Direct Evidence for the Formation of Precatenanes during DNA Replication*

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Background: Changes in DNA topology during replication are still poorly understood.

Results: Classical genetics and two-dimensional agarose gel electrophoresis showed that RIs tensioned in the absence of Topo IV.

Conclusion: The results indicated that replication forks swivel in vivo leading to the formation of precatenanes as replication progresses.

Significance: This conclusion ends a long lasting debate on the formation of precatenanes during replication.

Changes in DNA topology during replication are still poorly understood. Bacterial plasmids are negatively supercoiled. This underwinding facilitates strand separation of the DNA duplex during replication. Leading the replisome, a DNA helicase separates the parental strands that are to be used as templates. This strand separation causes overwinding of the duplex ahead. If this overwinding persists, it would eventually impede fork progression. In bacteria, DNA gyrase and topoisomerase IV act ahead of the fork to keep DNA underwound. However, the processivity of the DNA helicase might overcome DNA gyrase and topoisomerase IV. It was proposed that the overwinding that builds up ahead of the fork could force it to swivel and diffuse this positive supercoiling behind the fork where topoisomerase IV would also act to maintain replicating the DNA underwound. Putative intertwining of sister duplexes in the replicated region are called precatenanes. Fork swiveling and the formation of precatenanes, however, are still questioned. Here, we used classical genetics and high resolution two-dimensional agarose gel electrophoresis to examine the torsional tension of replication intermediates of three bacterial plasmids with the fork stalled at different sites before termination. The results obtained indicated that precatenanes do form as replication progresses before termination.

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§ The abbreviations used are: (−), negative or negatively; (+), positive or positively; RH, right-handed; LH, left-handed; Topo IV, topoisomerase IV; CCC, unreplicated covalently closed circle; OC, unreplicated open circle; CCRI, covalently closed replication intermediate; OCRI, open circle replication intermediate; L, linear form; CatA, catenane where both rings are nicked; CatB, catenanes where one ring is nicked and the other covalently closed; CatC, catenane where both rings are covalently closed.

The dynamics of DNA topology during replication are still poorly understood. Bacterial plasmids are negatively supercoiled. This underwinding facilitates strand separation of the DNA duplex during replication. Leading the replisome, a DNA helicase separates the parental strands that are to be used as templates. This strand separation causes overwinding of the duplex ahead. If this overwinding persists, it would eventually impede fork progression. In bacteria, DNA gyrase and topoisomerase IV act ahead of the fork to keep DNA underwound. However, the processivity of the DNA helicase might overcome DNA gyrase and topoisomerase IV. It was proposed that the overwinding that builds up ahead of the fork could force it to swivel and diffuse this positive supercoiling behind the fork where topoisomerase IV would also act to maintain replicating the DNA underwound. Putative intertwining of sister duplexes in the replicated region are called precatenanes. Fork swiveling and the formation of precatenanes, however, are still questioned. Here, we used classical genetics and high resolution two-dimensional agarose gel electrophoresis to examine the torsional tension of replication intermediates of three bacterial plasmids with the fork stalled at different sites before termination. The results obtained indicated that precatenanes do form as replication progresses before termination.

The results indicated that replication forks swivel in vivo leading to the formation of precatenanes as replication progresses. This conclusion ends a long lasting debate on the formation of precatenanes during replication.

In bacteria, DNA gyrase and topoisomerase IV (Topo IV) are the main type II DNA topoisomerases involved in the regulation of DNA topology during replication (12, 13). Both enzymes can relax supercoiled DNA molecules. Their mechanism of action, however, differs significantly (14). Topo IV can eliminate both the LH crossings of (+)-supercoiling as well as the RH crossings of (−)-supercoiling, although it is 20× more efficient doing the former (5, 15). In contrast, DNA gyrase is unique in its ability to introduce (−)-supercoils into covalently closed domains (16). When compared for their ability to remove (−)-supercoils, Topo IV and DNA gyrase eliminate them at roughly the same rate (6). However, Topo IV is 20× more efficient removing LH than RH crossings (5, 15). In other words, DNA gyrase is 20× more efficient introducing RH crossings than Topo IV is at removing them. DNA gyrase plays a critical role in maintaining replicating DNA (−)-supercoil and is essential for replication to proceed. Moreover, in the absence of Topo IV, replication progresses at almost normal rates, but fully replicated duplexes accumulate as catenated rings (5, 17). Despite that a conclusive explanation for the preference of Topo IV for LH crossings is still debated, there is general agreement that...
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FIGURE 1. Schematics illustrating a covalently closed and supercoiled molecule and two nicked molecules heavily intertwined. Note that the covalently closed molecule is (−)-supercoiled (A) where all the crossings are RH. In the catenated rings showed to the right (B), the duplexes intertwine in an RH manner, too. In the covalently closed molecule, the DNA duplex is depicted in blue and green, and the catenated duplexes are depicted in blue and red and green and red, respectively, indicating that they are the natural products of replication where the newly synthesized strands are depicted in red. Full black dots indicate the intramolecular nodes of the supercoiled form. Asterisks indicate the intermolecular nodes of the catenane.

Differences in the geometry of the crossings must play a major role (5, 8, 15, 18–23). DNA gyrase and Topo IV acting together ahead of the fork could be sufficient for replication to be completed. However, the processivity of helicase generating (+)-supercoiling might overcome DNA gyrase and Topo IV in their attempt to keep unreplicating DNA underwound. This potential problem was first recognized by Champoux and Been (24), who proposed that the (+)-supercoiling that transiently accumulates ahead of the fork could force it to swivel to diffuse this (+)-supercoiling behind the fork where Topo IV, the main decatenate in Escherichia coli (14, 25), would help to maintain the DNA of replicating molecules underwound. The foundations to analyze the structure of replication intermediates in vivo were established in the Cozzarelli and Marians laboratories in the late nineties of the last century. They called the putative intertwines of sister duplexes in the replicated region “precatenanes” to distinguish them from the supercoils of the unreplicated region (10, 11). Formation of precatenanes as replication progresses in vivo, however, is still questioned because if the replisome is anchored to the cell membrane, it would not be able to swivel (17, 19, 26–28). In any case, it is well known that in the absence of Topo IV fully replicated duplexes accumulate as catenated rings (17, 29). These rings intertwine in an RH manner (Fig. 1B). It should be noted, though, that once DNA is isolated and all proteins are removed, the forks have no impediment to rotate freely in vitro, and this rotation allows diffusion of the torsional tension from the replicated to the unreplicated regions and vice versa to achieve thermodynamic equilibrium (11). This redistribution of torsional tension occurs in such a way that the LH supercoils of the unreplicated region switch to RH precatenanes when diffused to the replicated region and vice versa. In other words, the naked DNA examined in vitro does not necessarily reflect the situation in vivo. We are only allowed to inspect naked DNA from equilibrated replication intermediates (RIs) but ignore whether or not their geometry changes due to deproteinization. In summary, one of the most puzzling uncertainties regarding the dynamics of DNA topology during replication concerns whether or not forks can swivel in vivo leading to the formation of precatenanes as the replication fork advances (10, 19, 30). In an attempt to shed new light on this issue, we used classical genetics and high resolution two-dimensional agarose gel electrophoresis to examine the RIs of the following three bacterial plasmids, pBR-terE@StyI, pBR-terE@AatII, and pBR-terE@DraI (29), isolated from E. coli strains with different combinations of topoisomerases. In most cases for these three plasmids, the replicating fork stalls as it reaches the TerE-Tus complex (31, 32). This blockage leads to the accumulation of partially replicated molecules with a mass ~1.2×, ~1.6×, and ~1.8× the mass of unreplicated molecules (Fig. 3).

The first possibility indicates that forks do not swivel during replication. This hypothesis precludes the formation of precatenanes during replication. In the case of unimpaired replication, fully replicated molecules would end up catenated in an RH manner solely due to the linking of the last ~200 bp of DNA for which it would not be possible for DNA gyrase and Topo IV to remove the links ahead of the replication fork at termination (6, 17).

According to this hypothesis, if RIs with the fork stalled at the ter-Tus complex were isolated from bacterial cells where Topo IV is active, molecules with the fork stalled would be (−)-supercoiled (showing RH crossings) in the unreplicated region and devoid of precatenanes (Fig. 2A). After deproteinization, though, some of the RH crossings of the unreplicated region would diffuse to the replicated one as LH precatenanes to equilibrate the torsional tension between unreplicated and replicated regions. In consequence, after deproteinization, these RIs would have RH crossings in the unreplicated region and LH precatenanes (Fig. 2B).

If RIs were isolated from cells where Topo IV is inactive, molecules would be (−)-supercoiled (showing RH crossings) in the unreplicated region and also devoid of precatenanes. As in the previous case, after deproteinization some of the (−)-supercoils of the unreplicated region would diffuse to the replicated one as LH precatenanes. In other words, if replication forks cannot rotate in vivo, partially replicated molecules with the fork stalled will look alike after deproteinization regardless of whether they were isolated from Topo IV-proficient or -deficient cells. The degree of torsional tension, however, would vary depending on the length of the region that remained unreplicated after fork stalling (Fig. 3). This region would represent only ~20% of the RIs for pBR-terE@DraI (full size ~7.98 kb), ~40% for pBR-terE@AatII (full size 7.12 kb), and ~80% for pBR-terE@StyI (full size 5.52 kb).

In summary, if the forks were not allowed to swivel during replication, the shorter RIs’ unreplicated region, DNA gyrase, would have less space to introduce RH crossings. This torsional tension introduced by DNA gyrase in vivo once the replication fork has stalled at the ter-Tus complex would redistribute
among the unreplicated and replicated regions only after deproteinization. For this reason, pBR-terE@StyI RIs would show the highest degree of torsional tension and pBR-terE@DraI the lowest. The important point here is that this would occur regardless of whether Topo IV was active or inactive in vivo.

The second possibility indicates that forks do swivel during replication. If true, forks would rotate clockwise or counterclockwise depending on the nature and degree of torsional tension ahead and behind the fork. If replication progresses unimpaired in a bacterial strain where Topo IV is active, (+)-supercoiling would continuously accumulate immediately ahead of the fork but only transiently. This local (+) torsional tension would force the fork to rotate in such a way to diffuse these (+)-supercoils to the replicated region as RH precatenanes (10, 11, 24). Topo IV would progressively remove these precatenanes. Upon stalling the replication fork, though, no more (+)-supercoiling would form ahead of the fork, and molecules would tend to equilibrate the torsional tension between unreplicated and replicated regions already in vivo. As mentioned previously, DNA gyrase is ~20× more efficient introducing RH crossings than Topo IV in removing them (5, 6, 15). For this reason, shortly after fork stalling, the unreplicated region would become saturated with (-)-supercoiling. DNA gyrase would keep introducing (-)-supercoils in the unreplicated region, and to keep the torsional tension equilibrated, these RH crossings from the unreplicated region would diffuse to the replicated one as LH precatenanes, where Topo IV would progressively remove them. It should be noted that Topo IV is thought to remove LH crossings as efficiently as

DNA gyrase introduces (-)-supercoiling (6, 33). This detrimental tug-of-war between DNA gyrase and Topo IV would start as soon as the replicating fork stalls at the terE-Tus complex and would likely continue until cell harvest. In consequence, partially replicated molecules isolated from Topo IV-proficient cells would be neither saturated with (-)-supercoiling in the unreplicated region nor heavily precatenated in the replicated one.

Note that for ColE1 plasmids in E. coli cells, there are 20–40 copies per cell, and this copy number is stringently regulated (34, 35). Moreover, stalling of the replication fork at the terE-Tus complex only occurs in ~80% of the cases. In the remaining ~20% (~5 plasmids per cell), the replicating fork goes by terE unimpaired probably due to a failure in the formation of a stable terE-Tus complex (36, 37). In these plasmids, replication is completed and segregation follows provided Topo IV is active. This small but consistent number of plasmids

![FIGURE 2: Schematics illustrating three partially replicated molecules. A, if the forks cannot swivel in vivo, the unreplicated region would be (−)-supercoiled showing RH crossings. The replicated region would be relaxed as the torsional tension ahead and behind the fork. B, after deproteinization, the forks are allowed to rotate (blue arrow) freely to achieve thermodynamic equilibrium. Some of the RH crossings of the unreplicated region in A will diffuse to the replicated region where they will adopt the form of LH precatenanes. The replicated region contains one continuous parental strand and one discontinuous nascent strand, each crossing in the replicated region corresponds to two precatenane crossings. C, if the forks can swivel in vivo, partially replicated molecules would be saturated with (−)-supercoiling (showing RH crossings) in the unreplicated region and heavily intertwined (showing LH crossings) in the replicated region.](image)

![FIGURE 3: Genetic maps and cartoons illustrating the three plasmids used in this study: pBR-terE@StyI, pBR-terE@AatII, and pBR-terE@DraI. The genetic maps show the name and mass of each plasmid. Inside, each map shows the relative position of its most relevant features as follows: the ColE1 unidirectional origin in green (ColE1 Ori); the E. coli terminator sequence in red (terE); and the rop gene and the ampicillin and tetracycline resistance genes in blue (ampR and terR). Outside, the relative position of the site recognized by AlwNI is indicated. To the right of the maps, schematics illustrate the partially replicated molecules with their corresponding masses. For comparison, all of them were drawn with a Δk = −4.](image)
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carrying the β-lactamase gene allows the cells to survive in the presence of ampicillin. In cells where Topo IV is inactive, however, although ~20% of the plasmids complete replication, the sister duplexes fail to segregate and catenanes accumulate (17, 29). As mechanical nicking is unavoidable during DNA isolation, these catenanes are detected in three different forms as follows: catenanes where both rings are nicked (CatAs); catenanes where one ring is nicked and the other covalently closed (CatBs); and catenanes where both rings are covalently closed (CatCs) (17, 29, 38, 39) together with partially replicated molecules.

If RIs were isolated from cells where Topo IV is inactive in vivo, upon fork stalling at the terE-Tus complex, no more (+)-supercoiling would form ahead of the fork. DNA gyrase, though, would keep introducing RH supercoils in the unreplicated region. As here we consider that forks do rotate in vivo, the RH crossings introduced by DNA gyrase in the unreplicated region would continuously diffuse to the replicated one as LH precatenanes. Here, however, Topo IV would not remove them. In consequence, these RIs would end up saturated with (+)-supercoiling in the unreplicated region and heavily precatenated (showing LH crossings) in the replicated one. In other words, if forks swivel in vivo, the RIs of the three plasmids isolated from Topo IV-proficient cells would be poorly torsioned as compared with those isolated from Topo IV-deficient cells. In both cases, though, the composition of the torsional tension would be different for the three plasmids. It would mainly include (−)-supercoiling for pBR-terE@StyI and predominantly left-handed precatenanes for pBR-terE@Dral (see Fig. 3).

Experimental Procedures

Bacterial Strains, Plasmids, and Culture Medium—The E. coli strains used in this study were DH5αF’ {F’ [gyrA96 (Nal’) recA1 relA1 endA1 thi-1 hisdR17 (rpsL-marg) glnV44 deoR Δ(lacZYA-argF)U169] F [lacProAC15 gyrB225 topA+ transduced]} and parE10 {W3110 [F rpsL-marg]}. Competent cells were transformed with monomeric forms of pBR-terE@StyI, pBR-terE@AatII, or pBR-terE@Dral and all derivatives of pBR322 with the polar replication terminator TerE (31, 32) cloned at variable distances from the unidirectional ColE1 origin as described before (29). DH5αF’ cells were grown in LB medium at 37 °C, and parE10 cells were grown at the restrictive temperature (43 °C). In all cases, 75 μg/ml ampicillin was added to the LB medium. Isolation of plasmid DNA was performed as described elsewhere (40–42).

Two-dimensional Agarose Gel Electrophoresis and Southern Transfer—The first dimension was in a 0.4% agarose (Seakem LE, Lonza) gel in TBE buffer (89 mM Tris borate, 2 mM EDTA) at 0.9 V/cm at room temperature for 25 h. The second dimension was in a 1% agarose gel in TBE buffer and was run perpendicular to the first dimension. The dissolved agarose was poured around the excised agarose lane from the first dimension, and electrophoresis was at 5 V/cm in a 4 °C cold chamber for 10 h. When necessary, 5 μg/ml chloroquine (Sigma) was added to the TBE buffer in both the agarose gel and the running buffer in the second dimension. Southern transfer was performed as described before (40–42).

Nonradioactive Hybridization—DNA probes were labeled with digoxigenin using the DIG-High Prime kit (Roche Applied Science). Membranes (nylon membranes, positively charged, Roche Applied Science) were prehybridized in a 20-ml prehybridization solution (2 × 0.5% Blotto, 1% SDS, 10% dextran sulfate, and 0.5 mg/ml sonicated and denatured salmon sperm DNA) at 65 °C for 4–6 h. Labeled DNA was added, and the hybridization lasted for 12–16 h. Hybridized membranes were sequentially washed with 2 × SSC and 0.1% SDS at room temperature for 5 min twice and with 0.1 × SSC and 0.1% SDS at 68 °C for 15 min twice as well. Detection was performed with an antidigoxigenin-alkaline phosphatase-conjugated antibody (Roche Applied Science) and CDP-Star (PerkinElmer Life Sciences) according to the instructions provided by the manufacturer. All experiments were performed twice to confirm the results obtained were reproducible.

Densitometry—Autoradiograms were scanned, and the region where unknotted and knotted RIs migrated was analyzed by densitometry using Image J64 (National Institutes of Health) to determine the ratio of knotted to unknotted molecules (29, 43).

Results

Our primary aim was to inhibit Topo IV without affecting DNA gyrase. For this reason, we first chose E. coli DH5αF’ cells as they bear the mutation gyrA96 that turns these cells resistant to derivatives of nalidixic acid (25, 44). Indeed, cells of this strain exposed to norfloxacine have been successfully used before to accumulate catenanes without affecting DNA gyrase (17, 45). In addition, DH5αF’ cells are RecA− avoiding the formation of multimers (46, 47). The convenience to use a RecA− strain is clearly illustrated in Fig. 4A. pBR-terE@AatII was used to transform W3110 cells that are RecA+. The combined detection of unreplicated and partially replicated forms of monomers and multimers, i.e. dimers, trimers, and so on, makes it difficult to identify the signals of interest. However, we found that molecules containing stalled forks tend to break in the presence of norfloxacine (Fig. 4B). The signals marked as “Broken RIs” are indicative of RIs broken at the forks (48, 49). For this reason, to inhibit Topo IV without affecting DNA gyrase we switched to parE10 cells that bear a Topo IV+ mutation (25, 26, 44). To check whether the topology of bacterial plasmids was somehow affected in DH5αF’ cells in the absence of norfloxacine compared with a wild-type strain, we determined the supercoiling density (α) of unreplicated forms of pBR-terE@AatII isolated from either DH5αF’ cells that are RecA−, Topo IV+, and gyrA96 or W3110 cells that are RecA+, Topo IV+, and Gyr+. The results obtained are shown in Fig. 5. In both cases σ was ~0.065 (modal ∆k = −27). Therefore, in the experiments that follow, in order to determine the torsional tension of RIs in the presence and absence of Topo IV, we decided to use DH5αF’ cells and parE10 cells exposed to the restrictive temperature for the last hour.

We transformed both cell strains with the bacterial plasmids pBR-terE@StyI, pBR-terE@AatII, and pBR-terE@Dral, cultured the cells until they achieved logarithmic growth, and iso-
lated the DNA from either *E. coli* DH5αF’ cells (where Topo IV is active) or parE10 cells grown for the last 15 min. To the right of each immunogram is an interpretative diagram of the most relevant features where unreplicated CCCs and OCs are depicted in black. CCRIs are depicted in red. Numbers refer to monomers (1) and multimers: dimers (2), trimers (3), etc. Broken RIs are indicated (48, 49).

**FIGURE 4.** Immunograms of intact forms of pBR-terE@AatII isolated from either *E. coli* W3110 or DH5αF’ cells (exposed to norfloxacin) analyzed by two-dimensional agarose gel electrophoresis. A, W3110 cells are RecA+, Topo IV+, and Gyr+ (44). B, DH5αF’ cells are RecA- and TopoIV- and bear the gyrA96 mutation that turns these cells resistant to derivatives of nalidixic acid. DH5αF’ were grown until the culture achieved logarithmic growth and were then exposed to 15 μM norfloxacin for the last 15 min. The most abundant topoisomer is indicated with a blue arrow. In both cases, the supercoiling density was $\theta = 0.065$ (modal Δα = −27).

**FIGURE 5.** Unreplicated forms of pBR-terE@AatII isolated from either DH5αF’ or W3110 *E. coli* cells analyzed by two-dimensional agarose gel electrophoresis. Representative immunograms of two-dimensional gels show where the first and second dimensions occurred in the presence of different concentrations of chloroquine to resolve all the population of topoisomers. The first dimension occurred in the presence of 1 μg/ml, and the second dimension was in the presence of 2 μg/ml chloroquine. The most abundant topoisomer is indicated with a blue arrow. In both cases, the supercoiling density was $\theta = 0.065$ (modal Δα = −27).

The results obtained are shown in Fig. 6. All the immunograms were aligned attending to the mobility of OCs. Note that chloroquine’s intercalation has no effect on the electrophoretic mobility of nicked unreplicated molecules (29, 37, 50).

The electrophoretic mobility of unreplicated forms of all three plasmids isolated from DH5αF’ cells analyzed without chloroquine was the same as expected for molecules almost identical in mass and shape as follows: 4385 bp for pBR-terE@StyI; 4449 bp for pBR-terE@AatII; and 4433 bp for pBR-terE@Dral (left column in Fig. 6). The only significant difference among pBR-terE@StyI, pBR-terE@AatII, and pBR-terE@Dral is the location of terE (Fig. 3). For this reason, the electrophoretic mobility of nicked RIs (OCRIs) varied for the three plasmids, as their masses were 5525 bp for pBR-terE@StyI, 7118 bp for pBR-terE@AatII, and 7979 bp for pBR-terE@Dral, respectively. Note that the CCRIs, depicted in red in the interpretative diagrams, appeared as just a few topoisomers with an electrophoretic mobility during the second dimension close to the mobility of unreplicated CCCs for pBR-terE@StyI. For pBR-terE@AatII, CCRIs appeared as an arc formed by a series of topoisomers that extended from a spot with electrophoretic mobility during the second dimension, slightly higher
than unreplicated OCs up to OCRIs. Finally, for pBR-terE@Dral, CCRIs also appeared as an arc formed by a series of topoisomers that extended from a spot with electromophoretic mobility during the second dimension, slightly lower than unreplicated OCs up to OCRIs as well. In this case, however, the arc of RI topoisomers showed an inflection for the last poorly torsioned molecules.

The electrophoretic mobility of unreplicated forms of all three plasmids isolated from parE10 cells analyzed by two-dimensional agarose gel electrophoresis. Two-dimensional gels where the second dimension occurred in the presence of 5 µg/ml chloroquine are indicated on top. To the right of each immunogram is an interpretative diagram of the most relevant features where CCRIs are depicted in red. Where it applies, CatAs (in light blue), CatBs (in dark blue), and CatCs (in green) are also shown. All immunograms were aligned according to the electromophoretic mobility of nicked monomers (OCs) and nicked replication intermediates (OCRIs) that are not affected by chloroquine. Blue arrows point to the most abundant topoisomer of unreplicated forms, and red arrows point to the most abundant CCRi in all cases.

In an attempt to compare the torsional tension of these RIs isolated from different cell strains in a different way, 5 µg/ml chloroquine was added only during the second dimension of the two-dimensional gel system. Chloroquine is a planar molecule that intercalates between the two strands of the DNA double helix. This intercalation changes DNA twist. As bacterial plasmids are (−)-supercoiled, chloroquine removes (−)-supercoiling first and adds net (+)-supercoiling only after all native (−)-supercoiling has been removed (37, 51). Moreover, it was repeatedly shown that in partially replicated molecules containing three-way forks, overwinding of the unreplicated region transforms three-way into four-way junctions called reversed forks or chicken feet (37, 50–53). Molecules with these four-
way junctions where the fourth arm varies in length are not (+)-supercoiled and show the same electrophoretic mobility as nicked RIs.

As mentioned previously, only chloroquine intercalation in the unreplicated region of the RIs is expected to affect DNA topology. For this reason, it would have a profound effect on pBR-terE@Styl but not on pBR-terE@Dral (29, 37). We chose 5 μg/ml chloroquine as we knew that for pBR322 with a ΔLk = 0, this concentration introduces ~23 (+)-supercoils.4 Indeed, the results obtained confirmed our expectations. For the three plasmids isolated from DH5αF’ cells, the effect of chloroquine’s intercalation on unreplicated molecules was alike (next to left column in Fig. 6). The effect on the shape of the arc corresponding to partially replicated molecules, however, differed for the three plasmids. For pBR-terE@Styl, the electrophoretic mobility of CCRIs during the second dimension in the presence of 5 μg/ml chloroquine changed in a way similar to the change observed for unreplicated CCCs. Taking into account that CCRIs cannot re-gain mobility after the removal of all their native (−)-supercoiling (37, 50), we may conclude that in the presence of 5 μg/ml chloroquine the modal topoisomer for pBR-terE@Styl CCRIs was close to 0 (indicated by the corresponding red arrow in Fig. 6). For pBR-terE@AatII, the arc of CCRIs changed its shape dramatically. It described a smooth arc without chloroquine. This arc, though, turned into an acute angle in the presence of chloroquine during the second dimension. In this case the modal topoisomer was −3 (indicated by the corresponding red arrow in Fig. 6). For pBR-terE@Dral, however, the change in the shape of the arc was subtle. If we compare these results with those obtained for the same three plasmids isolated from parE10 cells, the differences were obvious (see the right column on Fig. 6). pBR-terE@Styl CCRIs were not fully relaxed, and the majority of the molecules still showed an electrophoretic mobility higher than their corresponding OCRIs. The modal topoisomer in this case was −4 (indicated by the corresponding red arrow in Fig. 6). This means that for pBR-terE@Styl CCRIs isolated from parE10 cells, exposure to 5 μg/ml chloroquine during the second dimension was not enough to remove all the torsional tension. For pBR-terE@AatII, the shape of the arc of CCRIs was not as abrupt as in the case of the molecules isolated from DH5αF’ cells. Indeed, here most of the CCRIs were still torsionally tensioned and the modal topoisomer was −11 still showing an electrophoretic mobility significantly higher than their corresponding OCRIs (indicated by the corresponding red arrow in Fig. 6). For pBR-terE@Dral, however, the change in the shape of the arc was once again only subtle (the modal topoisomer was −13 for DH5αF’ versus −12 for parE10). Altogether, these observations indicated that 5 μg/ml chloroquine added during the second dimension affected the mobility of CCRIs differently. The results obtained confirmed that CCRIs isolated from parE10 cells were more torsionally tensioned than those isolated from DH5αF’ cells. The effect of chloroquine was notable for pBR-terE@Styl, less apparent for pBR-terE@AatII, and almost negligible for pBR-terE@Dral. Unreplicated plasmids isolated from cells where Topo IV is inactive are slightly more (−)-supercoiled than those isolated from cells where Topo IV is active (44, 54). As mentioned previously, here we used the RecA−DH5αF’ cells to avoid the formation of multimers due to recombination.

It was recently shown that torsional tension reduces the probability for Topo IV to create replication knots (29, 55–58). In contrast, it is well known that the level of DNA knotting is proportional to molecular mass (40, 59). To further confirm the results obtained so far, we decided to measure DNA knotting in the replicated region of the RIs isolated from DH5αF’ and parE10 cells. We anticipated that the number and complexity of replication knots would be higher for pBR-terE@Dral and lower for pBR-terE@Styl. To facilitate the quantitation of replication knots, we digested the RIs of the three plasmids with AlwNI. This restriction enzyme cuts the plasmids only once at the unreplicated region (see Fig. 3). The resulting digested molecules consisted of linear DNAs containing an internal bubble. These molecules were analyzed in two-dimensional gels allowing the identification of unknotted and knotted forms after hybridization with proper probes. Finally, the signals corresponding to unknotted versus knotted molecules were quantitated by densitometry (29, 43). The results obtained confirmed our expectations (Fig. 7). For both cell strains (DH5αF’ and parE10) the percentage of knotted bubbles increased with the size of the bubble (40). This observation suggests that torsional tension was higher for those CCRIs isolated from parE10 cells (29, 55–58).

Discussion

Formation of precatenanes as replication progresses has been discussed before (6, 8, 10, 19, 30, 33). However, the issue remains unsettled. Fully replicated molecules may become catenated just at the very end of replication before termination due to the linking of the last ~200 bp of DNA for which it would not be possible for DNA gyrase and Topo IV to remove the links ahead of the replication forks (6, 17, 38). However, the three major experimental observations supporting the formation of precatenanes as replication progresses are as follows: the excess of (+) -windings as a function of the extent of replication in vitro (6); the occurrence of knotted replication bubbles in bacterial plasmids (29, 37, 40, 43, 60, 61); and the detection of σ-shaped replication intermediates in Xenopus extracts after poisoning Topo II with etoposide and the purification by virtue of their covalent attachment to Topo II subunits (48).

Here, we obtained additional experimental evidence supporting the formation of precatenanes during the replication of bacterial plasmids. It is important to emphasize, however, that small bacterial plasmids are tiny topological domains significantly different from the big loops or compartments of prokaryotic and eukaryotic chromosomes (55, 62). Therefore, extrapolation of the results obtained with bacterial plasmids to bigger and more complex chromosomes should only be made with special care.

The results obtained here indicate that RIs isolated from parE10 cells grown for the last hour at the restrictive temperature (where Topo IV is inactive) were more torsionally ten-

4 J. B. Schwartzman, unpublished results.
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FIGURE 7. Comparison of knotted and unknotted RIs of the three plasmids isolated from either DH5αF’ or parE10 E. coli cells analyzed by two-dimensional agarose gel electrophoresis. DNA was isolated, digested with AlwNI, and analyzed in two-dimensional gels. The area of the immunogram where unknotted and knotted RIs migrated was scanned and analyzed by densitometry. In each case, immunograms are shown to the left, diagrammatic interpretations are in the middle, and densitometric profiles are to the right. The numbers at the top right corner of each profile indicate the ratio of knotted/unknotted molecules.

sioned than the same RIs isolated from DH5αF’ cells (where Topo IV is active). This observation was based on the electrophoretic mobility of the partially replicated molecules. As clearly observed by comparing the patterns of pBR-terE/AatII RIs analyzed in two-dimensional gels with no chloroquine (see Fig. 6), the electrophoretic mobility of these RIs was unambiguously displaced toward highly torsioned topoisomers in the case of molecules isolated from parE10 cells. As discussed above, this was expected provided the replication forks swivel in vivo during replication. This observation was not obvious for the other two plasmids, pBR-terE/Styl and pBR-terE/Dral. In these cases there was no significant difference in the distribution and intensity of the signals corresponding to RIs. As illustrated in Fig. 3, for pBR-terE/Styl the majority of the crossings are expected to be supercoils in the unreplicated region that contains ~80% of the molecule. For the same ΔLk, the electrophoretic mobility of catenanes would be roughly half that of supercoils (10, 63). These RIs migrated very close to unreplicated CCCs (see Fig. 6). For pBR-terE/Dral, however, most of the crossings are expected to be precatenates in the replicated region because the unreplicated region contains just ~20% of the molecule. Huge differences of torsional tension would be needed to significantly change the electrophoretic mobility in these two cases. On the contrary, for the RIs of pBR-terE/AatII, approximately half of the molecule remained unreplicated, although the other half was replicated (see Fig. 3). For this reason, even subtle changes in torsional tension have an immediate reflection in electrophoretic mobility. We wanted to confirm this observation and used 5 μg/ml chloroquine during the second dimension of the two-dimensional gel system. Chloroquine’s intercalation in the sister duplexes of the replicated region has no effect on the writhing because the nascent strands are already discontinuous (8). Only chloroquine’s intercalation in the unreplicated region of the RIs induces relevant changes in writhing. As for pBR-terE/Dral, the unreplicated region contains only ~20% of the molecule, and here the effect of chloroquine’s intercalation is expected to be negligible. The results obtained matched this prediction precisely. Moreover, for pBR-terE/Styl, the differences between RIs isolated from DH5αF’ and parE10 cells observed in electrophoretic mobility after chloroquine’s intercalation revealed that although not evidenced without chloroquine, the RIs isolated from parE10 cells were clearly more torsionally tensioned (see Fig. 6). Chloroquine’s intercalation completely relaxed a significant number of CCRIs in the case of molecules isolated from DH5αF’ cells, whereas those isolated from parE10 cells still retained significant electrophoretic mobility. A similar observation was evident for pBR-terE/AatII (Fig. 6). As mentioned previously, it should be noticed that for RIs containing three-way junctions, once all the native (−) supercoiling is removed, these molecules cannot acquire (+)-supercoiling because the nascent strands of the sister duplexes form a fourth arm leading to fork reversal with changes in neither mass nor electrophoretic mobility (50, 51). Finally, the number and complexity of replication knots were higher for the RIs isolated from DH5αF’ cells,
also suggesting that the degree of precatenation was higher for the RIs isolated from parE10 cells (see Fig. 7 and Ref. 29).

It could be argued that CCRIs isolated from DH5αF’ cells (where Topo IV is active) were less torsionally tensioned due to the removal of RH crossings by Topo IV in the unreplicated region. For this to be true, the efficiency of Topo IV removing the RH crossings of (−)-supercoiling should be higher than the efficiency of DNA gyrase introducing them. On the contrary, experimental data indicate that although Topo IV removes the LH crossings of (+)-supercoiling as efficiently as DNA gyrase introduces RH ones, DNA gyrase is about 20-fold more efficient introducing RH crossings than Topo IV is in removing them (5, 6, 15). For this reason, when simultaneously exposed to both enzymes, DNA would become progressively more and not less (−)-supercoiled with time.

It could also be argued that replisomes fall apart shortly after stalling in all cases. If this were true, torsional tension would equilibrate between the unreplicated and replicated regions in vivo but only after fork stalling. If RIs were isolated from DH5αF’ cells, the results expected would be indistinguishable from those observed if the forks swivel regardless of fork stalling (second possibility). The tug-of-war between DNA gyrase and Topo IV would turn RIs neither saturated with (−)-supercoiling in the unreplicated region nor heavily precatenated in the replicated one. If RIs were isolated from parE10 cells grown at the nonpermissive temperature, they would end up saturated with (−)-supercoiling in the unreplicated region and heavily precatenated in the replicated one, too. In other words, the results would be identical regardless whether replication forks swivel freely always or only after replisomes fall apart due to fork stalling. The crucial question is then how stable replisomes are after fork stalling? In other words, do replisomes stay attached to the RIs’ impeding fork swiveling long enough after stalling in vivo or do they disassemble quickly after stalling, thus allowing forks to swivel right away? This question has been approached in many laboratories in different systems. It is important to make a clear distinction between stalling of replication forks due to “accidental” or “natural” barriers (64). Nowadays it is generally accepted that the encounter of a replication fork with a damaged DNA template stalls the replisome and causes it to fall apart (65, 66). Moreover, stalling of replication forks at the so-called natural barriers, such as the ter-Tus complex during replication in E. coli, seems to be different (67, 68). Replication forks are blocked by Tus-bound Ter sites on the approach from one direction but not the other. In the former case, establishment of a stable locked complex involving a cytosine-specific pocket on the surface of Tus, explains Tus resistance to dissociation from Ter sites and the strength of the ter-Tus locks that are readily unlocked only when a later-arriving replication fork approaches the permissive face of the ter-Tus complex (69, 70). In addition, it was clearly shown that the DnaB helicase, which leads the replisome during replication, physically interacts with Tus and stabilizes it. This protein-protein interaction was confirmed in vivo and in vitro (71). Altogether, these observations indicate that replisomes stalled at ter-Tus complexes in the right orientation are fairly stable supporting the rationale of our approach.

We propose there are significant differences in the catalytic rate of DNA gyrase and Topo IV on the different substrates analyzed here. To confirm this idea, we investigated torsional tension in the RIs of pBR-terE@AatII isolated from E. coli SD108 (topA+ gyrB225). These cells have an attenuated DNA gyrase (72). To this end, as SD108 cells are RecA+, we analyzed the corresponding RIs in two-dimensional gels where the second dimension occurred in the presence of 5 µg/ml chloroquine. The results obtained are shown in Fig. 8A. Notice that RIs were more relaxed when isolated from SD108 cells, which bears an attenuated version of DNA gyrase, than when they were isolated from cells with a wild-type version of this topoisomerase (W3110 cells in Fig. 8B). This observation confirmed that the catalytic rate of DNA topoisomerases is crucial to determine torsional tension during replication.

In summary, here we used three different and independent criteria to show that in vivo the RIs isolated from DH5αF’ cells are less torsionally tensioned than those isolated from parE10
cells (Fig. 9). This observation strongly favors the idea that replication forks do swivel in vivo leading to the formation of precatenanes as replication progresses.

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