Positive Torsional Strain Causes the Formation of a Four-way Junction at Replication Forks*

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The advance of a DNA replication fork requires an unwinding of the parental double helix. This in turn creates a positive superhelical stress, a (+)-Lk, that must be relaxed by topoisomerases for replication to proceed. Surprisingly, partially replicated plasmids with a (+)-Lk were not supercoiled nor were the replicated arms interwound in precatenanes. The electrophoretic mobility of these molecules indicated that they have no net writhe. Instead, the (+)-Lk is absorbed by a regression of the replication fork. As the parental DNA strands re-anneal, the resultant displaced daughter strands base pair to each other to form a four-way junction at the replication fork, which is locally identical to a Holliday junction in recombination. We showed by restriction endonuclease digestion that the junction can form at either the terminus or the origin of replication and we visualized the structure with scanning force microscopy. We discuss possible physiological implications of the junction for stalled replication in vivo.

Unwinding of the parental strands by helicases during replication allows DNA polymerases access to their template for the synthesis of complementary strands. This unwinding behind the replication fork will cause an overwinding of the parental duplex in front of the fork. The paths of the DNA strands are best described in terms of the concept of linking number (Lk). Lk is a measure of the net number of crossings of the two strands of a topologically closed molecule of DNA. It is the sum of twist (Tw), or crossings in the double helix itself, and writhe (Wr), which results from one section of double helix crossing over another section of the same molecule (1). It can only be changed by breaking and resealing DNA strands. ΔLk is the difference between the Lk of a molecule and that of the same molecule in an unconstrained, relaxed state (Lk0). ΔLk can be either (+) or (−). During replication, ΔLk increases even though Lk remains the same, because separation of the parental strands lowers the value of Lk0. The ΔLk of replication increases by about one for every 10 base pairs of DNA that are replicated. The Tw of the DNA is converted to Wr when the strands are separated, which in turn must be removed to relax the DNA. Topoisomerases relieve this strain by catalyzing DNA passages, allowing the fork to move unhindered. In Escherichia coli, the most important such topoisomerase is DNA gyrase, which removes (+)-Lk by introducing (−)-ΔLk (2–4). The new winding of the parental and daughter strands introduced during replication do not contribute to DNA topology because the daughter strands are not topologically closed.

 Determination of the conformation of replication intermediates in response to a (+)-Lk is crucial to understanding DNA unlinking during replication. These conformations dictate the actual substrates for topoisomerases in replication. In a previous study of replication intermediates, we used partially replicated E. coli plasmids that had been stalled at a termination site, Ter (5). These DNA molecules have a homogeneous structure, are relatively easy to prepare, and model a replicating chromosomal domain. When these intermediates are isolated from cells or are formed in an in vitro replication reaction containing DNA gyrase, they have a (−)-ΔLk due to the activity of gyrase, perhaps, after replication halted. The (−)-ΔLk equilibrates between two forms. One has (−) supercoils ahead of the replication fork. The other, called precatenanes, has (−) crossings between the replicated DNA arms (Fig. 1A). Catenanes were originally postulated by Champoux and Been (6) and were subsequently analyzed theoretically (7). Additional studies have also presented evidence that precatenane are a potentially important structure during replication (8–11).

The conformation of partially replicated molecules with a (+)-Lk had not yet been examined, although this is thought to be the physiologically important form. We anticipated that partially replicated molecules with a (+)-Lk would look just like those with a (−)-ΔLk, except that the supercoils and precatenanes would have the opposite handedness (Fig. 1B). To generate a (+)-ΔLk replication intermediate, we added intercalating agents to unwind the parental strands of the partially replicated molecules described above. To our surprise, they contained no supercoils or precatenanes. Instead, the (+)-topological stress is relieved exclusively by a retreat of the replication fork and reannealing of parental strands. Any (+)-supercoil and precatenane links are thereby converted into an increased Tw of the parental strands. The displaced nascent strands subsequently base pair to form a four-way junction at the replication fork, producing the structure shown in Fig. 1C. We call this structure the "chickenfoot." Such a structure had been proposed by several authors to explain findings of daughter-daughter duplex DNA as detected by bromodeoxyuridine incorporation in eukaryotes (12–14), as well as double-strand breaks and homologous recombination at stalled replication forks in prokaryotes (15–17). This four-way junction previously has been called a reversed replication fork (18, 19). We provide
proof of the structure of this intermediate, and that it is promoted at a replication fork by a (+)-ΔLk. Our structural evidence combined with these in vivo studies suggests that the chickenfoot structure may be important when replication forks pause. This conclusion gains added significance from the recent realization that replication forks stall normally in aerobically growing cells (20), presumably from damage to the chromosomes.

EXPERIMENTAL PROCEDURES

Plasmid DNAs—Plasmids pREP83 and pREP48 have been described previously (5), and their names indicate the extent of replication allowed by the placement of Ter sites: for example, 83% of pREP83 is replicated. pREP83 was replicated bidirectionally in vitro and contains the E. coli origin of replication, oriC, and Ter sites to block each fork. The 5.8-kb, pBR322-based pREP48 was used to generate intermediates in vivo, and contains the unidirectional pUC origin of replication followed by one Ter site after 2.8 kb. pTus (5) is a plasmid which expresses the E. coli Tus protein under the control of the arabinose promoter. This protein blocks replication forks at Ter sites. In vitro and in vivo replication was as described previously (5, 21).

Preparation and Purification of in Vivo Replication Intermediates—E. coli harboring both pREP48 and pTus were used to generate partially replicated pREP48 molecules in vitro (5). Tus protein was induced in exponential phase and cells were allowed to grow for 1.5 h. Plasmid DNA was extracted from cells using a variation of the neutral extraction method (22). The partially replicated plasmid DNA was purified by gel electrophoresis, followed by electroelution, phenol extraction, and ethanol precipitation.

Gel Electrophoresis in the Presence of Intercalators—One-dimensional 0.9% agarose gels were run in TAE buffer with or without 5 µg/ml chloroquine to compare the (+)-ΔLk partially replicated molecules to those with a (+)+ ΔLk. For two-dimensional gels, DNA was first run in 0.9 or 1% agarose gels in TAE buffer at 79 V/cm. The gels were soaked for 3 h in TAE plus 10 or 11 µg/ml chloroquine, turned 90°, and run in the second dimension in buffer containing chloroquine at an additional 113 V/cm. The gels were then Southern blotted and probed with pBR322.

Enzymatic Reactions—DNA was relaxed with 20 units of wheat germ topoisomerase I (23) per 0.2–1 µg of partially replicated plasmid DNA in 50 mM Tris-HCl (pH 8), 2.5 mM EDTA, 50 mM NaCl, and 2 mM magnesium bromide. DNA was nicked with DNase I in a final concentration of 50 mM Tris-HCl, 50 mM NaCl, 10 mM MgCl₂, and 360 µg/ml ethidium bromide.

In restriction endonuclease reactions, 20–50 ng of DNA was preincubated in 10 mM Tris-HCl with or without 5 µg/ml chloroquine to compare the (+)-ΔLk partially replicated molecules to those with a (+)-ΔLk. For two-dimensional gels, DNA was first run in 0.9 or 1% agarose gels in TAE buffer at 79 V/cm. The gels were soaked for 3 h in TAE plus 10 or 11 µg/ml chloroquine, turned 90°, and run in the second dimension in buffer containing chloroquine at an additional 113 V/cm. The gels were then Southern blotted and probed with pBR322.

Denaturing Agarose Gels—DNA bands were excised from a 1% agarose gel in TAE and extracted using the QiaGene gel extraction kit (Qiagen). The DNA was precipitated with ethanol, resuspended in 50 mM NaOH and 1 mM EDTA, and run in a 1% agarose gel in 50 mM NaOH, 1 mM EDTA at 4 °C at 23 V/cm.

Scanning Force Microscopy (SFM)—Partially replicated plasmid DNA (2.5–3 ng) in 5 µM ethidium bromide was incubated for 10 min at room temperature. It was then brought to 2 mM MgCl₂, 10 mM NaCl, and 4 mM HEPES (pH 7.4), and the DNA was deposited onto freshly cleaved mica (24). After 2–5 min, it was washed with 3–5 ml of EM grade distilled water. The samples were then briefly dried with nitrogen prior to imaging.

All SFM images were obtained in air at room temperature with a Nanoscope III microscope (Digital Instruments Inc., Santa Barbara, CA, USA) operating in the tapping mode. Commercial diving board silicon tips (Nanosensor, Digital Instruments) with a force constant of 100 nN/m and a 250–300 kHz resonance frequency were used. An E type scanner (12 × 12 µm) was used for all imaging. Images were collected with a scan size between 1 and 4 µm at a scan rate of 1.9 Hz. Images were processed with a standard flatter filter using Nanoscope software.

RESULTS

Partially Replicated Plasmids with a (+)-ΔLk Have the Same Electrostatic Mobility as Those That Are Relaxed—We showed previously that partially replicated plasmids with a (+)-ΔLk have both a (+)-supercoiled unreplicated region and a (+)-precatenated replicated region as diagrammed in Fig. 1A (5). However, the actual substrate for topoisomerases during replication is thought to have (+), not (-), ΔLk. We prepared a (+)-ΔLk intermediate by adding intercalating dyes such as chloroquine or ethidium bromide to (+)-ΔLk forms. These dyes increase ΔLk by unwinding the parental strands, increasing Wr. Our first experiments used electrophoretic mobility to study the structure of the replication intermediates. Because Wr, composed of supercoiling and precatenanes, compacts DNA molecules, DNA with a higher Wr runs faster on an agarose gel than DNA with a lower Wr.

Our first experiments used pREP83 DNA replicated in vitro in the presence of Tus protein to halt synthesis at the Ter sites (25) (Fig. 2). The control reactions contained gyrase, which is known to introduce a (-)-ΔLk in the replication intermediates (lanes 1 and 3). The gyrase reaction gave the expected distribution of replication intermediate topoisomers, with different numbers of supercoils and precatenanes (see bands marked RI, lane 1). A second set of reactions contained topoisomerase I (Topo) IV as the sole topoisomerase (lanes 2 and 4). Because this enzyme removes both (+)- and (-)-supercoils, we expected the replication intermediates to be relaxed. The Topo IV reaction, however, yielded a single band (labeled RI) that comigrated with nicked replication intermediates rather than the expected ladder of relaxed topoisomers (lane 2). In an attempt to resolve these topoisomers, we increased the ΔLk of these DNA molecules by running the reaction mixtures on a gel containing 5 µg/ml chloroquine (lanes 3 and 4). This amount of chloroquine reduced the (-)-ΔLk of the partially replicated molecules from the gyrase reaction and thereby their electrostatic mobility (lane 3), but the intermediates from the Topo IV replication reaction (lane 4) remained unresolved and mi-
grated at the same rate as in the absence of chloroquine (lane 2). The unexpected electrophoretic mobility of the intermediates is not due to nicking by Topo IV because unreplicated plasmid DNA in these reactions ran as the covalently closed relaxed topoisomers (bands marked RC in lane 4). We concluded that the (+)-ΔLk intermediates exist in some form other than supercoiled topoisomers or precatenanes.

A definitive test of this conclusion employed two-dimensional agarose gel electrophoresis, in which the second dimension, introduces a more negative charge into the gel. In the first dimension, the molecules compensate for the supercoil but not the first, contains chloroquine. Negative topoisomers are resolved in the first dimension. Topoisomers that still have a (+)-ΔLk form the bottom portion of an arc. The middle topoisomer of the distribution takes up just enough chloroquine to be relaxed in the second dimension. The topoisomers with less (-)-ΔLk than the relaxed topoisomer become (+) supercoiled in the second dimension, forming the top part of the arc. (+), (-), and Rel. refer to whether the molecules have a (+)-ΔLk, a (-)-ΔLk, or are relaxed, respectively, in the second dimension. Two-dimensional gels of partially replicated pREP83 (B) and the in vivo replication intermediate of pREP48 (C) were run. Because replication intermediates do not adopt (+)-Wr, they do not form the top of the arc. Unidirectional and bidirectional origins of replication are indicated with arrows, and Ter sites are indicated with black flag symbols.

We obtained the same results with a partially replicated plasmid generated in vivo. pREP48 replicated from the unidirectional pUC origin of replication toward a single Ter site was run on a two-dimensional gel (Fig. 3C). These molecules have a (+)-ΔLk to begin. Once again, the (-)-ΔLk is present, but the (+)-ΔLk topoisomers migrate with the same mobility as relaxed partially replicated DNA. We conclude that partially replicated molecules produced both in vitro from a bidirectional origin and in vivo from a unidirectional origin do not have (+)-Wr even though they have a (+)-ΔLk.

A (+)-ΔLk Causes a Four-way Junction to Form at the Terminus and the Origin of Partially Replicated Plasmids—Since Wr = 0, partially replicated molecules with a (+)-ΔLk must have a (+)-ΔTw. The most likely cause of the (+)-ΔTw is that the molecules compensate for the (+)-ΔLk by rewinding the parental strands. This necessarily requires a concomitant unwinding of the most recently replicated DNA. The energy of base pairing would be conserved, however, if the displaced nascent strands of the unwound replicated DNA anneal to each other to generate a four-way junction at the fork (Fig. 1C). We named this four-way junction the chickenfoot, because of an

**Fig. 2.** One-dimensional gel electrophoresis of partially replicated DNA shows an absence of (+)-supercoiling and precatenation in the presence of chloroquine. pREP83 is schematized on top with a double-headed arrow symbolizing oriC, and the two Ter sites are indicated with black flag symbols. pREP83 was replicated in vitro as described (41) in the presence of Tus protein and either gyrase (lanes 1 and 3) or Topo IV (lanes 2 and 4). These reactions were run on 0.9% TAE gels in the absence (lanes 1 and 2) or presence (lanes 3 and 4) of 5 μg/ml chloroquine and Southern blotted. The replication intermediates relaxed with Topo IV comigrate with nicked replication intermediates in lanes 2 and 4. RI, replication intermediate; NRI, nicked replication intermediate; NC, nicked unreplicated circle; SC, supercoiled unreplicated circle; RC, unrelaxed relaxed circle topoisomers.

**Fig. 3.** Two-dimensional gel electrophoresis of partially replicated DNA. A, the expected result for plasmid DNA analyzed by two-dimensional gel electrophoresis, in which the second dimension, but not the first, contains chloroquine. Negative topoisomers are resolved in the first dimension. Topoisomers that still have a (-)-ΔLk in the second dimension form the bottom portion of an arc. The middle topoisomer of the distribution takes up just enough chloroquine to be relaxed in the second dimension. The topoisomers with less (-)-ΔLk than the relaxed topoisomer become (+) supercoiled in the second dimension, forming the top part of the arc. (+), (-), and Rel. refer to whether the molecules have a (+)-ΔLk, a (-)-ΔLk, or are relaxed, respectively, in the second dimension. Two-dimensional gels of partially replicated pREP83 (B) and the in vivo replication intermediate of pREP48 (C) were run. Because replication intermediates do not adopt (+)-Wr, they do not form the top of the arc. Unidirectional and bidirectional origins of replication are indicated with arrows, and Ter sites are indicated with black flag symbols.
obvious resemblance to the fowl appendage. If the chickenfoot model is correct, increase in $D_{Lk}$ from intercalating dyes will cause a linear duplex, resulting from the nascent-nascent pairing, to extrude from the replication fork. Cleavage with a restriction enzyme of a site in the replicated region provides a strong test of this model, as diagrammed in Fig. 4A. Because the site is in the replicated region, two double-strand cuts will be made. If no chickenfoot forms, these cleavages will result in a single-forked molecule and a short linear duplex after branch migration (right). B, partially replicated pREP48 molecules were cleaved directly (Form I), or first relaxed with wheat germ Topo I (Form IV) or nicked with DNase I (Form II). Molecules were cleaved either with SapI, which cuts 184 bp from the origin of replication, or with PvuII, which cleaves 366 bp from the Ter site. DNA was run in 1% agarose gels and Southern blotted. The two major bands correspond to single-forked or double-forked structures. C, partially replicated relaxed pREP48 was incubated with or without ethidium bromide, cleaved with PvuII and run on an agarose gel as in B. The top and bottom bands were excised, run on an alkaline agarose gel and Southern blotted. S, uncut sample DNA; T, top band DNA; B, bottom band DNA. The markings on the left denote the migration of linear fragments in kilobases. As expected, the bands in the lanes marked T run at 5.8 kb, 2.4 kb and .4 kb, and those in the lanes marked B run at 5.8 and 2.4 kb. D, the top and bottom bands shown in B were quantified using a PhosphorImager. Error bars indicate one S.D.

Two restriction enzymes were used: PvuII, which cuts 366 base pairs (bp) from the Ter site in the replicated region, and SapI, which cleaves 184 bp from the pUC19 origin of replication in the replicated region. In this way, we studied the structure at the terminus and origin independently. From the unwinding angle of ethidium bromide (27) and an average $D_{Lk}$ for pREP48 replication intermediates of $\sim 17$ (Form I) (5), we expect the average length of the extruded toe to be about 470 bp/molecule, which could potentially come from a single middle toe or two per molecule. We also relaxed the partially replicated molecules with wheat germ Topo I before adding dye (Form IV), which should increase the toe length per molecule to about 700 bp.

After cleavage with either restriction enzyme, two closely spaced low-mobility bands were detected (Fig. 4B). The bottom of these two bands predominated in the presence of ethidium bromide, while the top band predominated in its absence.

It seemed likely that the bottom band is the single-forked...
structure predicted by the chickenfoot model, and that the top is the double-forked structure derived from the usual three-way replication fork. Two experiments proved these assignments. First, we extracted these two bands from PvuII-treated, relaxed replication intermediates and analyzed them on an alkaline denaturing agarose gel (Fig. 4C). The bottom band (B) yielded only the two single-stranded products expected from the single-forked structure generated from the chickenfoot-containing molecule, corresponding to the 2.4-kb daughter strand from the long arm and the 5.8-kb parental strand. As predicted if the top band (T) is the double forked structure (see Fig. 4A), three fragments were detected corresponding to the 366 base daughter strand of the short arm, the 2.4-kb daughter strand of the long arm and the 5.8-kb parental strand.

Second, the small fragment released from the middle toe of the chickenfoot by restriction digestion was visible on a 2% agarose gel run for 24 h (data not shown). Its intensity correlated with the amount of the bottom band; i.e. it predominated when ethidium bromide was present. As expected, this high-mobility fragment comigrated with a 200-bp marker when the partially replicated DNA was cleaved with SapI and with a 370-bp marker when cleaved with PvuII.

Because the same amount, about 50%, of each partially replicated DNA was cleaved to form the bottom band indicative of the chickenfoot (Fig. 4D), we conclude that a chickenfoot was equally likely to be at the origin as at the terminus. Relaxation did not change the results, implying that the chickenfoot distribution did not change with increased middle toe length.

To make sure that the effect of ethidium bromide was due to an increase in ΔLk rather than unrelated chemical effects of the dye, we performed a control using nicked replication intermediates (Form II). There was no difference between restriction reactions with and without ethidium bromide for these molecules (Fig. 4B).

Visualization of the Chickenfoot—We visualized the chickenfoot directly by SFM. Partially replicated pREP48 molecules were incubated with ethidium bromide, deposited onto cleaved mica, and imaged by SFM using the tapping mode in air. Typical molecules are shown in Fig. 5, A-F. For 70% of topologically closed molecules, a chickenfoot was clearly evidenced by one or two long linear duplexes emerging from a three-way junction. This linear duplex does not appear without incubation in ethidium bromide, nor does it appear in plasmid DNA incubated in ethidium bromide (data not shown).

The mean total middle toe length per molecule is 472 bp (Table I). We expect the middle toes to equal 470 bp. This quantitative agreement is probably fortuitous, because there is a large variation in middle toe lengths as deposition of the molecules onto mica traps them in a single conformation of a dynamic structure.

The duplex emerges from either one or both of the three-way junctions. This confirms the restriction results that the chickenfoot can form at either the origin or the terminus. Unexpectedly, the molecules with chickenfeet appear supercoiled. Because the electrophoresis results showed clearly that the partially replicated molecules are not supercoiled, we believe this is an artifact of the deposition procedure for SFM. It is possible that the magnesium necessary for deposition on the mica displaced ethidium ions from the DNA (28). The chickenfoot may not have been re-absorbed because of the slow rate of branch migration in ethidium and magnesium (26, 29), but the DNA could have become supercoiled before deposition.

**DISCUSSION**

We showed that a (+)-ΔLk provokes the formation of a four-way junction at a replication fork in vitro. The result is a molecule without Wr, in which the (+)-ΔLk is taken up by an unwinding of parental DNA and a concomitant formation of a fourth arm of the junction. The evidence is: 1) gel electrophoresis indicates that partially replicated plasmids with a (+)-ΔLk are neither (+)-supercoiled nor precatenated and comigrate with relaxed replication intermediates; 2) a DNA duplex can be extruded from a junction by addition of ethidium bromide and detected by restriction enzyme digestion; and 3) the four-way junctions can be visualized by scanning force microscopy. This is the first definite proof of the structure of a (+)-ΔLk replication intermediate. We discuss next the energetics of chickenfoot formation, previous evidence for its occurrence in vivo, and the circumstances whereby a (+)-ΔLk may build up around a replication fork and favor chickenfoot formation.

**Thermodynamics and Kinetics of Chickenfoot Formation under Superhelical Stress—**Formation of alternative DNA structures under the action of a (+)-ΔLk is a well known phenomenon (30). These alternative structures have a higher free
thus resembles the cruciform structure, which is formed in superhelical stress (see Ref. 31, for example). Chickenfoot formation in (+)-ΔLk replication intermediates is no exception.

The chickenfoot has a junction of four double helices, and thus resembles the cruciform structure, which is formed in palindromes of (-)-supercoiled DNA, but it should form much more readily. Hairpin loops with unpaired bases in the cruciform contribute to the large free energy difference between the cruciform and linear structures. These hairpin loops, however, are absent in the chickenfoot structure. Another difference is that a cruciform forms from a linear DNA, whereas the chickenfoot is formed from a three-arm junction. Thus, very little additional irregularity is associated with chickenfoot formation, as opposed to cruciform extrusion. For cruciform extrusion the free energy cost is 20–28 kcal/mol (32), depending on ionic conditions. We expect that chickenfoot formation would be associated with only about 5 kcal/mol free energy change and thus can be formed at relatively low torsional stress in comparison with cruciform extrusion, as observed. Moreover, cruciforms form slowly due to the necessity of forming a large open region in the double helix as an intermediate (33–35). Nothing like this is needed to form the chickenfoot, and thus there is a much smaller kinetic barrier to the transition. Indeed, only a ΔLk of (+)-1 extrudes the chickenfoot (see Fig. 3). The ease of chickenfoot formation is probably due to the entropic gain resulting from the increased number of possible conformations of the four-way junction.

Our SFM and enzymatic results show that either one or two chickenfeet can form on a partially replicated plasmid. A single chickenfoot at either the origin or the terminus is enthalpically favored because there is one rather than two junction penalties. A chickenfoot at each junction, on the other hand, would be entropically favored by the increased number of possible conformations. There is an additional factor that influences whether one or two chickenfeet are formed. In molecules with fully ligated daughter strands, two chickenfeet would result in the replication bubble becoming a second topological domain, that would become (+)-supercoiled as the first domain, composed of parental strands, relaxed.

The cleavage data indicate that there is no preference for chickenfoot formation at the origin or terminus. This is interesting because the two three-way junctions have different properties, due to potentially incomplete Okazaki fragments near the terminus and RNA primers at the origin.

Physiological Implications of the Chickenfoot—The chickenfoot was first postulated in 1976 by Higgins et al. (14) who were studying human cells treated with methyl methanesulfonate. These authors proposed that the four-way junction allowed the repair of a methyl methanesulfonate-induced lesion on the leading strand template, as shown in Fig. 6A. Soon afterward, other researchers found similar evidence for mutagen-induced nascent-nascent duplex at replication forks (12, 13). Chickenfeet have also been observed in replicating DNA isolated from the eggs of Drosophila melanogaster (36).

It has more recently been proposed that the chickenfoot may play a role any time the replication fork stalls. In E. coli cells, the chickenfoot may emerge at replication forks stalled at the replication terminus (16), at a stalled RNA polymerase (17), or due to a mutant replicative helicase (15, 19). In addition, it has been postulated that the chickenfoot forms in yeast cells at replication fork blocks in ribosomal DNA (37).

Several ways that a stalled fork can be restarted are illustrated in Fig. 6B. It is possible that the three-way junction itself breaks without the intermediary of a chickenfoot, creating a substrate for RecBCD, the recombinogenic exonuclease (38), as shown in Fig. 6B, left. Recombination ensues with the sister arm, and a new replication fork is formed. In Fig. 6B, middle, is shown the regression of the replication fork to form the chickenfoot. This nascent-nascent duplex itself can be chewed back entirely by RecBCD, resulting in a reformed replication fork (15). Formation of a four-way junction at replica-
tion forks may be a method by which the cells create a recombinogenic end from a three-way junction, because a four-way junction is a natural intermediate in recombination. The four-way junction of the chickenfoot may be resolved by the Holliday junction processing proteins RuvA, -B, and -C (15) (Fig. 6B, right). This cleavage will result in a severed replicated arm, which can then become a substrate for recombination and replication restart much like the example on the far left. The role of recombination in replication restart has recently been extensively reviewed (20, 39).

This paper demonstrates that the chickenfoot forms spontaneously in DNA free of proteins. We find that it is stabilized by a (+)-DlK, but will also form at a low level in the absence of positive superhelical strain. About 10% of partially replicated molecules with a (+)-DlK formed a chickenfoot, and this number increased slightly if the molecules were nicked (Fig. 4B). Low levels of chickenfoot formation due to branch migration in vitro have been seen in molecules without a (+)-DlK (18).

Positive Supercoiling and Stalled Forks in the Cell—DNA replication causes a (+)-DlK in the domain surrounding the replication fork. If this (+)-DlK remains after a replication fork pauses, the chickenfoot would be a favored outcome. We believe that the domain surrounding the fork will have a (+)-DlK in all organisms, but certainly in eukaryotes and archaea which have no DNA gyrase. Thus, there will be constant pressure to form a chickenfoot when replication stops. In addition, (+)-DlK will build up fastest when two replication forks move toward each other (Fig. 6C, top), as at the terminus of chromosomal replication. Louarn et al. (16) have suggested that superhelical stress at the terminus may force a four-way junction to form at one of the replication forks. In eukaryotes, replication forks move toward each other much more frequently, whenever two adjacent domains of replication are completed.

(+)-DlK may also build up if a replication fork is moving in the direction opposite to an RNA polymerase. Simultaneous and oppositely oriented replication and transcription leads to pausing of the replication fork (40), and these pauses may allow for chickenfoot formation (Fig. 6C, bottom). Thus, chickenfoot formation due to a (+)-DlK may be important during replication in the cell.

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