not Impede the Activity of Helicase II. Does RTP serve as a contrahelicase to exclusively those helicases involved in Cairns-type DNA replication and not to those involved in conjugational DNA transfer (rolling circle replication) or DNA repair? We investigated the effect of RTP on the catalytic activity of helicase II, which is known to be involved in DNA repair (23), using the same M13mp18-BS3 and M13mp19-BS3 substrates. A typical set of results is shown in Fig. 5. We consistently observed that RTP over a wide range of concentrations, including up to a 90-fold molar excess over the substrate DNA, had no detectable effect on the DNA unwinding activity of helicase II. In contrast, as shown above, a 10-fold molar excess of RTP caused a 70% inhibition of DnaB helicase activity on the same M13mp19-BS3 substrate. We have recently found that the activity of helicase I, which catalyzes conjugational DNA transfer (23), was not inhibited by RTP (data not shown).

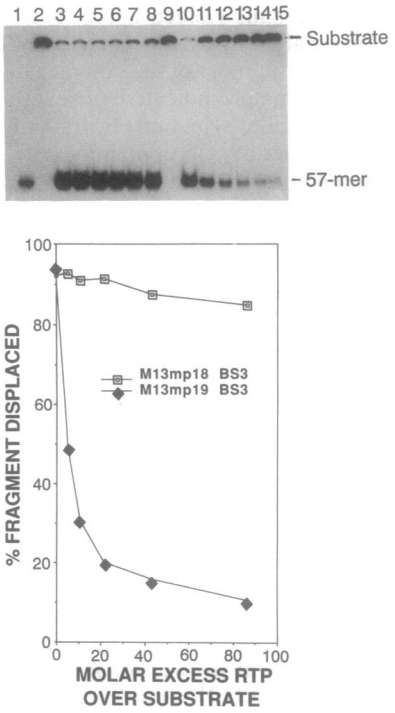

FIG. 4. Effect of RTP on the DNA unwinding activity of DnaB helicase. (*Upper*) Autoradiogram of an 8% polyacrylamide gel. Lane 1, boiled mp18-BS3 substrate; lane 2, substrate and helicase without ATP; lane 3, substrate and helicase with ATP; lanes 4–8, same as lane 3 but containing in addition 5-fold (lane 4), 10-fold (lane 5), 20-fold (lane 6), 40-fold (lane 7), and 90-fold (lane 8) molar excess of RTP over substrate. Note that RTP did not block DnaB-catalyzed unwinding of the mp18-BS3 substrate. Lane 9, mp19-BS3 substrate and DnaB without ATP; lane 10, substrate plus DnaB plus ATP; lanes 11–15, same as lane 10 but with 5-fold (lane 11), 10-fold (lane 12), 20-fold (lane 13), 40-fold (lane 14), and 90-fold (lane 15) molar excess of RTP over substrate. Note that RTP blocks unwinding activity of DnaB on the mp19-BS3 substrate.

DISCUSSION

Up to this time, the biochemical activity of only one RTP—namely, the ter protein of *E. coli*—has been reported (11, 12). Although ter is a contrahelicase to DnaB and to the helicase

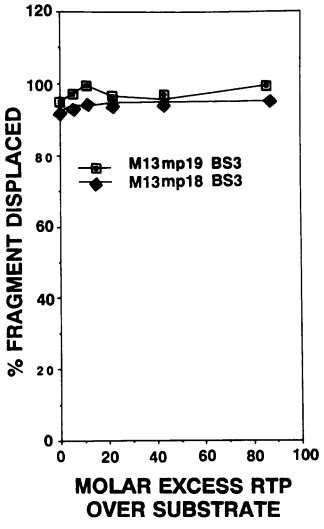

FIG. 5. Lack of inhibitory effect of RTP on the catalytic activity of helicase II. Note that neither the unwinding of mp18-BS3 nor that of mp19-BS3 catalyzed by purified helicase II was impeded by RTP over a wide range of concentrations.

activity of simian virus to large tumor antigen (30), it is mechanistically unclear as to how terminator proteins act to block helicases that have no apparent similarity in primary sequence. All attempts to crystallize ter thus far have been unsuccessful. In contrast, we have successfully crystallized RTP of *B. subtilis* (17) and have solved its 3D structure by x-ray crystallography (D. Bussiere, D.B., I.P., and S. White, unpublished data). Exploiting the crystal structure to elucidate the mechanism of action of RTP would require isolation of key mutant forms of the protein and an *in vitro* system of DNA replication and the identification and purification of the target replicative helicases. Since such advantages are presently missing in the *B. subtilis* system, our results are of considerable significance in providing an alternative system with which to mechanistically and functionally exploit the crystal structure of RTP.

How are two terminator proteins—namely, RTP and ter—that are so dissimilar in primary structure able to specifically block the same replicative helicase? It is very likely that the two proteins share a common 3D structural domain that is able to recognize a surface on DnaB helicase but not on helicase II or helicase I. We have recently found that both ter and RTP specifically bind to replicative helicases but not to helicases involved in DNA repair or in chromosome transfer by rolling circle replication (B.K.M., M. Lobert, I.P., R. Rahija, and D.B., unpublished data).

What is the physiological role of terminator proteins? The *tus* gene encoding the ter protein of *E. coli* can be deleted without any noticeable phenotype except for somewhat slower growth of the cells (D.B., unpublished data) This observation would seem to suggest that the ter protein is dispensable. It is conceivable that RTP could also be similarly dispensable. However, Nordstrom and coworkers (31) have observed that when DNA replication is initiated from an R1 plasmid ori integrated into the *E. coli* chromosome, the cell division is noticeably perturbed, generating elongated cells or filaments. Mutations in the *tus* gene reduce the frequency of filament formation (31). Replication forks initiated from an abnormal ori when arrested at the normal ter site perturb cell division, thus establishing a connection between termination and cell division. The τ sites may be keeping forks from approaching major transcription units from a direction opposite to transcription, thus, preventing collision between the replication and transcription complexes on DNA (32).

Although there seems to be indirect evidence against a cause and effect relationship between DNA replication and cell division (33), a potential role of termination in chromosome segregation cannot yet be ruled out. Finally, stringent response in *B. subtilis* causes arrest of forks at sites 200 kb away and at either side of the ori (9, 10). It remains to be seen whether RTP is responsible for this transitory arrest. If RTP is involved in this process, then one of its functions is probably to block overreplication of DNA.

There are indications of polar blocks to fork movement in eukarytic chromosomes (31–36). It is likely that terminator proteins probably exist in eukaryotes, and one of the functions of the proteins may be to limit gene amplification to designated regions of the chromosome, thereby controlling gross overreplication of chromosomal DNA.

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