The EMBO Journal vol.8 no.8 pp.2435 - 2441, 1989

Evidence of a *ter* specific binding protein essential for the termination reaction of DNA replication in *Escherichia coli*

Takehiko Kobayashi, Masumi Hidaka and Takashi Horiuchi

Department of Molecular Biology, Graduate School of Medical Science, Kyushu University, Maidashi, Fukuoka 812, Japan

Communicated by A.Kornberg

Activity binding specifically to the 22 bp of the DNA replication terminus (*ter*) sequence on plasmid R6K and the *Escherichia coli* genome was detected in the crude extract of *E.coli* cells. This activity was inactivated by heat or by protease but not by RNase treatments. Overproduction of the *ter* binding activity was observed when the extract was prepared from the cell carrying a plasmid with a chromosomal-derived 5.0 kb *Eco*RI fragment, on which one of the four *terC* sites, *terC2*, was also located. By mutagenesis of the 5.0 kb fragment on the plasmid with transposon Tn*3* and subsequent replacement of the corresponding chromosomal region with the resulting mutant alleles, we isolated *tau*$^-$ mutants completely defective in *ter* binding activity. These mutants simultaneously lost the activity to block the progress of the DNA replication fork at any *ter* site, on the genome or the plasmid. It would thus appear that the *ter* binding protein plays an essential role in the termination reaction, at the *ter* sites.

Key words: *ter* binding protein/termination reaction/*E.coli*

Introduction

A DNA replication terminus (*ter*) site on replicons is the position at which progress of the DNA replication fork is either arrested or is severely impeded. As the *ter* sites required for termination of DNA replication are present on the plasmid R6K genome and also the bacterial chromosome of *Escherichia coli* and *Bacillus subtilis* (Lovett *et al.*, 1975; Crosa *et al.*, 1976; Kuempel *et al.*, 1977; Louarn *et al.*, 1979; Weiss and Wake, 1983; Iismaa *et al.*, 1984; Monteiro *et al.*, 1984), *ter* may play some physiological role(s) in the process of segregation and partition of the replicons and/or in cell division. The DNA sequence of the *ter* sites and their arrangement have been determined, as described below; however, the mechanism and its physiological role(s) have not been elucidated.

In *E.coli* cells, DNA replication initiates at the unique origin (*oriC*) located at 84 min on the linkage map, proceeds bidirectionally on the circular chromosome, and the two replication forks meet at the directly opposite region of the *oriC*, named *terC* (Bird *et al.*, 1972; Prescott and Kuempel, 1972). In this region spanning *trp* (27 min) and *manA* (36 min) markers, there are two loci (T1 and T2; Hill *et al.*, 1987, 1988b; de Massy *et al.*, 1987), or four sites (*terC1*, *2*, *3* and *4*; Hidaka *et al.*, 1988; M.Hidaka and T.Horiuchi, unpublished) which have activity (called Ter activity) inhibiting movement of the replication fork.

We developed a 'Ter assay' and identified DNA sequences of the *ter* sites present on plasmids R6K, R100 (and its related plasmids) and *E.coli* genomes. All *ter* sequences are essentially the same and their consensus 22 bp sequence is 5'-(A/T)(G/T)TAGTTACAACAPy (A/T)C(A/T)(A/T)(A/T)(A/T)(A/T)(A/T)(A/T)-3'. They possess activity inhibiting travel of the replication fork in only one specific direction (Horiuchi *et al.*, 1987; Hidaka *et al.*, 1988; Horiuchi and Hidaka, 1988). Hill *et al.* (1988b) reported similar results. Arrangement and polarities of the *ter* sites on R6K and *E.coli* genomes are shown in Figure 10. The two *ter* (*terR*) sites of R6K are placed symmetrically on the R6K genome. Four *E.coli terC* sites are also symmetrically arranged on the chromosome (Hidaka *et al.*, 1988; M.Hidaka and T.Horiuchi, unpublished). In *B.subtilis*, the DNA sequence, also probably the *ter* site, was identified (Carrigan *et al.*, 1987). As there is homology between the *ter* sequences of *B.subtilis* and *E.coli* (Hill *et al.*, 1988b), a possible physiological role would have to be given attention.

Though the termination mechanism has not been elucidated, the high homology present in all the *ter* sequences, their arrangement and polarity suggested the possibility that in *E.coli* cells there might be some activity specifically binding to the *ter* sequence and it might block movement of the DNA replication fork, at the *ter* site. Hill *et al.* (1988a) identified a *trans*-acting gene, called *tus*, that maps near T2 (*terC2*) and is required for termination at T1 (*terC1*). Smith and Wake (1988) also reported an analogous gene in *B.subtilis*, the defect mutants of which showed a termination-less phenotype.

We now report evidence of activity of *ter*-specific binding protein in *E.coli* extract. The protein is apparently essential for the DNA termination reaction.

Results

Presence of ter binding activity in the E.coli crude extract

We first searched for *ter* binding activity in the *E.coli* crude extract, using a gel-retardation assay. A pair of *terR* sites of plasmid R6K are located on a 216 bp of *Alu*I DNA (Alu216) fragment (Horiuchi and Hidaka, 1988). This Alu216 fragment was used as DNA substrate for the gel retardation assay (Fried and Crothers, 1981; Carthew *et al.*, 1985). The crude extract prepared from wild-type *E.coli* (W3110; Bachmann, 1972) cell was mixed with ^{32}P-end-labeled Alu216 DNA fragment, left to stand for 30 min at room temperature and then examined on a polyacrylamide gel, followed by autoradiography. As shown in lanes 12, 13, and 14 in Figure 1a, the Alu216 DNA fragment (lane 1) was retarded only when it was mixed with crude extract, and new first upper and second upper discrete DNA bands

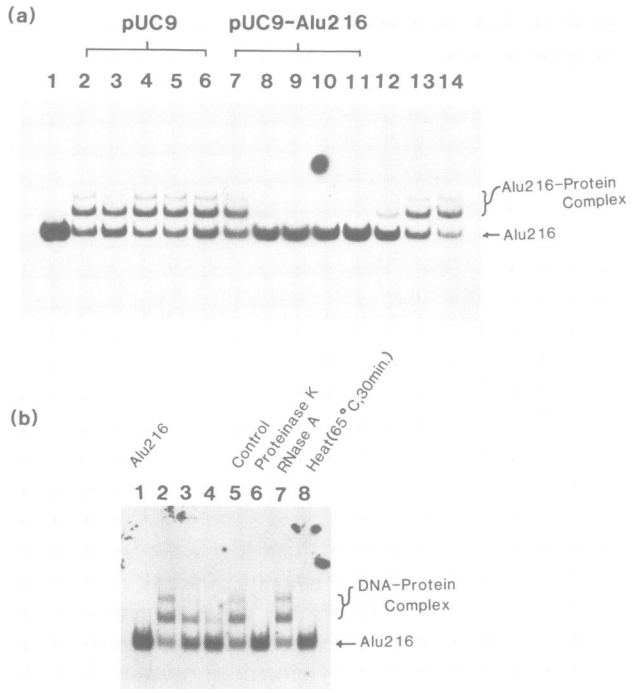

Fig. 1. Detection of Alu216 DNA specific binding activity in crude extract using gel-retardation assay and inactivation of the activity with heat or protease treatments. (**a**) Radiolabeled Alu216 DNA fragment (0.9 ng, 10 000 c.p.m.) was mixed with crude extract (3.7 μg protein) prepared from wild-type (W3110) *E.coli* cells, left to stand at room temperature for 30 min and analyzed through 5% polyacrylamide gel electrophoresis. The gel was dried and exposed to X-ray film for radioautography. Details are described in Materials and methods. **Lane 1**, complete reaction mixture minus cell extract; **lanes 2–6**, complete reaction mixture plus 0, 25, 50, 100 and 200 ng of pUC9 DNA as competitor respectively; **lanes 7–11**, complete reaction mixture plus 0, 25, 50, 100 and 200 ng of pUC9-Alu216 DNA as competitor respectively; **lanes 12–14**, the complete mixture contains 0.9, 1.8 and 3.7 μg protein of cell extract without competitor DNA respectively. Position of liner Alu216 fragment and its protein complex in the gel are also indicated. (**b**) **Lane 1**, complete reaction mixture minus cell extract; **lanes 2–4**, each reaction mixture contains 20, 10 and 5 μg protein of cell extract respectively; **lanes 5–8**, each reaction mixture containing 10 μg of cell extract was pretreated with none (at 30°C), proteinase K (400 μg, at 30°C), RNase A (400 μg, at 30°C) and heat (65°C) for 30 min before the addition of ^{32}P-labeled Alu216 DNA.

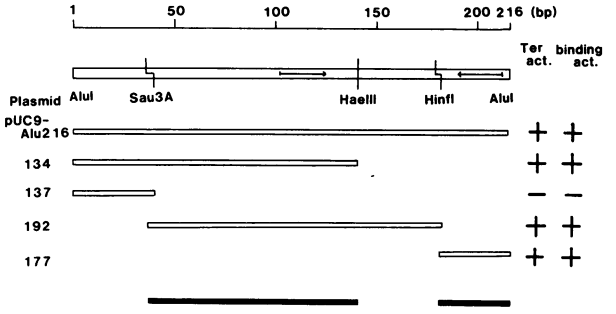

Fig. 2. Identification of the binding site on the Alu216 and its subfragments. The thick open bar at the top shows the restriction map of fragment Alu216. Arrows in the restriction map indicate the inverted 20 bp *terR* sequence. Ter and binding (competitive) activity of Alu216 and its subfragments, as indicated below, are shown on the right. The solid bar at the bottom shows the minimal region assumed to be required for the binding. Cloning of these subfragments and their Ter activities were as described in Horiuchi and Hidaka (1988).

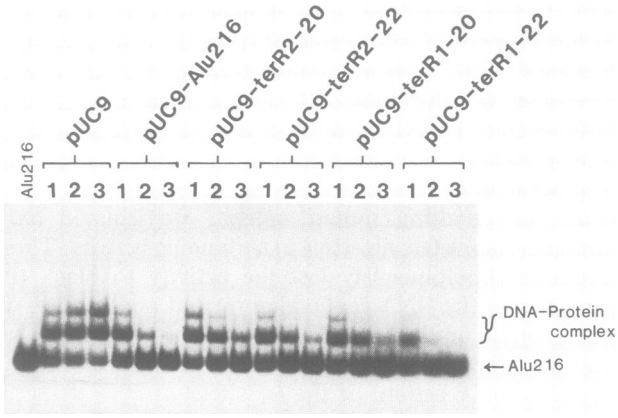

Fig. 3. Target sequence of the Alu216 binding protein. The procedures used are the same as in the legend to Figure 1. pUC9 derivative plasmids with a synthetic *terR* sequence, as indicated at the top of the figure, were added to the reaction mixture as competitor. 1, 2 and 3 indicates that 25, 50 and 100 ng of each competitor DNA was used respectively. pUC9-terR2-20 and others represent pUC9 plasmid derivatives into which synthetic 20 or 22 bp oligonucleotides with *terR1* or *terR2* sequences (described by Horiuchi and Hidaka, 1988) was inserted. Alu216 indicates the reaction in the absence of the cell extract.

appeared with increase in the amount of extract, thereby suggesting the presence of Alu216 binding activity in this extract. To determine whether or not the activity was Alu216 DNA fragment specific, a vector plasmid (pUC9) or Alu216 carrying pUC9 recombinant plasmid (pUC9-Alu216) DNA was added to the above reaction mixture, as a competitor. Though a large amount of pUC9 DNA (lanes 2–6) was added, the pattern of DNA bands corresponding to the DNA–protein complex remained unchanged. On the other hand, pUC9-Alu216 DNA (lanes 7–11) had the ability to compete, even at a low concentration. Thus, the DNA binding activity present in the extract is presumably Alu216 specific.

When the crude extract was pretreated with heat (at 65°C for 30 min) or with protease, the *ter* binding activity was completely inactivated, but the RNase had no effect (Figure 1b). These observations suggested that the activity might be carried by a protein factor.

Various subfragments derived from Alu216 DNA were tested for possible competition with the ^{32}P-radiolabeled parental fragment. The results are summarized in Figure 2. The binding sites are located on two separable DNA fragments: one is *Sau*3A–*Hae*III and the other is *Hin*fI–*Alu*I, on which the *terR1* and *terR2* sequences reside respectively (Horiuchi and Hidaka, 1988). This would suggest that the binding site might be the *terR* site itself. Figure 3 clearly shows that this is indeed the case; the synthetic 22 bp *terR* sequence and an even shorter 20 bp *terR* were adequate for an activity comparable to that of the Alu216 fragment. As the 22 bp of *terR* sequence alone possessed the potential to compete with almost all of the Alu216 DNA–protein complex, there might not be any other protein binding to sites other than *terR* sequence on the Alu216 fragment in the crude extract.

In addition, four different DNA fragments carrying *terC1*, *2*, *3* and *4*, which are terminus sites on the *E.coli* chromosome (Hidaka *et al.*, 1988), were also able to

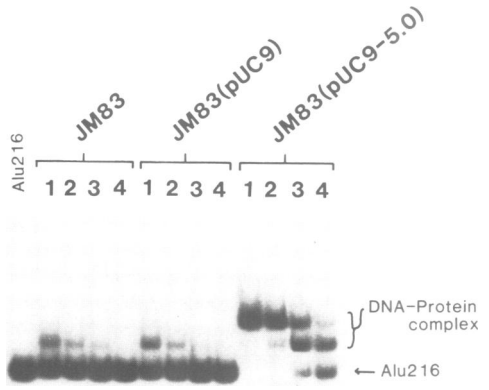

Fig. 4. Overproduction of *ter* specific binding activity. The procedures used are the same as in the legend to Figure 1. Cell extracts prepared from three different strains, as indicated at the upper part of the figure, were added to the reaction mixture at four different concentrations: 1, 2, 3 and 4 means that 20, 10, 5 and 2.5 μg of each extract was used respectively.

compete (data not shown). Thus, we concluded that the target site of the binding activity was 22 bp *terR* and probably also the *terC* sequence.

Overproduction of the ter binding protein

Hill *et al.* (1988a) reported that a *trans*-acting factor required for DNA termination was located on a 5.0 kb *Eco*RI DNA fragment very close to the *manA* gene (35.7 min) on the *E.coli* linkage map. On the same fragment was located one of the four terminus sites on the *E.coli* genome, *terC2* (T2) (Hidaka *et al.*, 1988; Hill *et al.*, 1988b). We prepared a cell extract from the strain carrying the plasmid with the 5.0 kb *Eco*RI fragment [pUC9−5.0(−); Hidaka *et al.*, 1988] and measured the *ter*-binding activity. As shown in Figure 4, the activity is at least 8-fold higher than that in the plasmid-free cells or in cells carrying the pUC9 vector alone. The increased activity also completely competed with the synthetic *terR* sequence (data not shown), hence the enhanced binding activity is also *terR* specific. All these observations suggest the presence of a gene, which controls the *ter* binding activity and which locates somewhere on the 5.0 kb DNA fragment. We tentatively termed the gene *'tau'*.

Isolation of the ter binding activity-less (tau⁻) mutants

We attempted to construct *E.coli* mutants completely defective in *ter* binding activity, using the method of Tn*3* (Ap^r) insertional inactivation of the *tau* gene on the chromosome. The following two-step procedures were used: first, the 5.0 kb *Eco*RI fragment, described above, inserted into ColE1 type vector was randomly mutagenized with transposon Tn*3* and 27 independent mutant plasmids with Tn*3* insertion at different sites were isolated (Kretschmer and Cohen, 1977; Horiuchi *et al.*, 1984). Using these Tn*3*-inserted plasmids, similar overproduction experiments to those described above were carried out. The results suggested that the region around the *terC2* site might be required for overproduction. Thus, we selected seven Tn*3*-inserted plasmids (nos. 9, 13, 39, 40, 41, 43, 49; see Figure 5), all insertion sites of which were located around the *terC2* site, as shown in Figure 5, and used these in the

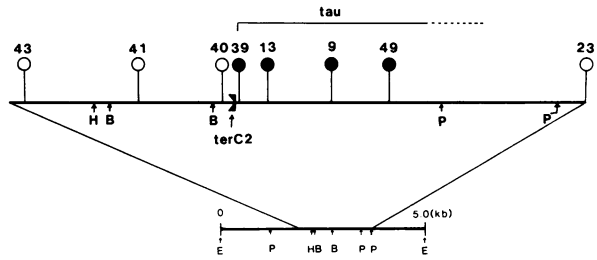

Fig. 5. Insertion sites of transposon Tn*3* on the 5.0 kb *Eco*RI fragment. The under bar represents a restriction map of the 5.0 kb *Eco*RI fragment, whose *terC2* and surrounding region are expanded at the upper site. E, H, B and P means the restriction sites of *Eco*RI, *Hin*dIII, *Bgl*I and *Pvu*II enzymes respectively. (♀) and (🌳) indicate insertion sites of transposon Tn*3*, mutants which showed *tau*⁺ and *tau*⁻ phenotypes respectively. The number above the circles represents the allele of each insertion mutant and its mutant strains. Determination of the Tn*3* insertion site and their phenotypes (except no. 23) were made by restriction analysis of the original Tn*3*-inserted plasmids and the experiment described in Figure 6 respectively. We classified mutant no. 23 into the *tau*⁺ group, as based on findings in the maxicell experiments (data not shown). (◥) show *terC2* sequence able to block the replication fork approaching from the left i.e. the clockwise replication fork on the chromosome. The deduced location of the *tau* gene is also shown.

next step. We replaced the wild-type allele on the chromosome with the Tn*3*-inserted mutant allele in the plasmid, by homologous recombination (Greener and Hill, 1980). The seven Tn*3*-inserted plasmids were transformed into *polA12* (temperature-sensitive *polA* mutation. Monk and Kinross, 1972) strain and Ap^rTc^r(Tc^r is vector marker) clones were selected at 30°C. After each Ap^rTc^r clone was grown from a single colony, Ap^r but Tc^s clones were selected at 42°C (*polA*⁻ condition). Because under these conditions ColE1 type plasmid is unable to replicate, the Ap^rTc^s clones were produced only by double crossing over at two homologous sites flanking Tn*3* between chromosomal and plasmid DNA. For confirmation, P1 transductional experiments were carried out. All Ap^r(Tn*3*) alleles were transduced with *manA* marker at 80−100% frequency; i.e. all Tn*3* were inserted into the host chromosome, at the original position. From these Man^+Ap^r transductants, each cell extract was prepared and the *ter* binding activity was measured. As shown in Figure 6a, extracts from nos. 40, 41 and 43 strains contained the *ter* binding activity, at the same level as that of the control *manA*⁻ recipient strain used for P1 transduction. On the other hand, nos. 9, 13, 39 and 49 derived extracts lost all *ter* binding activities, even when the amount of the extract was increased. From the location of these Tn*3* shown in Figure 5, a gene, we termed *tau*, and which controls the *ter* binding activity, is located at a region just right of the *terC2* sequence on the 5.0 kb fragment.

Ter activity of the ter site in the tau⁻ mutants

All *ter* sites have activity (called Ter activity; Horiuchi and Hidaka, 1988), which blocks the progress of DNA replication fork in wild-type cells. To investigate whether the *terR* site had Ter activity in the *tau*⁻ cells, we carried out the Ter assay as follows: pHSG299-terR plasmid DNA (pHSG299 is a Km^r derivative vector of the pUC plasmid; Takeshita *et al.*, 1987) was extracted from *tau*⁺ and *tau*⁻ cells, digested with *Eco*RI, analyzed through agarose gel electrophoresis, transferred to a nylon membrane filter and

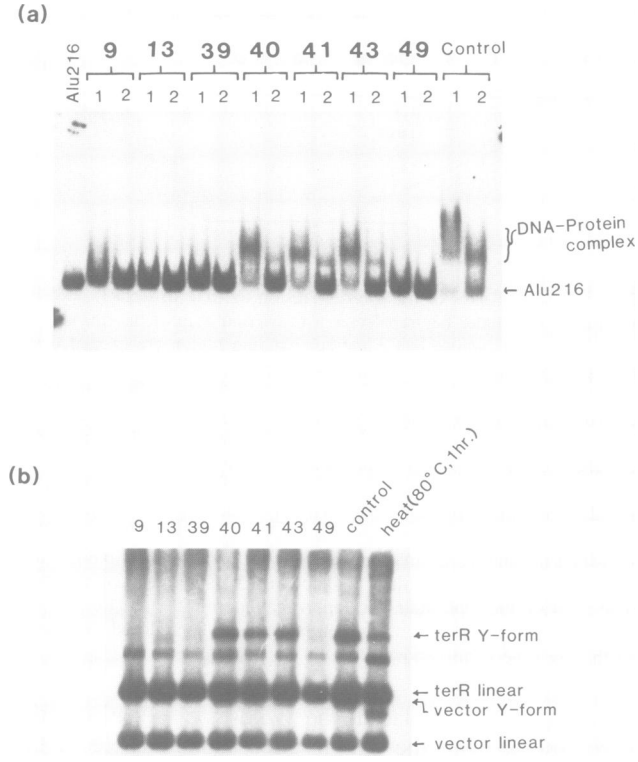

Fig. 6. Identification of mutants deficient in *ter* binding activity and Ter activity of *terR* site in the mutants. (**a**) The procedures used are the same as those for the legend to Figure 1. Cell extracts prepared from seven Tn*3*-inserted mutants, as indicated by strain number at the top of the figure, were added to the reaction mixture at two different concentrations; Samples 1 and 2 contain 3.6 and 1.8 μg respectively. Control strain is the Tn*3*-free isogenic *manA*$^-$ strain used as recipient for P1 transduction. (**b**) The procedure (Ter assay) used was as described (Horiuchi and Hidaka, 1988). pHSG299-terR plasmid DNA was extracted under neutral conditions from the seven Tn*3*-inserted mutants and a control strain (Tn*3*-free isogenic *manA*$^-$ strain), as indicated at upper side of the figure, digested with *Eco*RI, electrophoresed through an agarose gel, transferred to a nylon membrane filter and detected by DNA−DNA hybridization with pHSG299-terR DNA as probe. Sample heat (80°C, 1 h) contains heat-treated control sample at 80°C for 1 h after *Eco*RI digestion. At the right side of the gel the position of the DNA band corresponding to each molecule produced by restriction digestion of the plasmid DNA is shown. The two linear molecules of pHSG299 vector and *terR* fragment are 2.63 kb and 4.3 kb respectively.

hybridized with radiolabeled pHSG299-terR DNA as the probe. Because the *Eco*RI 4.32 kb *terR* fragment carried the Ter active site, the θ-shaped DNA replicative intermediate molecule, whose unidirectional replication fork initiating from the ColE1 origin halted at the *terR* site, accumulated in *tau*$^+$ cells and *Eco*RI digestion of the molecule produced two types of Y-shaped molecules, one derived from the *terR* fragment and the other from the vector, both of which were detectable by agarose gel electrophoresis. As shown in Figure 6b, in all samples, two major DNA bands, the smaller one corresponding to the 2.63 kb linear vector and the larger one corresponding to the 4.32 kb linear *terR* fragment respectively, were noted. Another minor band present in all samples probably represents linear molecules due to incomplete digestion. In addition, in only the samples extracted from the *tau*$^+$ cells was there a discrete, slow-moving DNA band which might correspond to the Y-shaped molecules derived from the *terR* fragment. Heat treatment of such a

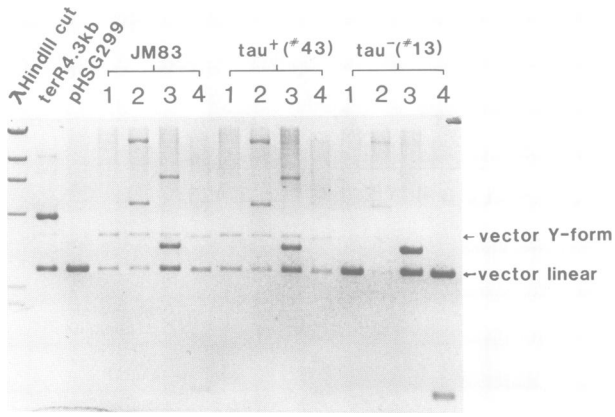

Fig. 7. Ter activity of four kinds of *terC* fragment in *tau*$^-$ mutant. The procedures used were much the same as those described in the legend to Figure 6b. **Lanes 1−4** indicate the DNA samples of *terC1, 2, 3* and *4* plasmid respectively, which were extracted from three different host strains. JM83, *tau*$^+$ (no. 43) and *tau*$^-$ (no. 13), as indicated at the top of the figure. After *Eco*RI or *Pvu*II (*terC4* plasmid only) digestion they were subjected to agarose gel electrophoresis and stained with ethidium bromide. pHSG299 and *terR* 4.3 kb indicate the two control DNA samples of pHSG299 vector alone and the 4.3 kb *terR* fragment carrying pHSG299 plasmid prepared similarly from *tau*$^+$ cells, respectively.

sample after digestion produced a new DNA band corresponding to the linear DNA, which originated from a pair of two arms of the Y-shaped terR molecule, by branch migration, as described (Hidaka *et al.*, 1988; Horiuchi and Hidaka, 1988), thereby confirming that the slowest DNA band was indeed the Y-form DNA of the *terR* fragment. On the other hand, in samples from *tau*$^-$ cells, the corresponding DNA band never appeared. Therefore, in the *tau*$^-$ cells, *terR* site is Ter inactive; that is, the DNA replication fork is not arrested at the *terR* site. In this gel, the other Y-form molecule derived from the vector is hard to detect, because its position overlaps with the band corresponding to the linear 4.32 kb *terR* fragment.

In the case of *terC* plasmids, similar results were obtained. Four kinds of *terC* plasmids each carrying the DNA fragment on which *terC1* (0.6 kb *Eco*RI), *terC2* (5.0 kb *Eco*RI), *terC3* (3.35 kb *Eco*RI) and *terC4* (1.0 kb *Pvu*II; unpublished data) were located were subjected to Ter assay. Figure 7 shows that when each *terC* plasmid DNA was extracted from *tau*$^-$ cells, in all samples, except *terC2*, none of DNA bands corresponding to the Y-shaped molecules (whether or not it was derived from the vector or insert) were visible, while they were detectable in those from *tau*$^+$ cells. In addition, the amounts of DNA in *terC*, in particular the *terC1*, *terC2* and *terC4* plasmids were very low in *tau*$^+$ cells, though the amount was normal in the *tau*$^-$ cells (except for *terC2* in this case also). These observations are consistent with the conclusion that the severe termination event was responsible for the decrease in copy number of Ter-active *terC* plasmid (Hidaka *et al.*, 1988). Only the *terC2* site behaved as Ter active even under *tau*$^-$ conditions. This is because the *terC2* plasmid carries the 5.0 kb *Eco*RI fragment on which the wild *tau*$^+$ gene is also located and is expressed regardless of the presence of the *tau* gene allele on the host chromosome.

These results were confirmed by Ter assay using synthetic 22 bp oligonucleotide *terR* and *terC* plasmids (Figure 8).

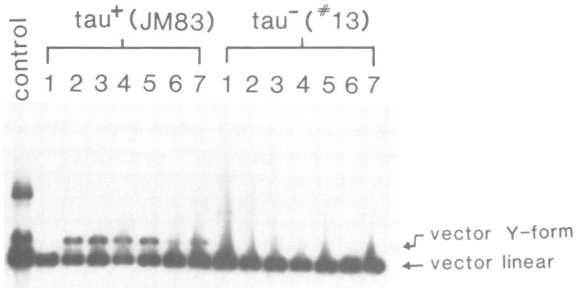

Fig. 8. Loss of Ter activity of synthetic oligonucleotide with the *terR* or *terC* sequence by *tau*⁻ mutation. All procedures were the same as those used in the Figure 6 experiment. Here, pUC9 DNA was used as the probe. Vector pHSG299 (Km^r derivative vector of pUC9) was used for the cloning of the following synthetic *ter* oligonucleotides; no insert (**lane 1**), *terC1* (22 bp, **lane 2**), *terC2* (22 bp, **lane 3**), *terC3* (57 bp, **lane 4**), *terC4* (22 bp, **lane 5**), *terR1* (22 bp, **lane 6**) and *terR2* (22 bp, **lane 7**). These plasmid DNAs were extracted from two host strains, *tau*⁺ (JM83) and *tau*⁻ (no. 13), and subjected to Ter assay. All oligonucleotides used were 22 bp, except in the case of the *terC3* sequence where a 57 bp oligonucleotide which contains *terC3* and its surrounding sequence was used, because *terC3* (22 bp) carrying the pHSG299 plasmid was structurally unstable.

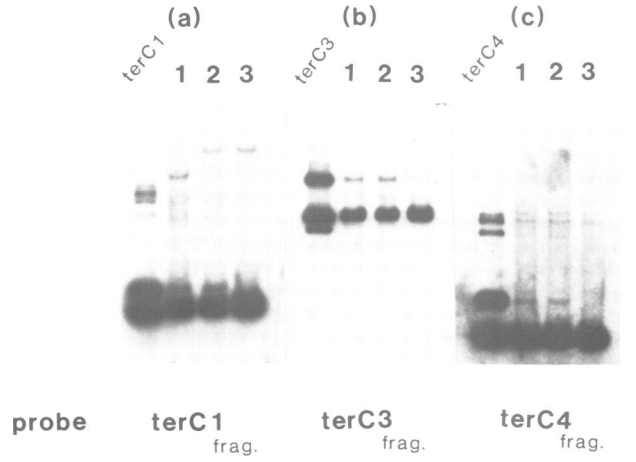

Fig. 9. Termination of three chromosomal *terC* sites in *tau*⁻ cells. The genomic DNAs were prepared from three *E.coli* strains, KHG300 and its Tn*3*-inserted *tau*⁺ (no. 43) and *tau*⁻ (no. 13) derivatives grown logarithmically at 37°C. Each derivative strain was constructed by introduction of each allele into the parental strain KHG300 by P1 transduction. After digestion with *Eco*RI or *Pvu*II (*terC4* only), the chromosomal DNA samples (**1**, wild-type; **2**, Tn*3*-*tau*⁺, **3**, Tn*3*-*tau*⁻) were separated into three sets and each was applied on the gel with the *Eco*RI-digested DNA sample of the corresponding *terC* plasmid, pHSG299-terC1 [0.6 (−) kb] (**a**), pHSG299-terC3 [3.35 (+) kb] (**b**) or pHSG299-terC4 [1.0 (−) kb] (**c**) respectively, and electrophoresed. The DNAs in gels were transferred to nylon membranes by capillary blotting for 15 h and hybridized with a radiolabeled 0.6 kb *terC1* fragment (**a**), a 3.35 kb *terC3* fragment (**b**) and a 1.0 kb *terC4* fragment (**c**) as the probe respectively.

tau⁺ cells had the ability to halt the replication fork at all *ter* sites, but this ability was completely lost in *tau*⁻ cells, even at the *terC2* site.

tau⁻ *cell is a termination-less mutant*

To investigate whether termination at chromosomal *terC* sites would occur in *tau*⁻ mutants, the Ter activities were measured, as described (Hidaka *et al.*, 1988). For this a *rnh*⁻*tau*⁻ double mutant was constructed, because the *rnh*⁻ (RNase H-defective) mutant can initiate a new type of DNA replication from sites somewhere in the *terC* region and their replication forks rapidly reached either of the *terC* sites, thereby facilitating detection of the Ter activity of chromosomal *terC* site in *rnh*⁻ mutant rather than in *rnh*⁺ wild-type strain (de Massy *et al.*, 1984; Hidaka *et al.*, 1988). As shown in Figure 9, chromosomal *terC1, terC3* and *terC4* sites in both of *tau*⁺ control strains are Ter active, because the Y-shaped molecules derived from chromosomal DNA, whose DNA band position is identical with that of control plasmid, were detectable. On the other hand, in *tau*⁻ cells, none of the corresponding DNA bands were detectable. We conclude that no chromosomal *terC* sites (*terC1, 3* and *4*) in the *tau*⁻ cells functioned. In this experiment we omitted the *terC2* site because the DNA band position of the Y-form molecules derived from three bacterial chromosomes differed due to different insertion sites of Tn*3* into the 5.0 kb fragment of the chromosomes: KHG300, no insertion; *tau*⁺ derivative, left side of the *terC2*; *tau*⁻ derivative, right side of the *terC2*.

Discussion

We identified the *tau* gene that controls at least a *ter*-binding protein, on the 5.0 kb *Eco*RI fragment near the *manA* gene in the *E.coli* chromosome. Hill *et al.* (1988a,b) reported that the *tus* gene, which is located on the same 5.0 kb *Eco*RI fragment, codes for the *trans*-acting factor essential for the termination reaction; that is the *tus* mutant was also a termination-less mutant. Thus, *tus* is probably identical with the *tau* gene we found in the present study. A probable interpretation is that *tau* is a structural gene for the *ter* binding protein. However, the possibility that *tau* might be a positive regulator gene essential for expression of a structural gene of the *ter* binding protein, or that *tau* gene product might be required for activation of an inactive *ter* binding protein would have to be excluded.

At any rate, the *ter* binding protein is essential for the termination process of DNA replication of both R6K and the *E.coli* genome. There may be other protein factor(s) included in the termination reaction, but the *ter*-binding protein might play a central role in the termination reaction because it has at least two key activities essential for termination: one is an activity binding to the *ter* sequence and the other one is blocking the replication fork at the *ter* site. This finding suggests that the *ter* binding protein recognizes the 22 bp *ter* sequence, binds firmly to it and the resulting DNA−protein complex itself has the ability to block progress of the replication fork. Elucidation of the blocking mechanism at the molecular level may help to explain how the replication fork proceeds on the usual double-stranded DNA molecule.

The *tau* gene is located in close proximity to the *terC2* sequence, as shown in Figure 5. In this region, we found one open reading frame, the transcription of which might start from a point just right of *terC2* sequence going to the right direction (data not shown). Our sequence data also suggested that the *ter* sequence overlapped with the promoter site of the presumed *tau* gene. Thus, the *ter* sequence might function as an operator site for *tau* gene expression and, if so, the *ter* binding protein might play the role of repressor for the expression. If the *tau* gene product was the *ter* binding

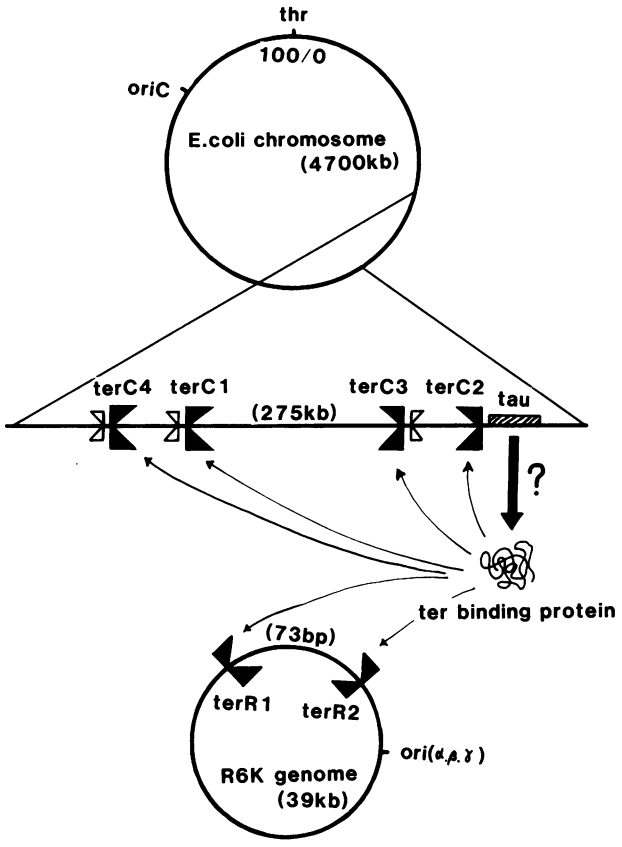

Fig. 10. A model of the *ter* system in *E.coli*. Upper and lower circles show schematically the circular *E.coli* chromosome (4800 kb) and R6K (39 kb) respectively. oriC and ori (α, β, γ) indicates the replication origin of *E.coli* and R6K genomes respectively. ( ) and ( ) indicate the DNA replication terminus (*ter*) sequence able to block the replication fork approaching from the right direction and the RNA transcriptional terminator-like sequence which would be able to terminate transcription from the right direction respectively. Distances between *terR1* and *terR2*, and *terC1* and *terC3* are shown. The *ter* binding protein (probably coded by the *tau* gene) can bind firmly to all *ter* sequences and the resulting DNA−protein complex blocks progress of the DNA replication fork.

protein, these regulatory systems would make an autoregulative circuit which would maintain constant the amount of *ter* binding protein, under various conditions. In addition, these exceptional structural features of the *terC2* site, compared with three other *terC* sites, might explain why at only the *terC2* site the transcriptional terminator-like sequence could not be found, as discussed later. Smith and Wake (1988) reported that in the *B.subtilis ter* system, an open reading frame, whose defective mutant showed a termination-less phenotype, is close to a *B.subtilis ter*-like sequence with an arrangement similar to that between *terC2* and *tau* gene on the *E.coli* genome. We do not yet know whether such a *tau*-like gene is present on the R6K genome. However, if present it would not be located on the R6K 4.32 kb fragment on which two *terR* sites are located, because the fragment showed the Ter-negative phenotype in the *tau*$^-$ cells.

The Tn*3*-inserted *tau*$^-$ mutants we isolated were completely defective but not leaky mutants, because (i) their mutation is the Tn*3* insertion type, (ii) their cell extracts showed no *ter* binding activity and (iii) none of the replicational intermediate molecules of either plasmid or chromosome accumulated in their cells. The Tn*3*-inserted *tau*$^-$ mutants were as easy to isolate as those of Tn*3*-inserted *tau*$^+$ strains. Moreover, *tau*$^-$ mutations were readily transduced to another strain with different genetic backgrounds by P1 transduction (data not shown) and the *tau*$^-$ mutants showed no distinct phenotype differences from the *tau*$^+$ strain. Thus the *tau*$^+$ gene may be non-essential for growth of the cell. A similar conclusion has been drawn by others for *E.coli* (Hill *et al.*, 1988b) and *B.subtilis* (Iismaa and Wake, 1987). To elucidate the physiological function of the *ter* system in *E.coli*, a more precise analysis of the *tau*$^-$ mutant is necessary.

In recent work (Hidaka *et al.*, 1988; unpublished), we noted at least three *terC* sites (*terC1, 3* and *4*), transcriptional terminator-like sequences located close to the *terC* sequence, in an orientation that prevents RNA polymerases from colliding against DNA polymerase, head to head at the site, as shown in Figure 10. This suggests the possibility that in the *E.coli ter* system there is a pair of two different kinds of terminators: one is the RNA transcriptional terminator and the other is a DNA replicational terminator (*terC*), both making one unit at the functional DNA terminus site (Hidaka *et al.*, 1988). Brewer and Fangman (1988) found a DNA replicational Ter active site in yeast. The opposite oriented transcription of 35S rRNA might terminate here. Their results can be explained well by our model on the structure of the *ter* site. The *ter* system may, therefore, not be restricted to prokaryotes. Physiological function and DNA structure common to both types of organisms may have to be considered.

Materials and methods

Bacterial strains, plasmids and phage

All bacterial strains used were derivatives of *E.coli* K12. W3110 (Bachmann, 1972), JM83 (Vieira and Messing, 1982), KHG300 (*rnh*$^-$::Tn*5*, unpublished), plasmid pUC9 (Vieira and Messing, 1982) and phage P1*vir* are from laboratory stocks. An *E.coli* strain GMS343 (*manA*$^-$; CGSC # 5496) was obtained from the *E.coli* Genetic Center (Yale University). pHSG299, a Km^r vector plasmid derived from pUC plasmid (Takeshita *et al.*, 1987), was purchased from Takara Shuzo Co. Ltd (Kyoto, Japan). Plasmids and strains used in the Tn*3* mutagenesis experiment were as described (Horiuchi *et al.*, 1984). All pUC plasmids carrying *terR* or *terC* (except *terC4*) sites were as described (Hidaka *et al.*, 1988; Horiuchi and Hidaka, 1988). Analogous pHSG299 derivatives were constructed by recloning of the insert obtained from the corresponding pUC derivatives.

Gel-retardation assay

Procedures used were essentially the same as the method described by Wang *et al.* (1987). Complete reaction mixture (30 μl) contained ^{32}P-end-labeled Alu216 (0.9 ng, 10 000 c.p.m.), poly(dI-dC)(dI-dC) (2 μg), 10 mM Tris−HCl, pH7.5, 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 10% glycerol and 1−20 μg protein of cell extract. When necessary, competitor plasmid DNA was added to the mixture, prior to adding the extract. The binding reaction was carried out at room temperature for 30 min, and then 1 μl of stop dye solution was added to the mixture followed by electrophoresis through 5% polyacrylamide gel (acrylamide/bisacrylamide ratio 29:1) containing 6.7 mM Tris−HCl, pH 7.5, 3.3 mM sodium acetate and 1 mM EDTA. The gel was pre-electrophoresed for 30 min at 11 V/cm and after application of the sample electrophoresis was carried out at the same voltage for 2 h at 4°C with buffer recirculation. The gel was then dried and exposed to X-ray film for radioautography.

Other methods

Ter assay, synthesis and cloning of oligonucleotide, bacterial DNA preparation, hybridization and general methods for DNA manipulation are as described (Horiuchi *et al.*, 1987; Hidaka *et al.*, 1988; Horiuchi and Hidaka, 1988). P1 transduction was as described by Ikeda and Tomizawa (1965). Tn*3* mutagenesis of the cloned *tau* gene and construction of *tau*$^-$ strains were carried out as described (Horiuchi *et al.*, 1984; Nakabeppu *et al.*, 1984). Preparation of cell extract for gel-retardation assay followed the method of Wickner *et al.* (1974), albeit on a small scale.

Acknowledgements

We thank Drs Daisuke Kitamura and Takeshi Watanabe for guidance on the gel-retardation assay, Dr Masahiro Akiyama for technical advice, Dr Bonita J.Brewer for informing us of results prior to publication, Drs Thomas M.Hill and Mark Smith for communicating their results, Dr Barbara J.Bachmann for providing the *E.coli* strain and Mariko Ohara for comments. This work was funded by Grants for Scientific Research from the Ministry of Education, Science and Culture, Japan.

References

Bachmann,B.J. (1972) *Bacteriol. Rev.*, **36**, 525–557.
Bird,R.E., Louarn,J., Martuscelli,J. and Caro,L. (1972) *J. Mol. Biol.*, **70**, 549–566.
Brewer,B.J. and Fangman,W.L. (1988) *Cell*, **55**, 637–643.
Carrigan,C.M., Haarsma,J.A., Smith,M.T. and Wake,R.G. (1987) *Nucleic Acids Res.*, **15**, 8501–8509.
Carthew,R.W., Chodosh,L.A. and Sharp,P.A. (1985) *Cell*, **43**, 439–448.
Crosa,J.H., Luttropp,L.K. and Falkow,S. (1976) *J. Bacteriol.*, **126**, 454–466.
de Massy,B., Fayet,O. and Kogoma,T. (1984) *J. Mol. Biol.*, **178**, 227–236.
de Massy,B., Louarn,S.B., Louarn,J.M. and Bouche,J.P. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 1759–1763.
Fried,M. and Crothers,D.M. (1981) *Nucleic Acids Res.*, **9**, 6505–6525.
Greener,A. and Hill,C.W. (1980) *J. Bacteriol.*, **144**, 312–321.
Hidaka,M., Akiyama,M. and Horiuchi,T. (1988) *Cell*, **55**, 467–475.
Hill,T.M., Henson,J.M. and Kuempel,P.L. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 1754–1758.
Hill,T.M., Kopp,B.J. and Kuempel,P.L. (1988a) *J. Bacteriol.*, **170**, 662–668.
Hill,T.M., Pelletier,A.J., Tecklenburg,M.L. and Kuempel,P.L. (1988b) *Cell*, **55**, 459–466.
Horiuchi,T. and Hidaka,M. (1988) *Cell*, **54**, 515–523.
Horiuchi,T., Maki,H. and Sekiguchi,M. (1984) *Mol. Gen. Genet.*, **195**, 17–22.
Horiuchi,T., Hidaka,M., Akiyama,M., Nishitani,H. and Sekiguchi,M. (1987) *Mol. Gen. Genet.*, **210**, 394–398.
Iismaa,T.P., Smith,M.T. and Wake,R.G. (1984) *Gene*, **32**, 171–180.
Iismaa,T.P. and Wake,R.G. (1987) *J. Mol. Biol.*, **195**, 299–310.
Ikeda,H. and Tomizawa,J. (1965) *J. Mol. Biol.*, **14**, 85–109.
Kretschmer,P.J. and Cohen,S.N. (1977) *J. Bacteriol.*, **130**, 888–899.
Kuempel,P.L., Duerr,S.A. and Seeley,N.R. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 3927–3931.
Louarn,J., Patte,J. and Louarn,J.M. (1979) *Mol. Gen. Genet.*, **172**, 7–11.
Lovett,M.A., Sparks,R.B. and Helinski,D.R. (1975) *Proc. Natl. Acad. Sci. USA*, **72**, 2905–2909.
Monk,M. and Kinross,J. (1972) *J. Bacteriol.*, **109**, 971–978.
Monteiro,M.J., Sergent,M.G. and Piggot,P.J. (1984) *J. Gen. Microbiol.*, **130**, 2403–2414.
Nakabeppu,Y., Kondo,H. and Sekiguchi,M. (1984) *J. Biol. Chem.*, **259**, 13723–13729.
Prescott,D.M. and Kuempel,P.L. (1972) *Proc. Natl. Acad. Sci, USA*, **69**, 2842–2845.
Smith,M.T. and Wake,R.G. (1988) *J. Bacteriol.*, **170**, 4083–4090.
Takeshita,S., Sato,M., Toda,M., Masahashi,W. and Hashimoto-Gotoh,T. (1987) *Gene*, **61**, 63–74.
Vieira,J. and Messing,J. (1982) *Gene*, **19**, 259–268.
Wang,J., Nishiyama,K., Araki,K., Kitamura,D. and Watanabe,T. (1987) *Nucleic Acids Res.*, **15**, 10105–10116.
Weiss,A.S. and Wake,R.G. (1983) *J. Mol. Biol.*, **171**, 119–137.
Wickner,S., Wright,M., Berkower,I. and Hurwitz. (1974) In Wickner,R.B. (ed.), *Methods in Molecular Biology: DNA Replication*. Marcel Dekker, New York, Vol. 7, p. 195.

Received on March 8, 1989; revised on May 5, 1989

Note added in proof

After we submitted our manuscript Hill *et al.* (*Proc. Natl. Acad. Sci. USA*, **86**, 1593–1597, 1989) reported similar observations: disruption of the *tus* gene results in simultaneous loss of ability of termination and activity binding to T2 terminator sequence.