Title: **“Replication Fork Barriers and Topological Barriers: Progression of DNA Replication Relies on DNA Topology Ahead of Forks”**

Authors: **Jorge B. Schwartzman* Pablo Hernández and Dora B. Krimer**

Address: Department of Cellular and Molecular Biology. Centro de Investigaciones Biológicas (CSIC), Ramiro de Maeztu 9, Madrid 28040, SPAIN.

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* Corresponding author: **Jorge B. Schwartzman**

E-mail: schwartzman@cib.csic.es

Abstract

During replication, the topology of DNA changes continuously in response to well-known activities of DNA helicases, polymerases and topoisomerases. However, replisomes do not always progress at a constant speed and can slow-down and even stall at precise sites. The way these changes in the rate of replisome progression affect DNA topology is not yet well understood. Here we discuss the interplay of DNA topology and replication in several cases where progression of replication forks reacts differently to changes in DNA topology ahead. We propose there are at least two types of replication fork barriers: those that behave also as topological barriers and those that do not. Two-dimensional agarose gel electrophoresis is the method of choice to distinguish between these two different types of replication fork barriers.

1. Introduction

According to the classical textbook “DNA Replication” by Kornberg and Baker [1]: “As DNA unwinds during replication, it acts as a coiled double-stranded rope. As one pulls the two strands apart at one end, twisting tension builds-up in the rest of the coiled portion. It is actually adding one twist to the remaining rope for each twist pull out of it. At some point one cannot separate the strands anymore. The remaining rope is too tightly twisted. If one relaxes the tension on the rope, it will twist around itself in a supercoil. It is releasing tension”.

In almost all prokaryotes, DNA is negatively (-) supercoiled (Figure 1A) and this (-) supercoiling favors melting of the parental DNA to be copied during replication [2], [3]. But as clearly explained by Kornberg and Baker [1], progression of the fork generates positive (+) supercoiling ahead (Figure 1B), and this (+) torsional tension, if not removed, will eventually block progression of the fork.

In bacteria, DNA gyrase, Topoisomerase IV (Topo IV) and swiveling of the fork cooperate to eliminate this (+) supercoiling and keep the DNA ahead of the fork (-) supercoiled [4], [5].

Fork swiveling, though, has an important consequence (Figure 1C). It causes newly replicated sister chromatids to twist around each other forming (+) pre-catenanes [6], [7], [8]. These pre-catenanes are eliminated by Topo IV later-on [9],

[4], [5]. It should be emphasized that although the formation of pre-catenanes during replication was observed in both prokaryotes and eukaryotes, there are significant differences between them. It was recently shown that the intrinsic mechanical properties of chromatin play a fundamental role in dictating pre-catenane formation and regulating chromatin topology throughout the cell cycle [10], [11]. Moreover, in eukaryotes fork rotation and pre-catenation are restricted during DNA replication by a conserved pair of replisome components (Tof1–Csm3 in budding yeast; Swi1–Swi3 in fission yeast and TIMELESS–TIPIN in humans) and only occur in hard-to-replicate contexts [12], [13].

2. DNA Replication Analyzed by Two-Dimensional Agarose Gel Electrophoresis

In 1987, Brewer and Fangman triggered a revolution in the field of DNA replication. At a Cold Spring Harbor meeting they introduced a new method to map replication origins in any specific DNA fragment. This method is now very popular and is known as “Two-Dimensional Agarose Gel Electrophoresis” or simply: 2D gels [14]. After restricting genomic DNA, two consecutive and perpendicular runs are performed (Figure 2). The first run takes place in a low percent agarose gel at a low voltage. The second run occurs in a higher percent agarose gel at a higher voltage. After these runs, the DNA is transferred to a hybridization membrane, and the fragment of interest is revealed by hybridization with an appropriate probe. Non-replicating forms lead to two well defined patterns: An arc of linear molecules at the bottom (depicted in black) and recombinants (represented in dark-blue). When a linear DNA fragment is replicated by a single fork that runs from one end to the other, it generates a simple-Y pattern (depicted in light-blue). When the fragment contains a bi-directional replication origin that is placed at the center of the fragment, it generates a bubble pattern (represented in red). And when the fragment is replicated by two converging forks that get into the fragment simultaneously and progress at the same speed, they generate a double-Y pattern (represented in magenta). These are the so-called simple patterns. Under these conditions in 2D gels, during the first run molecules separate mainly due to their mass. On the contrary, during the second run the electrophoretic mobility of replication

intermediates (RIs) is severely affected also by their three-dimensional shape. Note that for the simple-Y pattern, for example, the position of the fork along the molecule changes dramatically the length of the three arms (the parental and the new ones). At the beginning, the sum of the two new arms (in light-blue) is shorter than the un-replicated segment (in black). When half of the molecule has been replicated, the three arms look identical in length. After this point, the sum of the two new arms becomes longer than the un-replicated segment. These observations explain why the electrophoretic mobility of these RIs during the second run decreases from 1.0x to 1.5x and increases from 1.5x to 2.0x. For the bubble pattern, the position and size of the bubble (in red) determine the electrophoretic mobility of these RIs during the second run. Notice that for all X-shaped recombinants (in dark-blue) with almost exactly the same mass (close to 2.0x) their electrophoretic mobility is determined mainly by the position of the cross. The closer the cross to either of the two ends, the electrophoretic mobility increases while it becomes minimal when the cross is at the middle ^[15]. It should be noticed that in their original paper, Brewer and Fangman only proposed an interpretation for the various patterns observed ^[14]. The verification came later with a combination of 2D gels and electron microscopy ^[16].

The bi-directional replication origin may not be placed at the center of the fragment. In this case, the 2D gel pattern shows a transition from bubbles to simple-Ys. And if one replication fork gets into the fragment before another one that runs in the opposite direction, both convergent forks meet at a site that is neither centered. In this case, the 2D gel pattern shows a transition from simple-Ys to double-Ys. In short, the final pattern obtained describes the replication mode of any linear DNA fragment. Let us stress that the final pattern represents a population profile of a dynamic process. In other words, some molecules in the population of molecules analyzed are just starting to be replicated, others are in the middle of the process while still others already completed their replication. This means that for the simple-Y pattern, for example, not all the forks traverse the fragment synchronously. This is the reason to see, in the final autoradiogram, signals for all the steps of the process. The intensity of each signal is determined by the abundance of each particular form compared to all the forms. Note that usually the most intense signal corresponds to the un-

replicated form (1.0x). Most of the molecules of the sample that hybridize with the selected probe are not replicating.

3. DNA Replication Analyzed by Other Methods

Obviously, DNA replication can be studied using other various methods. It is important to distinguish between those methods that analyze single molecules, such as chromatin fiber autoradiography ^[17], dynamic molecular combing ^[18], ^[19], transmission electron microscopy ^[20], ^[21] and atomic force microscopy ^[22], ^[23]; and those methods, such as 2D gels, that allow the simultaneous examination of thousands of DNA molecules. Fork stalling can also be studied by examining some of its consequences: DNA lesion bypass ^[24], leading- and lagging-strand re-priming ^[25], ^[26], R-loop formation ^[27] and replication fork reversal ^[28], ^[29], ^[30], ^[31], ^[32]. Another set of newly developed methods, generally known as “genome wide” studies, allow identification of small variations in the genome itself or the association of specific proteins, such as polymerases and topoisomerases, to specific sites along the genome ^[33], ^[34], ^[35]. Although we recognize the value of the data obtained using all these different methods, here we restrict ourselves to discuss data obtained using 2D gels. For a detailed and excellent review of replication fork stalling at natural impediments see ^[36].

4. Replication forks stall at precise sites in ribosomal DNA repeats

Just a few months after Brewer and Fangman originally introduced 2D gels ^[14], this technique was used to identify Replication Fork Barriers (RFBs) in the ribosomal DNA repeats of the budding yeast, *Saccharomyces cerevisiae* ^[37], ^[38]. Indeed, the first RFBs were discovered before 2D gels in bacteria. In *Escherichia coli* and *Bacillus subtilis* circular chromosomes, initiation of DNA replication occurs bi-directionally at a specific origin, while termination is made sure to take place approximately 180° across from the origin, due to the presence of Ter DNA sequences that bind specific proteins ^[39], ^[40], ^[41], ^[42].

In *S. cerevisiae* ribosomal DNA repeats too, a specific DNA sequence binds a protein named Fob1 and this complex blocks progression of replication forks in

a polar manner ^{[43], [44], [45]}. In 2D gels ribosomal RFBs are detected as conspicuous spots on top of the corresponding pattern of RIs (Figure 4A)

Not all the potential Fob1p binding sites actually binds Fob1p, and many forks go through the potential barriers with no trouble ^{[46], [15]}. In any case, some forks do stall at the barriers, and the abundance and corresponding intensity of the signal of RIs containing the stalled fork become stronger in 2D gels (Figure 4A). The position of the stronger spot indicates the mass of the RI accumulated ^{[37], [38]}. A few years later, 2D gels were used to confirm the presence of RFBs in the ribosomal repeats of several plants and animals, and it was proposed this is a conserved feature in the mode of replication of eukaryotic ribosomal RNA genes. Curiously, although the feature is conserved, the DNA sequences and the proteins involved are not ^{[47], [48], [49], [50]}.

The precise mapping of RFBs at the ribosomal DNA repeats of the budding yeast remained a controversial issue until very recent. Kobayashi and co-workers showed that in the non-transcribed spacer between two 35S genes, there are actually three sequences (named RFB1, 2 and 3) that bind Fob1p *in vitro* ^[51]. This region was cloned in a circular yeast artificial chromosome (pYAC) and its replication mode examined in 2D gels. The replication forks stalled at only two close conspicuous sites and the intensity of the signal was stronger for the first barrier met by the replicating fork ^[15].

Curiously, the simple-Y signal continued beyond the stalled forks. This indicates not all the potential barriers block progression of the replicating forks. In an attempt to increase the efficiency of the barriers five copies of this region were cloned in tandem and the replication of linear fragments containing the barriers were examined in 2D gels. Five pairs of strong conspicuous spots were clearly detected, but now the intensity of the signal was always stronger for the second barrier of each pair met by the replicating fork. The situation reverted if replication of the same fragment was examined in DNA isolated from cells that over-expressed Fob1p. It was concluded that in the ribosomal DNA of *S. cerevisiae*, the abundance of Fob1p modulates the efficiency of ribosomal RFBs to stall replication forks ^[15].

5. Replication forks can stall at or close to replication origins

In 1990, Gahn and Schildkraut also used 2D gels to study the replication of p174 in human cells ^[52]. p174 is a circular mini-chromosome that contains the Epstein-Barr origin of replication: oriP, for “Origin of Plasmid Replication” (Figure 3A). oriP contains two relevant components: a “Dyad Symmetry” element and an array of tandem repeats, named the “Family of Repeats” (FR) located about 1 kb apart. The FR consists of 21 imperfectly conserved 30 bp direct repeats that include sequence motifs to which the initiation protein EBNA-1 binds with high affinity ^[53], ^[54].

Initiation of DNA replication occurs at or near the Dyad Symmetry element in a bi-directional manner. But the Family of Repeats acts as a Replication Fork Barrier and termination site (depicted in light-blue in Figure 3A). Thus, the replication of EBV mini-chromosomes proceeds in a predominantly uni-directional manner ^[52], ^[55].

Another interesting case concerns the replication mode of a peculiar bacterial plasmid: pPI21. This plasmid contains two perfect inverse repeats, both containing a uni-directional ColE1 origin named ori-alpha and ori-beta (represented in light-blue in Figure 3B). 2D gels were also used to find out how this plasmid replicates ^[56]. It turns out in pPI21 initiation of DNA replication occurs at a single origin. Progression of some but not all replicating forks initiated at either of the two potential origins stalls at the other inversely oriented and silent origin. The presence of inversely oriented ColE1 origins in circular plasmids was also used to investigate the mechanism involved in fork stalling as well as its involvement in the formation of knots ^[57], ^[58], ^[59], ^[60].

6. Replication forks stall due to head-on collision of transcription and replication

2D gels were also used to examine the consequences of head-on collision of transcription and replication machineries. In 2004, Krings and Bastia found that in *Schizosaccharomyces pombe* ribosomal repeats, there are four pausing sites

at the 3' end of the 35S genes. The first three barriers (1, 2 & 3) are caused by the binding of two specific proteins: Reb1 and Sap1 ^{[61], [62]}. The fourth and last conspicuous pausing site is due to the collision of transcription and replication machineries ^[63]. This collision occurs specifically at the 3' end of the first 35S gene met by the replicating fork (see below).

Approximately by the same time, Mirkin and co-workers were studying the mechanism of head-on transcription and replication collisions in bacteria. In *Escherichia coli* plasmids carrying constitutive or inducible promoters, they found no conspicuous barriers, but a whole replication pausing zone (Figure 4B) that coincided exactly with the transcribed DNA segment ^[64].

Comparing the results obtained by Krings and Bastia in *S. pombe* ribosomal repeats ^[63], with those obtained by Mirkin and co-workers in bacteria ^[64], one may conclude there are two classes of head-on transcription-replication collisions. In the case of tandemly repeated and highly transcribed genes, such as ribosomal DNA repeats, that are fully loaded with RNA polymerases ^[65], the head-on collision occurs precisely at the 3' end of the first 35S gene met by the replicating fork. In contrast, in the case of single and less intensely transcribed genes in bacteria, the collision can take place at any site along the transcribed gene. For this reason, here there is no conspicuous pausing site but a whole replication pausing zone that coincides exactly with the transcribed DNA segment (Figure 4B).

7. Replication forks stall at topological barriers

Finally, an interesting observation was made on triplex DNA segments that also act as RFBs ^[66]. A bacterial plasmid was constructed carrying a ColE1 unidirectional origin that contains eleven repeats of a GAA triplet (Figure 5). These triplets are prone to form triplex DNA ^[67]. The plasmids were linearized and the corresponding RIs were examined in 2D gels (Torres, Doctoral Thesis, 2005). The gradient or comet-like shape of the accumulated RIs on the simple-Y arc was evident (Figure 4C).

It is possible this comet-like shape of the accumulated RIs forms because triplex DNA is also a topological barrier that prevents swiveling of the forks. For this reason, the (+) supercoiling that builds-up ahead of the advancing fork is not eliminated efficiently and progressively accumulates slowing down the advance of the replicating fork.

To summarize, it appears there are at least three different types of RFBs: the conspicuous barriers, the pausing zones and the comet-like barriers (Figure 4).

But why does a replicating fork slow-down or even stall? There is a significant controversy in the literature concerning this issue. Santamaría and co-workers suggests the *E. coli* DnaB helicase is unable to dissociate the RNA-DNA hybrids that forms at ColE1 origins to start replication [59]. On the other hand, Kaplan proposed that the 3'-tail of a forked-duplex sterically determines whether one or two strands can pass through the central channel of a replication fork helicase [68].

Another interesting controversy is whether or not swiveling of the forks occurs all-the-way as replication proceeds as originally proposed by Champoux and Been [7] or only when forks come together at the termination of DNA replication [69], [70]. There is significant evidence suggesting that in small plasmids in bacteria [71] and *Xenopus* egg extracts [72], pre-catenanes form all the way as replication forks advance and this could only happen if fork rotation occurs continuously. However, in budding yeast chromosomes, replication bubbles are bi-directional, and every bubble is serviced by a single replication factory, which precludes rotation of the individual replication forks [73]. Moreover, the Baxter's laboratory demonstrated that in *S. cerevisiae*, fork rotation and pre-catenation are restricted during DNA replication by Tof1/Csm3 and only occur in hard-to-replicate contexts [12].

We would like to speculate that in the case of the conspicuous barriers observed near the 3' end of the 35S genes of ribosomal repeats as well as in EBV plasmids, pPI21, and the barriers that form due to transcription and replication collisions, progression of the replicating fork is not affected by the

topology of the parental DNA ahead. On the contrary, in the gradient or comet-like barrier that forms as the replicating fork approaches triplex DNA, the progressive accumulation of (+) supercoiling ahead of the forks is responsible for fork stalling.

In the chromosomes of higher animals, genomes are organized in TADs: Topologically Associating Domains ^[74], ^[75]. The transcription repressor CTCF is thought to define the boundaries of neighboring TADs ^[76]. In theory, they must behave as topological barriers for replicating forks. It would be interesting to check if replication forks do stall at these TADs and form comet-like signals in 2D gels.

In principle, fork stalling prevents completion of DNA replication. Prokaryotes and eukaryotes developed various mechanisms to overcome the different obstacles that may cause fork stalling to allow completion of DNA replication and avoid cell death. Discussion of this subject falls beyond the scope of this work. For a detailed and excellent review of the mechanisms that prokaryotes and eukaryotes developed to rescue stalled or damaged replication forks see ^[77].

8. Conclusions and outlook

The head-on collision of transcription and replication machineries is detrimental as it usually leads to the formation of R-loops that disrupt replication, inhibits gene expression, elevates mutagenesis and promotes genomic instability. For this reason, genomes evolved to minimize head-on collisions and favor the co-directionality of transcription and replication. However, in some essential genes that are highly transcribed and organized in tandem repeats, as is the case of ribosomal genes, these head-on collisions are unavoidable. Genomes evolved RFBs precisely to preclude such collisions. These ribosomal RFBs occur in plants and animals, and it was proposed to be a conserved feature in the mode of replication of eukaryotic ribosomal RNA genes. Curiously, although the feature is conserved, the DNA sequences and the proteins involved are not. Throughout evolution, though, deletions, inversions and translocations

generated structural genomic changes that make these head-on collisions unescapable. Prokaryotes and eukaryotes also developed diverse mechanisms to solve the detrimental consequences of unprogrammed fork stalling. On the other hand, progression of transcription and replication forks leads to changes in the topological organization of the DNA duplex ahead of the forks. The positive torsional tension that accumulates, if not removed, will eventually block its progression. In small circular bacterial plasmids and eukaryotic mini-chromosomes, topoisomerases and fork swiveling are sufficient to solve this problem. The increase of genome size, though, forced cells to organize their chromosomes into independent topological domains. This was accomplished by the setup of topological barriers that limit the so-called TADs. Here we review and discuss experimental data obtained using 2D gels that allowed the distinction of several types of RFBs and advances the question as to whether replication forks do stall at TADs and also form gradient or comet-like signals in 2D gels.

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Competing Interests

No competing interests are declared.

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Figure legends

Figure 1: Fork rotation and the formation of pre-catenanes. (A): In bacterial plasmids the DNA is (-) supercoiled. (B): Progression of the replicating fork causes the formation of (+) supercoiling immediately ahead. In prokaryotes this (+) supercoiling is continuously eliminated by the combined action of DNA gyrase, Topo IV and swiveling of the fork. (C): Fork rotation, though, causes newly replicated sister chromatids to twist around each other forming (+) pre-catenanes. Parental strands are represented in dark-blue and light-blue while nascent strands are depicted in red.

Figure 2: Analysis of linear replication intermediates (RIs) by two-dimensional agarose gel electrophoresis (2D gels). Patterns generated by linear RIs. Bubbles in red, Simple Ys in light-blue, Double-Ys in magenta, X-shaped recombinants in dark-blue and un-replicated fragments in black. 1.0x

indicates the un-replicated linear forms while 2.0x points to almost fully replicated forms with double the mass.

Figure 3: (A): genetic map of p174 showing its most relevant components. Initiation of DNA replication occurs bi-directionally at oriP somewhere between the Dyad and the FR (depicted in red). The replication fork that moves counterclockwise stalls at the FR and most of the minichromosome is replicated in a uni-directional manner by the fork that progresses clockwise. **(B): genetic map of pPI21 showing its most relevant components.** This bacterial plasmid contains two perfect inverse repeats, both containing an Ampicillin-resistance gene (in dark-blue) and a uni-directional ColE1 origin (in light-blue) named ori-alpha and ori-beta. The outer black arrows highlight these inverse repeats.

Figure 4: Cartoons illustrating Replication Fork Barriers (RFBs) as detected in 2D gels. (A): a conspicuous RFB on top of a simple-Y arc (in green). The intensity and relative position of the black spot (pointed by a light-blue arrow) indicate the efficiency and relative mass of the accumulated RIs. **(B):** A Replication Pausing Zone on a simple-Y arc (in green). The intensity and relative position of the black oblong signal (in black) caused by the head-on collision of transcription and replication machineries coincide exactly with the transcribed DNA segment. **(C):** a gradient or comet-like RFB on a simple-Y arc (in green). The shape of the signal (pointed by a light-blue arrow) indicates that the abundance of molecules containing stalled replication forks increases progressively as they approach the RFB. This observation suggests that this RFB (triplex DNA) is also a topological barrier.

Figure 5: Cartoon illustrating DNA replication fork stalling at triplex DNA. Triplex DNA forms when pyrimidine or purine bases occupy the major groove of the DNA double-helix forming Hoogsteen pairs with purines of the Watson-Crick base-pairs ^[78].

Figure 1

Figure 2

A **B** **Figure 3**

Figure 4