Received: 24 November 2020 | Revised: 3 February 2021

DOI: 10.1002/bies.202000309

BioEssays

PROBLEMS AND PARADIGMS

Prospects & Overviews

Changes in the topology of DNA replication intermediates: Important discrepancies between in vitro and in vivo

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Funding information
Paraguayan CONACYT-PROCIENCIA, Grant/Award Number: PINV15-573; Spanish Ministerio de Economia y Competitividad, Grant/Award Number: BFU2014-56835

Abstract

The topology of DNA duplexes changes during replication and also after deproteinization in vitro. Here we describe these changes and then discuss for the first time how the distribution of superhelical stress affects the DNA topology of replication intermediates, taking into account the progression of replication forks. The high processivity of Topo IV to relax the left-handed (+) supercoiling that transiently accumulates ahead of the forks is not essential, since DNA gyrase and swiveling of the forks cooperate with Topo IV to accomplish this task in vivo. We conclude that despite Topo IV has a lower processivity to unlink the right-handed (+) crossings of pre-catenanes and fully replicated catenanes, this is indeed its main role in vivo. This would explain why in the absence of Topo IV replication goes-on, but fully replicated sister duplexes remain heavily catenated.

KEYWORDS
catenation, DNA chirality, DNA topology, pre-catenation, replication, supercoiling

INTRODUCTION

To study the organization and function of living cells scientists strive to preserve their structure as much as possible. This was one of the reasons why acetic orcein was used to stain chromosomes.[1] When this is not possible, the artefactual changes should be clearly understood and defined, as in the case of the dehydration needed to study proteins and DNA by X-ray crystallography.[2]

The topology of DNA changes dynamically during replication in vivo,[3,4] and these changes significantly affect DNA chirality and the sign of nodes in covalently-closed domains such as DNA circles (CCCs). Moreover, in vivo DNA interacts with proteins,[5] further complicating its conformational possibilities. Most of the methods used to analyze DNA involve deproteinization, and it is well known that the removal of proteins affects DNA topology.[6] In other words, the topology of DNA examined by electrophoresis, electron microscopy or atomic force microscopy in vitro does not necessarily represent their situation in vivo. Here we discuss some of the changes experienced by partially replicated DNA molecules upon removal of proteins.

WHAT DO DNA CHIRALITY AND THE TOPOLOGICAL SIGN OF CROSSINGS MEAN?

First, two fundamental topological conventions to define chirality and sign assignment of the perceived crossings of DNA must be established. To determine the sign of these crossings or nodes, it is mandatory to define the orientation at which the analyzed segments are crossing. By convention for topological considerations needed to define DNA linking number (Lk), in the case of B-DNA the anti-parallel polynucleotide chains of the duplex are considered to run in the same direction (Figure 1A).

There are several ways to define DNA chirality.[7–9] Here we chose one of the methods proposed by Stone and co-workers.[7] The angle

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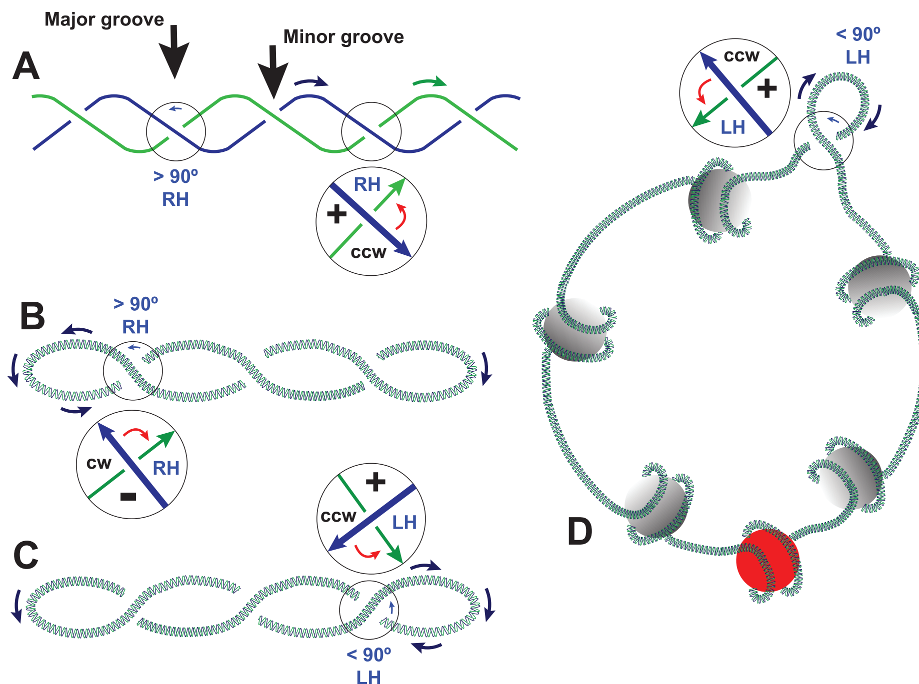

FIGURE 1 Cartoons illustrating some basic DNA topological features. (**A**) Schematic diagram of a double-stranded DNA linear molecule showing its major and minor grooves. For topological considerations both strands are given the same direction. By convention, at every crossing of the two strands, if the underlying segment must rotate counter-clockwise more than 90° to become parallel to the overlying segment, the node is right-handed. In addition, for right-handed DNA, the intra-duplex inter-strand crossings have a positive sign when the overlying segment must rotate counter-clockwise to be aligned with the underlying segment. For these reasons, all the nodes of the B-form DNA are right-handed with a positive sign. (**B**) Covalently-closed circle negatively supercoiled, where the crossings between two oppositely oriented segments are right-handed and have a negative sign. **(C)** Covalently-closed circle positively supercoiled, where the crossing between two oppositely oriented segments are left-handed and have a positive sign. The sign is also determined by convention. If the direction arrow closer to the observer needs to turn clockwise to overlay with the direction arrow further from the observer, the crossing has a negative sign. If the direction of turning is counter-clockwise, the crossing has a positive sign. The turning angle cannot be larger than 180°. Notice that the orientation of the overlying and underlying direction arrows are not independent of each other, but result from assigning a consistent direction (see the black arrows) along the whole DNA molecule analyzed. **(D)** In *Saccharomyces cerevisiae*, the DNA duplex wraps around nucleosomes in a left-handed manner except for the centromeric nucleosome (depicted in red), where it wraps in the opposite orientation. In addition, there could be regions that are positively supercoiled. The complementary strands of the DNA duplex are represented in blue and green

of two crossing segments, as both strands of the DNA double-helix encircled to the left of Figure 1A is determined by a counter-clockwise rotation of the underlying one. If the counter-clockwise rotation of the underlying strand in the process of becoming parallel to the overlying one is less than 90°, the node is called left-handed (LH). If it must rotate more than 90°, the node is referred to as right-handed (RH). Consequently, all the crossings of both strands of a B-DNA double-helix are RH (Figure 1A).

Contrary to the situation in defining chirality, there is total agreement in the literature on how to define the sign of a DNA node or crossing between two oriented segments, as both strands of the DNA double-helix encircled to the right of Figure 1A. If the overpassing strand needs to rotate clockwise to align with the overpassed one, the sign is negative (-). If the deflection is counter-clockwise, the sign is positive (+). As a consequence, all the crossings of both strands of a B-DNA double-helix have a (+) sign (Figure 1A).

The rules defined above apply to crossing DNA duplexes, too. In bacteria DNA is negatively supercoiled. Note that in covalently-closed circles (CCCs) the crossing duplexes have opposite directions. For this reason, these nodes are RH and have a (-) sign (Figure 1B). Progression of transcription and replication requires opening of the DNA double-helix, and this process generates (+) supercoiling ahead of the forks. In positively supercoiled DNA crossings are LH with a (+) sign (Figure 1C).

In eukaryotes DNA is associated with proteins, forming chromatin. DNA wraps itself about 1.65 times around nucleosomes in a LH manner to form the "beads on a string" chromatin fiber with a diameter of 11 nm. The wrapping orientation of DNA around nucleosomes forces DNA to under-wind.[6] For this reason, when nucleosomes are removed, the isolated naked DNA ends-up negatively supercoiled. Approximately one (-) supercoil develops for each nucleosome removed.[10]

There is one important exception to the aforementioned rule. As previously noted, in *Saccharomyces cerevisiae* the LH wrapping of DNA around regular nucleosomes forces DNA to under-wind.[6] However, DNA wraps around the centromeric nucleosomes in the opposite orientation.[11] For this reason, removal of centromeric nucleosomes induces (+) supercoiling (Figure 1D). Deproteinization leads to the reciprocal cancelling of all the resulting (-) and (+) supercoils. The

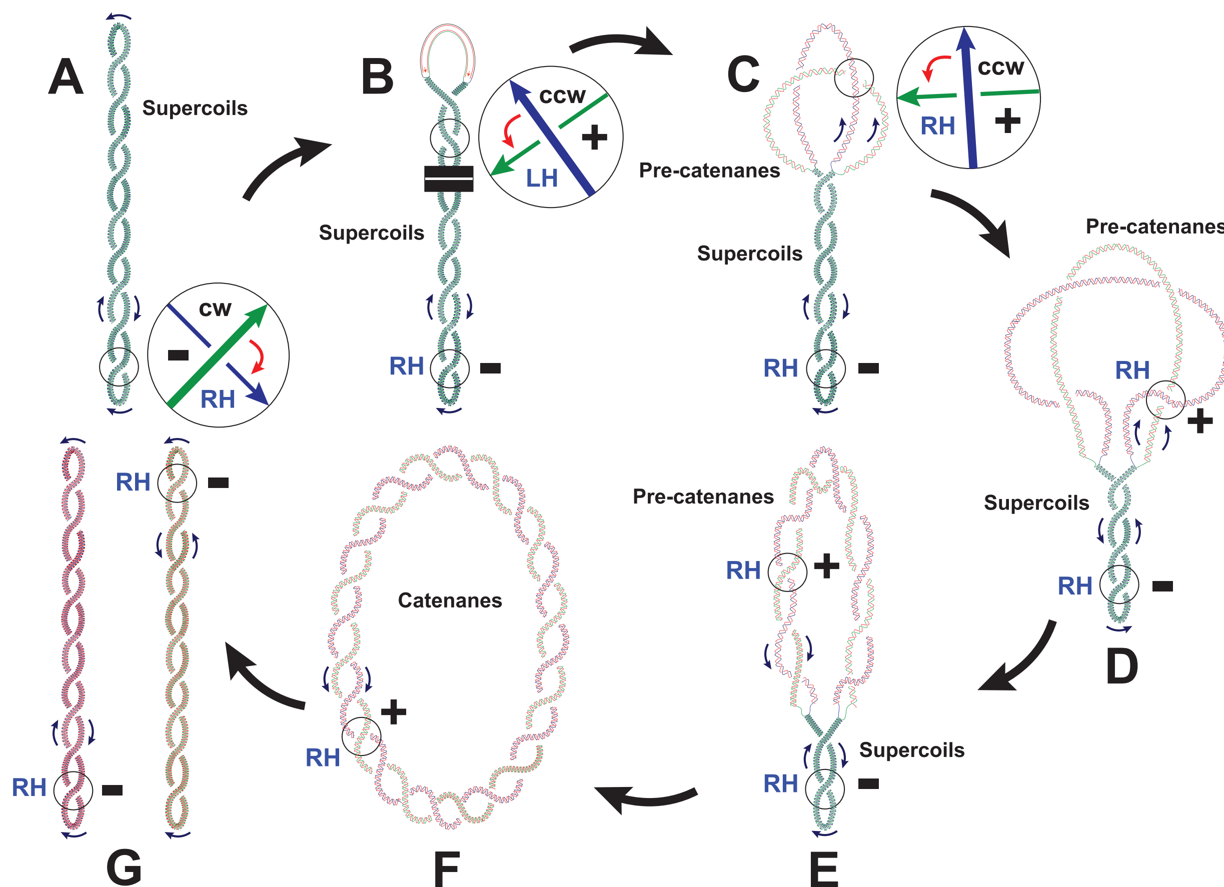

FIGURE 2 Cartoons illustrating the changes in topological sign and chirality that take place during the replication cycle. **(A)** Un-replicated negatively supercoiled form showing right-handed crossings with negative signs. **(B)** As replication starts, opening of the DNA double-helix generates positive supercoiling immediately ahead of the replication forks (upper part of the un-replicated region). The combined action of DNA gyrase, Topoisomerase IV and swivelling of the forks eliminate this positive torsional tension and keep the un-replicated region negatively supercoiled (lower part of the un-replicated region). **(C)** Swiveling of the forks diffuses some of the positive supercoiling that transiently accumulates immediately ahead of the forks from the un-replicated to the replicated region at the expense of generating right-handed positive crossings between the newly made sister duplexes. **(D)** As the replicating forks keep advancing the un-replicated negatively supercoiled region becomes smaller, and the size of the replicated region increases. At mid-replication both regions have approximately the same size, manifesting similar number of crossings. **(E)** At late replication stages, most of the molecule has been replicated, possessing numerous pre-catenane crossings in the replicated region and few negative supercoils in the un-replicated one. **(F)** Once replication finishes, the newly made sister duplexes remain heavily catenated. **(G)** Topoisomerase IV progressively eliminates catenation and allows the sister duplexes to segregate. The parental chains are represented in blue and green, while newly synthesized chains are depicted in red

cartoon in Figure 1D represents a circular mini-chromosome with five regular nucleosomes, one centromeric nucleosome and one (+) supercoil. After deproteinization this molecule would end-up as a plectoneme with three RH supercoils showing a (-) sign (Figure 1B). These are the yeast circular mini-chromosomes analyzed by electrophoresis, electron or atomic force microscopy. It is currently unknown whether this also applies to other eukaryotes in addition to yeast.

TOPOLOGICAL DYNAMICS IN THE UN-REPLICATED REGION OF REPLICATION INTERMEDIATES

The situation becomes even more complicated during replication. The cartoons in Figure 2 illustrate the changes in topological sign and chirality that occur as replication progresses in a circular covalently-closed (CCC) molecule in vivo. In bacteria, un-replicated circular molecules are maintained negatively supercoiled by the combined action of DNA gyrase introducing (-) RH supercoils and topoisomerase I keeping it under control (Figure 2A).

As replication starts, the advance of replication forks generates (+) supercoiling (over-winding of the DNA duplex) that transiently accumulates only immediately ahead of the forks (Figure 2B). RH (-) crossings and LH (+) ones cancel each other because they cannot co-exist in the same topological domain. Topo IV removes some of the LH (+) supercoils that form as a result of the advance of replication forks, and DNA gyrase introduces RH (-) supercoils to keep the un-replicated region negatively supercoiled. Note that in the cartoon represented in Figure 2B the upper part of the un-replicated region appears positively supercoiled while the lower part is negatively supercoiled. As mentioned above, this situation is inconsistent and it is presented here

only for didactical reasons. The advancing rate of the DNA helicase far exceeds the capacity of Topo IV and DNA gyrase to completely eliminate all the overwound LH (+) supercoiling that transiently accumulates immediately ahead of the advancing forks.[12] To solve this conundrum, Champoux & Been proposed that fork swiveling allows some of these LH (+) supercoils to migrate from the un-replicated to the replicated region.[12] This causes intertwining of the newly made sister duplexes. The nodes between sister duplexes in the replicated region are called pre-catenanes.[13] Migration of each crossing from the un-replicated region to the replicated one generates two pre-catenane crossings (Figure 2C).

It is important to note that replication intermediates contain two regions that in vivo behave as independent topological domains: the un-replicated and the replicated regions. The combined action of Topo IV, DNA gyrase and swiveling of the forks guarantee that the un-replicated region is always kept negatively supercoiled (Figure 2C).

TOPOLOGICAL DYNAMICS IN THE REPLICATED REGION OF REPLICATION INTERMEDIATES

The sister duplexes in the replicated region cannot be supercoiled because rotation of the free ends of the nascent strands of the duplex dissipates all the torsional tension that might form in vivo as well as in vitro. In replication intermediates, it is energetically favorable for supercoils of the un-replicated region to diffuse to the replicated one, and vice-versa, with opposite handedness.[4] For this reason, the LH supercoil crossings that transiently accumulate immediately ahead of the forks (Figure 2B) migrate to the replicated region as RH pre-catenane crossings. As crossing DNA duplexes run in opposite directions in the un-replicated region, whilst they run in the same direction in the replicated one, the (+) sign of the crossings is maintained after their diffusion to the replicated region (Figure 2C). Hence, replication intermediates in vivo comprise RH (-) crossings in the un-replicated region and RH (+) pre-catenane crossings in the replicated one (Figure 2C). As replication forks keep advancing, the un-replicated region becomes progressively smaller, with fewer (-) supercoils, while the replicated region becomes larger, with more and more RH (+) pre-catenanes (Figure 2C). Finally, once replication is completed, the fully replicated sister duplexes end-up heavily catenated (Figure 2F). Topo IV is responsible for the elimination of all catenane crossings leading to the segregation of the newly made sister molecules.[14] At the same time, DNA gyrase progressively introduces (-) supercoiling in the daughter CCCs[15] to start the cycle again (Figure 2G).

THE TOPOLOGY OF DNA REPLICATION INTERMEDIATES CHANGES AFTER DEPROTEINIZATION

DNA supercoiling, fork reversal, catenation, and knotting of circular molecules are analyzed by electrophoresis, electron or atomic force microscopy.[16–19] All these methods use naked DNA. Understanding the topological changes introduced during DNA isolation (deproteinization) is important to get a better and comprehensive understanding of DNA topology in vivo.

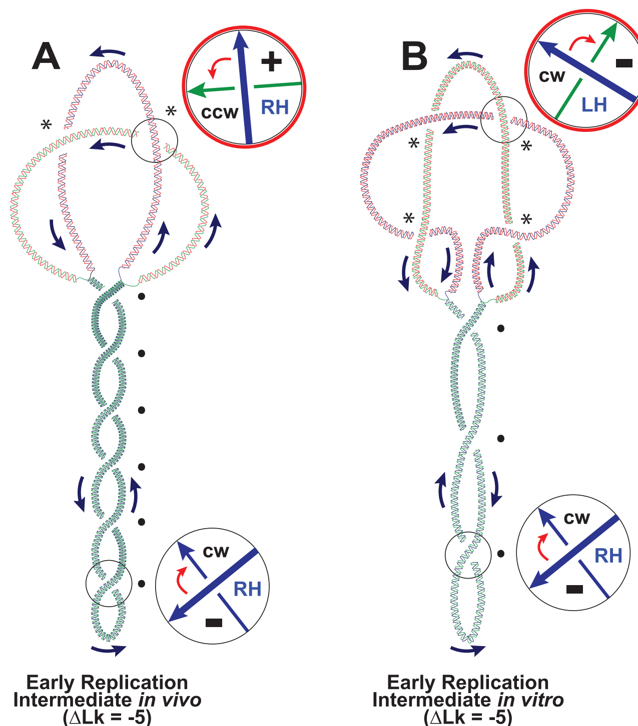

FIGURE 3 Cartoons illustrating the changes in topological sign and chirality that take place in a replication intermediate at an early stage of replication in vivo and after deproteinization. **(A)** Replication intermediate at an early stage of replication in vivo showing a relatively large negatively supercoiled un-replicated region with six right-handed crossings with negative sign. The relatively small replicated region shows one right-handed pre-catenane (two crossings) with positive sign. **(B)** After deproteinization in vitro, some of the negative supercoils from the un-replicated region diffuse to the replicated region with the opposite handedness in the process of reaching thermodynamic equilibrium. The first crossings to diffuse cancel the two pre-existing pre-catenane crossings, and the following ones establish new pre-catenane left-handed crossings with negative signs. Once thermodynamic equilibrium is achieved, the molecule ends up with three right-handed crossings of negative sign in the un-replicated region, and two left-handed pre-catenanes (four crossings) with negative sign in the replicated one. Black dots indicate supercoil crossings, and asterisks signify pre-catenane crossings. The parental chains are represented in blue and green, while newly-synthesized chains are depicted in red

At early stages of replication most of the molecule remains un-replicated and negatively supercoiled showing RH crossings with a (-) sign (Figure 3A). The small replicated region may show a few RH pre-catenane crossings with a (+) sign. When these early-replicated molecules are deproteinized, free swiveling of the forks allows the torsional stress of the un-replicated and replicated regions to attain thermodynamic equilibrium.[20] Because at these early stages of replication most of the molecule remains un-replicated and negatively supercoiled, the energy accumulated in this region forces the forks to

swivel, and pushes the abundant RH crossings with a (-) sign in the un-replicated region towards the replicated one. Each of these RH crossings with a (-) sign—now converted into two LH pre-catenane crossings with a (-) sign—rapidly cancels the few pre-existing RH pre-catenane crossings with (+) signs in the replicated region. As a consequence, once thermodynamic equilibrium is achieved, the molecule ends-up showing RH crossings with a (-) sign in the un-replicated region, and LH pre-catenane crossings with a (-) sign in the replicated one (Figure 3B). Note that ΔLk is maintained at -5 despite the number of crossings in both regions, and the chirality as well as the sign of pre-catenane crossings in the replicated region change after deproteinization (highlighted in red in Figure 3). These would be the early replicated deproteinized molecules analyzed by electrophoresis and visualized by electron microscopy or atomic force microscopy in vitro.

At mid-stages of replication, there would be a similar number of crossings in the un-replicated and replicated regions (see Figure 2D). The final conformation in vitro would depend on the number of RH (-) and LH (+) crossings in both regions just before deproteinization.

At late stages of replication, however, most of the molecule have already been replicated. The size and energy of the un-replicated region is not significant and the replicated region covers most of the molecule (Figure 4A). Consequently, there are few RH crossings of a (-) sign in the un-replicated region. Most of the crossings in these molecules are RH pre-catenanes with a (+) sign (Figure 4A). When these late-replicating molecules are deproteinized, the energy accumulated in the replicated region compels the forks to swivel, and pushes the more abundant RH pre-catenane crossings with a (+) sign of the replicated region towards the un-replicated one to reach thermodynamic equilibrium.[20] Each pair of these RH pre-catenane crossings with (+) signs of the replicated region converts into a single LH crossing with a (+) sign in the un-replicated region. Because the pre-catenane crossings in the replicated region are much more abundant, once they migrate to the un-replicated region, they rapidly cancel the few RH crossings with (-) signs that remain. As a consequence, at equilibrium the molecules may end-up with some LH crossings with a (+) sign in the un-replicated region and RH pre-catenane crossings with (+) signs in the replicated one (Figure 4B). Note that here ΔLk is maintained at +3 after deproteinization despite of the changes in the number of crossings, sign assignment and chirality in both regions (highlighted in red in Figure 4).

Once replication finishes, fully replicated catenanes only manifest RH crossings with (+) signs (Figure 2F), as confirmed by electron microscopy.[21]

In summary, with the advance of replication forks in vivo, some of the LH crossings with a (+) sign that transiently accumulate immediately ahead of the forks migrate to the replicated region as RH pre-catenanes with (+) signs.[12] In partly-replicated deproteinized molecules, free swiveling of replication forks allows the torsional stress in the un-replicated and replicated regions to attain thermodynamic equilibrium.[20] In molecules at early stages of replication, deproteinization causes migration of some of the RH crossings with a (-) sign from the un-replicated region to the replicated one as LH pre-

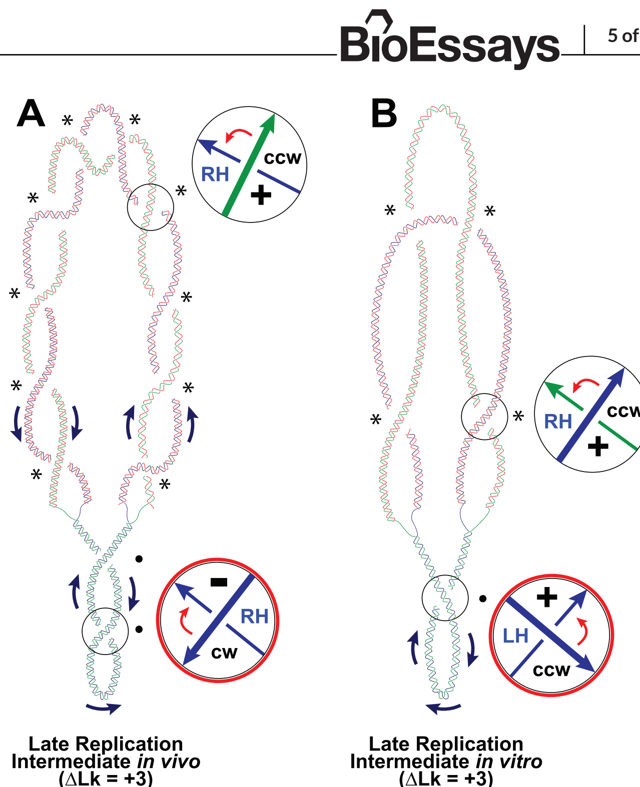

FIGURE 4 Cartoons illustrating the changes in topological sign and chirality that take place in a replication intermediate at a late stage of replication in vivo and after deproteinization. **(A)** Replication intermediate at a late stage of replication in vivo showing a relatively small negatively supercoiled un-replicated region with two right-handed crossings with a negative sign. Here the relatively large replicated region shows five right-handed pre-catenanes (ten crossings) with a positive sign. **(B)** After deproteinization in vitro, some of the positive pre-catenanes from the replicated region diffuse to the un-replicated region with the opposite handedness in the process of reaching thermodynamic equilibrium. The first two right-handed pre-catenanes (four crossings) to diffuse cancel both pre-existing right-handed negative supercoils, and the following one establishes a new left-handed supercoil with a positive sign. Once thermodynamic equilibrium is achieved the molecule ends up with two right-handed pre-catenanes (four crossings) of positive sign in the replicated region, and one left-handed supercoil with a positive sign in the replicated one. Black dots indicate supercoil crossings and asterisks signify pre-catenane crossings. The parental chains are represented in blue and green, while newly synthesized chains are depicted in red

catenanes with (-) signs. Because in these early-replicated molecules RH crossings with (-) signs are more abundant, they rapidly cancel and replace the few pre-existing pre-catenane RH crossings with (+) signs that might be present in the relatively small replicated region. As a consequence, once thermodynamic equilibrium is achieved, these early-replicated molecules end-up showing RH crossings with a (-) sign in the un-replicated region and LH pre-catenane crossings with (-) signs in the replicated one.

In molecules at late stages of replication, most of the molecule is already replicated. These molecules may still manifest a few RH crossings with a (-) sign in the un-replicated region and many RH pre-catenane crossings with (+) signs in the large replicated region. When these late-replication intermediates are deproteinized, free

swiveling of the forks allows the torsional stress in the un-replicated and replicated regions to reach thermodynamic equilibrium.[20] Some of the abundant RH pre-catenane crossings with (+) signs migrate to the un-replicated region with the opposite handedness. Here they rapidly cancel and replace the few pre-existing RH supercoils with a (-) sign. As a consequence, once thermodynamic equilibrium is achieved, these late-replicated molecules end-up showing a few LH crossings with a (+) sign in the small un-replicated region and RH pre-catenane crossings with (+) signs in the large replicated one. These are equivalent to the molecules analyzed by electrophoresis, electron- or atomic-force microscopy after deproteinization.

These observations may have important experimental consequences. Topo IV has been repeatedly shown to be the main *E. coli* de-catenase. In the absence of Topo IV activity, almost all fully replicated molecules remain catenated.[14,17,22] DNA gyrase may unlink sister duplexes in vivo, too, but it is at least 100-fold less efficient than Topo IV in the unlink of sister duplexes. Curiously, it was recently shown that Topo IV trails the replication forks and disentangles pre-catenanes primarily far behind the replisome.[23] The implementation of magnetic tweezers[24] facilitated the discovery that Topo IV relaxes the LH crossings of (+) supercoiling at a 20-fold faster rate than the RH crossings of (–) supercoiling on account of a 10-fold increase in processivity.[24–26] The mechanism underlying this chiral recognition of RH and LH crossings by Topo IV is still under debate, and several hypotheses have been proposed.[7,8,26–29] Regardless, if this observation holds for replication intermediates—as it should—it means that at early stages of replication, Topo IV would resolve the RH crossings of the un-replicated and replicated regions less efficiently as if they would be LH (Figure 3A). After deproteinization in vitro, this topoisomerase would resolve the few LH pre-catenane crossings of the replicated region more efficiently than the RH crossings of the un-replicated one (see Figure 3B). However, since at these early stages most of the molecules are still un-replicated, the different efficiency of Topo IV in the un-replicated and replicated regions would be negligible. At late stages of replication, the replicated region is significantly larger. Here, Topo IV would also relax the RH crossings of the replicated and un-replicated regions in vivo less efficiently as if they would be LH (see Figure 4A). After deproteinization in vitro, though, Topo IV would resolve the few LH crossings of the un-replicated region more efficiently than the RH pre-catenane crossings of the replicated one (Figure 4B). However, as at these late stages most of the molecules have been already replicated, the different efficiency of Topo IV in the un-replicated and replicated regions would be negligible as well. In other words, despite Topo IV may have a major processivity to relax LH (+) supercoils does not mean this is its main role in vivo. These observations would explain why once replication finishes, the fully replicated sister duplexes remain heavily catenated.[15,30] We propose that the high processivity of Topo IV to resolve the LH crossings with a (+) sign would be significant only in the relaxation of (+) supercoiling that transiently accumulates ahead of the replicating forks in vivo.

CONCLUSIONS AND OUTLOOK

The topology of replication intermediates changes significantly after deproteinization in vitro. In those molecules at early stages of replication, the handedness of pre-catenane crossings at the replicated region changes from RH to LH. In addition, the crossing signs change from (+) to (-). In contrast, in molecules at late stages of replication, the handedness of supercoil crossings at the un-replicated region changes from RH to LH and the sign of the crossings changes from (-) to (+). Therefore, the topology of replication intermediates after deproteinization in vitro does not reflect the situation in vivo. The topological aspect of replication intermediates analyzed by electrophoresis, electron microscopy or atomic force microscopy in vitro differs significantly from their appearance in vivo. These changes affect the efficiency of treatments with Topo IV in vitro.

ACKNOWLEDGMENTS

The authors acknowledge the critics and continuous support of Prof. Andrzej Stasiak and all the current and former members of their groups. This work was sustained by grant PINV15-573 from the Paraguayan CONACYT-PROCIENCIA program to M.J.F.N. and BFU2014-56835 from the Spanish Ministerio de Economía y Competitividad to J.B.S.

CONFLICT OF INTEREST

No potential conflict of interest was reported by the authors.

AUTHOR CONTRIBUTIONS

All the authors participated in the preparation and revision or the manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available.

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How to cite this article: Schvartzman, J. B., Martínez, V., Hernández, P., Krimer, D. B., & Fernández-Nestosa, M.-J. (2021). Changes in the topology of DNA replication intermediates: Important discrepancies between in vitro and in vivo. *BioEssays*, e2000309. https://doi.org/10.1002/bies.202000309