DNA replication in many prokaryotic chromosomes and at some eukaryotic chromosome regions is arrested at specific sequences called replication termini or Ter sites (6). Ter sites act as polar barriers to fork movement and act essentially as replication traps. In Bacillus subtilis, the Ter DNA interacts with a sequence-specific DNA-binding protein called replication terminator protein (RTP) which acts as a polar contrahelicase; i.e. it impedes helicase-catalyzed DNA unwinding when present in one orientation, whereas it lets the helicase pass through unimpeded in the opposite orientation (1–4). The bacterial chromosome is believed to exist in vivo not as naked DNA but as a DNA-protein complex, with most of the DNA-binding proteins remaining bound to the chromosome (5). Despite the fact that some of these proteins bind to DNA with relatively high affinity (e.g. lac repressor), the replication fork apparently has the ability to pass through these complexes unimpeded. The only region of the chromosome that is known to arrest effectively the replication forks is the terminus (6). The preceding observations suggest the following: (i) the replication apparatus apparently has an activity that allows it to pass through most protein-DNA complexes, some of which contain strong DNA-binding proteins, and (ii) since the replication terminus is able to arrest forks effectively, the terminator protein-DNA complex is likely to have special features that enable it to arrest replication forks. Thus, high affinity binding of terminator protein to Ter sites per se does not appear to be sufficient to cause the replication-terminating activity of RTP (12). We have hypothesized that DnaB (or the equivalent helicase of B. subtilis)-RTP interaction plays a key role in fork arrest (1). Despite the existence of in vitro evidence for RTP-DnaB interaction (7, 12), the validity of the protein-protein interaction model has been debated (11).

The raison d’être for carrying out this work was 2-fold: (i) to perform additional experiments, using independent approaches and different mutant forms of RTP, to reexamine the question of RTP-DnaB interaction in vitro and (ii) to investigate whether a common domain of RTP is involved in the arrest of both RNA polymerase and helicase. The observations presented here confirm the biologically meaningful interaction between RTP and DnaB and further extend the result by showing that a common domain of RTP seems to be involved in the arrest of both DnaB helicase and T7 RNA polymerase (and perhaps other RNA polymerases).

The replication termini of B. subtilis (Fig. 1) consist of overlapping core and auxiliary sites. A RTP dimer first binds to a core and then, by cooperativity, promotes the binding of a second dimer of RTP to the auxiliary site (8, 9). Interaction between two dimers is essential for fork arrest with the core end of the Ter site arresting the helicase and the auxiliary end, allowing the helicase to pass through unimpeded (7).

The RTP of B. subtilis is a homodimer belonging to the class of winged helix proteins (8, 9). The crystal structure of the RTP apoprotein has been solved at high resolution, and the structure contains four α-helices, three β-strands, and an unstructured, N-terminal arm (Fig. 2A) (9). Extensive random and site-directed mutagenesis of RTP had identified the N-terminal arm, the α-helix, and the β-strand to be the main DNA-binding elements (8), with the α helix contacting the major groove and the β-strand into the minor groove of Ter DNA (8). Affinity cleavage analysis that converted RTP to a site-directed chemical nuclease was used to determine amino acid to base contacts, and the results had confirmed that the α and β-helix contacted the major groove and β-strand, to the minor groove of Ter DNA (10, 26). X-ray crystallography had revealed an exposed hydropho-
bic patch that was suggested to be a possible docking surface for the helicase (9) (see Fig. 2B).

In this paper, we have used cross-linking, label transfer, and other techniques along with different mutant forms of RTP to present additional evidence in favor of the RTP-DnaB interaction model of replication termination. We also show that a common region of RTP is involved in arresting both the helicase and T7 RNA polymerase. Both helicase and RNA polymerases melt DNA, and the results suggest that there probably is a common structural motif in these enzymes that may be recognized by RTP.

EXPERIMENTAL PROCEDURES

Bacterial and Plasmid Strains—The Escherichia coli strains JM109 (sup E44, rel A1, recA, endA1, gyrA96, hsdR17, ~L~lac-proAB, [F’ tra D36, lacI^3(lacZM15, proA^R, rk, mk B^R)] was used for making M13 DNA; DH5a [F’ sup E44, lacU169 (480 lacZ Δ M15), hsdR17, recA1, endA1, gyrA96, thi-1, relA1] was used for cloning; and BL21(DE3)[F’ omp T, hsd S, rB^c^mB^g^al]) containing the plasmid pLysS was used for expressing proteins in pET vectors (Novagen). Wild type strain W3110 was used for preparation of cell extract in in vitro replication assays.

Gel Mobility Shift Assay—The assays were carried out as described in Ref. 13. Briefly, polymerase chain reaction products of Ter 1 and core binding sites were amplified from pUC18BS3 (53-base pair fragment having complete RTP binding site cloned as EcoRI–HindIII cassette) and pUC18core (fragment having the core binding site of RTP cloned as EcoRI–HindIII cassette) using universal M13 forward and reverse primers. These fragments were end-labeled with [γ-^32P]ATP and T4 polynucleotide kinase. 1 fmol of labeled DNA was used in each reaction, which was carried out in 20-μl volumes of 40 mM Tris-Cl (pH 7.5), 4 mM MgCl2, 50 μg/ml bovine serum albumin, 50 mM potassium glutamate, 5 μg of calf thymus DNA, and increasing amounts of RTP (0, 1, 2, 3, 4, 6, 8, 12, 16, 20, 40, 60, 80, 100, 200, 300, 400, 600, 800, and 1200 fmol). The reactions were carried out at room temperature for 30 min and resolved on 8% native polyacrylamide gels.

Radioactivity from gel bands corresponding to free, singly and doubly shifted ^32P-labeled IR1 DNA was quantified by a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). From these data, the fraction of IR1 oligonucleotide bound (FB) by two dimers of RTP protein was calculated and plotted as a function of the free RTP concentration (see Fig. 7). The data were then replotted as the log [FB/1 - FB] versus log [free RTP] (Fig. 7), based upon a logarithmic form of the Hill equation (Equation 1) (21).

experimental procedures

FIG. 1. Diagram showing the position of origin of replication and the terminator sequences. The stringent responsive replication checkpoints are present 200 kilobases downstream on either side of the origin and called the φc (left checkpoint) and φo (right checkpoint). The diagram is not drawn to scale, since it has disproportionately magnified the Ter region.

FIG. 2. Crystal structure of B. subtilis RTP. A, the overall structure of the monomeric protein consisting of four α helices, three β strands, and a disordered N-terminal arm. B, the exposed hydrophobic patch showing the hydrophobic residues and the location of the critical Tyr^33 residue (see Ref. 12).

$$
\log [FB/(1 - FB)] = H \log \text{[free RTP]} - \log K_{0.5}^H 
$$

where \( H \) is the Hill coefficient and \( K_{0.5} \) is a constant equal to the concentration of RTP at FB = 0.5. Plotted in this format, the line slope for points between 0.1 < FB < 0.9 is the Hill coefficient. At FB = 0.5, the following is true,

$$
\log K_{0.5}^H = H \log \text{[free RTP]} + \log K_{0.5}^H
$$

allowing direct calculation of the concentration of RTP required for half-maximal IR1 binding.

Hill Analyses of Wild Type, Y33F, and Y33N Replication Terminator Proteins—Listed are the Hill coefficients and the corresponding \( R^2 \) values derived for assessment of IR1-binding cooperativity by wild type, Y33F, and Y33N replication terminator proteins. Also listed are the values for \( K_{0.5} \) (defined here) and the concentrations of each RTP form required for half-maximal saturation of IR1 DNA (Table 1).

Purification of DnaB and RTP—The proteins were purified as described (7, 10).
Helicase Assay—Helicase assays were performed as in Ref. 1. Briefly, 10 pmol of complementary oligonucleotide with RTP binding sites were end-labeled using [γ-32P]ATP and T4 polynucleotide kinase and an end-labeled 10 pmol of complementary oligonucleotide with RTP binding sites were cross-linked to radiolabeled DNA by UV irradiation. B, wild type RTP; E56C, C, Y33C. Lanes 1–9 in B and C correspond to DNA, no UV (lane 1); DNA with UV (lane 2); DNA + underivatized RTP without UV (lane 3); DNA + underivatized RTP + UV (lane 4); DNA + derivatized RTP, no UV (lane 5); DNA + 5, 10, 25, and 50-fold molar excess of derivatized RTP + UV (lanes 6–9). Note that only derivatized E56C yielded cross-linked DNA-protein (arrows; C). Little or no cross-linked products were seen when either the wild type or Y33C RTPs were used (B and D).

Fig. 4. Binding of radiolabeled DnaB to wild type RTP-GST and various mutant forms of RTP-GST matrices. A, autoradiogram of a SDS-polyacrylamide gel showing the binding of increasing amounts of radiolabeled DnaB to wild type RTP-GST, Y33N-GST, and Y33F-GST affinity matrices. B, quantitation of the radioactivity from three sets of gels (values averaged) using a PhosphorImager. Note that all of the mutational substitutions at Tyr33 cause reduction in affinity for DnaB.
volumes containing a 5-, 10-, 25-, or 50-fold excess of derivatized proteins (wild type RTP, E56C, and Y33C) over 100 fmol of end-labeled DNA, 10 mM MOPS-NaOH, pH 7.3, 200 mM NaCl, and 50 μg of bovine serum albumin. The reaction mix was incubated in the dark at room temperature for 45 min. UV irradiation to samples was carried out using the long wavelength range of the UV lamp (model UVGL-25; Ultraviolet Products, San Gabriel, CA) held at a distance of 10 cm for 2 min. The UV-cross-linked samples were resolved on 10% SDS-polyacrylamide gels.

**Protein-Protein Interaction Studies by Glutathione S-Transferase (GST) Affinity Column Chromatography—**GST affinity chromatography assays were performed according to Refs. 7, 16, and 24.

**125I Label Transfer**—Reaction vials were coated in the dark with an 8-fold excess (1.2 μCi in chloroform, 100 μCi/vial of IODO-GEN (Pierce) and dried under liquid nitrogen. 0.15 mM S-[N-(4-azidosalicyloyl)cysteaminyl]-2-thiopyridyl (ACT) in chloroform was prepared, and 100-μl aliquots were dried in each tube in the dark and stored at −20 °C. Radioiodination was carried out in a 120-μl reaction volume containing 100 μl of 0.15 mM ACT, 100 mM sodium borate (pH 8.4), 100 μM potassium iodide in 0.1 M sodium borate buffer, pH 8.4, and 5 μCi of 125I. The reaction mix was transferred to an IODO-GEN-coated vial and reaction stopped after 30 s by transferring the reaction mix to a quench vial containing 15 μl of quench solution (1 mM tyrosine and 8 mM methionine in 0.1 M sodium borate, pH 8.4).

After a 10-min incubation at room temperature, the labeled 125I-ANT was used directly to react with RTP protein. 60 μl of 125I-ANT (6.7 nmol) from the iodination quench vial were mixed with 10 μmol of RTP in 40 μl and incubated for 20 min. The reaction mixture was loaded on 2 ml of Bio-Gel column (Bio-Rad) equilibrated previously in 40 mM Tris-Cl, pH 8.0, 100 mM KCl, 1 mM EDTA, 10 mM MgCl2, 5% glycerol (AT buffer) and eluted in the same buffer. 100-μl fractions containing derivatized 125I-RTP were pooled and used for photocross-linking. 80 μl of 125I-RTP, 160 nl of 200 mM DnaB helicase, and 560 μl of AT buffer were mixed and UV-irradiated using a Rayonet photochemical reactor, model RPR-100, for 20 s at room temperature in the dark. 5.9 mg of iodoacetamide were added for alkylation and reacted for 15 min at room temperature in the dark. The reaction mix was concentrated at 2000 × g to 20 μl using Ultrafree-CL centrifugal filters (Millipore Corp.). 20 μl of 80 mM iodoacetamide in 15 μl urea were added to concentrated sample and incubated for 15 min at room temperature in the dark. Two aliquots of concentrated samples (20 μl each) were prepared. To the first, 5 ml of 5× loading dye (0.5 mM Tris-Cl, pH 6.8, 50% glycerol, 10% SDS, and 0.1 mg/ml bromphenol blue) was added, and to the second sample, loading dye and 10% β-mercaptoethanol was added, and the samples were resolved on 30-cm-long 10% SDS-polyacrylamide gels.

**In Vitro Transcription**—In vitro transcription reactions were carried out as described (15, 17, 22, 23).

**RESULTS**

Despite the existence of some evidence for RTP-DnaB interaction in vitro (7) and in vivo (12), there has been continuing debate (11) as to whether RTP-DnaB helicase is really involved in replication fork arrest. This debate prompted us to reexamine this significant mechanistic question by using different approaches and additional mutants, including a conservative Y33F mutant form of RTP. Tyr32 was chosen for further investigation because previous work had implicated this as a critical residue for helicase arrest (7). We also wished to investigate whether the domain of RTP that is known to be involved in replication fork arrest also impedes the elongation of RNA polymerase. First we wished to determine if the Tyr32 residue of RTP was in contact with Ter DNA in solution as described below.

The Residue Tyr32 Does Not Contact DNA in an RTP-Ter DNA Complex—The exposed hydrophobic patch of RTP (Fig. 2B) was postulated to be a possible docking surface for DnaB helicase (9), and the Tyr32 residue, located near the patch, was found to be a critical residue in the RTP-DnaB interaction in previous experiments (18). Since the crystal structure of an RTP-Ter complex is not yet available, we wished to investigate whether the Tyr32 residue directly contacted Ter DNA in solution. We isolated the Y33C mutation by site-directed mutagenesis and coupled the photodynamic reagent azidophenacyl bromide to wild type RTP (which has a Cys residue in the dimerization domain, at coordinate 110), Y33C and E56C mutant forms of RTP. E56C was chosen for derivatization (Fig. 3A) because the residue is located in the α3 helix of RTP (Fig. 2A).
and is known to contact the major groove of Ter DNA (10, 26).

This derivative was therefore used as a positive control. Neither derivatized wild type RTP nor the Y33C form yielded significant amounts of cross-linked product with labeled Ter DNA (Fig. 3, B and D). In contrast, E56C protein readily yielded detectable amounts of protein-DNA cross-links, and two cross-linked species, corresponding to DNA bound to a single dimer or with two dimers of RTP, were readily detected (Fig. 3 C, arrows). Thus, on the basis of the result presented here, we concluded that the Tyr 33 residue neither contacted DNA in solution nor was located within 11 Å of the Ter DNA (the length of the cross-linker is 11 Å; see Ref. 19).

Interaction between RTP and DnaB as Revealed by Affinity Column Chromatography—For the purpose of critically reevaluating the interaction between RTP and DnaB further, we tagged DnaB with a kinase tag at the N-terminal end and purified the protein to near homogeneity and labeled it with \([\alpha-32P]\)ATP and muscle kinase (20). Previous work had used DnaB synthesized by coupled transcription-translation in vitro (7). We used authentic DnaB (kinase-tagged), produced in vivo that had full helicase activity (data not shown) in order to eliminate any chance of binding artifacts caused by misfolded or inactive DnaB produced in vitro. Wild type RTP and the various mutant forms were produced as fusion proteins with GST and immobilized separately onto glutathione-agarose beads. We made sure that equal molar amounts of wild type and each of the mutant forms of RTP were immobilized to a fixed amount of the affinity matrix by removing equal aliquots of each type of RTP affinity beads and estimating the amount of bound proteins by SDS-polyacrylamide gel electrophoresis.

We cleaved off the GST moiety from the wild type fusion protein and performed helicase-blocking assays to make sure that the protein was biologically active. It should be kept in mind that the Tyr 33 residue projects out of the surface of RTP apoprotein (Fig. 2 B), and mutational alteration at this site does not seem to affect the folding of the protein as suggested by its chromatographic behavior, solubility properties that were indistinguishable from that of the wild type protein. In fact, we were able to crystallize the mutant forms and collected a partial set of diffraction data but did not attempt to solve the structure.

Increasing amounts of labeled DnaB protein were bound to equivalent amounts of the wild type and mutant forms of the
RTP-affinity beads and washed, the labeled protein was step-eluted with increasing concentrations of NaCl, precipitated, resolved in SDS-polyacrylamide gels, and autoradiographed. The amount of bound proteins was quantitated with a PhosphorImager. The results showed that the wild type RTP matrix retained the labeled DnaB. Considering the fact that RTP is a basic protein, whereas DnaB is acid, the possibility of non-specific acidic and basic protein interaction was taken into account and eliminated by the following experiment (Fig. 4). We kinase-tagged and labeled another acidic protein (namely DnaG) and performed the affinity adsorption experiments with wild type RTP affinity beads. We did not observe any retention of DnaG by RTP-GST affinity matrix (not shown).

Nonspecific charge interaction causing binding artifacts is also not supported by our observation that replacement of an uncharged Tyr$^{33}$ residue by another uncharged F or A caused no alteration of the net charge but significantly reduced the protein-protein interaction. In fact, replacement of Tyr$^{33}$ with Asn, Ala, Cys, or Phe resulted in a reduction in protein-protein interaction (Fig. 3, A and B). The outcomes of the affinity binding experiments were consistent with the conclusion that there was specific protein-protein interaction between RTP and DnaB in solution and in the absence of Ter DNA and that the Tyr$^{33}$ residue played a key role in that interaction.

Cross-linking Label Transfer Experiment Supported a Critical Contribution of Tyr$^{33}$ to the Interaction—We also wished to reexamine possible interaction of DnaB to RTP by an independent method. We radioiodinated the bifunctional, photodynamic, azido cross-linker ACT (21) at position 3 to generate the 3$I^2$Iodo-ACT and coupled it to E30C RTP at the cysteine residue (Figs. 2B and 5A). This location was chosen because the crystal structure showed that both residues 30 and 33 were located close to each other on the protein surface and that both residues projected out at similar angles from the protein surface. Furthermore, derivatization at residue 30 only marginally reduced RTP-DnaB interaction (7). Similarly, wild type RTP (having a cysteine at position 110 in the dimerization domain) and the E30C,Y33N double mutant form of RTP were also derivatized in the dark with radioiodinated ACT. The rationale of the experimental approach is depicted in Fig. 5A. If RTP interacted with DnaB in vitro, the derivatized E30C protein should photocross-link to DnaB. Upon cleavage of the S–S bond by incubation with $\beta$-mercaptoethanol, the $\beta^2$I label should transfer to DnaB. The wild type RTP should neither cross-link nor transfer label to DnaB because the naturally occurring cysteine at position 110 is buried in the dimerization domain in the $\alpha_\beta$ helix (Fig. 2A). If the Tyr$^{33}$ residue contributed a critical contact with DnaB as suggested by the affinity adsorption experiments described above, then the double mutant form Y33N, E30C, derivatized with labeled ACT at the Cys$^{30}$ residue, should neither cross-link nor transfer label to DnaB.

The results of the experiment, in the absence of Ter DNA, are shown in Fig. 5, B and C. The wild type RTP, after incubation with the bifunctional cross-linking agent, showed no labeling of the protein, presumably because the naturally occurring cysteine in the dimerization domain was solvent-inaccessible (Fig.

### TABLE I

| Hill coefficient analysis of wild type (WT) RTP, RTP Y33F, and RTP Y33N | Hill coefficient | $R^2$ | Log$K_{d,H}$ | | |  
|---|---|---|---|---|---|  
| WT RTP | 1.8 | 0.96 | −15.7 | 1.9e−9 |  
| RTP Y33F | 1.9 | 0.96 | −16.7 | 1.6e−9 |  
| RTP Y33N | 0.7 | 0.97 | −6.5 | 5.2e−10 |  

5B, wild type, lanes 1–4). We incubated derivatized E30C with purified DnaB and irradiated the reaction mixture with UV to activate the azido group. The derivatized $\beta^2$I-labeled RTP, cross-linked to DnaB, generated a complex with a characteristic mobility in a SDS-8% polyacrylamide gel (Fig. 5, E30C, lane 4). Treatment of the reaction mixture with $\beta$-mercaptoethanol caused cleavage at the S–S bond and the $\beta^2$I label got transferred from the RTP to DnaB as indicated by a band of lower mobility that co-migrated with purified DnaB in the SDS gel (Fig. 5B; E30C, lane 3). If the reaction mixture was not irradiated with UV, no label transfer to DnaB occurred (Fig. 5B, E30C, lane 2). It should be noted that the difference in mobility between the DnaB-RTP complex is rather small, and thus it required a long run in an SDS gel to resolve the difference. A shorter run of the gel produced sharper bands but caused the RTP-DnaB complex to run at a position that was indistinguishable from that of DnaB (Fig. 5C, E30C, lanes 2 and 3).

We wished to determine whether the interaction between the two proteins was specific and if the Tyr$^{33}$ residue played a critical role in the interaction. The experimental results showed that when the Y33N,E30C double mutant form of RTP was derivatized by the radioiodinated ICP no label appeared in a DnaB-RTP complex or in DnaB (Fig. 5C, E30C,Y33N, lanes 2 and 3). The single mutant form E30C under identical conditions continued to show label transfer (Fig. 5C, lanes 2 and 3). Thus, the cross-linking, label transfer experiments were consistent with the conclusions derived from the affinity binding data described above that RTP interacted in vitro with DnaB even in the absence of Ter DNA.

The cross-linking in vitro between RTP and DnaB was also carried out in the presence of Ter DNA, and the results were identical to that obtained in the absence of DNA excepting that the amount of label transfer was considerably less (not shown). We wished to determine further the peptide of DnaB that was the recipient of the transferred label but were unable to do so due to the small yield of labeled material.

### DNA Binding Property of Wild Type and the Mutant Forms of RTP—To determine the DNA binding affinities and cooperativity of binding of wild type and the Y33F and Y33N mutant forms of RTP, oligonucleotide gel mobility shift experiments

---

**Fig. 8.** Helicase assay showing polar arrest of DnaB helicase by wild type RTP. Top panel, blocking orientation of Ter 1 in the helicase substrate. Note that wild type RTP but not the Y33F form could arrest DnaB-catalyzed unwinding (labeled oligonucleotide released from the partial heteroduplex) of the helicase substrate. Bottom panel, the control (nonblocking orientation of Ter 1) shows no helicase arrest by wild type RTP or Y33F.
were performed using 32P-labeled IR1 (Ter 1) DNA. The results from these experiments are shown in Figs. 6 and 7, plotted as the fraction of doubly occupied IR1 DNA as a function of the logarithm of the free RTP concentration. All three forms of the RTP bound to IR1 DNA with high affinities, with Y33N showing some reduction in affinity (less than 10-fold). The concentrations of free RTP for half-maximal saturation (i.e. two dimers of RTP) of IR1 were 1.9, 1.6, and 0.52 nM for the wild type, Y33F, and Y33N RTP forms, respectively. IR1 binding by the wild type and Y33F RTP forms was highly cooperative, as can be seen from the rapid rise in IR1 fraction bound with increasing RTP concentration and as shown in the logarithmic Hill plots (Fig. 7; see insets). The Hill coefficients for IR1 binding by wild type and Y33F RTPs were 1.8 and 1.9, respectively. In contrast, IR1 binding by the Y33N RTP mutant, although of high affinity, was less cooperative (Hill coefficient = 0.7; see Table I). Thus, the results showed that while the Y33F mutation either did not affect or only minimally affected the cooperativity and binding affinity of the mutant form of the protein to Ter DNA, it significantly reduced RTP-DnaB interaction in vitro. We have examined the off rate of Y33F protein in comparison with that of wild type RTP and have observed that the mutant form had a 3–4-fold higher off rate than that of the wild type protein (data not shown).

The Y33F Mutation Abrogated Helicase-arresting Activity in Vitro—Helicase assays were performed to analyze the ability of the wild type and the Tyr33 mutant form of RTP to block DnaB helicase activity. Single-stranded DNAs from two M13 clones containing blocking and nonblocking orientations of Ter 1 were hybridized to respective radiolabeled complementary oligonucleotides to generate substrate DNAs that contained the Ter site at the duplex region. The ability of DnaB helicase to melt and dislodge the oligonucleotide from the partial duplexes in the presence of increasing concentrations of wild type and the Y33F mutant form of RTP was analyzed on 8% nondenaturing polyacrylamide gels. Wild type RTP, as expected, was able to arrest DnaB in a polar fashion, whereas the Y33F mutant was almost completely defective in this function (Fig. 8). This result showed that position 33 is involved in interaction with DnaB and, as a consequence, also in helicase arrest in vitro.

**Fig. 9.** Effect of the wild type and mutant forms of RTP on replication fork progression in vitro. A, diagram showing the replication assay. The template that has a Col E1 ori and the Ter 1 site of B. subtilis in either orientation was replicated in the presence of RTP and resolved in a denaturing gel. The appearance of a band corresponding to the leading strand, of characteristic length (610 nt), that extends from the ori to the Ter was taken as positive evidence for replication fork arrest. B, autoradiogram showing replication arrest by wild type RTP in the blocking orientation of Ter 1 (arrow) but lack of arrest by Y33F and Y33N forms of RTP on the same template. The template containing the nonblocking orientation of Ter 1, as expected, did not give an arrested product.

**Fig. 10.** Autoradiograms showing that the polar arrest of transcription elongation promoted by RTP at the Ter site is abolished by mutant forms of RTP. Top, autoradiogram of a denaturing polyacrylamide gel showing that wild type (WT) RTP arrests T7 RNA polymerase-catalyzed transcription at the Ter site (lanes 3–5), whereas the mutant forms Y33A (lanes 6–8), Y33C (lanes 9–11), and Y33F (lanes 12–14) fail to arrest the RNA polymerase. An increasing range of 0.35, 0.7, and 1.4 fmol of RTP of the various forms were used, as indicated. The template used, pET22b-IR1, contained the Ter site in the blocking orientation with respect to the promoter. C (lanes 1 and 2), transcription without added RTP. Bottom, control experiments using the nonblocking orientation of the Ter site (pET22b-IR1 rev) showed no detectable arrest of the RNA chain elongation in the presence of RTP.

An increasing range of 0.35, 0.7, and 1.4 fmol of the wild type and each of the mutant forms of the protein were used in the experiments (e.g. in lanes 3–5, 6–8, 9–11, and 12–14).
**Y33F Mutation Abrogated Replication Arrest in Vitro—**In vitro replication assays with *E. coli* cell extracts were carried out with template DNA having the Ter 1 binding site in both orientations with respect to the ori. Wild type RTP and the Y33F, Y33N RTP mutant forms were tested for their ability to arrest the replication fork movement in vitro. The replication products were analyzed on 6% denaturing gels. The generation of a 610-nt-long (distance from ori to Ter) leading strand of replication intermediate was diagnostic of fork arrest by RTP. The blocking orientation of Ter 1 showed the arrested replication intermediate band in the presence of wild type RTP, but the band was not visible in the nonblocking orientation of Ter (Fig. 9B, Wt. RTP BLK and NBLK, respectively). The Y33F and Y33N RTP mutants were unable to arrest replication forks, showing an almost complete loss of this function (Fig. 9B, Y33F and Y33N). Thus, even the tyrosine to phenylalanine, conservative mutation at the residue 33 of RTP resulted in almost a complete loss of replication fork arresting activity.

** Mutations in the Tyr33 Residue Abrogate Arrest of Transcription Elongation—**Previous work had shown that RTP arrested RNA polymerases (T7 and *E. coli* RNA polymerases) in a polar mode. We wished to investigate whether a common region of RTP was involved in the arrest of both helicases and RNA polymerases. We set up transcription reactions using a template that had an upstream T7 promoter and a downstream Ter site (present in either orientation with respect to the promoter). Transcription initiated from the upstream promoter was arrested in the blocking orientation of the Ter site by wild type RTP. All mutant forms of RTP, Y33A, Y33C, and Y33F, were unable to arrest T7 RNA polymerase in the blocking orientation of the Ter site (Fig. 10, top). Templates having the Ter site in the opposite orientation failed, as expected, to arrest transcription elongation (Fig. 10, bottom). Thus, it appears that the Tyr33 residue is a key element not only in arresting helicase-catalyzed DNA unwinding but also in impeding RNA polymerase-catalyzed chain elongation.

** DISCUSSION **

Although the ability of replication terminator proteins to arrest replicative helicases (contrahelicase activity) and RNA polymerase has been known for some time (1, 2, 4) and we have published in vitro evidence for RTP-DnaB interaction (6), the details of the mechanism by which RTP arrests replication fork and helicases needed additional investigation. The reinvestigation of the RTP-DnaB interaction by independent methods and different mutants was also prompted in part by continuing debate in the literature as to whether RTP-DnaB interaction was involved in replication fork arrest (11). Another group had claimed that the mutations in the Tyr33 residue had caused a defect in DNA-protein interaction rather than the observed interaction between Tus terminator protein and DnaB of *E. coli* using the two-hybrid system. Furthermore, using a reverse two-hybrid analysis, we have isolated mutant forms of Tus that bind to Ter normally but are defective in interaction with DnaB and in helicase arrest. Although the Tus protein and RTP have significantly different crystal structures, the biochemical attributes of both proteins are similar, suggesting that they function by similar mechanisms (4, 6, 9). The results from both *B. subtilis* and *E. coli* systems, taken together, strongly support a mechanism of replication termination that involves not only Ter-terminator protein interaction but also mechanistically significant terminator protein-helicase interaction.

Recent structure-function analysis of the PcrA helicase supports an “inchworm” model of helicase action that involves ATP-dependent DNA melting and helicase translocation on single-stranded DNA (19). Future work should be able to settle whether terminator proteins block both of these steps by contacting the helicase.

Finally, the failure of mutant forms of RTP with amino acid substitutions at Tyr33 to arrest helicase and RNA polymerase would suggest a common inhibitory surface on RTP. It would be interesting in the future to isolate mutant forms of T7 RNA polymerase that escape arrest by RTP. Such mutant forms of T7 RNA polymerase, considered along with the crystal structure of the enzyme (25), should be a productive avenue for further investigations and should shed light on the mechanism that causes arrest of RNA chain elongation.

**Acknowledgment—**We thank Dr. Richard Ebright of Rutgers University for a gift of the cross-linker ACT.

**REFERENCES **

1. Khatri, G. S., MacAllister, T., Sista, P., and Bastia, D. (1989) Cell 59, 667–674
2. Lee, E. H., Kornberg, A., Hidaka, M., Kohayaishi, T., and Hortwich, T. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 9104–9108
3. Hill, T. M., and Marians, K. J. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 2481–2485
4. Kaul, S., Mohanty, B. K., Sahoo, T., Patel, I., Khan, S., and Bastia, D. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 11143–11147
5. Yamamoto, K. R., and Alberts, B. M. (1976) Annu. Rev. Biochem. 45, 721–746
6. Bussiere, D. E., and Bastia, D. (1999) Mol. Microbiol. 31, 1611–1618
7. Manna, A. C., Pai, K. S., Bussiere, D. E., and Bastia, D. (1996) Cell 87, 881–891
8. Pai, K. S., Bussiere, D. E., Wang, F., Hutchison, C., White, S. W., and Bastia, D. (1996a) EMBO J. 15, 3164–3177
9. Bussiere, D. E., Bastia, D., and White, S. W. (1995) Cell 80, 651–660
10. Pai, K. S., Bussiere, D. E., Wang, F., White, S. W., and Bastia, D. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 10871–10873
11. Duggin, I. G., Anderson, P. A., Smith, M. T., Wile, J. A., King, G. F., and Wake, R. G. (1999) J. Biol. Chem. 274, 8771–8777
12. Manna, A. C., Pai, K. S., Bussiere, D. E., White, S. W. A., and Bastia, D. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 3253–3258
13. Pendergast, P. S., Chen, Y., Ebright, Y., and Ebright, R. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 10287–10291
14. Kelman, Z., Naktinis, V., and O’Donnell, M. (1995) Methods Enzymol. 262, 430–442
15. Chen, Y., Ebright, Y. W., and Ebright, R. (1994) Science 265, 90–92
16. S. Mulugu, A. Potnis, S. Zaman, J. Taylor, K. Alexander, and D. Bastia, unpublished observations.
Replication Termination

17. Bastia, D., and Mohanty, B. K. (1996) in DNA Replication in Eukaryotic Cells (DePamphilis, M., ed) pp. 177–215, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
18. Wake, R. G., and King, G. (1997) Structure 5, 1-5
19. Soultanas, P., Dillingham, M. S., Wiley, P., Webb, M. R., and Wigley, D. B. (2000) EMBO J. 19, 3799–3810
20. Sahoo, T., Mohanty, B. K., and Bastia, D. (1995) J. Biol. Chem. 270, 29138–29144
21. Sahoo, T., Mohanty, B. K., and Bastia, D. (1995) EMBO J. 14, 619–628
22. Hill, A. V. (1913) Biochem. J. 7, 471–480
23. Mohanty, B. K., Sahoo, T., and Bastia, D. (1996) EMBO J. 15, 2530–2539
24. Ratnakar, P. V. A. L., Mohanty, B. K., Lobert, M., and Bastia, D. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 5522–5226
25. Jeruzalami, D., and Steitz, T. A. (1998) EMBO J. 17, 4101–4113
26. Mohanty, B. K., Bussiere, D. B., Sahoo, T., Pai, K. S., Meijer, W. J. J., Bron, S., and Bastia, D. (2001) J. Biol. Chem. 276, 13160–13168