JMB

COMMUNICATION

Formation of Knots in Partially Replicated DNA Molecules

José M. Sogo[1], Andrzej Stasiak[2]*, María Luisa Martínez-Robles[3] Dora B. Krimer[3], Pablo Hernández[3] and Jorge B. Schvartzman[3]

[1]*Institut für Zellbiologie ETH-Hönggerberg CH-8093 Zürich, Switzerland*

[2]*Laboratoire d'Analyse Ultrastructurale, Bâtiment de Biologie, Université de Lausanne, CH-1015 Lausanne-Dorigny, Switzerland*

[3]*Departamento de Biología Celular y del Desarollo, Centro de Investigaciones Biológicas Consejo Superior de Investigaciones Científicas Velázquez 144, 28006 Madrid Spain*

*Corresponding author

Bacterial plasmids with two origins of replication in convergent orientation are frequently knotted *in vivo*. The knots formed are localised within the newly replicated DNA regions. Here, we analyse DNA knots tied within replication bubbles of such plasmids, and observe that the knots formed show predominantly positive signs of crossings. We propose that helical winding of replication bubbles *in vivo* leads to topoisomerase-mediated formation of knots on partially replicated DNA molecules.

© 1999 Academic Press

DNA replication and transcription *in vivo* require the action of topoisomerases to relieve accumulated torsional stress in DNA molecules and to allow passage of DNA segments through each other (Adams *et al.*, 1992; Bates & Maxwell, 1997; DiNardo *et al.*, 1984; Rybenkov *et al.*, 1997; Wang, 1991). In a crowded cellular environment or in *in vitro* reactions topoisomerases can lead to inadvertent formation of DNA knots (Dean *et al.*, 1985; Shishido *et al.*, 1987). Under *in vivo* conditions, DNA knots are usually rare and short-lived as further action of specialised topoisomerases eventually resolves formed knots. However, as there is an equilibrium between knotting and unknotting reactions, the low proportion of naturally knotted DNA molecules can be separated from unknotted ones and analysed (Shishido *et al.*, 1987). By determining the type of the DNA knots formed it is possible to get valuable insights into the mechanism by which these DNA knots are formed (Dean, *et al.*, 1985; Stark *et al.*, 1992; Sumners, 1990;

Wasserman *et al.*, 1985) and to determine the topological state of the DNA at the moment of knotting (Spengler *et al.*, 1985). With this in mind, we decided to characterise the DNA knots formed *in vivo* within partially replicated plasmids with two head-to-head oriented ColE1 origins of replication. It has been observed that such plasmids accumulate replication intermediates in which several kb-long DNA portions between the two origin are entirely replicated and form well-defined replication bubbles (Martín-Parras *et al.*, 1992; Viguera *et al.*, 1996). In addition, it has been observed that these partially replicated molecules are frequently knotted (Viguera, *et al.*, 1996; Santamaría *et al.*, 1998). We decided, therefore, to analyse the type of knots formed in the well-defined replication bubbles between the two origins of replication. To do this we used the *Alw*NI restriction endonuclease to digest a preparation of partially replicated pHH5.8 plasmids so that the replication bubble, together with short flanking regions, was cut out from the rest of the plasmid (see Figure 1(a)). Two-dimensional (2D) agarose gel electrophoresis was then used to resolve differently knotted replication bubbles and other types of DNA fragments which are present after restriction digestion of partially
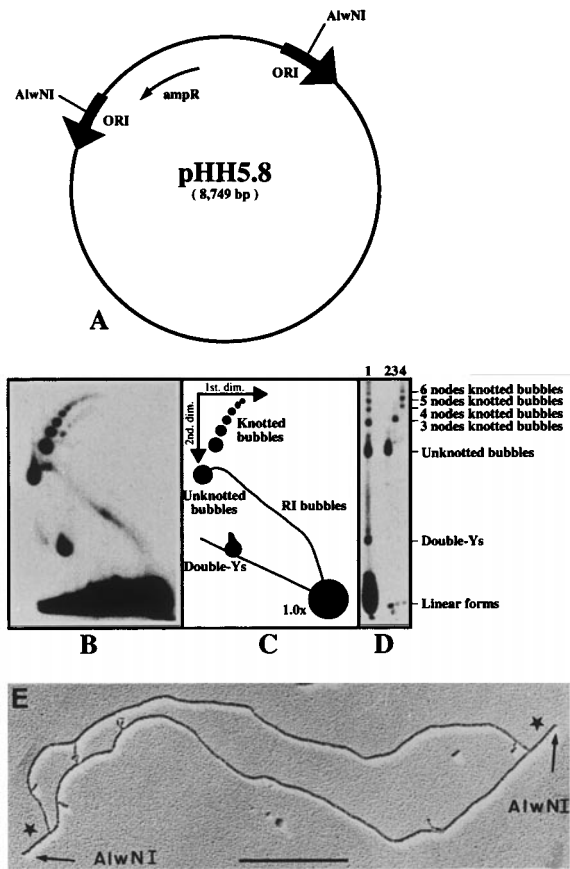
Figure 1. Characterisation of partially replicated DNA molecules. (a) Restriction map of the bacterial plasmid pHH5.8 containing two inversely oriented ColE1 origins. (b) Two-dimensional gel where knotted replication bubbles separate according to the number of nodes in the knots formed. (c) Diagrammatic interpretation of the autoradiogram shown in (b). (d) Standard agarose electrophoresis of the whole DNA sample (lane 1) and samples that were enriched for specific molecular species (lanes 2-4). (e) Electron micrograph of an unknotted partially replicated DNA molecule which was eluted from the region corresponding to the band of unknotted bubbles from a gel similar to the one represented in (d), lane 2. For better visualisation, the DNA was coated with RecA protein. The *Alw*NI cutting sites and the unreplicated portions (asterisks) flanking the bubble are indicated. The bar represents 1 kb length of RecA-coated DNA.

replicated DNA molecules (Viguera *et al.*, 1996; see Figure 1(b)). After isolation of specific DNA bands (Figure 1(b)-(d)) we used the RecA coating method in order to distinguish different types of DNA knots by electron microscopy (Krasnow *et al.*, 1983; Sogo *et al.*, 1987). Figure 2 shows different types of observed knots formed on the isolated replication bubbles (notice the protruding non-replicated flanking regions, which are indicated by asterisks). To correctly recognise the knot type it is necessary to determine at each crossing which segment passes under and which passes over. The more complicated the knot, the higher the chance that at least one of the crossings would be problematic in its interpretation, and this of course invalidates interpretation of the whole knotted molecule. Therefore, we initially decided to look at DNA knots isolated from the band expected to contain trefoil knots as it was the first band with knotted species (Figure 1(b)-(d)). We found 14 molecules which were unambivalent in their interpretation and all turned out to be right-handed trefoil knots, thus having three crossings with a positive sign (see the legend to Figure 3(a) for the sign convention). We then analysed the DNA preparation combined from the gel bands expected to contain more complicated knots (Figure 1(d)). Among the interpretable higher knots we observed several figure-of-eight knots with two right and two left-handed crossings, 5_2 knots with five right-handed crossings and 6_2 knots with four right-handed and two left-handed crossings (see Figure 2).

How do we explain the appearance of knots on the partially replicated DNA molecules, and why do these knots show predominantly positive crossings? To answer these questions one needs to remember that inadvertent knotting events resulting from topo II-type action tend to fix some of the crossings present in interwound supercoiled molecules into irreducible crossings of knotted molecules, whereby the sign of the fixed crossings remains unchanged (Wasserman & Cozzarelli, 1991). The knotting event by itself can introduce two additional crossings which can have positive or negative signs (Wasserman & Cozzarelli, 1991). Therefore, inadvertent knotting events occurring within negatively supercoiled DNA molecules (where DNA shows many negative crossings) are

Figure 2. Characterisation of DNA knots by electron microscopy of RecA-coated molecules. (a)-(f) Different types of observed knots. The trefoils (3_1 knots) were eluted from the region corresponding to the band of three noded knotted bubbles from a gel similar to that represented in Figure 1 (d), lane 3. The 4_1 and 5_2 and 6_2 knots were in the DNA eluted from a region containing four, five, and six noded knotted bubbles (see Figure 1(d) lane 4). The 3_1, 4_1, 5_2 and 6_2 terms are standard mathematical notations of the knots where the first number indicates minimal number of crossings of a given knot and the second (index) number indicates the tabular position of a given knot among the knots with the same minimal crossing number (Adams, 1994; Rolfsen, 1976). Schematic drawings show essential parts of the knots formed and are intended to help us to recognise the knot type. Consistent assignment of the direction along the knots is required to determine the sign of the crossings (see Figure 3). For more complicated knots, 5_2 and 6_2, we included their symmetrised diagrams positioned in such a way as to show good correspondence to the micrographs. For reasons of clarity, (g) is the tracing of the 6_2-knotted molecule shown in (f), where the unreplicated portions of the molecule are omitted as well as the fortuitous contact point (white arrowhead in (f)) between the strands. (h). Rearranged schematic drawing from (g) in a standard tabular form of 6_2 knot. The bar represents 0.5 Kb of RecA-coated DNA.

expected to produce knots with predominantly negative crossings. Indeed, the knots produced *in vivo* analysed by Shishido *et al.* (1987, 1989) showed mainly negative crossings, and this was interpreted to be a result of negative supercoiling (see Figure 3). *In vitro* action of type II topoisomerase on negatively supercoiled DNA molecules also results in the formation of twist-type knots
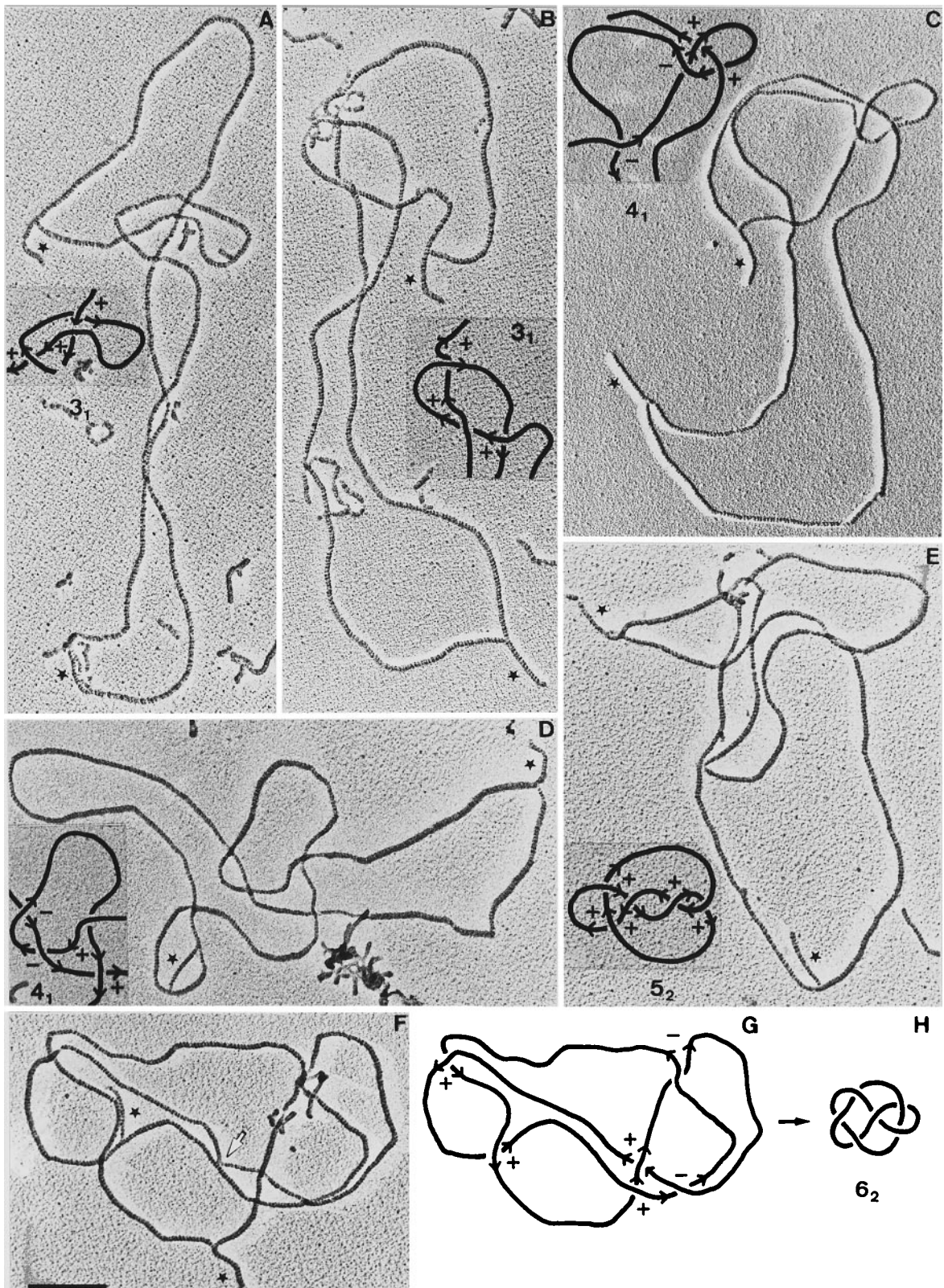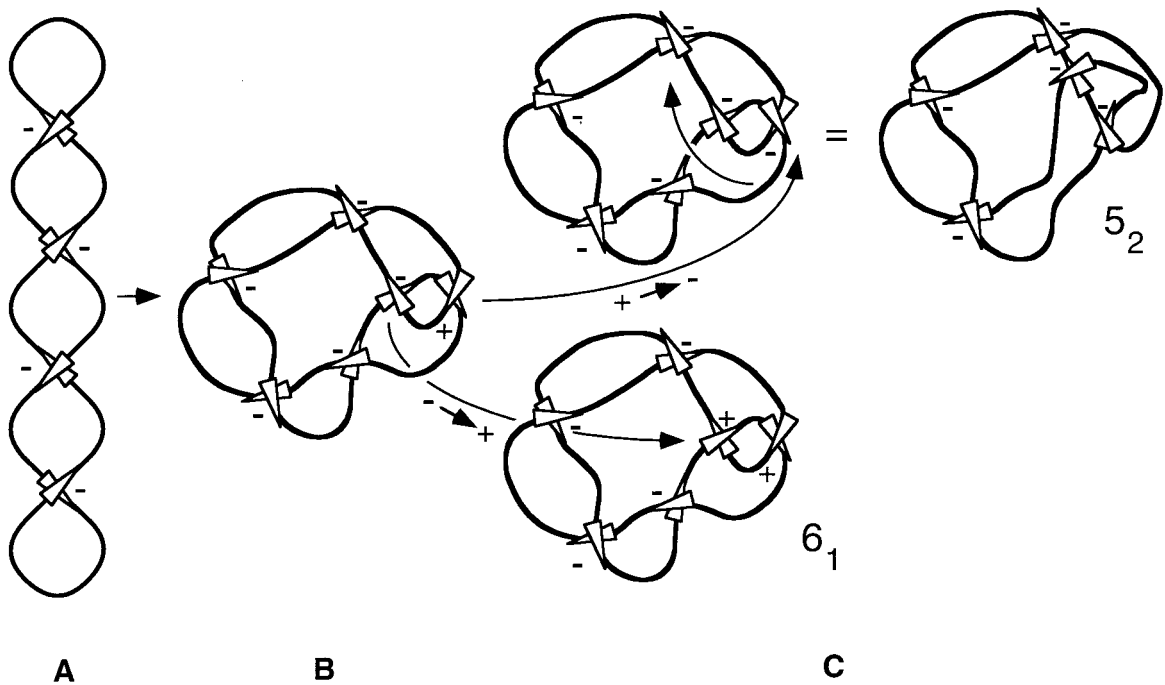

Figure 2 (*legend opposite*).

Figure 3. Inadvertent intramolecular interlockings in negatively supercoiled DNA molecules leads to formation of twist-type knots with a predominantly negative sign of the perceived crossings. (a) Schematic presentation of negatively supercoiled DNA molecule (the DNA double helix is not visible at this scale of the presentation). Note that the consistent assignment of the direction along the supercoiled DNA molecules shows that each crossing has a negative sign (despite the right-handed appearance of the superhelix). According to a mathematical convention (Bates & Maxwell, 1993), in a crossing with a negative sign the direction arrow which is closer to the observer would need to be turned in a clockwise direction in order to overlay it with the arrow which is further from the observer (the rotation has to be smaller than 180°). Of course in the case of positive crossings the corresponding rotation would be in a counter clockwise direction (see (b) and (c) for examples of positive and negative crossings). (b) Accidental overlap between coils of supercoiled molecules produces one positive and one negative crossing. (c) Topoisomerase-mediated accidental interlockings between overlapping coils of the negatively supercoiled DNA molecules lead to formation of twist-type knots with a negative sign of crossings in the portions between interlockings. When topoisomerase acts on the overlap by changing a positive sign into a negative one, all crossings in the knot created are negative. If the action of topoisomerase changes a negative crossing into a positive one, the knot created will have two positive crossings in addition to the negative crossings resulting from trapping of negative supercoils in inter-

with predominantly negative signs of crossings (Wasserman & Cozzarelli, 1991). How then are the positive crossings obtained when the continuous action of DNA gyrase introduces negative supercoiling into plasmid DNA molecules? The answer lies in the topology of partially replicated DNA molecules. If replication is stopped or blocked for some time, as is frequently the case of plasmids with two convergent ColE1 origins where one is a functional origin and the second acts as a polar replication fork pausing site (Viguera *et al.*, 1996; Santamaría *et al.*, 1998), then the continuous action of DNA gyrase establishes negative supercoiling in the unreplicated portion of the plasmid, and this causes a left-handed winding of torsionally relaxed newly replicated regions forming the replication bubble (Ullsperger *et al.*, 1995). Figure 4 shows that left-handed winding in the replication bubble results in the formation of crossings with a positive sign when the bubble is considered as an oriented circular domain. The topo II-type action on the overlap region within such an interwound replica-

tion bubble can then lead, for example, to the formation of trefoils with positive crossings and figure-of-eight knots with two positive and two negative crossings (see Figure 4(b)). The 5_2 knots can be formed analogously to a trefoil knot, with the interlockings occurring at a bigger distance from each other, and thus leading to the trapping of two more positive crossings from the helically wound replication bubble (see Figure 3, but notice the difference in the signs of crossings). The generation of 6_2 knots would require two consecutive topoisomerase-mediated intramolecular interlockings. First, a trefoil with positive crossings should be formed, and then the interlocking would be needed between a loop of the trefoil and an adequately positioned coil of the replication bubble twisted in a left-handed direction.

We have characterised here novel types of DNA knots which are generated *in vivo* and are localised in the newly replicated portions of plasmid DNA molecules. We propose that the formation of these types of knot is a consequence of helical left-
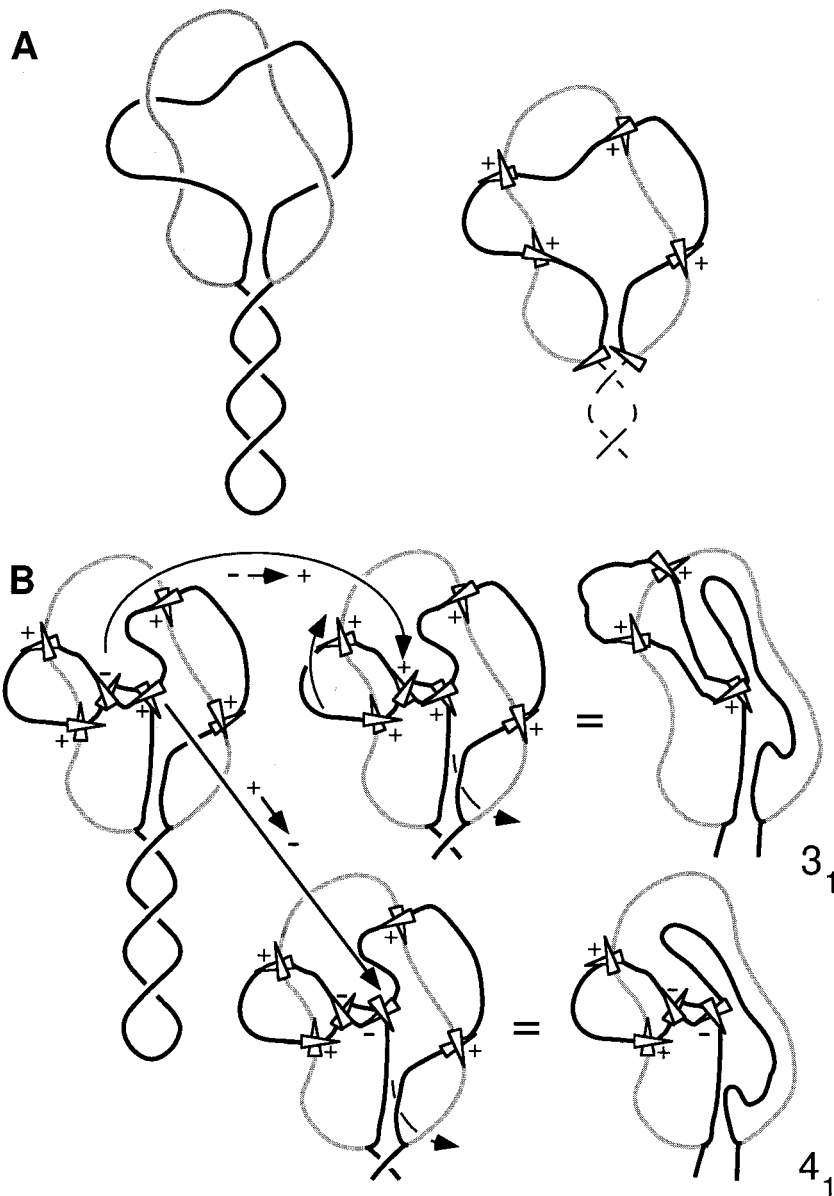
Figure 4. (a) Expected structure of replication bubbles in negatively supercoiled DNA molecules. In partially replicated molecules, parental strands are continuous and the action of gyrase negatively supercoils the whole molecule resulting in a right-handed appearance of the interwound unreplicated portion of the plasmid, and induces left-handed winding in the toroidally wound replication bubble. Continuous assignment of the direction on the "abstracted" twisted bubble reveals that all the crossings have positive signs (see Figure 3). (b) Formation of a trefoil knot (3_1) with positive crossings and a figure-of-eight knot (4_1) with two positive and two negative crossings by topoisomerase-mediated interlinking between overlapping coils of a replication bubble wound in a left-handed way. Formation of a right-handed trefoil knot requires that a negative crossing at an overlap region is changed by type II topoisomerase into a positive crossing. After elimination of nugatory crossovers, the knot formed is a trefoil and has three positive crossings. To form a figure-of-eight knot a positive crossing at the overlap region is changed into a negative crossing; this introduces two negative crossings, which together with two positive crossings trapped from the twisted portion of the replication bubble give rise to a chiral figure-of-eight knot, which is better visualised after elimination of nugatory twists. To facilitate visual tracing of supercoiled and knotted partially replicated DNA molecules, one of the newly replicated branches is drawn in grey.

handed winding of replication bubbles *in vivo*. Our results strongly support the model proposed by Ullsperger *et al*. (1995), where the replication bubbles in circular DNA molecules are helically wound. When this work was finished, we learned that very recent electron microscopy studies of purified DNA replication intermediates visualised helically wound replication bubbles (Peter *et al*., 1998). However, knowing that the level of supercoiling *in vivo* is about twofold lower than this in deproteinised DNA (Bliska & Cozzarelli, 1987), one can still ask the question whether the helical wind-

ing of replication bubbles observed by electron microscopy *in vitro* reflects a similar winding *in vivo*. Our results answer this question positively, since knotting events *in vivo* probe the actual topology and the resulting structure of DNA *in vivo* (Bliska & Cozzarelli, 1987). Further treatments of the DNA-like deproteinisation and subsequent RecA covering do not change the type of knots formed, and thus we can use this topological information to draw conclusions about the structural arrangement of partially replicated DNA molecules *in vivo*.

A caution is needed before extending our results to actively replicating DNA molecules, which are not stalled, in contrast to the case studied here. In actively replicating DNA molecules (transient intermediates; Peter *et al.*, 1998), the separation of DNA strands at the replication fork may completely remove negative supercoils continuously generated by the gyrase, or even cause an accumulation of positive supercoils (see Ullsperger *et al.*, 1995; Peter *et al.*, 1998). Therefore, actively replicating DNA molecules can be less prone to knotting or can even produce knots with predominantly negative crossings. However, our results demonstrate that helical winding of replication bubbles can arise *in vivo* when a torsional stress is generated in replicating DNA molecules.

Since the knots we observed and characterised are topologically fixed even if the unreplicated portion of a circular plasmid is cut with a restriction enzyme, it is possible that similar types of knots can exist and be topologically trapped also in replicating linear DNA of eukaryotic chromosomes, a possibility which was not considered before.

Acknowledgements

References

Adams, C. C. (1994). *The Knot Book*, W. H. Freeman and Company, New York.

Adams, D. E., Shekhtman, E. M., Zechiedrich, E. L., Schmid, M. B. & Cozzarelli, N. R. (1992). The role of topoisomerase IV in partitioning bacterial replicons and the structure of catenated intermediates in DNA replication. *Cell*, **71**, 277-288.

Bates, A. D. & Maxwell, A. (1993). *DNA Topology*, IRL Press, Oxford.

Bates, A. D. & Maxwell, A. (1997). Topoisomerases keep it simple. *Curr. Biol.* **7**, R778-R781.

Bliska, J. B. & Cozzarelli, N. R. (1987). Use of site-specific recombination as a probe of DNA structure and metabolism *in vivo*. *J. Mol. Biol.* **194**, 205-218.

Dean, F. B., Stasiak, A., Koller, T. & Cozzarelli, N. R. (1985). Duplex DNA knots produced by *Escherichia coli* topoisomerase I, structure and requirements for formation. *J. Biol. Chem.* **260**, 4975-4983.

DiNardo, S., Voelkel, K. & Sternglanz, R. (1984). DNA topoisomerase II mutant of *Saccharomyces cerevisiae*: topoisomerase II is required for segregation of daughter molecules at the termination of DNA replication. *Proc. Natl Acad. Sci. USA,* **81**, 2616-2620.

Krasnow, M. A., Stasiak, A., Spengler, S. J., Dean, F., Koller, T. & Cozzarelli, N. R. (1983). Determination of the absolute handedness of knots and catenanes of DNA. *Nature,* **304**, 559-560.

Martín-Parras, M. A., Hernández, P., Martínez-Robles, M. L. & Schvartzman, J. B. (1992). Initiation of DNA replication in ColE1 plasmids containing multiple potential origins of replication. *J. Biol. Chem.* **267**, 22496-22505.

Peter, B. J., Ullsperger, C., Hiasa, H., Marians, K. J. & Cozzarelli, N. R. (1998). The structure of supercoiled intermediates in DNA replication. *Cell,* **94**, 819-827.

Rolfsen, D. (1976). *Knots and Links*, Publish or Perish Press, Berkeley, CA.

Rybenkov, V. V., Ulsperger, C., Vologodskii, A. V. & Cozzarelli, N. R. (1997). Simplification of DNA topology below equilibrium values by type II topoisomerases. *Science,* **277**, 690-693.

Santamaría, D., de la Cueva, G., Martínez-Robles, M. L., KrimerD., B., Hernández, P. & Schvartzman, J. B. (1998). DnaB helicase is unable to dissociate RNA-DNA hybrids: its implication in the polar pausing of replication forks at ColE1 origins. *J. Biol. Chem.* **273**, 33386-33397.

Shishido, K., Komiyama, N. & Ikawa, S. (1987). Increased production of a knotted form of plasmid pBR322 DNA in *Escherichia coli* DNA topoisomerase mutants. *J. Mol. Biol.* **195**, 215-218.

Shishido, K., Ishii, S. & Komiyama, N. (1989). The presence of the region on pBR322 that encodes resistance to tetracycline is responsible for high levels of plasmid DNA knotting in *Escherichia coli* DNA topoisomerase I deletion mutant. *Nucl. Acids Res.* **17**, 9749-9759.

Sogo, J., Stasiak, A., DeBernardin, W., Losa, R. & Koller, T. (1987). Binding of proteins to nucleic acids as studied by electron microscopy. In *Electron Microscopy in Molecular Biology* (Sommerville, J. & Scheer, U., eds), pp. 61-79, IRL Press, Oxford.

Spengler, S. J., Stasiak, A. & Cozzarelli, N. R. (1985). The stereostructure of knots and catenanes produced by phage l integrative recombination: implications for mechanism and DNA structure. *Cell,* **42**, 325-334.

Stark, W. M., Boocock, M. R. & Sherrat, D. J. (1992). Catalysis by site-specific recombinases. *Trends Genet.* **8**, 432-439.

Sumners, D. W. (1990). Untangling DNA. *Mathematical Intelligencer,* **12**, 71-80.

Ullsperger, C. J., Vologodskii, A. V. & Cozzarelli, N. R. (1995). Unlinking of DNA by topoisomerases during DNA replication. *Nucl. Acids Mol. Biol.* **9**, 115-142.

Viguera, E., Hernández, P., Krimer, D. B., Boistov, A. S., Lurz, R., Alonso, J. C. & Schvartzman, J. B. (1996). The ColE1 unidirectional origin acts as a polar replication fork pausing site. *J. Biol. Chem.* **271**, 22414-22421.

Wang, J. C. (1991). DNA topoisomerases: why so many? *J. Biol. Chem.* **266**, 6659-6662.

Wasserman, S. A. & Cozzarelli, N. R. (1991). Supercoiled DNA-directed knotting by T4 topoisomerase. *J. Biol. Chem.* **266(30)**, 20567-20573.

Wasserman, S. A., Dungan, J. M. & Cozzarelli, N. R. (1985). Discovery of a predicted DNA knot substantiates a model for site-specific recombination. *Science,* **229**, 171-174.

Edited by M. Yaniv