Timely Release of Both Replication Forks from oriC Requires Modulation of Origin Topology*

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Initiation of DNA replication at oriC occurs bidirectionally both in vivo and in vitro. Although the proteins involved in establishing the replication forks are known, little is known about the events that ensure that initiation is bidirectional. We show here that in the absence of DNA gyrase, replication fork progression from oriC on a plasmid template in vitro is unidirectional, although both replication forks have formed at the origin. There was no bias in the release of one fork or the other, ruling out protein blockage of one fork as a possible reason for the asymmetric release. Timely release of both forks required the presence of either DNA gyrase or topoisomerase IV, suggesting that modulation of the topology of the origin region is the governing factor.

Replication of circular bacterial genomes occurs bidirectionally from the origin of chromosomal DNA replication, oriC. In Escherichia coli, replication from the origin has been reproduced in vitro using small plasmid DNA templates carrying the E. coli oriC (1). DnaA recognizes and binds to a number of repeated sequences in the minimal origin, “DnaA boxes” (2), organizing the origin DNA into a nucleoprotein structure that enables the interaction of some of the DnaA protomers with A + T-rich 13-mers that are just counterclockwise to the minimal origin. In the presence of the double-stranded DNA-binding protein HU, these 13-mers become denatured, presumably as a result of a weak affinity of DnaA for the single-stranded 13-mers (3).

Once this region of localized denaturation is established, replication fork assembly can proceed, governed by a series of protein-protein interactions. DnaB, the replication fork DNA helicase, is transferred to the DNA at a stoichiometry of two DnaB hexamers per oriC (4) from DnaB-DnaC complexes in solution (5) as a result of a protein-protein interaction between DnaB and GyrB, we cannot detect one between DnaB and GyrA. However, although we have been able to detect an interaction between DnaB and GyrB, we cannot detect one between DnaB and DNA gyrase. We therefore conclude that topological modulation of the origin region requires the presence of either DNA gyrase or topoisomerase IV (topo IV). This requirement for a topoisomerase could reflect the formation of some type of a complex at the origin that is necessary for proper bidirectional replication. However, although we have been able to detect an interaction between DnaB and GyrB, we cannot detect one between DnaB and DNA gyrase. We therefore conclude that topological modulation of the origin region is necessary for timely release of both replication forks during initiation.

MATERIALS AND METHODS

Replication Proteins and Antisera—Proteins for oriC replication, DnaA, DnaB, DnaC, DnaG, HU, and the single-stranded DNA-binding protein (SSB), timely release of both of these replication forks from the origin requires the presence of either DNA gyrase or topoisomerase IV (topo IV). This requirement for a topoisomerase could reflect the formation of some type of a complex at the origin that is necessary for proper bidirectional replication.

N.S. dedicates this paper to the memory of Elena Smelkova.

1 The abbreviations used are: pol III HE, polymerase III holoenzyme; topo IV, topoisomerase IV; kb, kilobase pair; SSB, single-stranded DNA-binding protein; ERI, early replication intermediate.

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min. Bis(sulfosuccinimidyl) suberate (freshly diluted in 50 mM HEPES-KOH (pH 7.8) and 100 mM NaCl) was added to a final concentration of 0.004%, and the reactions were incubated at 30 °C for 30 min. Cross-linking was stopped by the addition of 1 M glycine to a final concentration of 66 mM, and the incubation was continued for an additional 10 min. After addition of 25 μl of a 2× loading buffer (100 mM Tris-HCl (pH 6.8), 200 mM β-mercaptoethanol, 20% glycerol, 0.2% bromphenol blue, and 4% SDS), the samples were heated at 100 °C for 5 min and then chilled on ice for 5 min. Proteins were electrophoresed through a 6.5% SDS-polyacrylamide gel at 220 V for 4 h and visualized by silver staining. For Western blotting, proteins were separated as described above except that the amount of protein loaded was reduced by half. Proteins were transferred overnight at 4 °C at 25 V onto nitrocellulose membrane. Blots were probed with rabbit polyclonal antibodies raised against either DnaB or GyrB and were developed using an alkaline phosphatase-conjugated system.

RESULTS

Only One Replication Fork Is Active in Early Replication Intermediates—Plasmid DNA templates carrying oriC that are replicated in vitro by a combination of DnaA, DnaB, DnaC, DnaG, HU, SSB, and DNA gyrase produce replication products that are characteristic of bidirectional replication initiating at or near the origin sequence (Fig. 1A). On these templates, which are 6.7 kb in length (Fig. 1C), bidirectional replication produces two distinct populations of DNA when analyzed by denaturing alkaline agarose gel electrophoresis (Fig. 1A, lane 2) as follows: a leading strand population centered about 3 kb in length, indicative of the fact that the two replication forks that form at oriC meet roughly half-way around the template; and a lagging strand population centered about 0.5 kb in length. The addition of RNase H, DNA ligase, and DNA polymerase I (subsequently denoted RLP) to the reaction converts the products to full-length material (Fig. 1A, lane 3), indicating that the clockwise-moving leading strand could be ligated to the counterclockwise moving lagging strand on the same template molecule, for example (Fig. 1B). Circular nascent DNA is not observed because with gyrase as the only topoisomerase present, the bulk of the replicated DNA accumulates as a late replication intermediate, where there is still, on average, about 150 base pairs of unreplicated parental DNA (14). We have shown previously that either topoisomerase III (15) or IV (16) is required to support the terminal stages of replication when the late intermediate is converted to two daughter molecules.

In the absence of gyrase, initiation can occur because the template DNA is negatively supercoiled; however, replication fork progression proceeds only until it is inhibited by the accu-
mulation of excess positive linkages in the template. The DNA product formed is called an early replication intermediate (ERI). We have shown that the ERI is a true kinetic intermediate in the replication pathway (15). Subsequent completion of replication requires the release of the topological strain, which can be accomplished by either the addition of a topoisomerase or by cutting the template with a restriction enzyme.

Leading strands present in the ERI are about 1 kb in length (Fig. 1A, lane 1). At first glance, this size seems far too great. The plasmid template DNA contains about 40 negative supercoils; thus, if two replication forks formed and proceeded away from oriC in the ERI, one would expect the leading strands to be about 350 nucleotides in length, indicative of removal of the negative supercoils as the template was unwound as a result of replication, followed by generation of about 30 positive supercoils before replication ceased because of the accumulated topological strain. However, as we will show here, only one replication fork releases from the origin in the ERI, and as we have shown elsewhere (17), origin-proximal regression of the nascent DNA in the ERI allows more fork progression than one would have predicted based on the superhelical density of the starting template DNA.

That only one replication fork was active in the ERI is shown by the pulse-chase analysis displayed in Fig. 1A (lanes 4 and 5). Here the ERI was formed first and then the [α-32P]dATP was diluted out by a factor of 100 as the topological strain was released by the addition of either SmaI, a restriction enzyme that cuts the template DNA just to the left of oriC (Fig. 1C), or DNA gyrase. In each case the result was the same in that full-length DNA was the predominant product. If the ERI contained two paused replication forks with leading strands of about 1 kb, the final product length observed after the chase should have been somewhere intermediate between 5.7 and 3.3 kb.

To investigate the possibility that only one replication fork was active in the ERI more closely, an assay was devised to score release of both replication forks on one DNA molecule. This assay used the same template DNA as in the experiment shown in Fig. 1A, but we now included the replication fork arrest protein Tus. This protein will bind to the Ter sequences that are present 1.2 kb counterclockwise and 1.4 kb clockwise of oriC. The basis of the assay is shown in Fig. 1D. If two replication forks initiated, released from the origin, and progressed to the Ter sites, leading strands of 1.2 and 1.4 kb should be observed. In the presence of RLP, on any one molecule that has two active forks, the counterclockwise-moving leading strand should be joined to the counterclockwise-moving lagging strand, and vice versa, generating a 2.6-kb nascent DNA. If only one fork has released, the addition of RLP will not have any affect on the length of the nascent products.

In the absence of Tus, bidirectional replication requires the release of the topological strain, which can be accomplished by either the addition of a topoisomerase or by cutting the template with a restriction enzyme. We have shown that the ERI is a true kinetic intermediate in the replication pathway (15). Subsequent completion of replication requires the release of the topological strain, which can be accomplished by either the addition of a topoisomerase or by cutting the template with a restriction enzyme.

In the absence of Tus, two populations of nascent DNA were observed as follows: a leading strand population centered about 1.2 kb in length and a smaller lagging strand population (Fig. 1E, lane 1). The length of the leading strand population argues strongly that on most template molecules only one fork had progressed. Bidirectional leading strand synthesis on one DNA template would have been expected to result in leading strands that were, at best, half of the observed length when the superhelical density of the template and nascent strand regression were factored in. The addition of Tus led to the production of leading strand DNA products of defined length (Fig. 1E, lane 3). However, in the presence of RLP, although ligation of the nascent lagging strand fragments together could be observed, in neither case were any products larger than 1.2–1.4 kb observed (Fig. 1E, lanes 2 and 4). On the other hand, when Tus was present, the two defined leading strands were again observed in the presence of Tus, but now the addition of RLP converted the majority of the nascent DNA to a 2.6-kb product (Fig. 1E, compare lanes 5 and 6).

The kinetics of fork release from the origin in the absence and presence of Tus was investigated using the assay described above (Fig. 2). In the presence of Tus, the 2.6-kb band could be detected within 1 min after the start of the reaction, whereas even after 15 min of incubation in the absence of Tus only a small minority of the nascent DNA was converted to this product (Fig. 2B). Therefore, we conclude that in the absence of Tus only one replication fork progresses to any significant extent. This is also supported by the observation that roughly twice as much DNA was synthesized in the presence of Tus compared with the absence of Tus (Fig. 2C).

Even in the Absence of Tus, both Replication Forks Form at the Origin.—We next investigated whether the reason that only one fork progressed in the ERI was because only one fork, rather than the two required for bidirectional replication, was initiated under the conditions used. To do so, we used pulse-chase analysis as described above, except that Tus was present from the beginning of the reaction so that the conditions of the assay described in Fig. 1D applied. Thus, the ERI was formed first in the presence of [α-32P]dATP. The label was then diluted out by the addition of a 100-fold excess of cold dATP at the same time that the topological constraint was released by adding either DNA gyrase or Spdl, which cuts the template once outside of the region flanked by the Ter sites (Fig. 1C).

In either case, the 2.6-kb product diagnostic of bidirectional replication formed (Fig. 3A) with the same kinetics (Fig. 3B). These observations indicate that during the initial incubation...
not gyrase were incubated for 1 min in the presence of [α-32P]dATP at 37 °C. A 100-fold excess of unlabeled dATP and either gyrase (lanes 1–5) or SphI (lanes 6–10) was then added and the incubation continued. Aliquots (15 μl) were withdrawn at the indicated times, and the samples were processed and analyzed as described under "Materials and Methods." An autoradiogram of the denaturing alkaline agarose gel is shown. B, PhosphorImager analysis was used to quantify the fraction of the total radioactivity incorporated that was represented by the 2.6-kb DNA species.

Fig. 3. Both forks form at the origin during initiation. A, pulse-chase analysis of replication from pre-formed ERIs. Standard replication reaction mixtures increased in volume 6-fold containing Tus but not gyrase were incubated for 1 min in the presence of [α-32P]dATP at 37 °C. A 100-fold excess of unlabeled dATP and either gyrase (lanes 1–5) or SphI (lanes 6–10) was then added and the incubation continued. Aliquots (15 μl) were withdrawn at the indicated times, and the samples were processed and analyzed as described under "Materials and Methods." An autoradiogram of the denaturing alkaline agarose gel is shown. B, PhosphorImager analysis was used to quantitate the fraction of the total radioactivity incorporated that was represented by the 2.6-kb DNA species.

in the presence of the labeled dATP, both replication forks had, in fact, formed at the origin in the absence of gyrase. The fact that identical results were observed using either DNA gyrase or SmaI ruled out the possibility that, on any one molecule, nascent DNA made by one fork, e.g. the clockwise one, that had formed during the pulse-label became joined to nascent DNA made by a counterclockwise fork that had formed during the chase period. This is because initiation at oriC requires negatively superhelical DNA (15). Digestion by SmaI prevents subsequent initiation by relaxing the template. Thus, it seemed clear that the reason that both forks were not observed in Fig. 1A was that there was a delay in the release of one of the forks from the origin. Because the plasmid template is so small and the replication fork is known to move at roughly 1 kb/s, any delay in the neighborhood of 6 s will, in the absence of Tus, allow the first fork that released to transit all the way around the template before the release of the second fork. We conclude that gyrase is neither required for formation of replication forks during initiation nor for their progression.

Timely Release of Both Replication Forks from the Origin Requires Modulation of Template Topology—We considered three possible reasons for the observed delay in release of both replication forks from oriC in the absence of gyrase: (i) that in fact both forks did release, but there was a roadblock on one side or the other and close to the origin that prevented progression of one of the forks; (ii) that an interaction between gyrase and one of the proteins of the replisome was required; and (iii) that it was release of topological strain across the origin that was the determining factor. Of course, a combination of these is also possible.

To examine the possibility of blockage, we reasoned that such an event would be manifested as a bias in which of the forks appeared to release first. Indeed, cursory examination of the data in Figs. 1 and 2 supports this idea, because there appears to be a bias for the release of the counterclockwise moving fork in the absence of gyrase. This possibility resonates with the fact that because initiation of both forks occurs just to the left of the origin, the clockwise-moving fork would have to presumably transit through the region of the template where DnaA was bound. This could cause significant delay. Indeed, in the λ replication system, such a delay appears to be caused by the ΦO protein bound to the origin (18).

To examine this possibility more closely, replication reactions were performed in the presence of Tus and in either the presence or absence of gyrase. The replication proteins were then inactivated by heating at 65 °C, and the DNA products were digested with either the EcoRI or SacII restriction enzymes, which cut the template once between the origin and the Ter sites in the counterclockwise and clockwise directions, respectively (Fig. 4A). The amount of radioactivity in the bands at 1.2 and 1.4 kb was then determined. This restriction enzyme digestion was necessary to remove a background that compli-
icates the analysis, i.e., in the absence of this digestion, for example, incomplete leading strands from the clockwise-moving fork that were in excess of 1200 nucleotides would appear to contribute to the amount of radioactivity on the gel that could be attributed to the counterclockwise-moving fork. The restriction enzyme digestion removes these leading strands from that region of the gel.

The results of this analysis are shown in Fig. 4. In the presence of gyrase, the amount of radioactivity is roughly the same for each fork (Fig. 4, B and D). In the absence of gyrase, there is less than a 2-fold bias for the counterclockwise-moving fork at the earliest time points in the analysis (Fig. 4, C and E). We think that this is unlikely to indicate a significant bias in fork release because, as described above, the analysis is somewhat subjective with respect to how regions of the gel are chosen to represent one fork or the other and the data have not been normalized to the size of the DNA products (which would further reduce the apparent bias). If there actually were a 2-fold bias in fork release, we would have expected to observe a 2.6-kb band representing half the radioactivity in lanes 8–10 of Fig. 2A, for example. We therefore conclude that whereas there may be a slight bias to which fork releases in the ERI in the absence of gyrase, it is insufficient to account for the data shown in Figs. 1 and 2.

We also considered that gyrase could be playing an architectural role at the origin, contributing to the formation of a replisome complex at the origin that was necessary for proper bidirectional replication. We reasoned that this might be manifested by protein-protein interactions between gyrase and a component of the replication fork. An examination by cross-linking using the bifunctional cross-linking agent bis(sulfosuccinimidyl) suberate revealed an interaction between GyrB and DnaB (Fig. 5). Mixtures of these proteins at high concentrations (>1 μM) were treated with the cross-linking agent for 30 min and then analyzed by electrophoresis through 6.5% polyacrylamide gels containing SDS. A silver-stained gel (Fig. 5A) as well gels that were Western-blotted using either anti-DnaB antisera (Fig. 5B) or anti-GyrB antisera (Fig. 5C) are shown. In each case, a band (labeled with an asterisk) could be detected with a mobility of about 220 kDa that was dependent on the presence of both GyrB and DnaB. This is a clear indication of an interaction between these two proteins. From the estimated size of the cross-linked band, we suspect that the molecular species involved are GyrB2 and DnaB3, and both forms are known to exist in solution. Although this is a promising and interesting observation, we have been unable to demonstrate an interaction between DnaB and native gyrase; thus, because we cannot eliminate the possibility that the interaction surface on GyrB is unavailable for DnaB when GyrB is formed into native gyrase, we cannot ascribe any significance to the observed interaction.

To explore whether the requirement for gyrase for timely release of both replication forks from oriC related to timely modulation of the topology about the origin, we repeated the experiment described in Fig. 2 replacing gyrase with topo IV (Fig. 6). Although topo IV cannot supercoil DNA as gyrase does, it will relax positive supercoils and can support replication fork progression (19). We found that both replication forks released from the origin, allowing the formation of the 2.6-kb DNA fragment. Thus, we conclude that the observed requirement for gyrase for timely release of both replication forks from the origin relates to a requirement for topological modulation of the template DNA and is unlikely to be a specific requirement for the presence of gyrase itself.

**Fig. 5.** Protein-protein cross-linking detects an interaction between GyrB and DnaB. Protein-protein cross-linking was as described under “Materials and Methods.” Three gels are shown. A, silver-stained gel. B and C, Western blots of gels using either anti-DnaB antisera (B) or anti-GyrB antisera (C) as probes. The asterisk in all panels shows the position of the DnaB-GyrB cross-linked species.

**Fig. 6.** Topological modulation of the origin is required for the timely release of both replication forks. A standard replication reaction mixture containing Tus and topoisomerase IV was increased in volume 6-fold and incubated at 37 °C. Aliquots (15 μl) were removed at the indicated times, and the samples were processed and analyzed as described under “Materials and Methods.” Shown for comparison are the products of replication after a 5-min incubation in the presence of gyrase.

**DISCUSSION**

Replication of the E. coli chromosome occurs bidirectionally from oriC (20, 21). Although this reaction has been reproduced in vitro, thus identifying the enzymes required, little is known of the molecular events that actually occur at the origin to ensure that two replication forks have formed and can proceed
around the chromosome. In this report, we have shown that in the absence of topological modulation of the template DNA, even though two replication forks can be detected as having initiated at the origin, only one fork is released.

Because the plasmid template DNA used in vitro is negatively supercoiled, we have been able to develop a pulse-chase protocol that allows us to observe the action of a particular set of replication forks. In this procedure, an ERI is formed by initiating replication in the absence of a topoisomerase. During this period [$\alpha^{32}$P]dATP is included in the reaction. Replication fork progression proceeds in the ERI until excess positive supercoils have accumulated. These forks are stalled, but active, and replication fork progression can be resumed by the provision of either a topoisomerase or by cutting the template DNA with a restriction enzyme. In the latter run out, part of the procedure, excess unlabeled dATP is included to dilute the radioactive label. By using this procedure we have been able to show that either DNA gyrase (15), topo IV (19), or topoisomerase I (15), can support replication fork progression during $\theta$-type DNA replication, for example. We had noted, however, that the replication run out product under these conditions was invariably full length. This suggested that only one replication fork was active in the ERI. If both forks were active, it would not be possible to observe a full-length leading strand DNA; instead, one would expect two half-length leading strands.

To investigate this possibility further, we developed an assay that could definitively score bidirectional replication on the template DNA. This assay utilizes the replication fork arrest protein, Tus. Template DNAs were engineered such that the binding sites, Ter, for Tus were positioned close to and on either side of the origin in an orientation that would result in replication fork arrest in both directions when Tus was present. In addition, DNA polymerase I, RNase H, and DNA ligase were included in the reaction. This ensured that in the case of bidirectional replication, the leading strand that was being synthesized, e.g. in the clockwise direction from the origin, would be ligated with the Okazaki fragments being made in the counterclockwise direction from the same template strand. Thus, if these circumstances prevailed, a DNA fragment equal in length to the distance between the two Ter sites on the template would be produced. On the other hand, if one replication fork had moved clockwise on one template and another replication fork had moved counterclockwise on a different template, no such fragment could be generated.

By using this assay, it was clear that only one replication fork had progressed to any significant extent in ERIs. Interestingly, however, it could be demonstrated that both replication forks had formed during initiation. We considered three possible explanations for these observations. One obvious possibility was that one of the replication forks was blocked and prevented from moving a significant distance because of DnaA bound to the origin. This is because initiation at oriC actually occurs in an A + T-rich zone just counterclockwise of the minimal origin sequence (4). Thus, the clockwise-moving fork has to transit across the origin, a region of DNA that is presumably bound to multiple DnaA molecules in a nucleoprotein-like, protein-DNA complex (22). Indeed, during $\theta$-type replication of plasmid templates carrying the bacteriophage $\lambda$ origins of replication, such a blockage has been observed (18). Although we obtained evidence for a slight bias in fork release in the anticipated direction, it could not account for the data.

Another possibility that was considered was that formation of a replisome complex at the origin might require gyrase as a component, rather than require its activity as a topoisomerase, to be assembled correctly. This would be consonant with recent cell biological findings in vivo in Bacillus subtilis (23, 24) that suggest that replication of the chromosome occurs at a fixed point in the cell, where the template DNA is presumably drawn through a protein complex rather than the individual replication forks traversing each chromosome arm. Along these lines, we were able to demonstrate that one of the subunits of gyrase, GyrB, could be cross-linked to DnaB. However, a similar interaction between DnaB and native gyrase could not be demonstrated. Even so, if such an interaction occurred, we have no independent means for placing it at the origin. Thus, the possibility that a single replication factory synthesizes the DNA in a bacterial cell remains an attractive hypothesis.

In fact, we could demonstrate that gyrase was neither required for the assembly of the two forks at the origin nor for timely release of both forks. Topo IV worked just as well. This observation leads us to the conclusion that we were observing a requirement for timely topological modulation of the template DNA that allowed both forks to proceed. We do not know what aspect of origin structure this topological modulation is required to relieve. One possibility is that as both forks proceed from the origin, negative supercoils might become trapped in the region between them, distorting the DNA and preventing elongation by the fork that released second. In support of this, we have also observed that inclusion of topoisomerase III, which will relax only negative, not positive, supercoils under these conditions, also allows both replication forks to proceed (data not shown).

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