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Escherichia coli replication terminator protein impedes simian virus 40 (SV40) DNA replication fork movement and SV40 large tumor antigen helicase activity *in vitro* at a prokaryotic terminus sequence

(origin trap/termination of DNA replication/contrahelicase)

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ABSTRACT **We have discovered that the *Escherichia coli* terminator protein (Ter) impedes replication fork movement, initiated *in vitro* from the simian virus 40 replication origin by the large tumor antigen (TAg), at the terminator site (τR) of the prokaryotic plasmid R6K preferentially when τR is present in one orientation with respect to the origin. We also have discovered that Ter impedes helicase activity of TAg at the τR site, when τR is in this same orientation. In contrast with Ter, a mutant *Eco*RI protein (*Eco*RIgln111) that binds with high affinity to but does not cleave at *Eco*RI recognition sequences impedes both simian virus 40 fork movement and the helicase activity of TAg in an *Eco*RI-site-orientation-independent manner. These results suggest that a feature common to both TAg and prokaryotic helicases may recognize the Ter–τR complex resulting in a polarized pause in fork propagation and DNA unwinding. In contrast, the effect of *Eco*RIgln111–DNA complex on these reactions may be based on steric hindrance.**

DNA replication of *Escherichia coli* and its plasmid, R6K, terminates *in vivo* and *in vitro* at specific sequences called ter and τ, respectively (1–11). Replication forks, initiated from these prokaryotic origins *in vivo* and *in vitro*, are impeded by the *E. coli* terminator protein, Ter (Tus), only in one orientation of the ter or τ sequence with respect to the origin. The opposite orientation imposes no detectable barrier to fork movement (1, 7).

Retardation of fork progression is dependent on sequence-specific binding of Ter protein, encoded by the *E. coli tus* gene, to τ sites (1, 12). Ter also inhibits the DNA unwinding activity of *E. coli* DnaB helicase with the same τ polarity *in vitro* (8, 9). The polarity of Ter contrahelicase activity is reflected structurally in the asymmetric τ sequences to which a Ter monomer binds (12).

Aspects of eukaryotic DNA replication have been elucidated by studies of replication of the simian virus 40 (SV40) genome (13–18), which does not have a specific termination sequence (19). SV40 large tumor antigen (TAg) is the SV40 replication initiator protein. It also has DNA helicase activity and translocates on DNA mostly in the 3′ → 5′ direction (20, 21). This polarity of movement is similar to that of *E. coli* helicase II (22) but is different from that of DnaB helicase, which translocates on DNA in the 5′ → 3′ direction (23).

We investigated whether the effects of the interaction of *E. coli* Ter with τ sites in prokaryotic systems on replication fork movement and helicase activity could be extended to eukaryotic systems. Our rationale was as follows: In the short term, we were interested in exploring whether eukaryotic fork movement was impeded at a cloned τ site possibly because of some common features shared by prokaryotic and eukaryotic DNA helicases that were recognized by the Ter contrahelicase. In the longer term, we wish to explore whether inhibition of eukaryotic fork movement at τ sites can provide an approach to localization of eukaryotic chromosomal replication origin sequences. In principle, when Ter interacts with two τ sites positioned on either side of a putative bidirectional origin fork movement will be blocked at these sites, thereby creating an "origin trap." Analysis of newly synthesized DNA from the blocked replication intermediates will reveal the origin location.

In this report, we describe the analysis of the effects of the Ter–τ interaction on SV40 replication and TAg helicase activity *in vitro*. Also, we describe, for comparison with Ter, the effect of a mutant *Eco*RI restriction enzyme, *Eco*RIgln111, on the same activities. The mutant enzyme binds to *Eco*RI sites on DNA with very high affinity but with no detectable DNA cleavage activity (24). These experiments are the first step toward attaining the above objectives and using the Ter–τ interaction as an origin trap to localize eukaryotic origins.

MATERIALS AND METHODS

DNA Templates and Substrates. Plasmids containing the SV40 replication origin and the R6K terminator site (τR) in both orientations were constructed as described below.

The starting plasmid pOR.HSO has the *Hin*dIII–*Sph* I fragment of SV40 DNA containing the wild-type SV40 origin (S-ori) and was a generous gift from T. J. Kelly (25). The plasmid pCB.HsτcorrR was constructed by subcloning an ≈200-base-pair (bp) *Sph* I–*Kpn* I fragment containing the R6K τR site from a pUC19 derivative of pUC18τ80 (10) into *Sph* I/*Kpn* I-digested pOR.HSO. Plasmid pCB.HSτoppR, which contains τR in the opposite orientation, was constructed by subcloning the ≈180-bp *Xba* I–*Pvu* II τR-containing fragment from pUC18τ80 into *Xba* I/*Pvu* II-digested pOR.HSO.

Additional replication templates used in this study included: pUC.HSO (25), containing the S-ori fragment in the pUC19 polylinker region; and pOR.8-4 (25), containing the S-ori fragment but with a 4-bp deletion rendering the origin nonfunctional. These plasmids were a generous gift from T. J. Kelly. Other plasmids are described in the text.

Substrates for helicase assays were generated essentially as described (8). The partially duplex substrates to test Ter function, M13mp18τR and M13mp19τR, had τR in the functional orientation and the opposite orientation, respectively. Likewise, partially duplex substrates for studying the effect of *Eco*RIgln111 were prepared by hybridizing two 5′-end-labeled 26-base oligonucleotides, ER18 (5′-TACCGAGCTC-

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Abbreviations: SV40, simian virus 40; TAg, SV40 large tumor antigen; S-ori, SV40 origin; nt, nucleotide(s).

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GAATTCGTAATCATGG-3′) and ER19 (5′-GACGGC-CAGTGAATTCGAGCTCGGTA-3′), complementary to the single *Eco*RI restriction site in the recombinant τR-containing M13 templates. Each substrate, M13mp18ER18 and M13mp19ER19, respectively, had the *Eco*RI site in one of the two possible orientations.

Enzymes. TAg was obtained from Molecular Biology Resources (Cambridge, MA). *E. coli* Ter protein and DnaB helicase were prepared as described (8) and were generous gifts from members of the laboratory. Bacteriophage T4 DNA-dependent ATPase (Dda), helicase II, and *Eco*RIgln111 were generous gifts from B. Alberts (University of California, San Francisco), S. Matson (University of North Carolina, Chapel Hill, NC), and P. Modrich (Duke University), respectively. Enzymes were greater than 90% pure.

***In Vitro* SV40 DNA Replication Assays.** HeLa-cell cytoplasmic extracts were prepared as described by Wold *et al.* (26). The conditions for replication reactions were essentially as described by Wold *et al.* (26) except that 50 mM potassium glutamate also was included. Replication products were purified for analysis as described by Stillman and Gluzman (17).

Helicase Assays. All helicases were assayed under the same conditions essentially as described (8). In all reaction mixtures, 34 fmol of labeled DNA substrate was used.

RESULTS

Ter Protein Impedes Replication Fork Progression Initiated from the SV40 Origin *in Vitro* at a τR Site in an Orientation-Dependent Manner. The general scheme for studying the effect of Ter–τR interaction on SV40 DNA replication *in vitro* is shown in Fig. 1 (*Upper*). Replication forks initiated from the origin (ori) move bidirectionally but, if rightward fork movement is stalled at τR in the presence of Ter, then two types of newly synthesized DNA strands will be generated. Very early intermediates will have both the continuously synthesized leading-strand extending from ori to τR and the lagging strands (Okazaki fragments). On the other hand, if the Okazaki fragments have been ligated, cleavage at a unique restriction site within the replication loop (RE cleavage, Fig. 1 *Upper*) would generate newly synthesized DNA strands that extend from RE to τR. Thus, enrichment of a nascent DNA strand of the predicted length, after replication *in vitro* in the presence of Ter and cleavage at the unique restriction site, would be a clear demonstration that rightward fork movement is stalled specifically at τR.

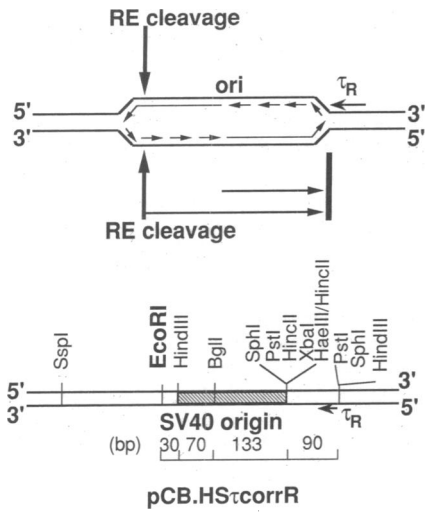

FIG. 1. (*Upper*) Replication intermediate with a bidirectional replication origin and a rightward replication fork stalled at the termination site (τR) in the presence of Ter. Cleavage at a unique restriction site (RE) within the replication loop would generate a newly synthesized DNA strand that extends from RE to the pause site at τR (indicated by a long horizontal arrow and a vertical bar, respectively), provided the Okazaki fragments (→→→) are ligated to each other. (*Lower*) Restriction map of SV40 DNA replication origin region of pCB.HSτcorrR. The numbers below the map indicate the distance in bp from the *Eco*RI site to τR. The complementary plasmid pCB.HSτoppR is described in the text.

Double-stranded supercoiled replication templates, pCB.HSτcorrR and pCB.HSτoppR, were used to study the effect of Ter on SV40 replication *in vitro*. The orientation of τR in pCB.HSτcorrR is indicated by the arrow shown in the restriction map; Ter bound to τR impedes movement toward the arrowhead (Fig. 1 *Lower*). As depicted, the τR site is in functional orientation relative to the rightward fork of S-ori. Functional orientation of τR is defined as that which imposes a barrier to prokaryotic replication fork movement in the presence of Ter (8, 10). Note that the unique *Eco*RI restriction site was located ≈323 bp from τR. The complementary plasmid pCB.HSτoppR was virtually identical, except that the τR site was in the opposite orientation relative to S-ori and ≈348 bp from the unique *Eco*RI site. The plasmid pUC.HSO (25) was a template used for *Eco*RIgln111 experiments, since the potential termination site (*Eco*RI restriction site) was approximately the same distance from the same S-ori replication fork as in the τR templates (≈248 bp). The use of templates with a different sequence background, which incorporate different amounts of dAMP, had no effect on the results of replication termination studies in prokaryotes (9–11) and did not seem to affect them in the SV40 system (unpublished data).

The extent of SV40 DNA replication was measured and expressed as pmol of dAMP incorporated (Table 1). As the averaged data from at least three experiments show (Table 1), the reaction was TAg-, ATP-, and S-ori-dependent, confirming that DNA synthesis was authentic replication initiated from S-ori (see pOR.8-4). Authentic replication also was confirmed by analysis of replication products on nondenaturing agarose gels (data not shown). Note also that the level of dAMP incorporation for the pBR322-based templates was consistently ≈5 times less than for pUC-based template (pUC.HSO) containing the same S-ori sequence, which correlates with reports in the literature (17, 25). This did not affect other characteristics of the SV40 replication reaction or its products (ref. 25; unpublished data).

Kinetic analysis of the effects of Ter and *Eco*RIgln111 on SV40 replication *in vitro* was performed. Synthesis of pCB.HSτcorrR and pCB.HSτoppR in the presence of 166 pmol (6 μg) of Ter (Ter/template molar ratio, 4150:1) was inhibited at most 6% relative to the synthesis of the same templates without added Ter (data not shown). In contrast with Ter, 2 pmol of *Eco*RIgln111 (*Eco*RIgln111/template molar ratio, 80:1) inhibited the rate and extent of pUC.HSO replication by 50% (data not shown). This inhibition probably was due to the propensity of *Eco*RIgln111 to bind nonspecifically to double-stranded DNA (24). All replication reac-

Table 1. Requirements for SV40 DNA replication *in vitro*

Change in reaction mixture	Template	DNA synthesis, pmol
None	pOR.HSO	17
	pCB.HSτcorrR	20
	pCB.HSτoppR	19
	pUC.HSO	96
− TAg	Any template	0.18
− ATP	pCB.HSτcorrR/pCB.HSτoppR	1.7
None	pOR.8-4	0.26

Replication reaction mixtures (25 μl) were incubated at 37°C for 2 hr (17, 25).

tions were TAg-dependent, indicating authentic DNA replication.

After incubation at 37°C for various periods of time, *in vitro* replication reaction products were cleaved with *Eco*RI and resolved by denaturing polyacrylamide gel electrophoresis. Analysis of *Eco*RI-cleaved newly synthesized DNA of pCB.HSτcorrR after a 30-min incubation in the absence or presence of Ter protein is shown in Fig. 2. The autoradiogram (Fig. 2 *Upper*) clearly shows that, with increasing amounts of Ter, a DNA band accumulated in the reaction products that was the expected size for an *Eco*RI to τR fragment [323 nucleotides (nt); Fig. 1]. The extent of enrichment of this band increased with increasing amounts of Ter, and this band was not visible in the replication products in the absence of Ter (Fig. 2 *Upper*, compare lane A with lanes C–F). The size of the enriched band was calculated by linear regression analysis of the size of the marker DNAs and was within 25 nt of the predicted value for the *Eco*RI–τR nascent chain. The extent of enrichment of the ≈323-nt band as a function of Ter concentration was quantified by densitometry.

Similar analyses were performed for the template with τR in the opposite orientation relative to S-ori (pCB.HSτoppR). Enrichment of a band migrating more slowly than the corresponding band for pCB.HSτcorrR increased with increasing amounts of Ter; its calculated size was within 25 nt of the predicted value of ≈348 nt (data not shown). The degree of enrichment of both of these bands, representing the stalled nascent DNA chain (*Eco*RI–τR) from each template ("functional" and "opposite" orientation of τR), is shown in Fig. 2 *Lower*. The results indicate that the fork was stalled preferentially when Ter was bound to the functional orientation of τR. In the presence of Ter, the τcorrR orientation was 2.5 times as effective at impeding fork movement as τoppR. Similar results were obtained in two additional experiments (data not shown). At present, it is not clear whether leading and/or lagging strands are stalled at τR.

The effect of *Eco*RIgln111 addition on fork movement of the pUC.HSO template replicated *in vitro* was measured after cleavage at a unique *Hin*dIII site. The mutant *Eco*RI protein also impeded fork movement at the *Eco*RI site as shown by enrichment of an ≈250-nt band, which is the predicted size of a *Hin*dIII–*Eco*RI nascent DNA chain (data not shown). Therefore, steric hindrance by a tight DNA-binding protein also can impede fork movement. However, the efficiency of the pause was at least 5 times less than that imposed by Ter. The overall detrimental effect of *Eco*RIgln111 on the extent of replication made it difficult to study its effects at higher *Eco*RIgln111/template ratios. Analysis of replication products of a template derived from pBluescript II KS+ (Stratagene), containing an *Eco*RI site in the opposite orientation relative to the same S-ori fragment, yielded similar results (data not shown). Thus, no *Eco*RI site orientation preference was observed. Although the double-stranded *Eco*RI recognition sequence is a palindrome, the site is devoid of symmetry when it is approached by the enzyme complex at the replication fork or the helicase on one DNA strand. When reference is made to the orientation of the *Eco*RI site, it is this aspect that is considered.

Ter Protein Impedes the Helicase Activity of SV40 TAg Preferentially when τR Is Present in One Orientation. How does Ter impede SV40 fork movement? Since Ter is a known contrahelicase of prokaryotic helicases (8, 9), an intriguing possibility is that Ter also impedes the DNA unwinding activity of TAg. To test this, two partially duplex circular substrates were constructed with the τR sequence in both orientations, and helicase assays were performed in the absence and presence of increasing amounts of Ter. The helicase assay quantifies the amount of labeled oligonucleotide that is displaced (unwound) from the partially duplex substrate by the helicase reaction. When Ter binds to τR in the functional orientation, its contrahelicase activity prevents displacement of the oligonucleotide by helicase.

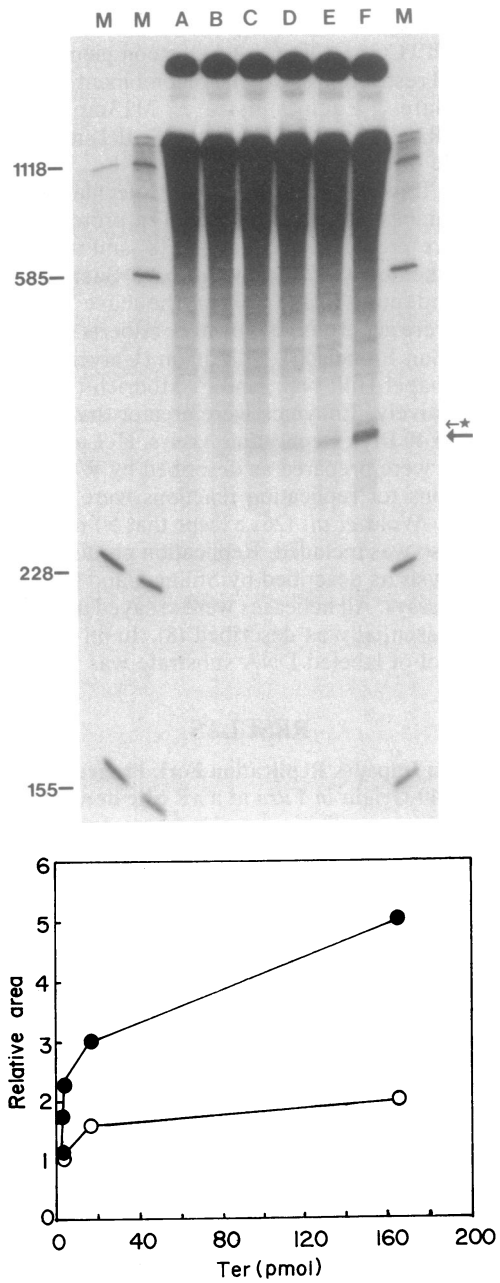

FIG. 2. Pause of SV40 replication fork movement by the Ter–τR interaction. (*Upper*) Autoradiogram of 4% polyacrylamide/50% urea denaturing gel showing the replication products of the template pCB.HSτcorrR (40 fmol/25 μl of reaction mixture), after a 30-min incubation at 37°C alone or with the addition of various amounts of Ter. TAg (1.4 μg) and extract (130 μg) were added to the 25-μl reaction mixture. The reaction products were cleaved at the unique *Eco*RI site (Fig. 1). Lanes: M, DNA size markers; A, *in vitro* replication reaction with no Ter protein added; B–F, 0.83, 1.66, 3.32, 16.6, and 166 pmol of Ter added, respectively. The same number of cpm was loaded in each lane. Numbers designate the size (nt) of marker DNA bands. Large arrow, major enriched band. Note the appearance of a larger enriched band (arrow with star) that may be due to some structural alteration in DNA caused by the addition of a large amount of Ter. (*Lower*) Quantification by densitometry of the newly synthesized DNA stalled at the correct or opposite orientation of τR. Relative area is defined as the percent of the total area under the densitometry tracing (curve) that is at the enriched band position divided by that at the equivalent position in the absence of Ter. Results for pCB.HSτcorrR (●) are compared with those for pCB.HSτoppR (○).

The results (Fig. 3) clearly demonstrated τR-orientation-dependent inhibition of the helicase activity of TAg by Ter; activity was inhibited 69% and 20% at 415 pmol (15 μg) of Ter with the M13mp18τR and M13mp19τR substrates, respectively.

Results of control experiments using *E. coli* DnaB helicase and helicase II revealed, as expected, that DnaB helicase activity was inhibited by Ter preferentially when the M13mp18τR substrate was used (Fig. 3). Helicase II was inhibited by Ter interacting with the opposite orientation of τR present in M13mp19τR (Fig. 3), which confirmed the observations of another group (9). Interestingly, bacteriophage T4 DNA-dependent ATPase (Dda) helicase, which functions in T4 replication *in vitro* to displace proteins bound to DNA (27), was not inhibited by Ter regardless of the orientation of τR (Fig. 3). The data from the helicase II experiments make it highly unlikely that the M13mp18τR substrate is more susceptible to inhibition by Ter due to unforeseen trivial reasons. The possible implications of these results will be discussed later.

***Eco*RIgln111 Protein Impedes DNA Unwinding by Various Helicases in an Orientation-Independent Manner.** The equilibrium dissociation constant of Ter bound to τR is 3 nM (12), which indicates moderate affinity for DNA. In contrast, *Eco*RIgln111 binds to its recognition site with a dissociation constant of ≈2.5 fM (24). This mutant *Eco*RI protein was chosen to investigate whether a strong protein–DNA interaction could impede helicase activity by steric hindrance. It was predicted that this steric effect would be independent of binding site orientation.

By using the appropriate partially duplex substrates that have the *Eco*RI site and flanking sequences in both orientations, helicase assays were performed for TAg, DnaB, helicase II, and Dda helicases in the absence or presence of *Eco*RIgln111. The results of these experiments for TAg and DnaB (Fig. 4) show that *Eco*RIgln111 bound to the *Eco*RI site in both orientations (M13mp18ER18 and M13mp19ER19) impeded the helicase activities of TAg and DnaB 90% and 80%, respectively, beginning at 40 pmol of *Eco*RIgln111 for both substrates. Helicase II and Dda helicases also were inhibited by *Eco*RIgln111 in an *Eco*RI-site-orientation-independent manner (data not shown). Helicase II activity was inhibited by 82%, beginning at 40 pmol of *Eco*RIgln111. However, only 20% inhibition of Dda helicase was observed at 40 pmol of *Eco*RIgln111, which increased to only 60% at 200 pmol of *Eco*RIgln111.

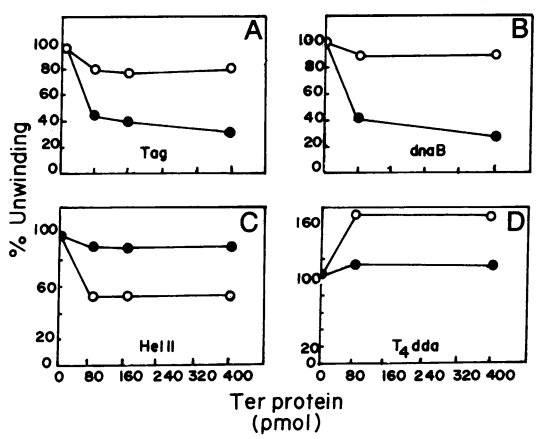

FIG. 3. Ter inhibition of helicase activity. (*A*) TAg. (*B*) DnaB. (*C*) Helicase II (Hel II). (*D*) Dda. Helicase activities tested were 1.4 μg of SV40 TAg (*A*), 0.5 μg of *E. coli* DnaB helicase (*B*), 0.2 μg of *E. coli* helicase II (*C*), 2.25 ng of bacteriophage DNA-dependent ATPase (Dda) (*D*). Thirty-four femtomoles of M13mp18τR (●) or M13mp19τR (○) was used as substrate for each assay (final volume, 20 μl). Percent unwinding was determined by densitometric scanning of autoradiograms. The extent of release of labeled annealed 34-base oligonucleotide by each helicase in the absence of Ter was taken as 100% for each set of experiments. The values are an average of at least four experiments. Note that, in striking contrast to the other helicases, T4 Dda helicase was not blocked by Ter.

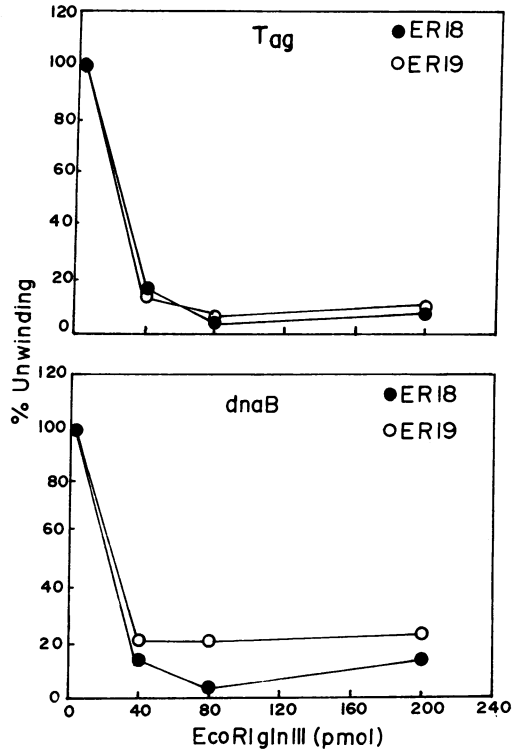

FIG. 4. *Eco*RIgln111 inhibition of helicase activity of TAg and DnaB. Values are an average of two sets of experiments. (*Upper*) Unwinding activity of TAg helicase versus pmol of *Eco*RIgln111 added. SV40 TAg (1.4 μg) was used with 34 fmol of M13mp18ER18 substrate (●) or 34 fmol of M13mp19ER19 substrate (○) for each 20-μl assay. Percent unwinding and other designations are as described in Fig. 3. (*Lower*) Unwinding activity of DnaB helicase versus pmol of *Eco*RIgln111 added. *E. coli* DnaB helicase (0.5 μg) was used. Other designations are as described in *Upper*.

Ter impeded the strand-displacement reaction of all the helicases tested, with the exception of Dda, in a polar fashion, whereas *Eco*RIgln111 imposed a nonpolar steric barrier to the strand-displacement reaction. Thus, the contrahelicase activity of Ter bound to τR could not be ascribed completely to steric hindrance. The data suggest that some feature common to TAg, DnaB, and helicase II may recognize the Ter–τR complex thereby impeding their activities.

DISCUSSION

The major findings we report are that the *E. coli* terminator protein Ter impeded SV40 DNA replication fork movement and the helicase activity of TAg *in vitro* in a τR-orientation-dependent manner. In contrast with Ter, *Eco*RIgln111 impeded the same activities in an *Eco*RI-site-orientation-independent manner.

Unlike the strictly orientation-dependent impediment to prokaryotic replication fork movement *in vivo* and *in vitro* by the Ter–τR interaction, impediment to SV40 fork movement was only partially orientation-dependent. It is possible that there is a steric component to the pause of fork movement imposed by the Ter–τR interaction in this system.

SV40 TAg is known to translocate mostly in the 3′ → 5′ direction on DNA. However, the same orientation of τR that impeded SV40 replication fork movement and the helicase activity of TAg also impeded prokaryotic replication fork

movement and the activity of DnaB helicase. This is curious because DnaB is known to translocate 5′ → 3′ on DNA (23). The exact molecular events surrounding the Ter–τR interaction, which are responsible for the pause/inhibition of fork movement and helicase activity, are unknown at present. One possible explanation for the observed results is that a 5′ → 3′ component of TAg helicase activity may be inhibited preferentially by the Ter–τR interaction. This actually may represent an ability of TAg to touch both DNA strands at the replication fork or as it displaces the oligonucleotide. Furthermore, this component of TAg activity may play a more important role in SV40 fork movement than the *in vitro* helicase assay data might indicate (20, 21). In this context, it is interesting to note that TAg has been shown to interact with the lagging-strand polymerase DNA polymerase α (28). A less likely alternative explanation is that the Ter–τR complex impedes activity in this heterologous system when TAg approaches the side of the τR DNA sequence opposite to the side that is approached by the other helicases and that results in inhibition (6, 9).

The observations that DNA unwinding activity of TAg and prokaryotic helicases are inhibited by Ter in a τR-sequence-orientation manner might suggest evolutionary conservation of a common domain that may recognize the Ter contrahelicase–τR complex. A functional domain of Ter may be exposed upon its binding to the asymmetric τR site and only this domain may be effective in impeding helicase activity.

It is interesting to note that catalytic amounts of Dda overcame the potential barrier imposed by the Ter–τR complex in helicase assays. This result suggests that Dda may not share the putative helicase domain of the other helicases tested that may be recognized by Ter.

Prokaryotic replication fork movement was impeded by Ter at a Ter/template molar ratio of 1:1 (8–11). In contrast, retardation of SV40 replication fork movement was detectable first at a molar ratio 41.5:1. The large amount of Ter required to block SV40 fork movement could be due to a Dda-like helicase present in cell extracts capable of displacing Ter. Other possible explanations for the observed results include (*i*) differences in accessibility of the helicase domain or (*ii*) inefficient recognition of the Ter–τR complex by TAg due to degeneracy.

In contrast with Ter, the *Eco*RIgln111 protein caused general inhibition of replication at a protein/template molar ratio of 80:1. This mutant *Eco*RI also has an ≈100-fold higher nonspecific DNA binding affinity than wild-type *Eco*RI (24). Thus, *Eco*RIgln111 also may be binding nonspecifically to the double-stranded DNA template and thereby inhibiting replication. That the DNA nascent chains were impeded less efficiently at a specific *Eco*RI site by *Eco*RIgln111 also could be due to its high general DNA-binding affinity.

Since replication fork movement can be impeded at (or near) τR in the presence of Ter in a representative eukaryotic replication system, Ter and τR should be useful reagents for localizing mammalian replication origins. If two τR sites are positioned on either side of a putative bidirectional origin, in principle, the approximate location of the origin firing region would be revealed by analysis of the replication intermediates. This origin-trap approach could supplement other methods for replication origin localization (29).

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