JOURNAL OF BACTERIOLOGY, July 1994, p. 4165–4167
0021-9193/94/$04.00+0

Vol. 176, No. 13

Flanking Sequences Affect Replication Arrest at the *Escherichia coli* Terminator *TerB* In Vivo

HÉLÈNE BIERNE,* S. DUSKO EHRLICH, AND BÉNÉDICTE MICHEL

Laboratoire de Génétique Microbienne, Institut National de la Recherche Agronomique, Domaine de Vilvert, 78352 Jouy-en-Josas Cedex, France

Received 14 December 1993/Accepted 5 April 1994

We have analyzed the effect of flanking sequences on Tus-induced replication arrest. pBR322 plasmid derivatives which carry the *Escherichia coli* replication terminator *TerB* at different locations were used. Efficiency of the replication arrest was estimated from the plasmid copy number and transformation frequency of *tus*$^+$ cells. We found that flanking sequences do affect replication arrest efficiency, a weak arrest being correlated with the presence of an AT-rich region which is replicated just before *TerB*. Some sequences located after the replication terminator can also affect replication termination. We propose that the AT-rich regions might impair binding of the Tus protein to the *TerB* sequence or facilitate helicase-induced unwinding of DNA and Tus displacement from the *TerB* site.

Replication of the *Escherichia coli* chromosome is arrested at specific sites located in the terminus region (5, 10). Six such sites have been characterized and designated *TerA* to *TerF* (for a review, see reference 9). An *E. coli* protein named Tus or Tau (12, 16) can bind to these sites. The Tus-*Ter* complex arrests DNA replication in a polar way, by inhibition of the DNA helicases (15, 20). It was shown that both chromosome and plasmid pBR322 replication can be arrested (11). The effect of sequences adjacent to the Tus-*Ter* complex on the arrest of replication has never been examined. In this study, we observed that on pBR322-derived plasmids, the efficiency of the replication arrest is decreased in all cases by an AT-rich region preceding *TerB* and that the sequences located after *TerB* can also affect the Tus-*TerB* complex activity.

Position of *TerB* affects the activity of the Tus-*TerB* complex. We compared the efficiencies of replication arrest on pBR322 derivatives which carry the *E. coli* replication terminator *TerB*, cloned at four different sites within an Em^r gene (pBR-TcC, -N, -H, and -B; Table 1). The arrest at *TerB* was monitored by following the accumulation of specific replication intermediate molecules, which have a theta shape (14) (not shown). As expected, these molecules were present in the *tus*$^+$ cells but not in the Δ*tus* cells. However, they were reproducibly more abundant with pBR-TcC than with other *TerB*-carrying plasmids. This finding suggested that *TerB* is particularly efficient in pBR-TcC. To further test this conclusion, we made use of the observation that *E. coli Ter* sites and the *Bacillus subtilis Ter* region decrease plasmid copy number (8, 26). The copy numbers of the four plasmids carrying *TerB* were compared with that of the parent pBR-Tc. *TerB* had a moderate effect (20 to 25% reduction) in all plasmids except pBR-TcC, in which case a fivefold decrease was observed. This finding confirms a higher efficiency of replication arrest in this plasmid.

We observed that plasmid pBR-TcC transforms *tus*$^+$ cells poorly and thought that this could be due to a higher activity of the Tus-*TerB* complex on this plasmid. The transformation efficiencies of different *Ter*-carrying plasmids were therefore compared in *tus*$^+$ and Δ*tus* strains (Fig. 1). pBR-TcN, -H, and -B transformed both strains equally well. In contrast, the transformation efficiency of pBR-TcC decreased nearly 30-fold in the *tus*$^+$ strain. This test was used to further analyze the reasons for a more efficient replication arrest in this plasmid.

TABLE 1. Plasmids used[a]

Plasmid	Description
pBR-Tc	pBR322 deleted for the *Ava*I-*Pvu*II region (which carries the *rop* gene) and carrying, in the *Cla*I site, the Em^r gene of pE194 (nt 1940–2900 [13]) under the control of an *E. coli* promoter (pCO [19]). The promoter of Tc^r gene is inactive.
pBR-TcC	pBR-Tc with *TerB* in *Cla*I, adjacent to the pBR322 Tc^r gene
pBR-TcN	pBR-Tc with *TerB* in *Nsi*I, 55 bp from *Cla*I
pBR-TcH	pBR-Tc with *TerB* in *Hpa*I, 200 bp from *Cla*I
pBR-TcB	pBR-Tc with *TerB* in *Bcl*I, 575 bp from *Cla*I
pBR-Cm	pBR-Tc having the Tc^r gene (*Hin*dIII-*Sty*I) replaced by the pACYC184 Cm^r gene (*Hin*dIII-*Pvu*II fragment of the Pharmacia vector pKK232-8) devoid of promoter and carrying λt0 (25) in *Hpa*I
pBR-CmC	pBR-Cm with *TerB* in *Cla*I
pBR-CmC1	pBR-CmC with a 446-bp fragment of pUB110, 67% AT, preceding *TerB* (nt 3686–4032 [21])
pBR-CmC2	pBR-CmC with 443 bp of *B. subtilis* chromosome sequence, 51% AT, preceding *TerB* (nt 898–1371 [24])
pBR-CmH	pBR-Cm with *TerB* in *Hpa*I followed by the λt0 transcription terminator
pBR-CmH1	pBR-Cm with *TerB* in the *Hpa*I site, preceded by 397 bp, 40% AT (nt 972–1369 of pBR322), devoid of λt0
pBR-CmH2	pBR-Cm with *TerB* in the *Hpa*I site, preceded by 454 bp, 38% AT (nt 972–1425 of pBR322), and followed by 600 bp, 37% AT (nt 375–972 of pBR322)

[a] The sequence of the oligonucleotide used to insert *TerB* (11) was

5′-CTGCAGAATAAGTATGTTGTAACTAAAGTAG-3′
3′-GACGTCTTATTCATACAACATTGATTTCATC-5′

The sequence of *TerB* was determined for all of the pBR-Tc series, for pBR-CmC, and for pBR-CmH2. nt, nucleotides.

* Corresponding author. Mailing address: Laboratoire de Génétique Microbienne, Institut National de la Recherche Agronomique, Domaine de Vilvert, 78352 Jouy-en-Josas Cedex, France. Phone: 33-1 34 65 25 14. Fax: 33-1 34 65 25 21.

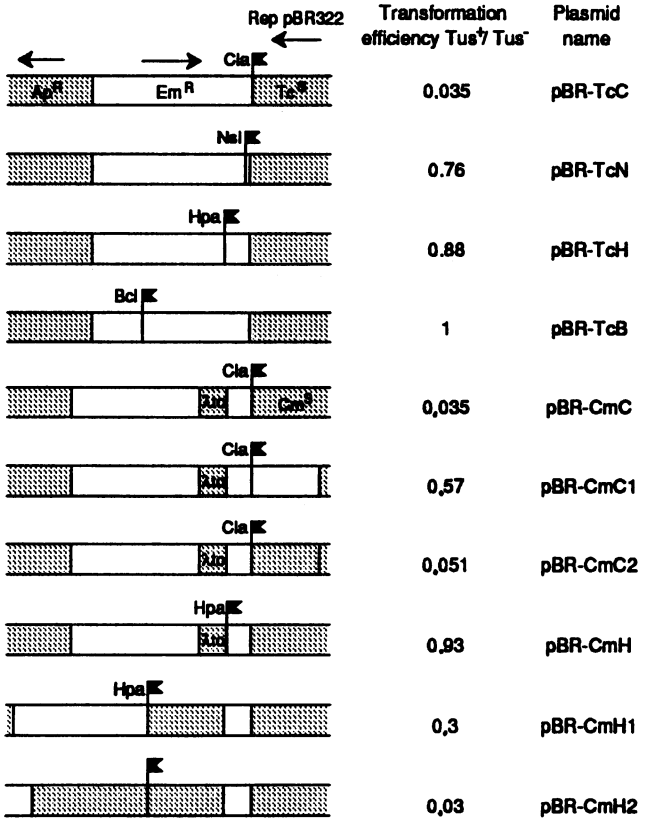

FIG. 1. Schematic representation of the *TerB* region and transforming efficiency of pBR-Tc and pBR-Cm derivatives. Only the Em^r gene and its adjacent sequences are shown; the direction of replication of pBR322 is indicated. Sequences represented as white and grey boxes contain over 63% and less than 52% AT, respectively. The replication terminator *TerB* is represented as a black flag. The transcription terminator λt0 is indicated. Transformation efficiencies were measured as follows. The bacterial strains used were *E. coli* JJC40 (an AB1157 *hsdR* derivative) and JJC33, a JJC40 Δ*tus*::Kan^r derivative (1). Cells were made competent by $CaCl_2$ treatment, transformed at nonsaturating DNA concentrations, and plated on ampicillin (40 μg/ml). Cell competence was measured with pBR-Tc, which transforms both strains equally well. All plasmids conferred the same level of Ap^r in JJC33. Transformation efficiency is the average of 5 to 10 independent determinations. The ratios were determined by dividing the average transformation efficiency in the *tus*$^+$ strain by that in the Δ*tus* strain.

Replication arrest depends on the sequences preceding *TerB*. *TerB* is in a transcribed region in all plasmids except pBR-TcC, since the transcription terminator of Em^r gene is located between *Cla*I and *Nsi*I sites. Transcription may affect Tus-induced replication arrest, since potential transcriptional terminators bracket some *Ter* sites in the *E. coli* chromosome (8). To check the effect of transcription on replication arrest, we introduced a transcription terminator (λt0 [25]) between the promoter of the Em^r gene and *TerB* in pBR-TcN. The resulting plasmid transforms *E. coli* with the same efficiency as pBR-TcN (not shown). Transcription through *TerB* therefore appears not to influence the efficiency of arrest.

The lower transformation efficiency of pBR-TcC is not due to decreased distance between the replication origin and the terminator (not shown). It does not result from a specific effect of the Tc^r gene sequence, replicated before *TerB* in pBR-TcC, since plasmid pBR-CmC (Table 1) transformed *tus*$^+$ cells with 30-fold-lower efficiency than Δ*tus* cells (Fig. 1).

We considered the possibility that the AT content of the region replicated before *TerB* is important. When *TerB* is cloned at the *Cla*I site, this region originates from pBR322 (pBR-Tc) or pACYC184 (pBR-Cm), which both contain 47% AT. When *TerB* is within the Em^r gene (in pBR-TcN, -H, and -B), it is preceded by a region containing 64 to 74% AT. To test the effect of AT content, two DNA fragments of the same length but with different AT contents (67 and 51%) were introduced before *TerB* (plasmids pBR-CmC1 and pBR-CmC2, respectively; Table 1 and Fig. 1). Only the AT-rich fragment allowed efficient transformation of *tus*$^+$ cells (Fig. 1). Furthermore, a plasmid carrying *TerB* within the Em^r gene of pBR-Cm, at the *Hpa*I site, efficiently transformed *tus*$^+$ cells, as expected from the high AT content (74%) of the preceding region (pBR-CmH; Fig. 1).

For all plasmids tested, a low transformation efficiency correlates with the presence of a GC-rich sequence before *TerB*. However, when a pBR-CmH derivative in which *TerB* is preceded by a GC-rich region was used, the transformation efficiency decreased only threefold compared with that of pBR-CmH (pBR-CmH1; Fig. 1). Therefore, the presence of a GC-rich region preceding *TerB* does not systematically favor replication arrest. With the pBR-CmH derivatives, the transformation efficiency decreased 30-fold only when GC-rich sequences were present on both sides of *TerB* (plasmid pBR-CmH2; Fig. 1).

Conclusion. We have characterized two classes of *TerB*-carrying plasmids. Those of the first class transform a *tus*$^+$ and a Δ*tus* strain with similar efficiencies, and their copy number is only slightly affected by the presence of the Tus protein. All plasmids in which the replication terminator is preceded by an AT-rich region belong to this class. Since four different AT-rich regions were tested, a sequence-specific effect can be ruled out. Plasmids belonging to the second class transform a *tus*$^+$ strain 20- to 50-fold less efficiently than a Δ*tus* strain, and their copy number is decreased 2- to 5-fold by the presence of Tus. Three of the plasmids in which *TerB* is preceded by a GC-rich region belong to this class. However, the presence of a GC-rich region before *TerB* does not always impair transformation, since in the pBR-CmH series of plasmids, GC-rich sequences have to be present on both sides of the replication terminator to decrease the transformation efficiency (pBR-CmH2).

Several hypotheses can be considered to account for these observations. It is known that AT-rich sequences have a low melting point and correspond to plasmid regions hypersensitive to single-stranded DNA nucleases (17, 27; reviewed in reference 22). Their melting can induce strand separation of adjacent regions (22). The Tus protein binds efficiently to *Ter*-carrying oligonucleotides of 33 bp in vitro and does not protect more than the terminator site in footprinting experiments (6). However, denaturation of the *Ter* site, induced by adjacent AT-rich regions, could modify Tus binding in vivo. Alternatively, interaction between the helicases and the Tus-*Ter* complex could be modified by the AT content of the preceding region. It is known that the complex acts by arresting progression of the helicases associated with the replication fork (15, 20). Since the free-energy requirement for unwinding of AT-rich regions is relatively low, progression of the helicases might be facilitated by high AT content. The effect of GC-rich sequences may be that of clamping, thus increasing *TerB* efficiency. However, one of the four GC-rich sequences did not have such an effect, which suggests that functions other than primary DNA sequence may intervene. These could be topology, bending, or other features of the secondary or tertiary DNA structure. Finally, a protein which counteracts Tus has

been found (23). Its action may depend on the composition of the flanking sequences.

We did not find any bias in the sequence composition of the regions flanking terminators *TerA*, *TerB*, *TerC*, and *TerF*, which contain between 45 and 53% GC in the *E. coli* chromosome. In contrast, the terminus region of the *B. subtilis* chromosome is particularly AT rich (3). It would be interesting to know whether the efficiency of *B. subtilis Ter* site is also influenced by flanking sequences. Replication pause sites other than *Ter* sites have been described in procaryotes (18, 28) and in eucaryotes (2, 4, 7). It is possible that the AT content of the flanking regions also affects the efficiency of replication arrest at such sites.

We are very grateful to M. Uzest for skillful technical assistance and to D. Vilette for helpful discussions throughout this work. We thank C. Bruand, L. Jannière, D. Vilette, and C. Anagnostopoulos for helpful comments on the manuscript and F. Haimet for the artwork. B.M. is on the CNRS staff.

This work was supported in part by grant BIOT CT91-0268 from the European Community.

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