DNA Knotting Caused By Head-on Collision

Of Transcription And Replication

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ABSTRACT

Collision of transcription and replication is uncommon, but the reason for nature to avoid this type of collision is still poorly understood. In *Escherichia coli* pBR322 is unstable and rapidly lost without selective pressure. Stability can be rescued if transcription of the tetracycline-resistance gene (*Tet*^{R}), progressing against replication, is avoided. We investigated the topological consequences of the collision of transcription and replication in pBR322-derived plasmids where head-on collision between the replication fork and the RNA polymerase transcribing the *Tet*^{R} gene was allowed or avoided. The results obtained indicate that this type of collision triggers knotting of the daughter duplexes behind the fork. We propose this deleterious topological consequence could explain the instability of pBR322 and could be also one of the reasons for nature to avoid head-on collision of transcription and replication.

Collision of transcription and replication is generally avoided in nature with very few exceptions (1; 2). Moreover, in some cases where this is unavoidable, such as in the eukaryotic rDNA loci, a specific mechanism developed to stall the replication fork progressing against transcription just prior to the 3' end of the transcriptional unit (3; 4; 5). The fact that this feature is conserved from bacteria to vertebrates implies it has an unambiguous evolutionary advantage (6; 7). But the reason for cells to avoid this type of collision is not fully understood. As in prokaryotes DNA polymerase moves along the DNA template at least 10x faster than RNA polymerase, the possibility exists for the replication complex to encounter a transcription complex when both are co-oriented as well as when they progress against each other. Both types of collision were studied using the bacteriophages T4 and Φ29 *in vitro* systems (8; 9; 10; 11). The results obtained indicate that the replication fork pauses when it meets a head-on RNA polymerase. Experimental evidence that this is true also *in vivo* was obtained for the transfer RNA (tRNA) genes in *Saccharomyces cerevisiae* (2). But the pause caused by the physical contact of an RNA polymerase with the proteins at a replication fork may not be the primary cause for evolution to avoid head-on collision of transcription and replication (1). The DNA template accumulates (+) ΔLk ahead of an actively transcribing gene (12). Unwinding of the helix by DNA helicase during replication also

leads to positive superhelical turns in the unreplicated template ahead of the fork (13; 14; 15). Bacterial DNA gyrase introduces (-) ΔLk in this region but this is not enough to compensate all the (+) ΔLk that builds up ahead of the fork, at least during replication. Champoux and Bean (16) suggested that the (+) ΔLk generated ahead of the fork during replication distributes both ahead of and behind the replication fork. To distinguish between ΔLk in the unreplicated portion and in the replicated one, Peter and co-workers (14) call "supercoils" to the first and "precatenanes" to the latter. It is now accepted that during DNA replication in bacteria, topo IV (removing precatenanes in the replicated region) helps DNA gyrase (introducing negative supercoils in the unreplicated region) to eliminate all the (+) ΔLk that builds up during replication (13; 14; 15; 17). The net ΔLk of a plasmid at any time results from the balance between the activities of these three enzymes.

pBR322 is not a natural *E. coli* plasmid. Bolívar and co-workers (18) constructed this plasmid as a multipurpose cloning system. It has a unidirectional ColE1 replication origin and codes for two antibiotic resistance genes: ampicillin (Amp^R) and tetracycline (Tet^R). The Amp^R gene is co-oriented with the ColE1 origin but the Tet^R gene is inversely oriented (Figure 1). As the Tet^R gene transcribes constitutively, collision with the

replication fork is unavoidable during replication. Moreover, in bacteria grown in the presence of ampicillin, the simultaneous transcription of Amp^R and Tet^R genes in non-replicating plasmids leads to the formation of twin supercoiling domains where (+) ΔLk accumulates in the intergenic region (19). pBR322 DNA showing net (+) ΔLk was clearly identified in chloroquine 2D gels after inhibition of DNA gyrase with novobiocin and this positive supercoiling depends on the presence and orientation of the Tet^R gene (19). pBR322 knotted forms was observed in *E. coli* topoisomerase mutants and most of the nodes of these knots have a negative sign (20). Formation of these knotted plasmids also depends on the presence and orientation of the Tet^R gene (21). In agreement with these observations, pBR322 DNA isolated from DH5αF' cells, carrying no topoisomerase mutations, reveals significant amounts of knotted plasmids as well as molecules showing low levels of supercoiling when analyzed by the Brewer-Fangman neutral/neutral (N/N) two-dimensional (2D) agarose gel electrophoresis (22). Altogether, these observations indicate that in pBR322, transcription of the Tet^R gene is responsible for the particular topological characteristics of this plasmid in *E. coli* cells (19; 20; 21). Stability of pBR322-derived plasmids during growth of their *E. coli* host in the absence of antibiotics has been studied in detail. pBR322 was found to be very unstable under these conditions and was lost within ~60

generations. But a number of derivatives where the promoter of the *Tet*^{R} gene had been deleted, were stable under the same conditions (23). This observation indicates that transcription of the *Tet*^{R} gene is responsible also for the instability of pBR322 in the absence of selective pressure.

It was recently shown that ColE1 plasmids containing a stalled fork could be knotted. But these knots occur in the replicated region behind the fork (24). Most of the nodes of these knotted bubbles have a positive sign (25) indicating that they resulted from *in vivo* action of a type II topoisomerase on negatively twisted precatenanes (26).

N/N 2D agarose gel electrophoresis was originally designed to separate branched from linear molecules (27), but this technique can be used also to resolve the different forms undigested circular DNA can adopt (22; 28; 29; 30). In the present report we used this technique to compare the patterns observed for several pBR322-derived plasmids where transcription of the *Tet*^{R} gene was on or off and where progression of the DNA replication fork was blocked either before or after the *Tet*^{R} gene.

pBR18-TerE@*Sty*I and pBR322-TerE@*Sty*I were constructed inserting the 23 bp that constitutes the *E. coli* polar replication terminator

TerE (31; 32) in its active orientation between the unique *Sty*I and *Ava*I sites of pBR18 or pBR322 (Figure 1). pBR18 is a derivative of pBR322 where the nucleotides between the unique *Eco*RI and *Hin*dIII sites had been replaced with the polycloning fragment of pUC18 (33). In doing so, the promoter for the Tet^R gene is lost. Thus, the main difference between pBR18 and pBR322 is that pBR18 lacks the promoter for the Tet^R gene (Figure 1). We anticipated that in both plasmids, replication forks would stop at the TerE-TUS complex leading to the accumulation of specific RIs containing an internal bubble and with a total mass 1.26x the mass of non-replicating plasmids. It was previously shown that in order to reveal the presence of knotted bubbles, ΔLk has to be eliminated (24; 25; 33; 34). *E. coli* DH5αF' cells were transformed with either pBR18-TerE@*Sty*I or pBR322-TerE@*Sty*I and plasmid DNAs were digested with *Sca*I, a restriction enzyme that cuts both plasmids only once and outside the putative replicated region. Then linearized molecules were analyzed by N/N 2D agarose gel electrophoresis (35). Autoradiograms of these gels are shown in Figure 2 with corresponding diagrammatic interpretations to their right. These autoradiograms revealed that in both cases the vast majority of partially replicated plasmids had a fork stalled at TerE. In other words, after digestion with *Sca*I, pBR18-TerE@*Sty*I and pBR322-TerE@*Sty*I occurred in only two basic forms: the non-replicating (1.0x) linear form

and the partially replicated RI containing an internal bubble (1.26xBubble). To the right of the accumulated 1.26xBubble, a rather short "beads-on-a-string" signal was observed. This signal corresponded to knotted bubbles (24; 25; 33; 34). No significant difference in the number and complexity of knotted bubbles was observed in the autoradiograms corresponding to pBR18-TerE@*Sty*I and pBR322-TerE@*Sty*I.

pBR18-TerE@*Aat*II and pBR322-TerE@*Aat*II were constructed inserting TerE (31; 32) at the unique *Aat*II site of pBR18 or pBR322 (Figure 1). 2D gel autoradiograms of these plasmids after restriction digestion with *Alw*NI are shown in Figures 3A and B, with diagrammatic interpretations to their right. Signals detected below the accumulated bubbles were likely due to single-stranded breakage of replication intermediates (RIs) containing an internal bubble and trailing during the first dimension due to overloading was responsible for the tails detected for the most abundant molecular species in the autoradiograms (36). A densitometric analysis of the "beads-on-a-string" signal corresponding to unknotted and knotted bubbles is included above each autoradiogram. The most significant difference between these two plasmids was the number and complexity of knotted bubbles. The densitometric profiles confirmed this observation. When the strength of the signal corresponding to

unknotted bubbles of both profiles was made equal, there were 64% more knotted bubbles in pBR322-TerE@*Aat*II.

The excess of knotted bubbles observed for pBR322-TerE@*Aat*II could be due to the head-on collision of transcription and replication. But the possibility existed also that this extra knotting could be caused by transcription of the Tet^R gene itself regardless of whether it occurred against or co-oriented with replication fork progression. To test this latter possibility, a new plasmid was made where the *Eco*RI-*Sty*I restriction fragment of pBR322-TerE@*Aat*II was inverted (see Figure 1). In the new plasmid (pBR322-TerE@*Aat*II-inv) transcription of the Tet^R gene was co-oriented with progression of the replication fork. The corresponding 2D gel autoradiogram after restriction digestion with *Alw*NI is shown in Figure 3C, with a diagrammatic interpretation to its right. Note that in the new plasmid the number of knotted bubbles was even lower than for pBR18-TerE@*Aat*II (Figure 3A), where the promoter of the Tet^R gene had been deleted. This isn't unexpected, though, as in pBR18-TerE@*Aat*II some transcription could still take place, although at a very low rate, driven from cryptic promoters located upstream the deleted one (36). Note that this putative low level of transcription, however, was not sufficient to turn the cells resistant to tetracycline, probably because these transcripts were not

translated properly. The observation that pBR322-TerE@*Aat*II-inv exhibited the lowest number of knotted bubbles strengthens the idea that the excess of knotted bubbles observed for pBR322-TerE@*Aat*II was indeed caused by head-on collision of transcription and replication.

Knotted bubbles were originally detected in ColE1 plasmids where replication forks pause or are permanently blocked at a Ter site or at another ColE1 origin with the opposite orientation (24; 25; 33; 34). This type of knots reflects the number and pattern of DNA crossings trapped between the two segments participating in the strand passage event (25; 26). As knotted bubbles occur in the replicated portion of partially replicated plasmids, the two segments involved are the two daughter duplexes. In those cases where replication and transcription progress against each other, (+) ΔLk accumulates in the region between the two advancing forks. This change in topology rapidly diffuses to the replicated portion where it changes the number of precatenanes facilitating DNA knotting. Blockage of the replication fork at TerE with the concomitant formation of some knotted bubbles occurred in all the plasmids we have studied alike. But the increased number of knotted bubbles observed for pBR322-TerE@*Aat*II was due to some extra-knotting that took place only

in this plasmid where the replication fork progressed against the RNA polymerase transcribing the *Tet*^{R} gene (Figure 4B).

Why most of the nodes of non-replicating knotted plasmids of pBR322 have a negative sign (20) while the sign of the nodes of knotted bubbles are predominantly positive (25)? Knotted bubbles form when a type II topoisomerase crosses two successive precatenanes (26). For this reason positive supercoiling leads to DNA knots having predominantly negative nodes while negative supercoiling leads to knots with positive nodes (37; 38). In pBR322 the opposing orientation of *Amp*^{R} and *Tet*^{R} genes leads to the accumulation of (+) ΔLk in the intergenic region (19). The nodes of the DNA knots formed in these non-replicating plasmids have a negative sign (20) because the template was positively supercoiled (Figure 4A). On the other hand, due to the combined action of DNA gyrase and topo IV, partially replicated ColE1 plasmids display negatively twisted precatenanes (Figure 4B). For this reason the sign of the nodes of knotted bubbles are predominantly positive (25).

The biological significance of knotted bubbles is still unknown, but DNA knotting has potentially devastating effects on cells (39). It was

recently shown that topo IV alone is responsible for unknotting DNA in *E. coli* cells (40). It is conceivable that topo IV should be able to eliminate most knotted bubbles *in vivo*, but too many knots are likely to delay or severely interfere with normal segregation. The excess of knotted bubbles observed for pBR322-TerE@*Aat*II could explain the instability of pBR322 in *E. coli* cells in the absence of selective pressure (23). Altogether, these observations led us to propose the deleterious consequence of this excess of DNA knotting, and not just the physical collision of an RNA polymerase with the proteins at a replication fork, as the main reason for nature to avoid head-on collision of transcription and replication.

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LEGEND TO FIGURES

Figure 1: Maps of the plasmids used in this study showing the relative position of their most relevant features: the ColE1 unidirectional origin, the *E. coli* terminator TerE, the Amp^R, Tet^R and *rop* genes and the recognition sites for a number of restriction endonucleases. To construct pBR18-TerE@*Sty*I and pBR322-TerE@*Sty*I, two oligos:

5'-CTTGGGGCTTAGTTACAACATACTTTAAC-3' and

5'-CCGAGTTAAAGTATGTTGTAACTAAGCCC-3' containing the 23 bp that constitutes the *E. coli* TerE terminator with a 3' *Sty*I and a 5' *Ava*I tails were annealed to each other and inserted between the unique *Sty*I and *Ava*I sites of pBR18 or pBR322. To construct pBR18-TerE@*Aat*II and pBR322-TerE@*Aat*II, two different oligos:

5'-CGTCGACGGCTTAGTTACAACATACTTTAAGACGT-3' and

5'-CTTAAAGTATGTTGTAACTAAGCCGTCGACGACGT-3' with two *Aat*II tails and one *Sal*I site were annealed to each other and inserted at the unique *Aat*II site of pBR18 or pBR322. Construction of pBR322-TerE@*Aat*II-inv was performed inverting the *Eco*RI-*Sty*I fragment of pBR322-TerE@*Aat*II. Transcription of the Tet^R gene in pBR322, pBR322-TerE@*Sty*I, pBR322-TerE@*Aat*II and pBR322-TerE@*Aat*II-inv was

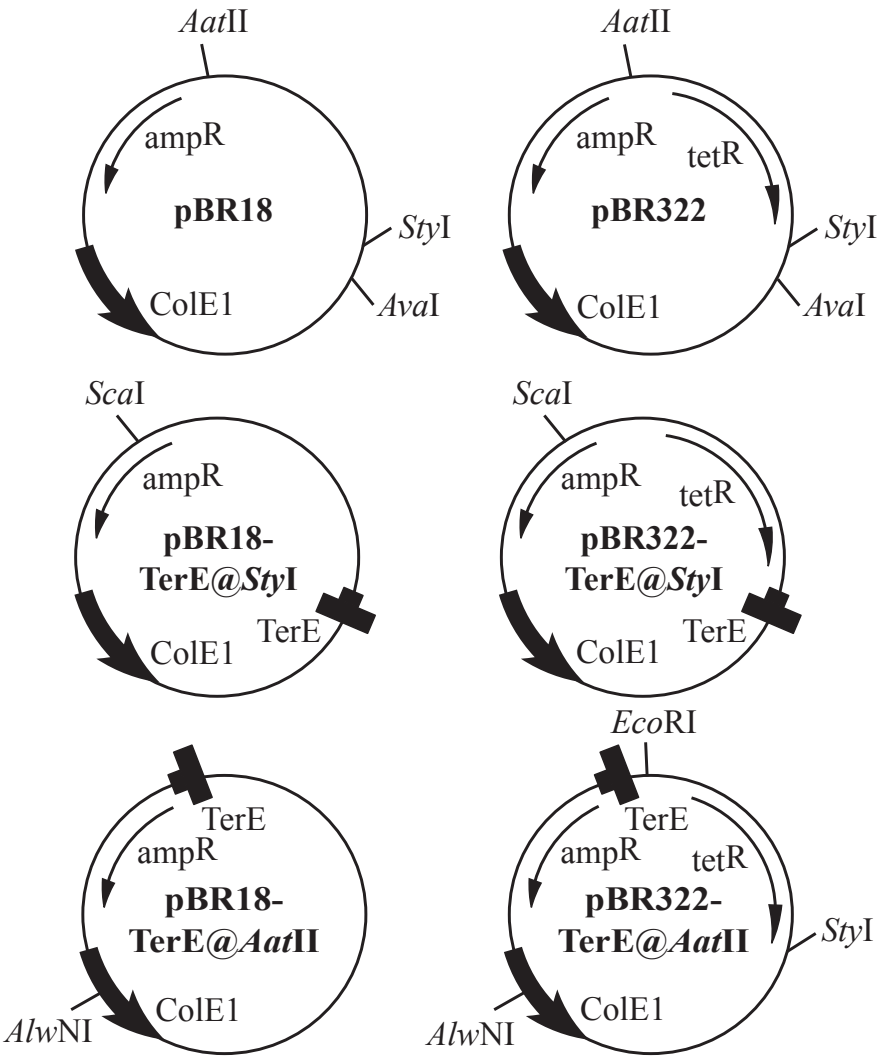
confirmed growing the cells transformed with these plasmids at 37°C in LB medium containing 12.5 μg/ml tetracycline.

Figure 2: Autoradiograms of 2D gels corresponding to pBR18-TerE@*Sty*I (A and B, upper panels) and pBR322-TerE@*Sty*I (C and D, lower panels) after digestion with *Sca*I. No significant differences were observed between both plasmids. The *E. coli* strain used was DH5αF'. Competent cells were transformed with monomeric forms of the plasmids as described (24; 34; 36). Cells were grown at 37°C in LB medium containing 50 mg/ml ampicillin. Isolation of plasmid DNA, N/N 2D agarose gel electrophoresis, Southern transfer and hybridization were performed as described elsewhere (22; 24; 33; 34).

Figure 3: Autoradiograms of 2D gels corresponding to pBR18-TerE@*Aat*II (A, upper panel), pBR322-TerE@*Aat*II (B, mid panel) and pBR322-TerE@*Aat*II-inv (C, lower panel) after digestion with *Alw*NI. Note the increased number and complexity of knotted bubbles in pBR322-TerE@*Aat*II (B, mid panel). To help visualization of this difference, a densitometric profile of unknotted and knotted bubbles (made using version 1.61 of NIH Image) is shown above each autoradiogram with the profile corresponding to pBR322-TerE@*Aat*II shaded and superimposed on the

profiles of the other two plasmids. The *E. coli* strain used was DH5αF'. Competent cells were transformed with monomeric forms of the plasmids as described (24; 34; 36). Cells were grown at 37°C in LB medium containing 50 mg/ml ampicillin. Isolation of plasmid DNA, N/N 2D agarose gel electrophoresis, Southern transfer and hybridization were performed as described elsewhere (22; 24; 33; 34).

Figure 4: Topological consequences of the opposing orientation of two actively transcribing genes (A) and head-on collision of transcription and replication (B) are schematically presented. Head-on orientation of two actively transcribing genes leads to the formation of (+) ΔLk in the intergenic region. This causes topo IV to knot the template. As the DNA was positively supercoiled, the nodes of these knots have predominantly negative signs (upper right corner). Head-on collision of transcription and replication also leads to the accumulation of (+) ΔLk. But this positive supercoiling rapidly diffuses behind the replication fork, changing the twisting degree of the two daughter duplexes. This change in topology facilitates the formation of knotted bubbles. As due to the combined action of DNA gyrase and topo IV precatenanes were negatively twisted, the nodes of these knotted bubbles have predominantly positive signs (lower right corner).


AatII
ampR
pBR18
StyI
AvaI
ColE1
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tetR
pBR322
StyI
AvaI
ColE1
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pBR18-
TerE@StyI
TerE
ColE1
ScaI
ampR
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pBR322-
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TerE
ColE1
TerE
ampR
pBR18-
TerE@AatII
AlwNI
ColE1
EcoRI
TerE
ampR
tetR
pBR322-
TerE@AatII
StyI
AlwNI
ColE1

Figure 1

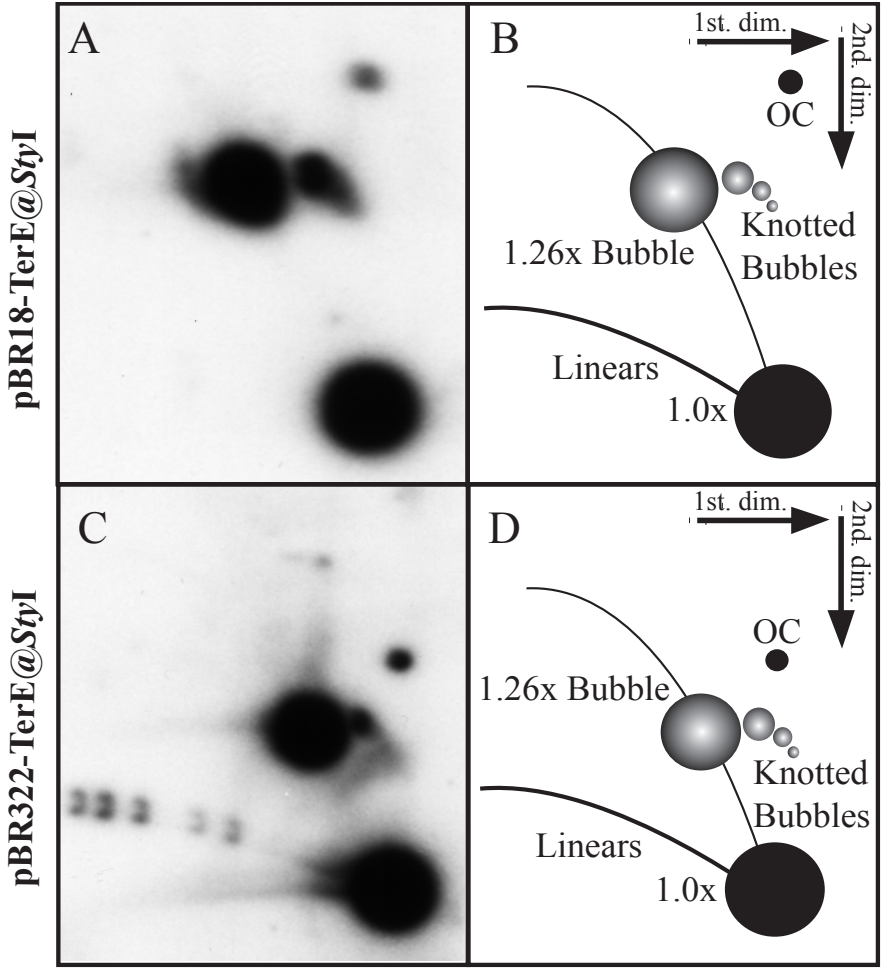
pBR18-TerE@StyI
pBR322-TerE@StyI
A
B
C
D
1st. dim.
2nd. dim.
OC
1.26x Bubble
Knotted
Bubbles
Linears
1.0x

Figure 2

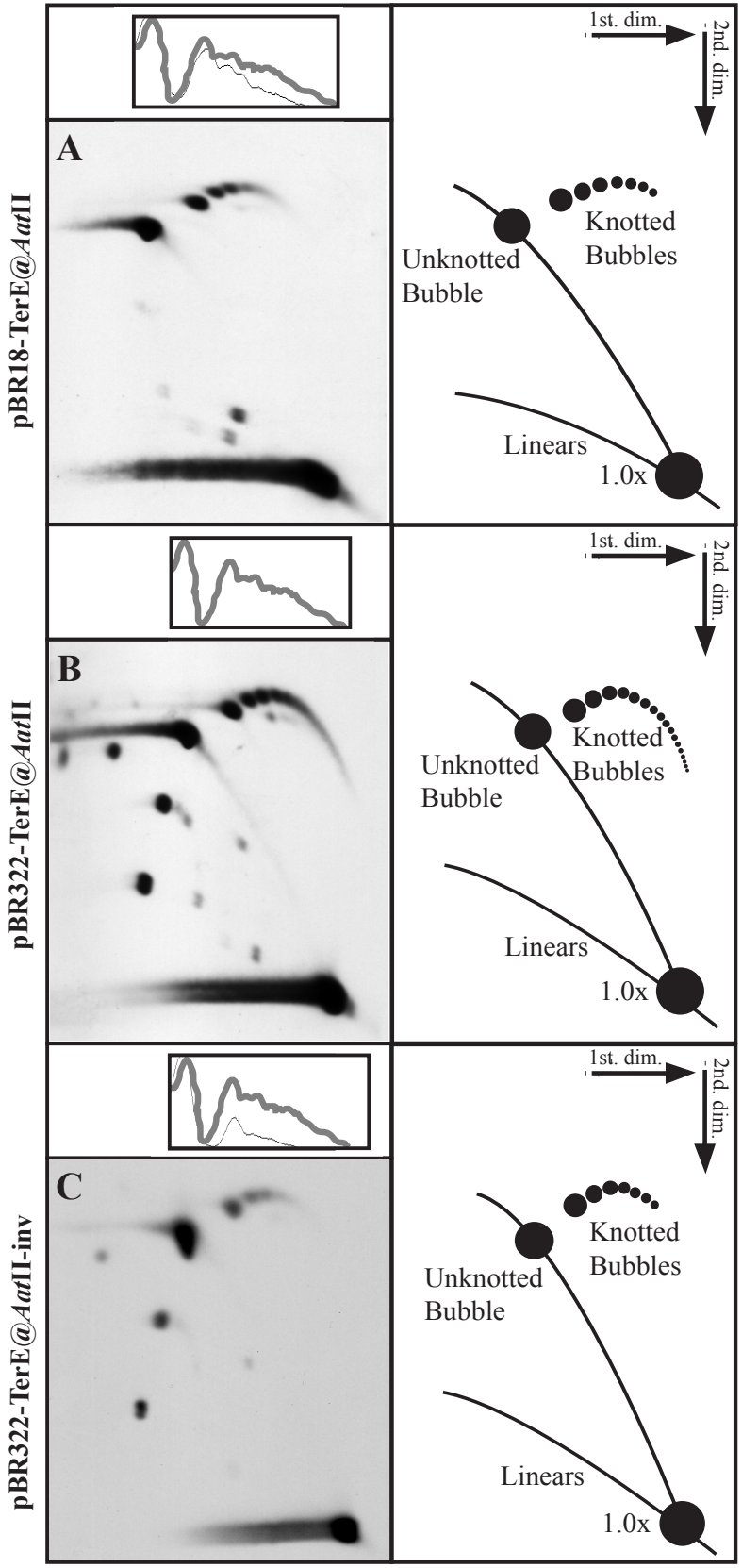
pBR18-TerE@AatII
A
1st. dim.
2nd. dim.
Unknotted Bubble
Knotted Bubbles
Linears
1.0x
pBR322-TerE@AatII
B
1st. dim.
2nd. dim.
Unknotted Bubble
Knotted Bubbles
Linears
1.0x
pBR322-TerE@AatII-inv
C
1st. dim.
2nd. dim.
Unknotted Bubble
Knotted Bubbles
Linears
1.0x

Figure 3

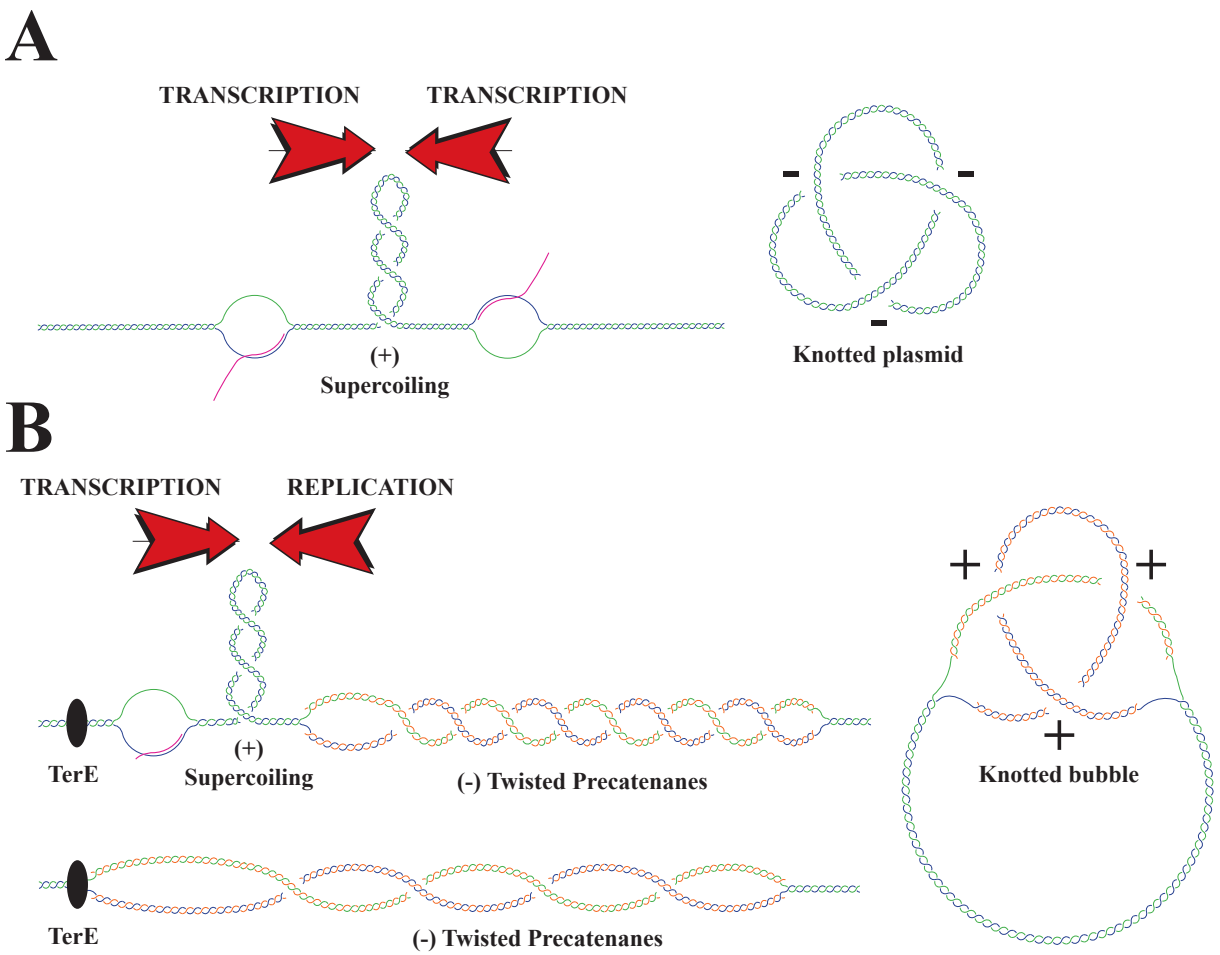
A
TRANSCRIPTION
TRANSCRIPTION
(+)
Supercoiling
Knotted plasmid
B
TRANSCRIPTION
REPLICATION
TerE
(+)
Supercoiling
(-) Twisted Precatenanes
Knotted bubble
TerE
(-) Twisted Precatenanes

Figure 4