Replication Fork Reversal Occurs Spontaneously after Digestion but Is Constrained in Supercoiled Domains*

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Replication fork reversal was investigated in undigested and linearized replication intermediates of bacterial DNA plasmids containing a stalled fork. Two-dimensional agarose gel electrophoresis, a branch migration and extrusion assay, electron microscopy, and DNA-psoralen cross-linking were used to show that extensive replication fork reversal and extrusion of the nascent-nascent duplex occurs spontaneously after DNA nicking and restriction enzyme digestion but that fork retreat is severely limited in covalently closed supercoiled domains.

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gel in Tris-borate-EDTA buffer containing 0.3 μg/ml EthBr run perpendicular with respect 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. Southern transfer was performed as described before (7, 29).

Psoralen Cross-linking—To perform psoralen cross-linking, 50–100 ng of DNA in a total volume of 20 μl was incubated with 10 μg/ml 4,5’,8 trimethylpsoralen (Sigma) for 1 h at room temperature in the dark in a 96-well open plate and subsequently irradiated with a 500-watt high pressure mercury lamp (model TQ 700; Original Hanau) on ice in a open plastic dish for 15 min. The lamp was placed 7 cm above the plastic dish, and the light was filtered through a Pyrex glass to eliminate radiation below 300 nm. Psoralen stock solution was prepared in 100% ethanol. This procedure was performed either before or after DNA digestion. Subsequently, the two-dimensional agarose gel electrophoresis was carried out as described before.

Non-radioactive Hybridization—pBR322 that only hybridizes to the plasmid was used as a probe. DNA was labeled with the random primer fluorescein kit (PerkinElmer Life Sciences). Membranes were prehybridized in a 20-ml prehybridization solution (2× saline/sodium phosphate/EDTA, 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 hybridization lasted for 12–16 h. Hybridized membranes were sequentially washed with 2× SSC and 0.1% SDS, 0.5× SSC and 0.1% SDS, and 0.1× SSC at room temperature except for the last wash, which took place at 65 °C. Detection was performed with an antifluorescein-AP conjugate and CDP-Star (PerkinElmer Life Sciences) according to the instructions provided by the manufacturer.

Preparation of DNA Samples Enriched for Specific RIs—Specific molecules were isolated from agarose gels following the procedure described by Olavarrieta et al. (7) with minor modifications. After restriction digestion, DNA isolated from exponentially growing cells was analyzed in a one-dimensional agarose gel, the lane was cut and incubated with 0.1 mM NaCl in TNE buffer (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, 100 mM NaCl) at 65 °C for 4 h, and the selected DNA sample was electroeluted out of the agarose gel and resuspended in distilled water.

Electron Microscopy—The purified DNA sample was spread on EM grids under non-denaturating conditions in redistilled water by the benzylidimethyl-alkyl ammonium chloride (BAC) method (34).

Branch Migration and Extrusion Assay—The agarose lane of the first dimension containing the DNA sample was incubated with 0.1 mM NaCl in TNE buffer (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, 100 mM NaCl) at 65 °C for 4 h without 0.3 μg/ml EthBr or in the presence of 0.3 μg/ml EthBr. Subsequently, the second dimension was performed as described before.

RESULTS AND DISCUSSION

It is generally thought that RFR is repressed in (−) supercoiled molecules and favored by (+) supercoiling (7, 8, 35). It was recently shown, however, that formation of Holliday-like junctions at both forks of a replication bubble creates a topological constraint that prevents further regression of the forks (33). To confirm this observation for a different plasmid, we used a modification of two-dimensional gels where the agarose gel run perpendicular with respect 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. Southern transfer was performed as described before (7, 29).

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Replication Fork Reversal

FIGURE 2. Exposure of undigested partially replicated plasmids to 65 °C in the presence of 0.1 M NaCl enhances branch migration and leads to total extrusion of the nascent-nascent duplex but only for nicked forms. Autoradiograms of two-dimensional gels corresponding to pBR18-TerE@AatII where the second dimension occurred either without 0.3 μg/ml EthBr or in the presence of 0.3 μg/ml EthBr are shown. For the autoradiograms shown to the right, the agarose lane of the first dimension (1st dim) containing the DNA sample was incubated at 65 °C with 0.1 M NaCl in TNE for 4 h either without 0.3 μg/ml EthBr (top) or in the presence of 0.3 μg/ml EthBr (bottom) before proceeding with the second dimension. A diagrammatic interpretation is shown to the right of each autoradiogram. The signals resulting from total extrusion of the nascent-nascent duplex are depicted in gray and indicated by arrows. The dotted lines indicate the relative position of open circle (OC) replication intermediates (OCRIs) after the first and second dimensions. CCC, covalently close circle; Ls, linear.

The observation that extensive RFR and complete extrusion of the nascent-nascent duplex are prevented in (−) as well as (+) supercoiled RIs prompted us to reinvestigate whether or not the DNA molecules containing reversed forks that are putatively responsible for the cone signal identified in two-dimensional gels indeed form in vivo (10–13). To this end, we combined two-dimensional gels with psoralen cross-linking, the branch migration and extrusion assay described above, and EM. If RFR occurs in vivo, the signal should be detected in restriction fragments of all sizes.

exposed to very high (0.3 μg/ml and above) concentrations of EthBr (7, 33), suggesting that RFR is favored by low and moderate levels of (+) supercoiling but is inhibited when the torsional stress reaches certain threshold. Postow et al. (8) used atomic force microscopy to study the topology of RIs containing stalled forks in the presence of 5 μM EthBr (equivalent to 1.97 μg/ml). They noticed that under these conditions, RIs become heavily supercoiled but interpreted that this supercoiling was an artifact induced during deposition of the molecules onto mica. It was later shown, however, that RIs recover electrophoretic mobility and are able to acquire (+) supercoiling when exposed to 0.3 μg/ml and higher concentrations of EthBr (7) due to the topological locking mechanism activated as soon as RFR forms at both forks of a replication bubble (33).

The two nascent strands (nascent-nascent duplex). During the second dimension, the new molecular species migrated as open circles and linear forms of 2627 bp, precisely the distance between the ColE1 origin and the TerE site in pBR18-TerE@AatII, indicating that the new linear fragment corresponded in fact to the extruded double-stranded fourth arm. In the very same autoradiograms, however, no extrusion occurred for CCRIs. Therefore, we concluded that extensive branch migration and extrusion of the fourth arm was impeded in CCRIs regardless of whether the DNA was (−) or (+) supercoiled. This was unexpected as it is generally thought that (+) supercoiling actually favors RFR and complete extrusion of the nascent-nascent duplex (8, 35). This observation, on the other hand, agrees with the finding that RIs are able to acquire electrophoretic mobility and become (+) supercoiled when regardless of the extent of replication (Fig. 3). Moreover, if RFR forms and retreats unconstrained once a replication fork stalls at a DNA lesion and these lesions occur at random, the mass of the RI with the stalled fork would vary between 1.0 and 2.0×. As clearly depicted in Fig. 3, the signal expected for a mix of molecules where the fork stalls and retreats from different sites is not the cone depicted in Fig. 3B as a gray triangle but rather a smear covering the whole area limited by X-shaped recombinants to the left, and the ascending portion of the simple-Y pattern to the right, as also painted in Fig. 3D in gray. We used two-dimensional gels to examine restriction fragments of different sizes (4.3, 4.4, 3.1, and 3.6 kb, respectively) where the RIs containing stalled forks were double-Ys of 1.26× (1.26 times the mass of unreplicated fragments) for pBR18-TerE@StyI digested with AflIII and 1.60× for pBR18-TerE@AatII also.
We also examined simple-Ys of 1.81× for pBR18-TerE·AatII digested with AflIII and PvuI and 1.70× for the same plasmid digested with PstI and EcoRI (Fig. 1, circular and linear maps). The spikes emanating from the prominent spot on top of the simple- or double-Y arcs (Fig. 4, represented in red in the diagrams) were easily recognized, although their location, intensity, and extension varied from one gel to the other. Moreover, they did not necessarily fit into the so-called cone signal described elsewhere (10–13). This spike extended almost exclusively below the accumulated spot for the 1.26× RI, and it also extended below the accumulated spot but showed a small bulge above it for the 1.60× RI and extended both above and below the accumulated spot for the 1.81× RI. Similar signals have been observed for RIs of specific masses in other systems as well (15, 39). It is important to note, however, that only a small fraction of the accumulated RIs experienced RFR (Fig. 4). The strength of the spot generated by accumulated RIs indicated that most of them were pretty stable, and a discrete signal for molecules that experienced RFR was detected only for RIs that accumulated due to fork stalling. To confirm that these signals were generated by molecules displaying reversed forks, the agarose lane containing the DNA that came out of the first dimension was heated at 65°C in the presence of 0.1 M NaCl for 4 h before the second dimension took place. The results obtained are shown in Fig. 4, middle vertical panel, with corresponding interpretative diagrams. Note that heating between the first and second dimensions eliminated the original diagonal signals and generated novel ones that ran perpendicular to the first dimension in all cases (this was remarkable for the 1.81× RI) and are represented in Fig. 4 in red in the middle vertical diagrams. It seems likely that the bulk of materials that run at the position of the TerE-stalled RIs contained molecules where the fork retreated to some extent but where the nascent-nascent duplexes were too small to affect their mobility in an appreciable manner. These molecules were nevertheless susceptible to branch digested with AflIII.
Migration and extrusion by heat. This interpretation would account for both the new vertical spike, if not all such molecules completely extruded in response to heat, as well as the new spots. We speculated that molecules where the replication fork stalled at TerE and have undergone various extents of RFR, which in turn altered both their first and their second dimension mobilities, generated the original diagonal spikes. As DNA heating was performed after the first dimension was completed, it could not alter first dimension mobilities, but after total extrusion of the nascent-nascent duplexes, the molecules that originally gave rise to the diagonal spikes would now yield a horizontal bulge that should be detected aside of the new signals. In fact, this was clearly the case for both of the two new spots observed, particularly in the case of the 1.81 × sample (Fig. 4, indicated with blue arrows in the middle bottom diagram).

For all the fragments studied, the identification of prominent spots that migrated together with the accumulated RI during the first dimension and as linear molecules with the same electrophoretic mobility of unreplicated forms during the second dimension indicated that these molecules resulted from complete extrusion of the fourth arm. Moreover, in the autoradiogram corresponding to the 1.81 × RI, a second smaller spot was detected that corresponded to the extruded nascent-nascent linear duplex of 2567 bp. This linear duplex was not detected for the other two fragments examined due to their smaller size. To further confirm that these vertical signals were indeed generated by molecules containing reversed forks, a DNA sample enriched for the 1.60 × molecules (Fig. 4, encircled by a black dotted line in the corresponding middle autoradiogram) was prepared and examined at the EM (shown in Fig. 4, at the far right panel). As this sample included molecules of different
sources (plasmid as well as chromosomal) and the extent of fork retreat was expected to vary significantly from molecule to molecule, the criterion employed to select those molecules that experienced RFR was the following one. Molecules containing a reversed fork are only those where two arms are identical and the increase in length of each of the other two compensate the progressive loss in length of the first pair (Fig. 3). This criterion allowed us to select a number of molecules where the fourth arm was readily identified (Fig. 4, right panel, indicated by black arrows in the interpretative diagrams) and confirmed that these were indeed RIs where the replication fork had retreated to different extents (7, 40, 53).

Once we confirmed that molecules containing reversed forks generated the vertical signal emanating from the accumulated spots, we investigated whether RFR occurred at the stalled fork in vivo or in vitro. To this end, we used two different and complementary approaches. First, pBR18-TerE@AatII was digested with PstI and EcoRI to generate RIs of 1.70/H11003 containing no replication fork stalled at TerE. In this case, the fork of the RI that accumulated corresponded to the ColE1 unidirectional origin (Fig. 1, D and H). Analysis of these RIs by two-dimensional agarose gel electrophoresis revealed a spike and a pattern that were almost identical to the one shown in Fig. 4 at the bottom left (data not shown). This observation suggested that RFR was likely to occur after digestion in vitro.

To test this hypothesis, we used psoralen cross-linking to prevent any further branch migration (41). DNA molecules corresponding to the 1.81× sample (Figs. 1 and 5) were cross-linked with psoralen either after or before DNA digestion and analyzed in two-dimensional gels. Notice that the pattern corresponding to the sample cross-linked with psoralen after DNA digestion (Fig. 5B) was almost identical to the one generated by the untreated sample (Fig. 5A) except that the spike generated by molecules containing reversed forks was now decorated with a number of regularly distributed extra spots. Psoralen intercalation and cross-linking do not occur in a uniform fashion (41). The extra spots might well correspond to sites where psoralen cross-linking occurred in a preferential mode. Branch migration could still shift between cross-links but could not move across them. Surprisingly, no signal for molecules containing reversed forks was detected when psoralen cross-linking took place before DNA digestion (Fig. 5C). In addition, in this case, each of the spots was duplicated. It is well known that the amount of intercalating agents is reduced almost by half in nicked as compared with covalently closed circles (42). The detection of doublets in this autoradiogram was a consequence of this phenomenon. For each doublet, the spot showing slower electrophoretic mobility derived from molecules that were covalently closed at the time of psoralen cross-linking and, in consequence, captured almost double the amount of psoralen (43, 44).

It is important to note, however, that our results do not indicate that RFR does not occur in vivo. It has been well established that in E. coli, once a replication fork hits a DNA lesion, RecA (45) and/or RecG (4) promotes regression of the stalled fork, generating a Holliday-like junction (HLJ) that is subsequently processed by the RuvABC complex to allow replication restart by PriA (3, 46, 47). This retreat of the forks that occurs in vivo, however, is severely constrained by DNA supercoiling and probably cannot extend very long (33). The results we showed here question to what extent the cone signal detected in two-dimensional gels (10–13) reflects the limited retreat of the forks that may occur in vivo as opposed to the extensive branch migration that takes place in vitro after restriction enzyme digestion.

**FIGURE 5.** Replication fork reversal occurs spontaneously but only after restriction enzyme digestion. Autoradiograms of two-dimensional gels where the RI that accumulated was 1.81× are shown together with their corresponding interpretative diagrams to the right. A, an untreated sample is shown on top. The DNA sample was cross-linked with psoralen either after (B) or before restriction enzyme digestion (C). Notice that no spike was detected when psoralen cross-linking occurred before restriction enzyme digestion. Arrows at the bottom right indicate the signals expected for linear molecules of 0.80× (2567 bp).
Replication Fork Reversal
digestion. Furthermore, delocalized termination of DNA replication, which also generates a triangular smear (16–26), could be enhanced by fork stalling at DNA lesions and might certainly contribute to the so-called cone signal.

In summary, here we showed that extensive RFR and extrusion of the fourth arm occurs spontaneously but only after nicking or DNA restriction enzyme digestion. These results strengthen the observation that the extent of fork retreat is severely constrained in supercoiled domains, probably due to the topological locking that triggers when RFR forms at both forks of a replication bubble (33).

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