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The relationship between sequence-specific termination of DNA replication and transcription

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In *Escherichia coli* and *Bacillus subtilis* replication fork arrest occurs in the terminus at sequence-specific sites by the binding of replication terminator proteins to the fork arrest sites. The protein–DNA complex causes polar arrest of the replication forks by inhibiting the activity of the replicative helicases in only one orientation of the terminus with respect to the replication origin. This activity has been named as polar contrahelicase. In this paper we report on a second novel activity of the terminator proteins of *E.coli* and *B.subtilis*, namely the ability of the proteins to block RNA chain elongation by several prokaryotic RNA polymerases in a polar mode. The replication terminator proteins ter and RTP of *E.coli* and *B.subtilis* respectively, impeded RNA chain elongation catalyzed by T7, SP6 and *E.coli* RNA polymerases in a polar mode at the replication arrest sites. The RNA chain anti-elongation and the contrahelicase activities were isopolar. Whereas one monomer of ter was necessary and sufficient to block RNA chain elongation, two interacting dimers of RTP were needed to effect the same blockage. The biological significance of the RNA chain anti-elongation activity is manifested in the functional inactivation of a replication arrest site by invasion of RNA chains from outside, and the consequent need to preserve replication arrest activity by restricting the passage of transcription through the terminus–terminator protein complex.

Keywords: contrahelicase activity/transcription blockage

Introduction

Since DNA replication and transcription occur in the chromosomes at the same time, the possible mechanistic impact of one process on the other is of considerable interest. Several laboratories have studied the effect of replication fork propagation on RNA transcription (Brewer, 1988; Brewer *et al.*, 1992; Liu *et al.*, 1993; Liu and Alberts, 1995; Lucchini and Sogo, 1995). Observations on *in vivo* replication of human hemoglobin genes have suggested a temporal relationship between transcriptional activity and replication during early S phase of the cell cycle (Dhar *et al.*, 1988). DNA replication consists of three steps: initiation, ongoing replication, and termination. Although the possible relationship between initiation and elongation of a replication fork, and transcription has been studied, hitherto no information was available on the relationship between termination of DNA replication and transcription.

With a few exceptions (e.g. early stage of phage λ replication; see Valenzuela *et al.*, 1976) most prokaryotic chromosomes terminate their replication at sequence-specific termini (Crosa *et al.*, 1976; Kuempel *et al.*, 1977; Louarn *et al.*, 1977; Kolter and Helinski, 1978; Bastia *et al.*, 1981; Lewis *et al.*, 1990; Carrigan *et al.*, 1991; Smith and Wake, 1992). Sequence-specific arrest of replication forks has also been observed in eukaryotes at ribosomal DNA loci of yeast (Brewer and Fangman, 1988; Linskens and Huberman, 1988; Brewer *et al.*, 1992), human (Little *et al.*, 1993), plants (Hernandez *et al.*, 1988), in Epstein–Barr virus (Gahn and Schildkraut, 1989) and at yeast centromeres (Greenfeder and Newlon, 1992).

The arrest of the replication forks at each terminator site is polar in prokaryotes (Hidaka *et al.*, 1988; Sista *et al.*, 1989; Smith and Wake, 1992) and in yeast rDNA (Brewer *et al.*, 1992). The polar arrest of the replication forks at each site is caused by the binding of the terminator protein ter (or Tus) to the fork arrest site called Ter or τ in *Escherichia coli* and R6K plasmid (Hill *et al.*, 1989; Sista *et al.*, 1989). Similarly, the fork arrest at the terminus of *Bacillus subtilis* is caused by binding of its replication terminator protein (RTP) to the sites known as BS3 or Ter sites (the two initially discovered Ter sites, TerI and TerII, are also known as IRI and IRII sites; Smith and Wake, 1992; Kaul *et al.*, 1994; Sahoo *et al.*, 1995a). The terminator proteins, when bound to their cognate sites, arrest progression of replication fork approaching only from one direction, not from the other and thus the arrest is called polar or orientation-dependent.

Both *E.coli* and *B.subtilis* have at least six replication fork arrest sites each. In *E.coli* the two contiguous sites of opposite polarity called τ1 (TerA) and τ3 (TerC) are located 270 000 bp apart [τ1 (TerA) and τ2 (TerB) are located 350 000 bp apart] whereas the corresponding sites of *B.subtilis* are only 59 bp apart (Hill *et al.*, 1988; Lewis *et al.*, 1990; Hidaka *et al.*, 1991; Hill, 1992; Franks *et al.*, 1995). Replication forks approaching the sites from right to left will travel past the τ1 or the IRII sites and will be arrested at τ3 (or τ2) or IRI. The converse is true of the forks that travel left to right. However, once the first fork reaches a site and is arrested, the stalled replication complex at the arrested first fork apparently blocks the second fork so that the intervening region of the chromosome between the two termini is not replicated twice (see Figure 1). *In vivo* studies show that the τ1, τ2 and τ3 are all needed for replication fork arrest in *E.coli* (Pelletier *et al.*, 1988; Louarn *et al.*, 1991).

The ter protein of *E.coli* is a 36 kDa monomer (Sista *et al.*, 1991) and RTP of *B.subtilis* is a dimer with a subunit molecular weight of 14 500 Da (Carrigan *et al.*, 1991).

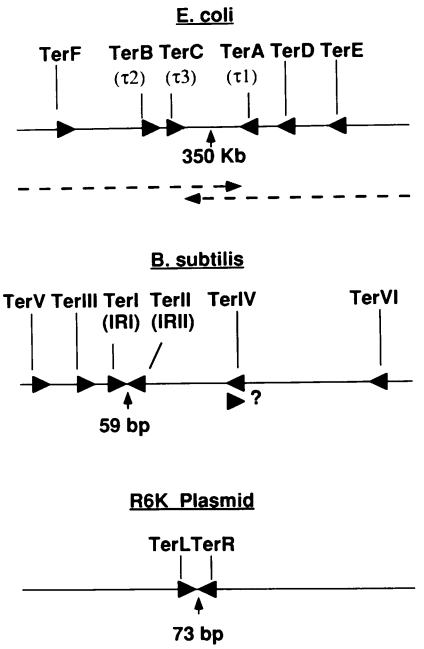

Fig. 1. Schematic representation of the replication termini regions of *E.coli* and *B.subtilis* and of the plasmid R6K. Replication termination occurs between the termini τ1 (TerA) and either τ2 (TerB) or τ3 (TerC) in *E.coli*. Termination of *B.subtilis* occurs between IRI and IRII (TerI and TerII) and termination in plasmid R6K occurs between TerL and TerR. The separation between τ1 and τ2 is 350 000 bp whereas that between τ1 and τ3 is 270 000 bp. The two termini IRI and IRII are separated by 59 bp whereas the termini TerL and TerR of R6K are separated by 73 bp. The dashed arrows show the mode of progression and the points of arrests of replication forks indicating the polar arrest of fork movement. Note that in *E.coli*, as contrasted with R6K and *B.subtilis*, the contiguous termini of opposite polarity are separated by a considerable distance.

We have recently reported the crystal structure of the RTP apoprotein at 2.6 Å (Bussiere *et al.*, 1995). The structure shows an antiparallel coiled-coil dimerization domain and a predicted 'winged helix' DNA-binding domain. No terminator protein from a eukaryote has hitherto been identified.

The first *in vitro* termination of DNA replication at a τ site in a cell-free system was reported by Germino and Bastia (1981). Subsequent work showed that the ter protein is a polar contrahelicase that impedes the DNA unwinding activity of DNA helicase in only one orientation of the τ sequence with respect to the replication origin (Khatri *et al.*, 1989; Lee *et al.*, 1989). The polar contrahelicase activity is helicase-specific (Khatri *et al.*, 1989; Hiasa and Marians, 1992; Sahoo *et al.*, 1995b). The RTP of *B.subtilis* functions *in vivo* and *in vitro* in *E.coli* and is also a polar contrahelicase of DnaB (Kaul *et al.*, 1994) and PriA (Sahoo *et al.*, 1995b) helicases of *E.coli*. The ter protein also acts as a polar contrahelicase of SV40 T antigen and impedes SV40 replication *in vitro* (Bedrosian and Bastia, 1991; Amin and Hurwitz, 1992).

We wished to investigate if the ter protein and RTP had any other biochemical activity in addition to the known polar contrahelicase activity. In this paper we have shown that both of the terminator proteins impose polar blocks to elongation of transcription *in vitro*, i.e. the proteins upon binding to their cognate termini (τ for ter and BS3 for RTP; see Sahoo *et al.*, 1995a,b) block RNA chain elongation catalyzed by T7, SP6 and *E.coli* RNA polymerases in a polar fashion. The contrahelicase and the polar transcriptional anti-elongation activities were isopolar.

We have investigated the biological relevance of the RNA chain blockage activity and have shown that invasion of a terminator protein–terminus DNA complex by an RNA transcript *in vitro* abrogated the contrahelicase activity. The passage of an RNA transcript through a replication terminus *in vivo* released the arrested replication forks. Transcription approaching a terminus towards the blocking end did not override the arrest of the replication fork. Thus, we believe that the blocking of RNA transcription by ter and RTP protects the replication termination function from possible transcriptional inactivation *in vivo*.

Results

Ter and RTP impose polar blocks to elongation of transcription in vitro

Although some have claimed that ter (Tus) impedes many proteins that translocate on DNA (Lee and Kornberg, 1992), recent studies have shown specific arrest of only the replicative helicases (i.e. DnaB, PriA and SV40 T antigen; Khatri *et al.*, 1989; Bedrosian and Bastia, 1991; Amin and Hurwitz, 1992; Hiasa and Marians, 1992; Kaul *et al.*, 1994; Sahoo *et al.*, 1995b) by ter and RTP. Since the movement of RNA polymerases on duplex DNA causes partial melting of the DNA duplex and the enzyme movement has polarity, features that are shared with helicases, we wished to investigate whether the ter protein of *E.coli* and RTP of *B.subtilis* would also block RNA chain elongation in a polar mode at the cognate replication termini. We constructed DNA templates that included a T7 promoter or an SP6 promoter or the A1 promoter of phage T7 recognized by *E.coli* RNA polymerase and positioned a BS3 (IRI) terminus of *B.subtilis* (Lewis *et al.*, 1990; Sahoo *et al.*, 1995a) or the τ2 terminus of *E.coli* (Hill *et al.*, 1988) downstream of these promoters in either orientation. For example, the transcriptional template for T7 RNA polymerase containing the BS3 terminus is shown in Figure 2A. The templates were digested with *Blp*I (*Esp*I) and transcribed with T7 RNA polymerase and [α-^{32}P]NTPs in the absence or presence of various concentrations of, exogenously added, purified RTP. In the absence of added RTP, the run-off transcript of the expected length (~330 nt), that was initiated from the T7 promoter and terminated at the *Blp*I (*Esp*I)-generated end of the linear templates, was detected in autoradiograms (Figure 2B, lanes 1 and 5). In the presence of RTP, one orientation of BS3 site showed arrest of the RNA chain at the replication terminus, whereas no such arrest was seen when the BS3 site was reversed with respect to the T7 promoter (Figure 2B, compare lanes 2, 3 and 4 with 6, 7 and 8 respectively). Similar results were obtained using the τ2 site and ter protein of *E.coli*, using both T7 and SP6 RNA polymerases and the cognate promoters (data not shown). Similar results were also obtained using the BS3 site, RTP, SP6 RNA polymerase and its cognate promoter. Thus, both RTP and ter proteins acted as polar blockers of RNA chain elongation by T7 and SP6 RNA polymerases *in vitro*, when bound to the respective replication terminus sequences.

We also investigated the ability of RTP to block RNA

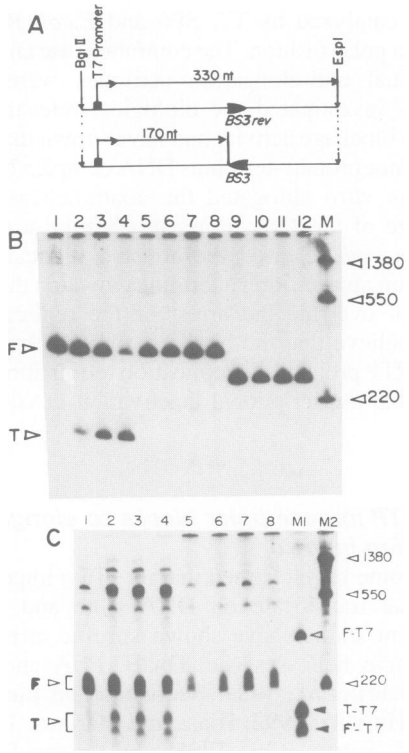

Fig. 2. Polar inhibition of elongation of transcription by RTP of *B.subtilis*. (**A**) The DNA template used in the run-off transcription experiment contained a T7 promoter and a downstream BS3 sequence present in either orientation. The DNA was linearized with *Esp*I. (**B**) Autoradiogram of a 6% polyacrylamide–7 M urea gel showing polar blockage of T7 RNA polymerase catalyzed RNA chain elongation by RTP at BS3 site. Lane 1, full-length transcript (F) synthesized in the 'blocking' orientation of BS3, in the absence of RTP; lanes 2–4, RNA transcripts synthesized on the template with the blocking orientation of BS3, in the presence of equimolar, 2-fold excess and 4-fold excess of RTP over DNA substrate, respectively. Note the arrest of the RNA chains (T) at the BS3 site in the presence of RTP. Lane 5, full-length transcript synthesized by T7 RNA polymerase on template containing the 'non-blocking' orientation of BS3; lanes 6–8, RNA transcripts synthesized on the same template as in 5, but in the presence of equimolar, 2-fold excess and 4-fold excess of RTP over DNA. Note that RTP is unable to block RNA chain elongation on this template. Compare lanes 2 and 6, 3 and 7, 4 and 8. Lane M, molecular size markers. Note that the control DNA template (pET22b linearized with *Eco*RI) without the BS3 terminus did not block the RNA transcript (lanes 9–12). (**C**) Autoradiogram of a 6% polyacrylamide–7 M urea gel showing the polar RNA chain anti-elongation activity of RTP on *E.coli* RNA polymerase-catalyzed transcription. Lane 1, RNA chain initiated from the A1 promoter on a template containing the functional orientation of BS3, but in the absence of RTP; lanes 2–4, same as 1 but in presence of 5-fold, 10-fold and 20-fold excess RTP over DNA substrate. Note the arrest of RNA chain at BS3 (transcripts labeled T) site; lane 5, RNA chains initiated from the A1 promoter on a template containing the non-functional orientation of BS3, but in the absence of RTP; lanes 6–8, same as 5 but in the presence of 5-fold, 10-fold and 20-fold molar excess of RTP over DNA substrate. Lane M1, markers; F-T7, full-length transcript as in (B); T-T7, arrested transcript as in (B); F'-T7, a run-off transcript from pET22b linearized at *Eco*RI site; M2, molecular size markers.

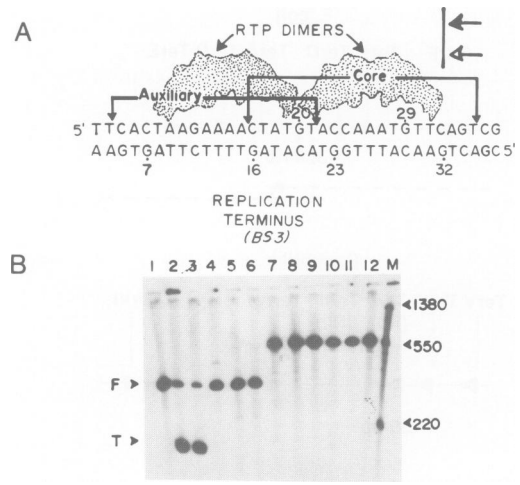

Fig. 3. Run-off transcription showing that a single RTP dimer bound to a core sequence cannot arrest RNA chain elongation. (**A**) The sequence of BS3 terminus of *B.subtilis* showing the overlapping core and auxiliary sites, each site binding one dimer of RTP. The template contained a T7 promoter and a downstream core site of BS3, that was cloned in either orientation with respect to the T7 promoter. Open arrow, direction of transcription block; filled arrow, direction of replication block. (**B**) Autoradiogram of a 6% polyacrylamide–7 M urea gel showing run-off transcripts on T7 promoter-BS3 and T7 promoter-BS3 core templates. Lanes 1–3 contained BS3 in the blocking orientation and included no RTP, 4-fold and 8-fold molar excess RTP over DNA, respectively. Lanes 4–6 show run-off transcripts from a T7 promoter-BS3 template with BS3 present in the non-functional orientation and included no RTP, 4-fold and 8-fold molar excess RTP over DNA, respectively. Lanes 1–3 and 4–6 served as positive and negative controls for the experiment shown in lanes 7–12. Lanes 7–9 show run-off transcript generated from a linear DNA fragment that contained a T7 promoter and the core sequence in one orientation and without RTP, with 4- and 8-fold molar excess of RTP over DNA, respectively. Lanes 10–12 show transcripts from the DNA template with the core sequence in opposite orientation and with no RTP, 4-fold and 8-fold molar excess of RTP over DNA, respectively. Note that the transcript on the 'core template' is longer in comparison with that shown in lanes 1–6 because of the length of the template used. Note that the core sequence is unable to arrest RNA chains in the presence of RTP in either orientation with respect to the T7 promoter.

chain elongation catalyzed by *E.coli* RNA polymerase. Suitable templates were constructed containing the efficient T7A1 promoter and the BS3 replication terminator sequence, present downstream of the A1 promoter, and run-off transcription experiments performed. The results showed that RTP was able to block *E.coli* RNA polymerase-catalyzed RNA chain elongation at the BS3 terminus in a polar mode (compare lanes 2, 3 and 4 with 6, 7 and 8 respectively in Figure 2C). Note that the full-length and the arrested transcripts are twin bands that, we believe, are generated by transcription starting from both the promoter and the single-stranded sticky end of the restriction fragment located near the promoter.

Polar arrest of transcription requires two interacting dimers of RTP bound to the BS3 terminus

Previous work has shown that the replication termini of *E.coli* and plasmid R6K bind to single monomers of ter protein (Sista *et al.*, 1991). In contrast, each functional replication terminus of *B.subtilis* binds two interacting dimers of RTP (Lewis *et al.*, 1990; Sahoo *et al.*, 1995a). The core sequence of BS3 binds a single RTP dimer and then by cooperative protein–protein interaction, promotes binding of a second dimer to the auxiliary site (Lewis *et al.*, 1990; Sahoo *et al.*, 1995a; see Figure 3, top). The isolated core readily binds a single RTP dimer but is

known to be ineffective *in vitro* in promoting helicase and replication fork arrest (Sahoo *et al.*, 1995a).

DNA templates were then constructed containing a T7 promoter and the core sequence of BS3, present in either orientation with respect to the promoter. Run-off transcription experiments, using linearized templates and T7 RNA polymerase, showed that the core sequence by itself, in the presence of bound RTP, was unable to impede RNA chain elongation in either orientation with respect to the promoter (Figure 3, bottom). Thus, a single RTP dimer bound to DNA was unable to elicit polar blockage of RNA chain elongation activity. This observation, considered together with the results shown in Figure 2, led us to conclude that RTP dimer–dimer interaction is necessary to elicit polar arrest of RNA chain elongation *in vitro*.

The transcriptional blockage by the replication terminator proteins raises questions about the mechanism of the transcriptional block, i.e. one might ask whether the RNA transcript simply pauses transiently at the terminus or whether the arrest is more long-lived or if the transcript is actually terminated. These questions will be dealt with in a future report because the principal theme of the present work is the mechanism of termination of DNA replication.

Transcriptional abrogation in vitro of contrahelicase activity of RTP

The experiments described in this section were provoked by two questions. What is the biological significance of the ability of terminator proteins to block transcription? What would be the impact on replication termination of an RNA transcript that is allowed to invade a replication terminus? Before considering these questions it may be worthwhile to recall the arrangement of the replication termini of *E.coli* and *B.subtilis* on their respective chromosomes and of plasmid R6K (Figure 1). Since the termini impede RNA chain elongation and replication fork movement in the same polarity, transcripts moving left to right would pass through the first set of termini but should be blocked by the second set having the opposite polarity. The converse should hold good for transcripts moving in the opposite direction. Therefore, a transcript in a tus^+ cell could penetrate a terminus only by approaching it from its non-blocking end. Keeping this consideration in mind and in order to seek an answer to the two related questions posed above, we constructed a helicase substrate called M13mp19BS3T7Pr (see Figure 4A). The template DNA included a T7 promoter and a downstream BS3 terminus sequence of *B.subtilis* (Sahoo *et al.*, 1995a). The orientation of BS3 was such that DnaB helicase of *E.coli* translocating clockwise in a 5′→3′ direction on the circular DNA would be blocked by RTP bound to the double-stranded BS3 terminus sequence of the substrate. The T7 promoter was located such that the transcript originating from it would invade the BS3 site. The substrate was prepared by extension of an upstream primer on the single-stranded circular DNA template in the presence of ddTTP, [α-^{32}P]dATP, dGTP, dCTP, dTTP and T7 DNA polymerase. Labeled extension products of various lengths were thus generated forming heteroduplexes on the circular template. A constant amount of the heteroduplex substrate (20 fmol) was used in all the experiments. Addition of

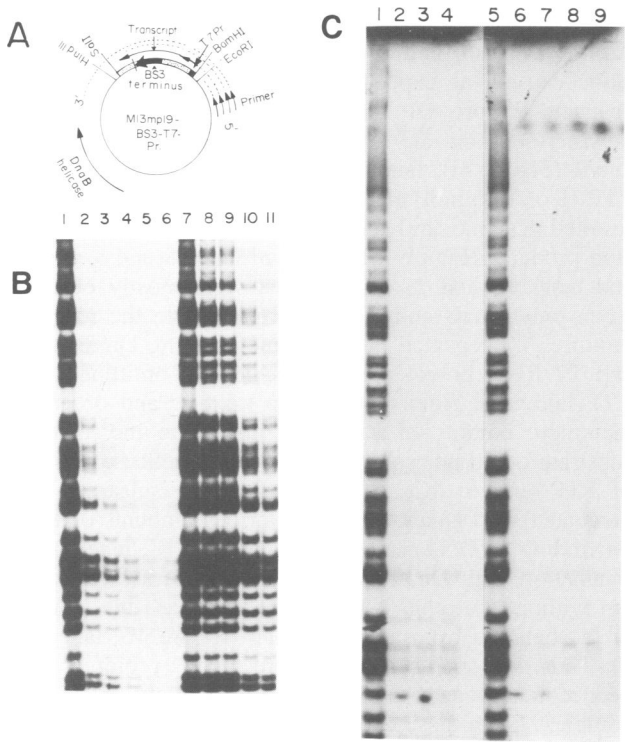

Fig. 4. Release of DnaB helicase activity arrested at the BS3 terminus by transcriptional invasion. (**A**) Schematic representation of the experimental strategy. The primer was extended on an M13mp19BS3T7Pr template in the presence of dideoxy-TTP and [α-^{32}P]dATP to generate a set of extension products. The heteroduplexed substrate was used to monitor the helicase activity of DnaB and its arrest by the addition of RTP. The effects of an RNA transcript, initiated at the T7 promoter (Pr) and invading the BS3 terminus were also monitored. (**B**) Autoradiogram of a 8% non-denaturing polyacrylamide gel showing release of DnaB activity from arrest by RTP. Lane 1, substrate (20 fmol) + 2 pmol DnaB; lanes 2–4, blockage of DnaB activity by the addition of 0.75, 1.0 and 1.5 pmol of RTP; lanes 5–6, substrate + 200 and 300 fmol of T7 RNA polymerase, respectively; lane 7, substrate + DnaB + T7 RNA polymerase; lanes 8–9, same as in lane 3 excepting that T7 RNA polymerase was added to initiate transcription from T7 Pr; lanes 10–11, same as in lane 4 excepting that T7 RNA polymerase was added to the reaction mixture. Note the blockage of DNA unwinding by the addition of RTP (compare lanes 2–4 with lane 1). Note also that the release of arrested helicase by transcription (compare lanes 8–9 with 3, and 10–11 with 4). (**C**) Autoradiogram of 8% non-denaturing polyacrylamide gel showing that active process of transcription rather than mere binding of RNA polymerase to the T7 promoter is needed to release arrested DnaB helicase. 20 fmol of DNA substrate, 2 pmol of DnaB and 200 fmol of T7 RNA polymerase were used in all of the experiments. Lane 1, substrate + DnaB; lane 2, substrate + DnaB + 0.75 pmol RTP; lane 3, substrate + DnaB + 1.0 pmol RTP; lane 4, substrate + DnaB + 1.25 pmol RTP; lane 5, substrate + DnaB + T7 RNA polymerase; lane 6, substrate + 300 fmol T7 RNA polymerase; lane 7, substrate + DnaB + RTP (0.75 pmol) + RNA polymerase; lane 8, substrate + DnaB + RTP (1.0 fmol) + RNA polymerase; lane 9, substrate + DnaB + RTP (1.25 pmol) + RNA polymerase. All lanes had samples with ATP but without CTP, GTP and UTP, thus preventing active transcription.

DnaB (2 pmol) to the substrate caused extensive DNA unwinding resulting in the release of the extension products that were resolved into a ladder of bands in a non-denaturing 6% polyacrylamide gel (Figure 4B, lane 1). Addition of 750 fmol, 1 pmol and 1.5 pmol of RTP, as expected, caused the arrest of the helicase activity as manifested in the blockage of the release of the extension

products from the heteroduplex (Figure 4B, lanes 2–4). Addition of 200 and 300 fmol of T7 RNA polymerase alone caused, as expected, no release of the extension products (Figure 4B, lanes 5 and 6) nor did T7 RNA polymerase show any effect on the helicase activity of DnaB (Figure 4B, lane 7). Addition of DnaB (2 pmol), RTP (1 or 1.5 pmol) and T7 RNA polymerase (200 fmol) resulted in significantly enhanced release of labeled extension products (Figure 4B, compare lanes 8 and 9 with 3 and lanes 10 and 11 with 4) that were mostly blocked when only DnaB and RTP were present in the reaction mixture. We repeated the experiments using DnaB, RTP and T7 RNA polymerase, with ATP but omitting GTP, CTP and UTP from the reaction mixture and observed that mere binding of RNA polymerase to the helicase substrate could not counteract the contrahelicase activity of RTP (Figure 4C). When the helicase substrate was incubated with DnaB, ATP and various amounts of RTP, unwinding of DNA was blocked (Figure 4C, lanes 2–4). Incubation with DnaB, RTP, RNA polymerase and ATP but without the other NTPs failed to release the activity of the helicase from arrest by RTP (Figure 4C, lanes 7–9). Thus, active transcription from the T7 promoter was needed to abrogate the helicase arrest at the BS3–RTP complex. From these experiments we conclude that invasion of a terminus–terminator protein complex by an RNA transcript *in vitro* causes abolition of contrahelicase activity of the replication terminator protein.

Since the arrest of RNA transcript is polar, an RNA transcript that approaches the terminus towards its blocking end should not affect the arrest of helicase at the BS3 site. This expectation could not be clearly tested *in vitro* because of the incomplete nature of the arrest of the RNA transcripts at the replication terminus *in vitro* (see Figures 2 and 3). This question was examined *in vivo* and has been presented later.

Transcriptional invasion causes release of replication forks arrested at the terminus in vivo

We wished to investigate if the transcriptional abrogation of the contrahelicase activity *in vitro*, described above, also occurs *in vivo*, as manifested in the release of replication forks arrested at a replication terminus. We constructed three plasmid replicons each one of which contained a unidirectional, ColE1 (pUC18/19) origin of replication and the β-lactamase (ampicillin resistance) marker (Figure 5A). The plasmids pUCτ2 and pUCτ2rev contained the very efficient τ2 replication terminator of *E.coli* (Hill *et al.*, 1988) in the functional and non-functional orientation, respectively. The plasmid pUCτ2Pr included, in addition to the τ2 in the functional orientation, the A1 promoter of phage T7 that directed an RNA transcript (of *E.coli* RNA polymerase) passing through the τ2 sequence and terminating at a transcription terminator that prevented the transcript from entering the replication origin (Figure 5A).

The three plasmids were transformed into the tus$^+$ *E.coli* host (strain TH423) and grown in LB medium containing ampicillin. Replication intermediates were isolated as described in a later section. The DNAs were digested with *Hin*dIII and analyzed in Brewer–Fangman 2D gels (see Brewer and Fangman, 1987; Khatri *et al.*, 1989). A representative set of autoradiograms is shown

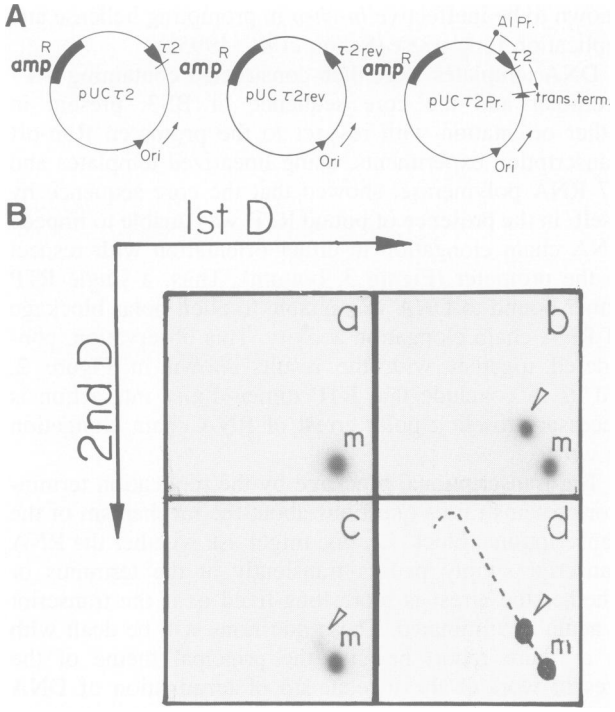

Fig. 5. Effect of transcription on replication termination *in vivo*. (**A**) The plasmids pUCτ2, pUCτ2rev and pUCτ2Pr contained the β-lactamase marker (amp^R), origin of replication (Ori) and the replication terminator τ2. The pUCτ2Pr also included the T7A1 promoter and a transcriptional terminator (▲) that prevented the transcript from T7A1Pr from entering the replication origin. Each plasmid also contained a lac promoter (not shown) that was kept repressed in a lacI^q background. (**B**) Autoradiogram of a 2-dimensional Brewer–Fangman gel showing severe reduction in the ability of a replication terminus to block fork movement *in vivo* as a result of an invasion by an RNA transcript. (**a**) Replication profile of pUCτ2rev; note the prominent monomer (m) spot but the total absence of any termination intermediate. (**b**) In contrast, the pUCτ2 sample has a very prominent termination intermediate (arrowhead). (**c**) The invasion by RNA polymerase of the τ2 sequence in the pUCτ2Pr DNA significantly inactivates the τ2 terminus thus resulting in a feeble termination spot (arrowhead) as contrasted with (b). (**d**) Diagrammatic representation of the expected, ideal replication profile of pUCτ2 DNA; m denotes monomer, the arrowhead denotes the termination intermediate. Note that the termination spot in panel c is at a slightly different location compared with panel b because the pUCτ2Pr DNA has an extra length of DNA that includes the T7A1 promoter and transcription terminators.

in Figure 5B. The pUCτ2rev DNA, as expected, did not show any detectable arrest of replication fork because of the non-functional orientation of the replication terminus with respect to the origin (Figure 5B, panel a). The τ2 terminus in pUCτ2 plasmid caused efficient arrest of the replication fork yielding a termination spot that was at least as strong as the monomer spot that represents the fully replicated plasmid DNA (Figure 5B, panel b). The pUCτ2Pr plasmid, in contrast, yielded a much lower level of termination intermediates (~5% of the plasmid DNA) that was consistent with abrogation of termination function of τ2 by the invading A1 transcript (Figure 5B, panel c; compare with panel b). The question of the effect of a transcript approaching the blocking end of the replication terminus on replication termination is described below using a lac promoter.

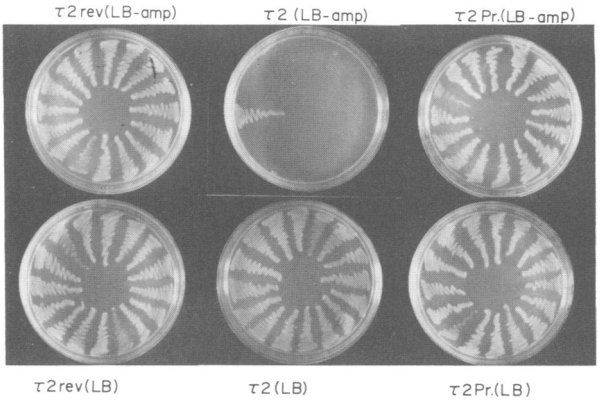

Fig. 6. Photograph showing an *in vivo* test of the transcriptional abrogation of replication termination. The plasmids shown in Figure 5 (top) were transformed into the tus$^+$ *E.coli* strain DH5α and plated on LB plates containing ampicillin. Colonies were picked and subcultured on LB plates without ampicillin for several generations and then streaked on LB plates and LB plates containing ampicillin. Top row shows cultures on LB plates containing ampicillin; bottom row shows cultures growing on LB plates. Note that 15 out of 16 colonies have lost the pUCτ2 plasmid. In contrast, all 16 colonies have retained the pUCτ2Pr. The control pUCτ2rev plasmid has not been lost during growth in non-selective medium.

The inhibitory effect of a strong replication terminus on plasmid maintenance is overridden by the passage of an RNA transcript through the terminus sequence

We wished to confirm further the ability of an RNA transcript functionally to override a replication terminus *in vivo* as manifested by the increased frequency of survival of a plasmid carrying a strong replication terminus. The following experiment is based on the observation that unidirectionally replicating plasmids containing the τ2 terminus in a functional orientation, with respect to the *ori*, are eliminated unless there is a strong selection pressure to maintain the plasmid in the host cell (Hidaka *et. al.*, 1988). We have observed that the plasmid remains stable in isogenic tusΔ host, thereby showing that the fork arrest at τ2 was the reason for the instability (data not shown).

For the plasmid maintenance experiments, we used the same set of three plasmids, pUCτ2, pUCt2rev and pUCτ2Pr, described above (Figure 5A). *Escherichia coli* DH5α (tus$^+$) cells were transformed separately with the three plasmids under selection for ampicillin resistance. Sixteen colonies from each of the three sets of transformants were picked at random and grown in LB medium without ampicillin for three cycles, each cycle of growth being 16–20 h. The cultures were then streaked on LB plates containing ampicillin to check for the frequency of plasmid loss, and on LB plates to check cell viability. The results showed that only one out of the 16 colonies containing the pUCτ2 plasmid survived after growth in non-selective medium whereas all 16 of the colonies of pUCτ2Pr survived under identical conditions (Figure 6). All 16 of the pUCτ2rev-containing colonies survived under the same conditions. As an additional control we have compared the stability of the same plasmid with the T7 A1 promoter located at the same position with respect to the origin but without a τ site and have observed that the activity of the promoter *per se* had no detectable effect

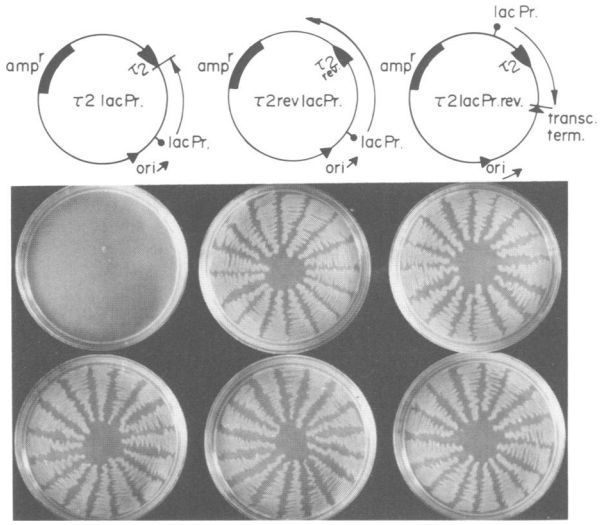

Fig. 7. A promoter directing a transcript at the blocking end of τ2 terminus was unable to overcome replication fork arrest at the terminus. Top row: structure of τ2lacPr., τ2revlacPr. and τ2lacPr.rev. plasmids. Note that in τ2lacPr. and τ2lacPr.rev. the promoter (lacPr) directs transcript at the blocking end and the non-blocking end of the τ2 respectively. Middle row: frequency of loss of the plasmids τ2lacPr. (left), τ2revlacPr. (center) and τ2lacPr.rev. (right) on ampicillin plates. Note all 16 randomly picked clones have lost the τ2lacPr plasmid, whereas all 16 have retained the τ2lacPr.rev. plasmid. As expected, all 16 clones retained the τ2revlacPr. plasmid after growth without ampicillin selection. Bottom row: cells streaked on LB plates to check cell survival. Left, center and right plates correspond to the left, center and right plasmids shown in the top row, respectively.

on plasmid stability (data not shown). Thus, the high frequency of loss of the pUCτ2 plasmid, caused by the efficient fork arrest at τ2, was overridden by the RNA transcript initiated from the A1 promoter and passing through the terminus sequence.

It may be noted, when the plasmids were first transformed into the tus$^+$ *E.coli* strain DH5α and grown on LB plates containing ampicillin, the plasmid pUCτ2 yielded very few and small colonies whereas both pUCτ2rev and pUCτ2Pr plasmids yielded colonies with high frequency and with normal growth characteristics. The plasmids did not show any such change in a tusΔ strain (data not shown). It may also be noted that the plasmid pUCτ2Pr grown in the tus$^+$ strain DH5α retains the promoter and the τ site intact.

The experiments and analyses presented above strongly support the proposition that passage of an RNA transcript, *in vivo* and *in vitro*, can functionally inactivate a replication terminus by abrogating the contrahelicase activity of the terminator protein when the RNA transcript approaches and enters the replication terminus from the non-blocking end.

RNA transcripts approaching the replication terminus towards the blocking end in vivo fail to override fork arrest

We constructed three plasmids, each of which included the ColE1 *ori* and the ampicillin resistance marker. The τ2lacPr plasmid contained the replication terminus in the orientation to block both the replication fork originating from the ColE1 *ori* and transcription from a lac promoter (Figure 7, top row, left). The plasmid τ2revlacPr contained

the terminus in the opposite orientation so that neither the replication fork nor the transcription from the lac promoter would be blocked (Figure 7, top row, center). The third plasmid, τ2lacPr.rev, contained a lac promoter directing a transcript towards and through the non-blocking end of the τ2 sequence whereas the replication fork from the ColE1 origin moved towards the blocking end of the terminus (Figure 7, top row, right). The transcript was prevented from entering the origin by a suitably positioned transcription terminator. We decided to use the lac promoter because, for technical reasons, we were unable to recover a recombinant plasmid having the A1 promoter in close proximity to the ColE1 *ori*. The plasmids were transformed into the tus$^+$ *E.coli* host MC1000 (Casadaban and Cohen, 1980) that had a complete deletion of the lac operon including the repressor gene, thus allowing full constitutive expression of the lac promoter of the plasmids. Plasmid survival after growth in non-selective medium was checked as described above in Figure 6, with slight modifications. The results showed that the lac promoter, directing a transcript towards the blocking end of τ2 could not override fork arrest (Figure 7, middle row). As expected, the promoter directing a transcript through the non-blocking end could prevent plasmid loss, presumably by releasing arrested forks.

Discussion

In this paper we have investigated the mutual interaction between termination of DNA replication and transcription. The results presented show that in addition to the known polar contrahelicase activity, the replication termination proteins of both *E.coli* and *B.subtilis* cause polar arrest of transcription elongation catalyzed by the T7, SP6 and *E.coli* RNA polymerases, at the terminator protein–terminus DNA complex. The contrahelicase and transcription-blocking activities of ter and RTP were isopolar, i.e., the same orientation of the terminus sequence, with respect to the promoter or the origin, blocked both RNA and DNA chain elongation and helicase-catalyzed DNA unwinding, upon binding to the terminator proteins.

The observation that RTP and ter impose polar blocks on replicative helicases and RNA polymerases and considering the fact that there is little or no primary sequence homologies between helicases and RNA polymerases, one might wonder if the terminator proteins are simply non-specific, polar clamps that would block the progression of any helicase or RNA polymerase on DNA. Contrary to the claim that the replication terminator protein of *E.coli* blocks most proteins that slide on DNA (Lee and Kornberg, 1992), we and others have reported that both ter (Khatri *et al.*, 1989; Bedrosian and Bastia, 1991; Hiasa and Marians, 1992) and RTP (Kaul *et al.*, 1994; Sahoo *et al.*, 1995a,b) can block the activity of replicative helicases and not that of helicases involved in DNA repair (e.g. helicase II) and conjugational DNA transfer (e.g. helicase I). The helicase-specific contrahelicase activity would suggest protein–protein interaction between the helicases (or RNA polymerases) and the contrahelicases. Although presently, we have no direct evidence for RNA polymerase–ter (RTP) interaction, we have recently observed DnaB–ter (RTP) and SV40 T antigen–ter (RTP) interactions *in vitro* (B.K.Mohanty and D.Bastia, manuscript in preparation). The ter protein was reported to block DNA polymerase I of *E.coli*. However, the extent of the polarity was only 2-fold, suggesting a non-specific road block to polymerase movement (Lee and Kornberg, 1992). We have repeated these experiments and have observed only non-specific blockage of DNA polymerase I by ter protein (T.MacAllister and D.Bastia, unpublished results). The question of specificity of impedance is also addressed by our recent work that shows that a specific point mutant of RTP failed to block DnaB, PriA helicases and DNA replication forks but still imposed polar blocks to elongation of RNA transcripts by T7 RNA polymerase. This result strongly supports the existence of a specific region on RTP that recognizes replicative helicases and further suggests that the requirements for transcriptional block and helicase block are separate. The biochemistry and crystal structure of the mutant form of RTP will be published elsewhere (A.Manna, K.S.Pai, D.E.Bussiere, S.White and D.Bastia, manuscript in preparation).

The Reb1 protein of yeast (Lang and Reeder, 1993; Lang *et al.*, 1994) blocks RNA chain elongation by RNA polymerase I in a polar mode, at a specific binding sequence. We have discovered that Reb1p bound to its cognate site is unable to block T7 RNA polymerase and the DNA duplex unwinding by DnaB helicase (B.K.Mohanty, T.Sahoo and D.Bastia, unpublished results). Thus, any DNA binding protein which is a polar blocker of RNA and/or DNA chains is not necessarily capable of acting as a general polar road block to chain elongation by a different RNA polymerase or to DNA unwinding by a replicative helicase. Precise mapping of the surface of the putative blocking domain of RTP (and ter), isolation of appropriate non-blocking mutants and solving the crystal structure of the non-blocking mutant forms of the protein should settle the issue.

What is the biological significance of the polar arrest of transcription elongation by ter and RTP? In this paper we have also shown that invasion of the terminus–terminator protein complex by an RNA transcript abrogates the helicase and replication fork blocking activities of the terminus–terminator protein complex. The results also show that transcription approaching the replication terminus towards its blocking end fails to disrupt termination of replication because of the polar transcription anti-elongation activity of the terminator proteins. The physiological significance of the results presented in this paper can be understood by considering the results presented here along with some previously published observations discussed below. Taken together, the results strongly suggest that the terminator proteins protect the replication termini from possible inactivation by transcripts invading at least from one direction. The replication termini TerA (τ1), TerB (τ2) and TerC (τ3) of *E.coli* are the most frequently used termini (Pelletier *et al.*, 1988; Louarn *et al.*, 1991). It is interesting to note that each one of these is flanked by sequences that resemble ρ-independent transcription terminators at the non-blocking ends (Hidaka *et al.*, 1988). Thus, both ends of the most frequently used termini of *E.coli* seem to be protected from transcriptional invasion (Figure 8A). It is also worth noting that the τ2 terminus of *E.coli* is located in the path of a transcript that is initiated from the P1 promoter (Figure 8B; Roecklein and Kuempel, 1992). In the tus$^+$ *E.coli*, the transcript is

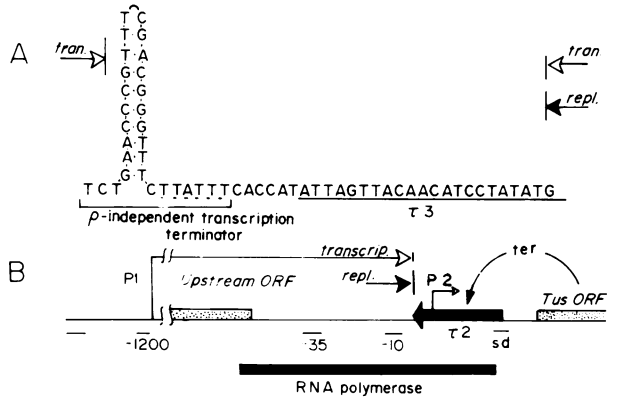

Fig. 8. Diagram showing the sequence around the τ3 (TerC) terminus of *E.coli* and autoregulation of ter (tus) gene expression. (**A**) The τ3 terminus, like τ1 and τ2, has a GC-rich hairpin and T-rich sequence that characterizes a ρ-independent transcription terminator. The directions of ter-mediated block of replication and transcription are shown. (**B**) The promoter region of the *tus* gene of *E.coli*. The ter protein binds to the τ2 operator and displaces the bound RNA polymerase to autoregulate ter synthesis at the transcription initiation step (Natarajan *et al.*, 1991). The transcription initiated from the upstream P1 promoter will be blocked in a polar fashion by ter protein bound to τ2, thus effecting autoregulation at the transcription elongation step (Roecklein and Kuempel, 1992). If the transcripts from P1 were allowed to invade the τ2 sequence, functional impairment of replication termination at τ2 could have resulted.

impeded at the τ2 site whereas in tusΔ cells the transcript proceeds through the replication terminus (Roecklein and Kuempel, 1992). Thus, this example provides a clear case where the terminator protein protects a frequently used replication terminus from transcriptional inactivation.

In the case of *B.subtilis*, the two termini IRI and IRII (TerI and TerII; Figure 1) are located only 59 bp apart (Lewis *et al.*, 1990). It would take two transcripts invading the IRI and IRII from both directions to inactivate the two termini, a situation that probably would be rendered unlikely by natural selection. The fact that IRI is the most frequently used terminus *in vivo* would suggest that it probably is not invaded by a transcript from the non-blocking end. Although most transcription units are terminated at suitably positioned transcription terminators *in vivo*, it should be worth keeping in mind that few transcriptional terminators are non-leaky (Reynolds *et al.*, 1992). Thus, the RNA chain anti-elongation activity of the replication terminator proteins should provide additional insurance against possible transcriptional inactivation.

It may be worth noting that both DnaB helicase and RNA polymerase are moving on the same strand of the helicase substrate used in the experiment shown in Figure 4 but in opposite directions, on a collision course. It appears from the results that the helicase is able to continue unwinding the DNA past the promoter sequence. We do not know what happens to the RNA polymerase after its encounter with the helicase. In a series of very elegant studies B.M.Alberts, E.P.Geiduschek and their co-workers have shown that replication forks generated *in vitro* can pass RNA polymerase molecules that are moving in the same direction as the fork without displacing the transcription complex (Liu *et al.*, 1993). When replication and transcription are on a collision course, in the absence of helicase, the forks stall for several seconds at the RNA polymerase–DNA complex (Liu and Alberts, 1995). Addition of helicase reduces the time of arrest without displacing the RNA polymerase, which switches to the newly synthesized strand and continues making RNA.

In *B.subtilis*, a special set of replication arrest sequences block replication forks at 200 kb from the origin on both arms of the replicon only under stringent conditions (Levine *et al.*, 1995). These conditionally active replication fork-arresting sequences also require RTP. How are the replication arresting sequences selectively turned on under high concentrations of ppGpp? It is tempting to suggest that under relaxed conditions, a promoter directing a transcript into the non-blocking end of the replication arrest sequences could keep the arrest sites in an inactive state. Under high ppGpp concentrations the promoter is shut off (e.g. ribosomal promoters), thus activating the arresting sequences.

In conclusion, we have shown that the replication terminator proteins of both *E.coli* and *B.subtilis* impose polar blocks to elongation of RNA transcripts catalyzed by several prokaryotic RNA polymerases. The possible raison d'etre for such an activity seems to be the compelling need to protect the replication termini from transcriptional inactivation. The ability to block RNA chain elongation probably also contributes to the autoregulation of the *tus* gene of *E.coli*.

Materials and methods

Plasmids and strains

All cloning and plasmid preparations with the plasmids containing the *E.coli* τ2 site were carried out in the tusΔ strain JS117 of *E.coli*. For the BS3 clones all cloning and transformations were carried out in *E.coli* host DH5α. MC1000 is a strain of *E.coli* which lacks the entire lac operon including *lacI* (Casadaban and Cohen, 1980). Construction of plasmids containing BS3 site for T7 and SP6 RNA polymerase transcriptions was carried out as follows: a 59 bp *Eco*RI fragment containing the BS3 site was cloned into pET22b (Novagen) at the *Eco*RI site, in both orientations. For SP6 RNA polymerase reaction *Xba*I–*Hin*dIII fragments containing the BS3 site in either orientation were isolated from the above clones and were cloned into the vector pSP65 (Promega). Construction of plasmids containing the τ site for T7 and SP6 RNA polymerase transcriptions was carried out as follows: a 150 bp *Hin*dIII fragment containing the τ2 site was cloned in both orientations at the *Hin*dIII site of pET22b plasmid. Similarly, for SP6 RNA polymerase reaction the same fragment was cloned into the plasmid pSP65. Construction of clones containing the BS3 site for transcription by *E.coli* RNA polymerase was carried out as follows: *Xba*I–*Sal*I fragments containing the BS3 site in either orientation were cut out from the pET22bBS3 clones described above and cloned into M13mp18. A *Kpn*I–*Xba*I fragment containing the promoter of the T7A1 gene was cut out from the plasmid pRL418 (Chan and Landick, 1989; kindly provided by Dr Rajendra Kumar, NIH) and was cloned in front of the BS3 site in M13mp18BS3. DNA fragments were cut out from the RF DNAs containing the T7A1 promoter and BS3 site in both orientations and were purified from the gels run without ethidium bromide, extracted successively with phenol, phenol:chloroform:isoamyl alcohol, chloroform:isoamyl alcohol and precipitated with ethanol. Construction of DNA substrate for transcription induced release of helicase blockage was done as follows: a *Bgl*II–*Bsp*EI fragment containing the T7 promoter and the BS3 site in reverse orientation with regard to transcription blockage was derived from pET22bBS3rev and was cloned into a *Bam*HI–*Ava*I digest of M13mp19 to construct the clone M13mp19BS3T7Pr. Construction of DNA clones for plasmid stability assay were made as follows: a *Hin*dIII fragment containing the *E.coli* τ2 site was cloned in both replication blocking and non-blocking orientations at the *Hin*dIII site of pUC18 to make the clones pUCτ2 and pUCτ2rev respectively. An *Xba*I fragment containing the τ2 site was cloned into pRL418 at *Xba*I site in replication blocking and transcription non-blocking orientation to construct the clone pUCτ2Pr.

Protein purification

T7 RNA polymerase (Davanloo *et al.*, 1984) was purified from the overproducer pAR1219 (kindly provided by Dr A.Rosenberg, Brookhaven National Laboratory, NY) according to the published procedure (Grodberg and Dunn, 1988). RTP was purified according to Mehta *et al.* (1991). *Escherichia coli* RNA polymerase purification has been published elsewhere (Burgess and Jendisak, 1975; Natarajan *et al.*, 1991). Ter was purified from the overproducer plasmid pGK1 (Khatri *et al.*, 1989) by the following procedure: after induction with IPTG for 3 h the cells were lysed with lysozyme by freeze–thaw. After a 70% ammonium sulfate precipitation step the pellet was dissolved in buffer A containing 20 mM Tris–HCl pH 7.5, 20 mM NaCl, 1 mM EDTA, 10% glycerol, 10 mM β-mercaptoethanol and was dialyzed against the same buffer for 10–12 h. The dialysate was centrifuged and loaded on a DEAE–cellulose column equilibrated with the same buffer. The flow-through was loaded on a heparin agarose column and eluted with an NaCl gradient of 0.02–1 M in buffer A. The peak containing ter was pooled, dialyzed against buffer A and loaded on a S–Sepharose column equilibrated with buffer A and eluted with a linear gradient of 0.02–1 M NaCl in the same buffer. The peak was dialyzed against buffer A and loaded on a MonoS column (Pharmacia). The protein was eluted with a linear gradient of 0.02–1 M NaCl in buffer A. The peak fractions were pooled, dialyzed against buffer A, made to 50% glycerol and frozen in liquid nitrogen and stored at –70°C.

In vitro transcription

All the components of transcription reaction for T7 and SP6 RNA polymerases were assembled at room temperature to avoid precipitation of DNA as the reaction buffer contained spermidine. A typical reaction mixture for T7/SP6 RNA polymerase contained in 20 µl: 40 mM Tris–HCl, pH 7.9, 6 mM $MgCl_2$, 2 mM spermidine, 10 mM DTT, 20 units of RNasin (Promega or Pharmacia), 0.5 mM each of ATP, CTP, UTP, 12 µM GTP and 20 µCi [α-^{32}P]GTP (800 Ci/mmol) and 0.2 pmol of linearized DNA. Before the addition of the RNA polymerase, RTP or ter was added to the reaction mixture and incubated at room temperature for 10 min. The reaction was started by addition of 20 units of commercial T7 RNA polymerase (NE Biolabs), 1 pmol of purified T7 RNA polymerase or 20 units of SP6 RNA polymerase (NE Biolabs) and incubated at 37°C for T7 RNA polymerase and at 40°C for SP6 RNA polymerase. After 1 h, 1 unit of RNase-free DNase I (Pharmacia) was added and further incubation was done for additional 30 min. The reaction mix was extracted once with phenol:chloroform:isoamyl alcohol and once with chloroform:isoamyl alcohol. RNA was precipitated, dried and dissolved in diethyl pyrocarbonate (DEPC)-treated water (Sambrook *et al.*, 1989). Equal counts were mixed with RNA sample buffer, heated at 60°C for 5 min and loaded on a 6% polyacrylamide–7 M urea gel. The gels were dried and exposed to X-ray films.

The transcription with *E.coli* RNA polymerase was performed as follows: a 25 µl reaction mixture containing 40 mM Tris–HCl, pH 8.0, 10 mM $MgCl_2$, 5 mM DTT, 50 mM KCl, 50 fmol DNA containing T7A1 promoter and BS3 site in either orientation, 400 µM each of ATP, CTP and UTP, 10 µM cold GTP plus 20 µCi of [α-^{32}P]GTP, 50 µg/ml gelatin and 250 fmol *E.coli* RNA polymerase. The reaction mixture was assembled without RNA polymerase but with RTP in appropriate tubes and incubated at room temperature for 10 min and then RNA polymerase was added and reaction was carried out at 37°C for 30 min. The reaction was stopped by addition of 1 unit of RNase-free DNase I and a further incubation was carried out at 37°C for 20 min. The RNA was extracted with phenol:chloroform:isoamyl alcohol, chloroform:isoamyl alcohol and precipitated with one-third volume of 10 M ammonium acetate and 2 volumes of ethanol. After 30 min at –20°C the RNA was precipitated and dissolved in 20 µl of DEPC-treated water. Equal counts were taken in the RNA sample buffer and run in a 6% acrylamide–7 M urea gel. The gels were dried and exposed to X-ray films. Transcription-induced helicase deblocking assay was as follows: ssDNA of the M13mp19-BS3T7Pr construct was prepared according to Sambrook *et al.* (1989). The M13 universal primer was annealed and extended by Sequenase version 2.0 (United States Biochemicals) in the presence of [α-^{32}P]dATP and dideoxy-TTP as described in Sahoo *et al.* (1995a). The reaction mixture in 20 µl volume contained 20 fmol DNA substrate (typically 60 000 c.p.m.), 1.0 or 1.5 pmol RTP, 200 fmol T7 RNA polymerase, 2 pmol DnaB in the transcription/helicase buffer containing 40 mM Tris–HCl, pH 7.9, 6 mM $MgCl_2$, 50 mM potassium glutamate, 5 mM DTT, 2 mM ATP, 0.5 mM each of CTP, GTP, UTP and 50 µg/ml BSA. The reaction was assembled with all the reagents without the RNA polymerase and DnaB but with RTP at room temperature for 15 min to bind RTP to the BS3 site on DNA; the reaction was then started by adding RNA polymerase and DnaB. After 30 min incubation at 37°C the reaction was stopped by addition of the stop dye containing a final concentration of 5% sucrose, 50 mM EDTA, 0.4% SDS and bromophenol blue. The reaction mixture was analyzed by a non-denaturing gel containing 8% polyacrylamide run with 1× TBE buffer.

Plasmid stability assay

The assay was done according to Hidaka *et al.* (1988) with minor modifications. Briefly, plasmids were individually transformed into the tus$^+$ strain DH5α of *E.coli* and cells were grown on LB plates containing 50 µg/ml ampicillin. All plasmids except the one with τ2 site in replication-blocking orientation resulted in large numbers of normally growing colonies on the plates. Colonies were picked up for each type of plasmid and serially subcultured on LB plates three times. The plates were incubated at 30°C. After subculturing, the cells were streaked on LB plates and LB plates with ampicillin and incubated at 37°C overnight. All experiments were repeated at least two or three times. The plasmid stability assay with both lac promoter and replication origin firing from the same direction (see Figure 7) was done in the *E.coli* strain MC1000 that does not produce lac repressor.

Identification of in vivo replication termination intermediates

The replication intermediates of the plasmids pUCτ2, pUCτ2rev and pUCτ2Pr were prepared from the *E.coli* tus$^+$ host strain TH423 (a gift from Dr T.Hill, Drexel University, Philadelphia, PA) according to Horiuchi *et al.* (1987), digested with *Hin*dIII and resolved by 2D gel electrophoresis (Brewer and Fangman, 1987, 1988). Southern transfer and hybridizations were according to Sambrook *et al.* (1989). The DNAs were probed with pUC18 labeled with [α-^{32}P]dATP by random primer labeling according to the manufacturer's recommendations (New England Biolabs).

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