Probing the activation of the replicative origin of broad host-range plasmid R1162 with Tus, the *E.coli* anti-helicase protein

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ABSTRACT

The *E.coli* Tus protein is an anti-helicase involved in the termination of chromosome replication. The binding site for this protein, *ter*, was cloned into derivatives of the broad host-range plasmid R1162. The *ter* site caused the orientation-specific termination of plasmid replication fork movement in cell extracts containing Tus. Plasmids were constructed so that two sites for initiation of R1162 replication flanked the iteron-containing domain of the origin. In these plasmids, the site next to the AT-rich region within the iteron-containing domain was more active. In addition, when *ter* was placed between the more active site and the iterons, initiation of replication from this site was specifically inhibited. The data support a model for entry of the essential, plasmid-encoded helicase at one side of the direct repeats, and for its movement primarily in one direction away from these repeats to activate the initiation sites for DNA replication.

INTRODUCTION

The replicative origin of the broad host-range plasmid R1162, and the nearly identical plasmid RSF1010, consists of two functional domains (Fig. 1). The first of these is made up of three and one-half direct repeats (DRs) and adjacent GC- and AT-rich regions of DNA (designated GC and AT in Fig. 1). The direct repeats are the primary binding site of the plasmid-encoded replication protein RepIB (RepC in RSF1010) (1, and unpublished results). In addition, RepIB promotes localized strand separation in the AT-rich segment (Kim and Meyer, accepted for publication). The second domain contains two, oppositely-facing sites for the initiation of DNA synthesis, termed *oriL* and *oriR*, which are located at the ends of a large inverted repeat (2–4). These sites are the primary positions for the initiation of each plasmid strand. If one of the sites is deleted, the plasmid is substantially disabled, since only one of the two strands is then efficiently synthesized during replication (4).

Both of the origin domains are required for efficient plasmid replication, but the distances between them, and their relative

orientations, can be changed (2). An important question is how these domains are nevertheless functionally linked. We have used the *E.coli* Tus–*ter* system to visualize the interactions between the two domains that make up the origin. Oppositely-facing *ter* sites are located at the replicative terminus of the *E.coli* chromosome (5–7) and the plasmid R6K (8,9). These sites consist of about 20 bp of DNA and have a similar sequence (8–10). The host Tus protein binds to *ter* (11) and acts as an anti-helicase, thus interfering with progression of the replication fork (12–14). The activity of Tus is polar, and depends on the orientation of *ter* (5,12–14). We have found that when a *ter* site is cloned near the replicative origin of R1162, or between the two origin domains, it interferes with the replication of plasmid DNA in Tus⁺ cell extracts. The effects of position and orientation of the *ter* site suggest a model in which the initiation sites for DNA synthesis are activated by one or more proteins that travel primarily in one direction from the domain containing the direct repeats.

MATERIALS AND METHODS

Bacterial strains, plasmids, and *ter* DNA

The bacterial strains used in this study are MV10 (*thr leu lacY thi supE44 fhuA ΔtrpE5*), a derivative of C600 (15), and MV10 *tus*[–]. The Tus[–] strain was constructed by transduction with P1vir grown on PK2607 (9, kindly provided by P. Kuempel), which has a kanamycin-resistance marker disrupting the *tus* gene.

Transductants were selected for resistance to kanamycin, and subsequently screened for the Tus[–] phenotype by testing cell extracts for replication of pUC18 and pUC19 (16) derivatives containing the *ter* sequence. The pUC19 derivative contains *ter* in the active orientation, and its replication is thus specifically inhibited in the Tus⁺ extract (14). Extracts that no longer showed this specific inhibition were assumed to be derived from Tus[–] strains.

All the plasmids used in this study have the same general structure, but differ in the region of the origin of replication. For each plasmid, the configuration of the origin is shown in Fig. 1, and the underlying constructions are outlined in Table

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1. With the exception of the origin region, the plasmids are all nearly identical to pUT860, which has been described in detail (4). In brief, the plasmids contain an origin region (approx. 600 bp) that has been modified to contain restriction sites useful for cloning (3). This is linked to an approximately 5000 bp R1162 DNA fragment containing the essential replication genes, and a 1600 bp fragment derived from the IncP-1 plasmid R751 and encoding trimethoprim-resistance (Tp^r).

A DNA fragment containing the *ter* sequence T2 (9) was also prepared by annealing two chemically synthesized oligonucleotides. The sequence of the resulting fragment is shown in Fig. 2.

General procedures

Cell extracts for the replication of plasmid DNA in vitro were prepared and used generally according to the method of Scherzinger *et al.* (17), as described previously (3). Overall DNA synthesis was measured by adding 4 μ Ci [3 H]dTTP (81 Ci/mmole) to 25 μ l reaction mixtures containing 0–0.8 μ g supercoiled plasmid DNA, purified by CsCl-ethidium bromide centrifugation (18), and determining the number of acid-insoluble counts by liquid scintillation counting. For the detection of nascent DNA strands, reaction mixtures contained 0.20–0.25 μ g supercoiled plasmid DNA, 10 μ Ci [α - 32 P]dATP, and 100

pmoles ddCTP. Newly-synthesized strands were analyzed after denaturation by electrophoresis on 6% polyacrylamide–6M urea sequencing gels.

RESULTS AND DISCUSSION

The Tus–*ter* system brings about orientation-specific termination of R1162 DNA replication

We constructed plasmids containing the *E. coli ter* sequence on either side of the two initiation sites for R1162 DNA replication, *oriL* and *oriR*, and in each orientation (pUT1154, pUT1155, pUT1157 and pUT1159, Fig. 1). Plasmid pUT1154 contains *ter* oriented so that if it was active against the helicase associated with the replication fork, it would block progression of the nascent DNA strand initiated from *oriR*. Similarly, *ter* in pUT1157 would block synthesis from *oriL*. The plasmids with *ter* in the opposite orientation (pUT1155 and pUT1159) would have a lesser effect on synthesis, since they would affect the movement of a replication fork only after most of the corresponding plasmid strand had been synthesized.

Table 1. Plasmids used in this study.

Plasmid	Construction
1. pUT1106	Identical to pUT860 (4), but deleted for 950 bp BamHI fragment containing pBR322 and R751 DNA.
2. pUT1154, pUT1155	By insertion of <i>ter</i> synthetic sequence (Fig. 1) into XbaI-SmaI sites in pUC18 (16), followed by excision of the fragment with EcoRI+ HindIII. The single-stranded ends were filled in, and the fragment ligated to similarly flush-ended, EcoRI-digested pUT1106.
3. pUT1157, pUT1159	Same as for pUT1154 and pUT1155, but <i>ter</i> DNA inserted into EcoRV site within origin region (Fig. 1).
4. pUT1161	By replacement of small EcoRI-SmaI <i>oriL</i> fragment in pUT1106 with EcoRI-HindIII fragment from M13mp19 (19). The HindIII end was filled in prior to the replacement. A DNA fragment consisting of The EcoRV-SmaI <i>oriR</i> fragment, linked to a PvuII-SmaI fragment from M13mp19, was then inserted into the BamHI site in the origin region.
5. pUT1162, pUT1197	Same as pUT1161, but contains <i>ter</i> fragment, excised from pUC18 derivative by SmaI-HincII digestion, cloned into EcoRV site in origin region.
6. pUT1172	Same as pUT1161, but EcoRV-BamHI fragment containing DRs and AT-rich region cloned in opposite orientation.

Figure 1. Origins of replication for plasmid used in this study. The general organization of the naturally occurring R1162 replicative origin is shown at the top. Abbreviations for the functional domains are defined in the text. Relevant restriction sites (B = BamHI, R = EcoRI, Rv = EcoRV and Sm = SmaI) are also indicated.

Figure 2. DNA fragment containing *ter* site T2 (within dashed lines, 9) cloned into plasmids as shown in Fig. 1. The fragment was prepared by annealing two chemically synthesized oligonucleotides. In the orientation shown, the site would block helicase entering from the left.

Plasmid DNA replication was tested in cell extracts prepared from isogenic MV10 *tus*⁺ and *tus*⁻ strains containing the plasmid pMS94, which encodes the R1162 replication proteins (20). We did not observe differences in DNA synthesis in the *Tus*⁺ and *Tus*⁻ extracts for either pUT1154 or pUT1157 (Table 2). There was also no difference for pUT1155 (*ter* in inactive orientation relative to *oriR*) or pUT1106 (control plasmid without *ter*). In contrast, there was noticeable relative inhibition of pUT1159 DNA replication in the *Tus*⁺ extract. In this plasmid, *ter* is situated between the DNA initiation sites and the iterons, in an orientation that would block the movement of helicase or other proteins from the iteron domain toward *oriL* and *oriR* (Fig. 1).

Although overall DNA synthesis was not noticeably inhibited in *Tus*⁺ extracts in the case of pUT1154 and pUT1157, we looked for termination of replication fork movement by a more sensitive method. Nascent strand synthesis initiated from *oriR* in *Tus*⁺ and *Tus*⁻ extracts was examined for both pUT1154 (*ter* in the active orientation with respect to *oriR*), and pUT1155 (*ter* in the inactive orientation) (Fig. 3). After replication of the DNAs

Figure 3. Nascent strand DNA synthesis from *oriR* in *Tus*⁺ (lanes A-D) or *Tus*⁻ (lanes E-H) extracts. Lanes A,E: pUT1154, no digestion with restriction enzyme, lanes B,F: pUT1154, digested with PstI, lanes C,G: pUT1155, no digestion, lanes D,H: pUT1155, digested with KpnI.

Table 2. Replication of R1162 derivatives in *Tus*⁺ and *Tus*⁻ extracts.

Plasmid	DNA (nmol nucleotide) ^a	Incorporation of dTMP <i>Tus</i> ⁺ / <i>Tus</i> ⁻ (pmol) ^b	Ratio (<i>Tus</i> ⁺ / <i>Tus</i> ⁻)
1. pUT1106	0.6	53.4 / 51.5	1.0
	1.2	85.0 / 70.5	1.2
2. pUT1154	0.6	40.8 / 29.7	1.4
	1.2	68.5 / 58.2	1.2
3. pUT1155	0.6	61.0 / 82.7	0.7
	1.2	68.5 / 82.5	0.8
4. pUT1157	0.6	48.6 / 63.5	0.8
	1.2	49.4 / 62.1	0.8
5. pUT1159	0.6	14.7 / 69.8	0.2
	1.2	33.7 / 89.0	0.4

^a Amounts of plasmid DNA used were within experimentally-determined range for optimal DNA synthesis.

^b Background incorporation (no plasmid DNA) was less than 1 pmol and was subtracted from each value.

in cell extracts in the presence of ddCTP and [α -³²P]dATP, the DNA was digested with restriction endonuclease (PstI for pUT1154 and KpnI for pUT1155), subjected to denaturing polyacrylamide gel electrophoresis, and the newly-synthesized DNA visualized by autoradiography (3,21). In each case, a nascent strand of 230 bases was observed after the DNA was cleaved at the PstI or KpnI site located just before the *ter* sequence in the direction of travel of the replication fork from *oriR* (Fig. 3, lanes B, D, F, H). There were roughly similar amounts of this strand for both pUT1154 and pUT1155 in the *Tus*⁻ extract (lanes F and H), indicating that in the absence of *Tus* protein, *oriR* was equally active in both plasmids. In the *Tus*⁺ extract, the amount of nascent strand synthesis was generally higher for pUT1155 than for pUT1154 (lanes B and D). The reason for the difference in the *Tus*⁺ background is not clear. One possibility is that, even in the presence of ddCTP, enough strands complete replication in the absence of *Tus*-mediated termination to allow a detectable amount of reinitiation.

When digestion with restriction endonuclease was omitted, a band corresponding to a 240-base fragment of DNA was detected for pUT1154 DNA replicated in the *Tus*⁺ but not the *Tus*⁻ extract (Fig. 3, lanes A and E). This band was absent when pUT1155 was replicated in either extract (lanes C and G). The distance between *oriR* and the closer end of the *ter* site is 240 base-pairs, and therefore the DNA in this band likely represents nascent DNA synthesis initiated from *oriR* and terminated at *ter*. We conclude that the *Tus-ter* system can block the R1162 replication fork, but that it does so weakly.

The *Tus-ter* system inhibits activation of the initiation sites for R1162 replication

Unlike the other *ter*-containing plasmids, replication of pUT1159 DNA was significantly inhibited in *Tus*⁺ extracts (Table 2). The

Figure 4. Replication of (A) pUT1161 and (B) pUT1162 in cell extracts. Filled symbols: MV10 (*Tus*⁺) extract; hollow symbols: MV10 *tus*⁻ extract.

location and orientation of *ter* in this plasmid suggested that this site was interfering directly with activation of the initiation sites by the iteron-containing domain. To explore this possibility in greater detail, we constructed the plasmids pUT1161 and pUT1162 (Fig. 1), which contain the iteron domain bracketed by two, oppositely-facing copies of *oriR*. Because each plasmid strand has an *oriR* site for initiation, the plasmid DNA is replicated well both in vivo and in vitro, although the overall structure of the origin has been substantially altered (4). The plasmid pUT1162 also contains a copy of *ter* between one *oriR* and the iteron domain (Fig. 1).

Both pUT1161 and pUT1162 were tested for replication in Tus^+ and Tus^- extracts (Fig. 4). Plasmid pUT1161 was replicated to a similar extent in both cases (panel A), whereas replication of pUT1162 was inhibited when *Tus* protein was present (panel B). We examined nascent DNA synthesis from each *oriR*, in order to determine the degree of inhibition at each location. Plasmid DNAs were replicated in cell extracts containing ddCTP, and then digested with *EcoRI*. The expected sizes of the nascent DNA strands are shown in Fig. 5. A nascent strand of 115 bases would be generated by synthesis from the left *oriR*, as defined in the figure, and a strand of 155 bases by synthesis from the other *oriR*. In the Tus^- extract, DNA synthesis was initiated from each *oriR* in pUT1161 and pUT1162 (Fig. 6, lanes E and F). In both plasmids, there was substantially more initiation from the left *oriR*. The difference in the activity of each *oriR* was due to its position with respect to the iteron-containing domain. When this domain was inverted (pUT1172), the right *oriR* became most active (Fig. 6, lane H).

Different results were obtained when nascent strand synthesis in the Tus^+ extract was visualized. There was no effect on the relative activity of each *oriR* for pUT1161 or pUT1172, which

Figure 5. Location and sizes of nascent DNA strands expected for each *oriR* in pUT1161 and pUT1162 after digestion of plasmid DNA with *EcoRI*. The thickness of the arrows denotes the amount of synthesis from each origin in the Tus^+ extract, indicated from Fig. 6.

lack the *ter* sequence (Fig. 6, lanes A and D). However, for pUT1162, initiation from the left *oriR* was specifically inhibited (lane B). This effect depended on the orientation of *ter*. Plasmid pUT1197 (Figs. 1 and 5) is identical to pUT1162, but contains *ter* in the inverted orientation. The densities of the bands for pUT1197 in both the Tus^+ and Tus^- extracts were essentially the same as for pUT1161, which lacks the *ter* sequence (Fig. 6, lanes C and G).

Figure 6. Nascent strand DNA synthesis from each *oriR* in Tus^+ (lanes A-D) and Tus^- (lanes E-H) extracts. Plasmid DNAs are pUT1161 (lanes A,E), pUT1162 (lanes B,F), pUT1197 (lanes C,G) and pUT1172 (lanes D,H).

Figure 7. Scheme of events for activation of *oriR* in pUT1162.

Our results show that the *oriR* on one side of the iteron-containing domain, the side containing the AT-rich DNA (Fig. 1), is preferentially activated. In addition, binding of Tus at a properly oriented *ter* site between this initiation site and the iteron domain inhibits activation. We interpret this to mean that one or more proteins required for activation of *oriR* enter the DNA in the domain containing the direct repeats, and move predominantly in one direction away from these repeats. As drawn in Figs. 1 and 5, this movement would be leftward in pUT1161, pUT1162 and pUT1197, and rightward in pUT1172, which has the inverted DR domain. The favored side for initiation is occupied by *oriL* and *oriR* in the naturally-occurring R1162 (2). Replication of R1162 is DnaB-independent (22), but requires a plasmid-encoded helicase (1), which is therefore a likely component of the activation machinery. This helicase is apparently required for the transient melting of DNA at each *ori*, because it is no longer required for initiation of DNA synthesis if the origin is present in single-stranded form (23).

A reasonable sequence of events leading to activation of the R1162 origin is diagrammed in Fig. 7. The R1162 protein RepIB first binds to the direct repeats and causes localized melting of the adjacent AT-rich region, which serves as the point of entry for a helicase (24), plasmid-specific in this case. This helicase then moves predominantly away from the direct repeats to activate the initiation sites for replication. A *ter* sequence in the active orientation blocks movement of the helicase when Tus protein is supplied, thus interfering with activation of the downstream initiation site.

For pUT1161 and pUT1162, why does the *oriR* on the unfavorable side of the direct repeats nevertheless show weak activity? Activation of *oriR* presumably involves transient melting of the DNA, making the site accessible to the plasmid-encoded primase. This could come about by occasional migration of the helicase from its entry site through the direct repeats, a route opposite from the normal direction. The weak *oriR* might also be activated by an entering replication fork initiated from the other *oriR*. The first explanation is probably correct, because inhibition of the strong *oriR* by the Tus protein does not appear to reduce coordinately the activity of the weak *oriR* (Fig. 6, 155-base band in lanes A and B). Further experiments will be required to explain the molecular basis for the directional specificity of the R1162 helicase. One possibility is that either the GC-rich tract between the AT-rich region and the direct repeats (Fig. 7), or the direct repeats themselves with bound RepIB protein, or both, provide physical barriers to progressive unwinding and translocation by the helicase.

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