The terminus region of the \textit{Escherichia coli} chromosome contains two separate loci that exhibit polar inhibition of replication

(P2 sig5 prophage/plasmids/DNA-DNA hybridization/DNA synthesis)

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**ABSTRACT** The terminus region of the chromosome of \textit{Escherichia coli} contains two separate sites, called \textit{T1} and \textit{T2}, that inhibit replication forks. \textit{T1} is located near 28.5 min, which is adjacent to \textit{trp}, and \textit{T2} is located at 34.5–35.7 min on the opposite side of the terminus region, near \textit{manA}. The sites act in a polar fashion, and replication forks traveling in a clockwise direction with respect to the genetic map are not inhibited as they pass through \textit{T1} but are inhibited at \textit{T2}. Similarly, counterclockwise forks are not inhibited at \textit{T2} but are inhibited at \textit{T1}. Consequently, forks are not inhibited until they have passed through the terminus region and are about to leave it. Studies with deletion strains have located \textit{T2} within a 58-kilobase interval, which corresponds to kilobase coordinates 387–445 on the physical map of the terminus region.

The terminus region of the chromosome of \textit{Escherichia coli} is located directly opposite the origin of replication on the circular genetic map (1). One of the most interesting features of this region is that it inhibits replication forks that are traveling in either a clockwise or counterclockwise direction with respect to the map (2–5). When origins of replication located near the terminus region were used (2–5), inhibition of replication forks occurred somewhere in the interval between \textit{trp} (28 min) and \textit{manA} (36 min). Although the function of this inhibition is unknown, its presence suggests that the terminus might encode partitioning, decatenation, or cell-division control sites, and impediment of replication forks in this region evolved to ensure efficient use of such sites (6).

More recent experiments using replication cycles initiated at \textit{oriC} (7) indicated that the last DNA to be replicated, and consequently the region where forks meet most frequently, was located near 31.2 min. Based on these results, it generally has been assumed that the replication block (terC) is located at this position (1). As discussed by Bouché \textit{et al.} (7), however, other interpretations of the data can be made. Therefore, identification of the region of most frequent fork encounter does not necessarily identify the site of replication-fork inhibition.

We report here, as do de Massy \textit{et al.} in an accompanying report in this issue (8), that replication forks are not inhibited at a single site in the middle of the terminus region. Instead, there are two inhibition sites, \textit{T1} and \textit{T2}, which are located at the outer edges of the terminus region. These sites are polar, and they only inhibit replication forks that have passed through the terminus region and are about to leave it. Specifically, clockwise traveling replication forks are inhibited at \textit{T2} at 34.5–35.8 min, near \textit{manA}, and counterclockwise traveling forks are inhibited at \textit{T1} at 28.5 min, near \textit{trp}.

**MATERIALS AND METHODS**

**Bacterial Strains.** All replication fork assays were performed with strains PK998 or PK1012 and derivatives containing the indicated deletions. These temperature-sensitive strains are \textit{dnaA} mutants and contained temperature-sensitive P2 \textit{D4 c5 sig5} bacteriophage, which we call P2 \textit{sig5} prophage, integrated near \textit{galK} (16 min) or \textit{metG} (47 min), respectively. They were identical to PK583 and PK304, which have been described (2, 3), except that the \textit{rac} cryptic prophage has been removed (9).

**Genetic Procedures.** Fig. 1 shows the locations of the deletions described in this paper. The end points were determined by Southern hybridizations with appropriate probes (11). PK1441, containing deletion \textit{1441}, was isolated from a strain that contained phage \textit{d} reverse \textit{c1857} (inserted at kilobase (kb) 164) and \textit{zdc-235::Tn10} (at kilobase 290). Derivatives were selected that were temperature resistant and tetracycline sensitive (12). PK1463 was isolated from a strain containing \textit{zdd-230::Tn9} and \textit{zdc-235::Tn10} (13), and PK1738 was isolated from a strain containing \textit{zdd-230::Tn9} and \textit{zdc-234::Tn10}. In both cases, tetracycline-sensitive derivatives were isolated and screened further. PK2035 and PK2038 were derived from a \textit{pgS} strain that contained \textit{zdc-235::Tn10} (at kb 290) and \textit{zdf-237::Tn10} (at kb 445). Presence of \textit{pgS} allowed selection for \textit{dgsA} (at kb 425) mutants, which grew anaerobically on glucose as the carbon source (14).

Bacteriophage \textit{P1} was used to transduce deletions into PK998 and PK1012. The presence of the deletion in recipient strains was verified by both genetic and Southern blot analysis.

**DNA-DNA Hybridization Assay.** Cells were grown at 28°C in M9 minimal medium supplemented with required amino acids (80 \mu g/ml) and thymine (2 \mu g/ml). At \textit{A}_{550} of =0.45, cells were collected on filters (HAWP, Millipore), washed once with M9 minimal medium, and resuspended at the same concentration in medium lacking all required amino acids except arginine. Incubation was continued for 170 min at 28°C to allow preexisting forks to complete replication cycles. The culture was then shifted to 42°C for 10 min to inactivate the \textit{dnaA} product and induce initiation from the P2 \textit{sig5} prophage. Required amino acids and \textit{\textsuperscript{3}H}thymine were then added (final specific activity, 8.1 Ci/mmol). Samples (2 ml) were removed at indicated times, washed four times with an equal volume of TE buffer (25 mM Tris-HCl/10 mM EDTA, pH 8.0), and resuspended in 1.0 ml of TE buffer containing lysozyme (5 mg/ml). After incubation for 5 min at room temperature, Pronase and NaDodSO\textsubscript{4} were added to a final concentration of 100 \mu g/ml and 1%, respectively, and the samples were incubated at room temperature overnight.

Samples were prepared for hybridization in the following manner. Four hundred microliters (1–2 \times 10\textsuperscript{7} cpm) of cellular \textit{\textsuperscript{3}H}DNA was hydrolyzed with 250 mM HCl in a final volume of 1.0 ml; 200 \mu l of 2.5 M NaOH was added to neutralize the
acids and denature the DNA. At this time 2 × 10^7 cpm of
32P-nick-translated chromosomal DNA (obtained from a
stationary culture of the same strain) was added to the
sample. After 5 min at room temperature, 100 μl of 2.5 M HCl
was added to neutralize the base, and 200 μl of 2 M sodium
phosphate buffer (pH 6.8) was added to stabilize the pH. The
sample was then brought to 4× NaCl/Cit (1× = 0.15 M
NaCl/0.015 M sodium citrate, pH 7), 4× Denhardt’s solution
(11), 50% formamide, and 1% NaDodSO4 in a final volume of
8.0 ml.

Plasmid probes were prepared by the alkaline hydrolysis
method essentially as described by Maniatis et al. (11). A list
of the plasmid probes used in these experiments is presented
in Table 1. Plasmids were banded in CsCl gradients at least
twice to remove contaminating chromosomal DNA. Purified
probes were loaded on nitrocellulose by using a slot-blot
apparatus (Schleicher & Schuell) by the following procedure.
Plasmid, 0.6 pmol in 200 μl, was hydrolyzed for 5 min in 0.25 M
HCl. The acid was neutralized, and the fragmented
plasmid was denatured by the addition of 0.4 M NaOH to
a final volume of 500 μl. Tris/salt (1.0 ml; 0.75 M Tris-HCl/1.5
M NaCl, pH 7.5) was added, and 500 μl of DNA was loaded
per slot (three slots per plasmid probe; 0.2 pmol of plasmid
per slot). Each slot was then washed with 500 μl of Tris/salt
and, after drying the blots, they were baked for 2 hr at 80°C
and prehybridized overnight at 42°C in 50% formamide
containing 5× NaCl/Cit, 5× Denhardt’s solution, 50 mM
sodium phosphate buffer (pH 6.8), and 1% NaDodSO4. After
prehybridization, the solution was replaced with the hybrid-
ization mixture containing the labeled DNAs and allowed to
hybridize for 36 hr at 42°C. Blots were washed four times in
2× NaCl/Cit containing 0.1% NaDodSO4 for 5 min per wash
at room temperature and twice in 0.2× NaCl/Cit containing
0.1% NaDodSO4 at 55°C for 45 min each wash. Slots were cut
out and assayed in a scintillation cocktail (Omnifluor;
New England Nuclear) to determine the 3H/32P ratios for each
plasmid.

Triplicate samples of plasmid pBR322 were included on
each blot to serve as a control for background hybridization of
both 3H and 32P. The average number of cpm bound to the
pBR322 control was subtracted from the experimental values
prior to calculating the 3H/32P ratio for each slot. The mean
and standard error were then determined from the three
corrected values obtained for each plasmid probe.

RESULTS

In all experiments, the P2 sig5 prophage was used to initiate
replication forks from positions close to the terminus region.
P2 sig5 prophage inserted at 16 min (near gal) was used to
study clockwise-traveling replication forks in the terminus
region, and an insertion at 47 min (near metG) was used to
study counterclockwise-traveling forks. As described in
previous publications (2, 3, 21), initiation at the P2 sig5
prophage was induced in conditions that prevented initiation
of replication forks from oriC. The asymmetric placement of
the P2 sig5 prophage with respect to the terminus region was
advantageous because replication forks originating from the
prophage could be monitored as they passed through the
terminus without interference from forks approaching from
the other side.

We have used a greatly expanded repertoire of plasmids in
our assay to determine the amount of replication in the
terminus and at other positions around the chromosome
details of these plasmids are given in Table 1). Consequently,
we have increased the sensitivity of the marker-frequency
analysis considerably. Additionally, all strains used in the
experiments reported here lacked the rac prophage, which is
located at 30 min (1). This avoided complications due to the
potential induction of this prophage, which could lead to its
excision (9) or to replication initiated at oriF (28).

Fig. 2 shows the results of an experiment in which replication
was initiated from the P2 sig5 prophage located near the gal locus. Samples were taken at 90, 135, and 180 min
after induction of the prophage replication origin. The clock-
wise-traveling replication forks proceeded normally through
most of the terminus region but were severely inhibited in the

Table 1. Plasmids used in DNA-DNA hybridization assay

<table>
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<th>Plasmid number</th>
<th>Position, min</th>
<th>Plasmid designation</th>
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</tr>
<tr>
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<td>pLC36-1</td>
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<td>4</td>
<td>27.7</td>
<td>pLN48</td>
<td>18</td>
</tr>
<tr>
<td>5</td>
<td>28.3</td>
<td>pμE</td>
<td>W. Epstein</td>
</tr>
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<td>19</td>
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<tr>
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<td>20</td>
</tr>
<tr>
<td>8</td>
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P2 lg cc vir22 (27) was used as the probe for determining replication
at the P2 sig5 prophage origins located at either 16 or 47 min.
Previously observed, however, the replication severely inhibited the travel of forks subsequent to the arrival of the prophage. The plasmids used were 2-9, 11-13, 15-19, and 21 in Table 1.

A comparable experiment was done to determine the location of the region in which counterclockwise-traveling replication forks were inhibited (Fig. 3). In this experiment, the P2 sig5 prophage was inserted near metG. Counterclockwise-traveling forks traversed most of the terminus region but were severely inhibited in the region between 29.1 min (plasmid pGS27) and 27.7 min (plasmid pLN48). This region corresponds to kb 70-140 on the Bouché map (10). As observed with the clockwise block, the inhibition site was not altered by arrival of subsequent replication forks, and forks did proceed through the block. The counterclockwise-traveling forks met the clockwise-traveling forks in the region near 55 min.

Comparison of Figs. 2 and 3 shows that the site that blocks counterclockwise-traveling forks (T1) has no effect on clockwise-traveling forks; similarly, the site that blocks clockwise-traveling forks (T2) has no effect on counterclockwise-traveling forks. This indicates that the inhibition sites act in a polar fashion.

In order to characterize the inhibition sites further, we isolated and tested a number of deletions in the terminus region. Our goal in these studies was to obtain deletions that define T1 and T2 to regions of 15 kb or less, at which stage it should be possible to study the sites further in plasmids. Such a deletion approach is feasible, since the terminus

![Diagram](image_url)
region is not essential, and it is possible to isolate deletions in which neither inhibition site functions (21).

By use of various deletions (1463, 1738, 2035 and 2038; see Fig. 1) we can presently limit T2 to a region of <60 kb. Although deletion 2035 (at kb 290–445) did not provide a more specific localization than the experiments described above, it set the limits for further analyses, since loss of that interval also removed T2 (data not shown). Deletions 1463 (at kb 290–342) and 1738 (at kb 348–387) subdivided the region corresponding to the left-hand part of deletion 2035. One (1738) removed the interval in which inhibition occurred, and the other (1463) deleted the region immediately upstream. Neither of these deletions affected T2, however, and Fig. 4 shows the results obtained with a strain containing deletion 1738. T2 was no longer functional in strains harboring deletion 2038 (at kb 360–445), which removed the region corresponding to the right-hand side of 2035 (Fig. 5). Combining the results obtained with deletions 2038 and 1738 places T2 between kb 387 and 445.

Although we have isolated a number of deletions that remove parts of the left-hand side of the terminus region, we have not yet isolated small deletions that remove T1. The most informative deletion is 1441 (at kb 123–290), in which T1 still functioned (data not shown). de Massy et al. (8) have

![Fig. 4. Termination of replication in strain PK218, which contains deletion 1738 (kb 350–390) and P2 sig5 prophage located at 16 min. The boundaries of the deletion are indicated by the vertical lines. The points represent the amount of DNA replication 180 min after induction of the prophage. The plasmids used were 1–18 and 21 in Table 1.](image)

![Fig. 5. Termination of replication in strain 2216, which contains deletion 2038 (kb 360–445) and P2 sig5 prophage located at 16 min. The boundaries of the deletion are indicated by the vertical lines. The points represent the amount of replication 180 min after induction of the prophage. The plasmids used were 1, 2, 4, 6–18, 20, and 21 in Table 1.](image)
isolated comparable deletions that removed kb 119–162 and 133–246, and T1 still functioned in strains harboring those deletions. This shows that T1 is to the left of kb 123.

DISCUSSION

We demonstrate here that clockwise- and counterclockwise-traveling replication forks are not inhibited in the same part of the terminus region of the E. coli chromosome. Clockwise-traveling forks are inhibited at T2, which is between 34.5 and 35.7 min on the side of the terminus region near manA. Counterclockwise-traveling forks are inhibited at T1, which is on the opposite side of the terminus region at 28.5 min near Trp. The two regions that inhibit replication forks are consequently separated by ≈6 or 7 min on the genetic map. The mechanism by which replication forks are inhibited is presently unknown, but it should be stressed that the blocks act in a polar fashion. Clockwise-traveling forks are not affected as they travel through T1, but they are inhibited by T2. Similarly, counterclockwise-traveling forks are not affected by T2, but they are inhibited by T1.

The location of the replication blocks indicates that normal replication cycles usually terminate with the collision of replication forks in the interval between T1 and T2. The clockwise genetic distance from oriC to T2 is ≈51 min, and the counterclockwise distance from oriC to T1 is ≈56 min. If these distances are actually physically equivalent (ca. 53.5 min of DNA), then one fork would have to proceed at least 15% faster than the other (53.5 vs. 46.5 min of DNA replicated) for an inhibition site to be used. Autoradiographic studies have shown that the majority of chromosomes exhibit bidirectional replication rates that differ by <15% (refs. 30 and 31; unpublished data). If replication cycles are frequently terminated between T1 and T2 without a block site being used, the questions arise of how essential the sites are, how often they are used, and why they evolved. It should be added that, if the physical distances from oriC to T1 and T2 actually were 56 and 51 min, respectively, T2 would be used more frequently than T1. This does not fit the data of Bouché et al. (7), who demonstrated that the average position of fork encounter was near 31.2 min, midway between T1 and T2.

The arrangement of the replication blocks in E. coli is considerably different from that observed in other terminus regions that have been studied. In plasmid R6K, there is only one termination site, which inhibits replication forks traveling in either direction (32). A similar situation possibly occurs in Bacillus subtilis in which replication forks traveling counterclockwise (33–35) and clockwise (36) halt in the same restriction fragment, and only one block site has been identified. A second site might exist, however, since counterclockwise-traveling replication forks are the first to arrive at this site (33), and their presence would inhibit counterclockwise-traveling forks. Strains in which the counterclockwise-traveling forks are the first to arrive in this region have not yet been tested. Regardless, a comparison of the features of the terminus regions of E. coli and B. subtilis should provide insights into the important features of these regions.

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