Termination Complex in *Escherichia coli* Inhibits SV40 DNA Replication in Vitro by Impeding the Action of T Antigen Helicase*

(Received for publication, September 23, 1991)

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DNA replication terminus (ter)-binding protein (TBP) in *Escherichia coli* binds specifically to the terminus (ter) site, and the resulting complex severely blocks DNA replication in an unique orientation by inhibiting the action of helicases. To generalize the intrinsic nature of the oriented ter-TBP complex against various helicases, we tested the potential of the complex to inhibit the action of three helicases, DNA helicase I, simian virus 40 (SV40) large tumor (T) antigen, and helicase B, derived from F plasmid, SV40, and mouse FM3A cell, respectively. The complex impeded the unwinding activities of all tested helicases in a specific orientation, with the same polarity observed in case of blockage of a replication fork, and, as a result, there was a block of SV40 DNA replication in both crude and purified enzyme systems in vitro. As the specificity in polarity of inhibition extends to heterologous systems, there may be common structure/mechanism features in helicases.

Much knowledge has been gained regarding the mechanism of termination of DNA replication. A DNA replication termination site, which severely arrests the DNA replication fork, is present on the plasmid R6K (Bastia et al., 1981; Horiuchi and Hidaka, 1988) and on the genomes of *Escherichia coli* (de Massy et al., 1987; Hill et al., 1987) and *Bacillus subtilis* (Carrigan et al., 1987; Lewis et al., 1989). In eukaryotic cells, functionally similar sites have been found in yeast (Brewer and Fangman, 1988) and plant (Hernandez et al., 1988) cells.

To block progress of the DNA replication fork in *E. coli*, two factors are required. One factor is the ter sequence of ~22 bp, 1 pair or more of which is arranged in an inverted position on the DNA molecule (Hidaka et al., 1988; Hill et al., 1988). The sequence has the potential to inhibit travel of the replication fork in a specific direction. Another factor essential for the termination reaction is the ter-binding protein (TBP), encoded by the tau(tus) gene, which binds specifically to the ter sequence (Germino and Bastia, 1981; Hidaka et al., 1989; Hill et al., 1989; Kobayashi et al., 1989; Sista et al., 1989).

From the in vitro reconstructed oriC plasmid replication system of *E. coli* with the ter sequence and purified TBP, it was found that no protein other than TBP appears to be necessary for the termination reaction. Termination reaction, dependent on both the ter sequence and TBP, can occur in an in vitro reconstructed system in the same manner as in vivo (Lee et al., 1989).

DNA helicase, an enzyme with the potential to unwind the double-stranded DNA to become a single strand, is thought to head the DNA replication machinery, and DNA synthesis thus proceeds. The effect of the ter-TBP complex on the helicase activity was investigated, using a partial duplex DNA substrate (Lee et al., 1989). Three tested helicases, dnaB, rep, and uvrD proteins, were all inhibited by the complex only in one direction, with the same polarity as observed for blockage of a replication fork in vivo. These observations suggested that DNA fork blockage by the complex is responsible for inhibition of the helicase.

To examine the generality of the ter-TBP complex to inhibit various types of helicases, we applied the TerB-TBP complex in *E. coli* to the simian virus 40 (SV40) in vitro DNA replication system. We obtained evidence that it blocked the SV40 DNA replication by impeding the action of SV40 large tumor (T) antigen. Bedrosian and Bastia (1991) independently examined the effectiveness of the *E. coli* termination system in SV40 DNA replication in *vitro* and reported similar results. However, the polarity of inhibition of the SV40 T antigen by the complex which they determined is exactly the opposite of what our evidence suggests.

**MATERIALS AND METHODS**

Preparation of the DNA Helicase Substrates—The 5.0-kb EcoRI fragment containing the TerB sequence was inserted into the EcoRI- cleaved site of M13mp10 DNA with both orientations, from which the viral, single-stranded circular M13TerB-CCW or M13TerB-CCW DNA was prepared. A 10-fold excess (molar ratio) of 30-mer oligonucleotides containing the TerB sequence (5'-TATAAAATAG- TATGTGTGAACTAATAAGTG-3' or 5'-CCACTTTAGTTACAA-

CATACCTAATTITATA-3'), the 5'-end of which was labeled with P, was annealed to either single-stranded DNA, respectively, in 10 mM Tris-HCl (pH 8.0), 100 mM NaCl, and 1 mM EDTA at 100 °C for 2 min and cooled slowly to 50 °C. Incubation was continued for 1 h at 50 °C, and then the samples were cooled to room temperature. The template, with the 30-mer oligonucleotide annealed to it, was purified by a Bio-Gel A-5m (1.0 ml) gel filtration, extracted with phenol and chloroform, precipitated with ethanol, and dissolved in TE (10 mM

5361
Tris HCl (pH 8.0), 1 mM EDTA solution.

**Assays of Helicases:** DNA Helicase I, SV40 T Antigen, and Helicase B—Assays of helicases were as described (Lee et al., 1989). In brief, the reaction mixture (20 μl) contained 40 mM Tris-HCl (pH 7.6), 5 mM dithiothreitol, 2.5 mM MgCl₂, bovine serum albumin (50 μg/ml), 2.5 mM ATP, and 0.05 pmol (as partial duplexed circle molecules) of TBP, followed by incubation at 37 °C for 10 min, after which the reaction was terminated by adding an equal volume of a stop solution containing 50 mM EDTA, 1% sodium dodecyl sulfate, 20% glycerol, 0.02% xylene cyanol, 0.05% α-phenyl ethyl alcohol, and finally in the fragment (165 bp) opposite the origin (Fig. 2). In the absence of TBP, the label proceeded bidirectionally. The histogram taken at 2 min, based on the amount of label in each fragment (corrected for

**Enzyme and DNA—Preparation of topoisomerase I, the polymerase α-prime complex, and SSS from HeLa cells was as described elsewhere (Ishimi et al., 1988). SV40 T antigen was purified from SV40 T antigen-infected insect cells infected with recombinant baculovirus, as reported (Ishimi et al., 1991). DNA helicase I (Abdel-Monem et al., 1976), helicase B (Seki et al., 1988), and TRP (Hidaka et al., 1989) were also as described previously. T4 polymerase kinase, T7II11, Accl, Ddel, Asel, and Ssp1 were purchased from Takara Shuzo Co., Ltd. (Kyoto, Japan). pSV01AEP (ori-ter) and unfractionated HeLa cell extract were prepared as previously described (Wobbe et al., 1988). Phage M13mp10 was from our laboratory stock.

**RESULTS**

**Ter-TBP Complex Impedes the Action of DNA Helicase I, SV40 T Antigen, and Helicase B—In a report by Lee et al. (1989), the ter-TBP complex was found to impede the action of all three E. coli helicases tested, dNaB, rep, and uvrD proteins, although Khatri et al. (1989) reported opposite results regarding the latter two helicases, rep and uvrD proteins. Thus, the possibility arose that inhibition by the complex of the actions of helicases could apply to various types of helicases in various species, rather than be specific protein-protein interactions.**

We examined the effect of the complex on three other helicases, DNA helicase I (Abdel-Monem et al., 1976), SV40 T antigen (Stahl et al., 1986), and helicase B (Seki et al., 1988), derived from F plasmid, SV40, and mouse FMSA cell, respectively, using the partial duplex DNA fragment containing the terB sequence. Two substrates were constructed, in which 30-mer TerB oligonucleotides were annealed to single-stranded circles to generate the duplex TerB sites in an orientation to block either clockwise (CW) or counterclockwise (CCW) replication (Fig. 1) (see “Materials and Methods”). Helicase activities can be monitored by displacement of oligonucleotides. DNA helicase I, required for the transfer of F plasmid DNA, and helicase B, the function of which is unknown, translocate on the DNA strand in the 5'→3' direction (CW). In contrast, SV40 T antigen, essential for its DNA replication, translocates in the 3'→5' direction (CCW). In the absence of TBP, all three helicases displaced the oligomer with both substrates; hence the ter sequence alone has no effect on the helicase action, as expected (Fig. 2). In contrast, in the presence of TBP (TBP/template molar ratio, 100:1), DNA helicase I and helicase B failed to displace the oligomer when ter was oriented to block in the CW direction; conversely, SV40 T antigen was unable to displace the oligomer when ter was oriented to block in the CCW direction (Fig. 2). Thus, the E. coli ter-TBP complex also blocks the action of two helicases functioning in mammalian cells in a specific direction, and the polarity is the same as observed for blockage of a replication fork in E. coli *in vitro*.

**Construction of pSV01AEPterB (ori-ter) Plasmid—Since the rate of replication fork movement depends on the rate of unwinding, our results led to the notion that the ter-TBP complex can block SV40 DNA replication. We tested this in the SV40 DNA *in vitro* replication system. For the template of replication in vitro, pSV01AEPterB, which contains the TerB sequence, was constructed as follows. About a 2.8-kb pSV01AEP containing the SV40 origin fragment (EcoRI G fragment) (Wobbe et al., 1988) was digested with TthII11 and Accl enzymes, and the synthetic 24-mer TerB oligonucleotide was inserted with the orientation expected to block the CW replication fork (Fig. 3). The presence of the oligonucleotides in the hybrid plasmid was confirmed by DNA sequencing (Sanger et al., 1977).

**SV40 DNA Replication Is Inhibited at the Ter Site in a Crude System—After preincubation of the template DNA with T antigen and HeLa crude extract in the presence or absence of TBP (TBP/template molar ratio, 28:1) at 37 °C for 15 min, DNA synthesis was initiated by adding dNTP, and the incubation was continued at 37 °C for 1, 2, 5, and 10 min. The reaction was terminated at the indicated times, and DNA synthesis was monitored by [α-32P]dCTP incorporation. Purified DNA was subjected to both acrylamide gel and alkaline-agarose gel electrophoresis.

Before acrylamide gel electrophoresis, DNA was digested with DdeI and Asel, a procedure which produces eight fragments, as shown in Fig. 4c. In the absence of TBP, the label first appeared in origin proximal fragments (570 bp, 240 bp) and, with time, sequentially in fragments distal to the origin, and finally in the fragment (165 bp) opposite the origin (Fig. 4d). This would suggest that replication initiated at the origin and proceeded bidirectionally. The histogram taken at 2 min, based on the amount of label in each fragment (corrected for

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**FIG. 1.** Substrates for the three helicases. Left, in M13TerB-CCW, the TerB sequence is oriented to block CCW replication; Right, in M13TerB-CW, the TerB sequence is oriented to block CW replication.
Inhibition of SV40 DNA Replication in Vitro

5363

50 CCW(-) CCW(+)

10 CW(+)

0.6 1.2 1.6 2.4 DNA helicase I (μg)

0 1 2 3 SV40 T antigen (μg)

Helicase B ATPase activities (unit)

FIG. 2. Ter-TBP complex inhibition of helicase activities, in a specific or preferential orientation. The proportion of the displaced oligomers was quantified using a Fuji Bio-Image analyzer BAS2000 and plotted.

FIG. 3. Construction of pSV01ΔEPTerB plasmid. Two complementary synthetic oligomers of 26 (5′-ATACCTTTAGTTTACAAC ATACCTATT-3′) and 25 (5′-CAAATAAGTAATTGTAACATA GT-3′) nucleotides were annealed to generate a duplex containing the TerB sequence and restriction nuclease Thh111I and Acl cohesive ends, and then inserted into the Thh111I and Acl site of pSV01ΔEP plasmid. The TerB site was expected to inhibit the CW replication fork.

size), also indicates a bidirectional DNA synthesis (Fig. 5a). However, the appearance of labeled fragments differed when TBP was present. Labeling of the fragments containing the ter sequence and just right of ter was delayed significantly.

FIG. 4. Fragment labeling of SV ori+ ter DNA in the in vitro replication. After preincubation without deoxynucleotides, DNA synthesis was initiated, and incubation was continued, with or without 50 ng of TBP, at 37 °C for the time indicated above the lanes. Samples were digested with Ddel and AseI, applied to nondenaturing 5% polyacrylamide gel electrophoresis, dried, and autoradiographed. a, DNA synthesis in the crude system, containing SV40 T antigen and the HeLa crude extract. b, DNA synthesis in the pure system, containing SV40 T antigen, DNA polymerase α-primase, HeLa SSB, and topoisomerase I. c, a restriction map of pSV01ΔEPterB is presented. Sizes of the fragments produced by Ddel and AseI digestion are indicated in bp. Replication origin and TerB sites are also shown.

FIG. 5. TBP-dependent inhibition of DNA synthesis at the TerB site. The autoradiogram of each fragment in Fig. 4 was scanned, and the density values were corrected for fragment size. The values shown are for a 2-min replication in the crude system and 10 min in the pure system. The eight fragments generated by the Ddel and AseI digestion are shown on the linearized plasmid (top). Origin and TerB site are also shown.

The product of DNA synthesis was analyzed through alkaline-agarose gel electrophoresis (Fig. 6) before which each sample of DNA was digested with SspI to linearize replicated DNA molecules. After 5 min, the completely replicated linear 2.8-kb fragment was seen both in the absence and presence of TBP. In addition, only in the presence of TBP was a fragment of about 870 bp observed in all samples, and even at 1 min, this length corresponded to that of the origin-containing fragment between SspI site and TerB (No. 1) at which DNA synthesis is arrested (Fig. 6, a and c).

Ter-TBP Complex Inhibits SV40 DNA Replication in a Reconstructed System—To confirm finding of a direct effect of the ter-TBP complex on DNA replication, the same experi-
Inhibition of SV40 DNA Replication in Vitro

The replication products were denatured and electrophoresed on a 1.5% agarose gel in 30 mM NaOH and 1 mM EDTA. The products were digested with Ssp1 prior to electrophoresis but only in the crude system. The gel was fixed, dried, and autoradiographed. Arrowheads indicate unique bands, and the deduced corresponding molecules are shown on the plasmid on the right.

iments were performed in a reconstructed SV40 DNA in vitro replication system, in which only polymerase α is responsible for DNA synthesis (monopolmerase system) (Ishimi et al., 1988; Hurwitz et al., 1990). The template DNA was preincubated with polymerase α/primase. T antigen, HeLa SSB, and topoisomerase I in the presence or absence of TBP (TBP/template molar ratio, 28:1), followed by DNA synthesis reaction for 1, 3, 10, and 30 min. Label in the fragments produced by the digestion of synthesized DNA with DdeI and AseI showed the same results as obtained in the crude system, although the rate of DNA synthesis was slower than that in the crude system (Fig. 4b). In the presence of TBP, DNA synthesis on the 570- and 470-bp fragments is delayed (Fig. 4b), and inhibition of CW DNA replication at the ter site is evident on the histogram at 10 min (Fig. 5b); however, the bidirectional replication is less clear than that in the crude system in the absence of TBP because DNA synthesis proceeds to a large extent at 10 min.

The nascent DNA strands synthesized in this pure system can be identified using alkaline gel electrophoresis (Fig. 6). In the absence of TBP, two major products of long leading strands of 1.4 kb and short lagging strands (Okaazaki fragments) were synthesized in this system (Ishimi et al., 1988). The products of leading strand synthesis in the presence of TBP differed drastically from those in the absence of this protein, though Okaazaki fragments of about 200 bp are common to both situations. The synthesized leading strands of half the length (1.4 kb) of the template plasmid (pSV12EPTerB), initiated from the origin bidirectionally with each meeting at the position opposite the origin, were almost totally converted to two types of molecules of about 530 and 2270 bp. They seem to correspond to the CW leading strand, arrested immediately at the TerB site and the CCW one which reached the TerB site, respectively, as deduced from their length. These results show the complete inhibition or pausing by the ter-TBP complex of at least leading strand synthesis of SV40 DNA in an in vitro reconstructed system.

DISCUSSION

The terminator protein, TBP, a key protein of DNA replication termination reaction, binds specifically to the ter sequence with a specific orientation and inhibits the one-way replication movement. The target for blockage by TBP was found to be the inability of dnaB helicase to separate the duplex DNA (Lee et al., 1989; Khatri et al., 1989). Other helicases, rep and uvrD proteins, which translocate on the DNA strand in a direction opposite that of dnaB protein, are also blocked but only when the TBP is oriented in the other direction. However, Khatri et al. (1989) reported opposite results regarding the latter two helicases, rep and uvrD. In their experiments, the TBP did not block DNA unwinding by rep and uvrD protein; rather a stimulatory effect was noted. Thus, the question of whether or not the interaction between the TBP and helicases as the basis for the blockage is specific had to be addressed.

In the present work, we applied the ter-TBP complex in E. coli to the heterogeneous SV40 in vitro crude and reconstructed replication systems in which the SV40 T antigen acts as a helicase essential for DNA replication. We obtained evidence that the complex does indeed inhibit the 3'→5' unwinding activity of SV40 T antigen and does simultaneously block the DNA synthesis in both systems for SV40 DNA replication. In the reconstructed system, it was clear that leading strand synthesis was strongly inhibited at the ter site and asymmetrical leading strands were synthesized (Fig. 6b). In the crude system, even when the TBP was present, full-length DNA molecules appeared at 10 min and to the same extent as seen in the absence of TBP (Fig. 5a). These molecules are considered alternative ones, produced by asymmetrical fusion of bidirectional (clockwise and counterclockwise) replication forks at the TerB site. Because of the delay of the full-length linear molecules in the presence of TBP compared with those in the absence of TBP (Fig. 5a) and the asymmetrical replication mode when TBP was present (Fig. 5), it is probable that the lagging strands synthesis was also impeded at the ter site. Bedrosian and Bastia (1991) reported similar results that TBP, forming the complex with the TerR derived from the R6K plasmid, impeded SV40 DNA replication fork movement and the helicase activity of T antigen in vitro, in an orientation-specific manner. But a large difference exists between their findings and ours regarding orientation of the ter sequence at which TBP inhibits the helicase activity of T antigen. In in vitro helicase assays, the orientation of the ter sequence forming the partially duplex DNA is opposite in their experiments and ours, when TBP impeded the action of T antigen. The ter sequence present in CCW orientation (see Fig. 1) which inhibited T antigen in our experiments did not do so in theirs, while the CW orientation one which inhibited T antigen in their experiment never did so in our experiment. T antigen translocates 3'→5' on the single-stranded DNA (Goetz et al., 1988; Wiekowski et al., 1988); hence, our notion that the ter-TBP complex can inhibit the helicase activity of T antigen in the same orientation as it blocks DNA replication seems reasonable. In contrast, in their report, the action of T antigen encountering the ter-R-TBP complex was prevented only in the orientation of the ter sequence which did not block DNA replication, when T antigen translocates in a 3'→5' direction. They assumed a 5'→3' component of T antigen helicase activity and thought that it may be inhibited preferentially by the ter-R-TBP complex. The different experimental procedures used may explain the discrepancy. In helicase assays, they titrated the TBP, and a large amount of TBP (1,000–12,000-fold TBP per DNA template) was added, while we titrated the helicases with a smaller amount of TBP (100-fold TBP per DNA template). Another possibility is the difference in the ter sequence used; we used TerB in E. coli which binds TBP more tightly than the terR they used (Kuempel et al., 1989; Sista et al., 1991).

The complex also inhibited DNA helicase I, necessary for strand transfer of F plasmid DNA between E. coli cells (Abdel-
Inhibition of SV40 DNA Replication in Vitro

5365

Monem et al., 1983), and helicase B, partially purified from the mouse FM3A cells, in an orientation-dependent manner. Moreover, it blocked polyoma virus T antigen (Seki et al., 1990 and RecQ protein (Umez u et al., 1990), a helicase encoded by the RecF gene family, recQ. Thus, the complex inhibited all eight helicases tested, derived from virus and mammalian cells as well as E. coli and functioning in repair and recombination as well as replication. The TBP probably blocks the basic action of the helicases to separate strands of the duplex. It is not likely that the TBP blocks helicases by a specific protein-protein interaction. Many or all helicases seem to share a common step to separate double-stranded DNA at which the ter system inhibits the action. If the TBP and ter sequence are used, one can inhibit the helicase activity or progression of the replication fork at a specific site. This approach would be useful for analyzing the mechanisms of action of helicase and components included in replication fork-protein complex, and so on.

On the TBP side, there seems to be a domain inhibiting the basic action of helicases. This helicase-inhibiting domain and the DNA-binding domain might be separable, and if a protein inhibiting the replication fork is present in cells other than E. coli, it might carry the domain with the potential to block helicases. In Bacillus subtilis, the protein essential for DNA replication termination reaction has been isolated, and the primary amino acid sequence deduced from the DNA sequence was determined (Carrigan et al., 1987). Although the sequence has no homology to that of TBP (Hill et al., 1989), there may be homology at the tertiary structure level. The structure of TBP is now being determined in crystallization experiments.

In bacterial conjugation, the rate of DNA transfer from donor to recipient cell, in which DNA helicase I is needed to separate the double strands, is not delayed around the terminus region. The genetic map from the Hfr cross-complementation and the physical map from Bouché (1982) and Kohara et al. (1987) are in good agreement. This is inconsistent with the finding that TBP inhibits the action of DNA helicase I. This helicase may possibly bypass inhibition by the TBP in the Hfr cross by functioning in conjugation with other factors in vivo (Reyers et al., 1991).

Acknowledgments—We thank Drs. A. Kornberg and E. H. Lee, H. Nakayama, and K. Umez u for kindly providing Rep and DnaB proteins as controls for helicase assays and RecQ protein, respectively. A. Kikuchi for the opportunity to collaborate with Y. I., and M. Ohara for helpful comments.

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