Knotting Dynamics
During DNA Replication

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The topology of plasmid DNA changes continuously as replication progresses. But the dynamics of the process remains to be fully understood. Knotted bubbles form when topo IV knot the daughter duplexes behind the fork in response to their degree of intertwining. Here we showed that knotted bubbles can form during unimpaired DNA replication, but they become more evident in partially replicated intermediates containing a stalled fork. To learn more about the dynamics of knot formation as replication advances, we used two-dimensional agarose gel electrophoresis to identify knotted bubbles in partially replicated molecules where the replication fork stalled at different stages of the process. The number and complexity of knotted bubbles rose as a function of bubble size, suggesting that knotting is affected by both precatenane density and bubble size.
INTRODUCTION

Segregation of the products of plasmid DNA replication is a rather complex topological problem. Nature designed a special kind of enzymes, DNA topoisomerases, specifically to deal with this problem. As replication proceeds, unwinding of the parental strands by DNA helicase leads to the formation of $\Delta Lk$ ahead of the fork, which must be removed for the fork to keep progressing (Kornberg & Baker, 1992). This $\Delta Lk$ diffuses across the replication fork so that it is redistributed both ahead of and behind the fork (Champoux & Been, 1980; Peter et al., 1998). In bacteria two specific type II DNA topoisomerases act in different ways and at different places to reduce $\Delta Lk$ during replication: DNA gyrase, introduces $-\Delta Lk$ ahead of the fork and topoisomerase IV (topo IV) removes precatenanes in the replicated region (Alexandrov et al., 1999; Lucas et al., 2001; Peter et al., 1998; Ullsperger et al., 1995). Negative supercoiling assists any process that requires double helix opening (Kanaar & Cozzarelli, 1992). Moreover, $+\Delta Lk$ can lead to replication fork reversal through the formation of Holliday-like junctions, at least in vitro (Olavarrieta et al., 2002; Postow et al., 2001). These observations suggest that plasmids should remain negatively supercoiled throughout the process for replication to go on, but there is no experimental evidence indicative
for the latter. As $\Delta L_k$ distributes both ahead of and behind the fork (Champoux & Been, 1980; Peter et al., 1998), and the replicated portion enlarges as replication advances, this (-) $\Delta L_k$ would progressively switch from (-) supercoils to negatively twisted precatenanes. In other words, the number of precatenanes is expected to rise as a function of bubble size. But measuring the number of precatenanes \textit{in vivo} as replication advances is not an easy task. There are ways, however, to infer changes in the number of precatenanes as replication progresses. Knotted bubbles form when a type II topoisomerase crosses two successive precatenanes (Postow \textit{et al.}, 1999; Sogo \textit{et al.}, 1999). For this reason they reflect the number and pattern of DNA crossings trapped between the two segments that participated in the strand passage event. As knotted bubbles occur in the replicated portion of partially replicated plasmids, the two segments involved are the two daughter duplexes. Plasmids containing a stalled fork, however, might have an excess of (-) $\Delta L_k$, due to a possible residual action of DNA gyrase when DNA helicase has already stopped (Olavarrieta \textit{et al.}, 2002). This is unlikely, though, at least in the case of plasmids containing forks stalled at a Ter/TUS complex. In this case, the newly replicated nascent leading strands go as far as 3-5 bp behind the TUS binding site (Mohanty \textit{et al.}, 1998) leaving no physical space for DNA helicase and DNA gyrase to remain bound to the unreplicated
parental duplex, at least if DNA helicase and gyrase are physically linked and act in a coordinate manner (Duguet, 1997). Interestingly, in partially replicated ColE1 plasmids containing a stalled fork, most of the nodes of knotted bubbles have a positive sign (Sogo et al., 1999). Knots generated by type II DNA topoisomerases reflect not only the number but also the pattern of DNA crossings trapped within a single topological domain. For this reason positive supercoiling leads to DNA knots with negative nodes while negative supercoiling leads to knots with positive nodes (Krasnow et al., 1983; Wasserman & Cozzarelli, 1991). The observation that in partially replicated molecules containing a stalled fork most of the nodes of knotted bubbles had a positive sign (Sogo et al., 1999) indicates that in vivo these molecules had precatenanes that were negatively twisted (Postow et al., 1999). This would be valid only for molecules containing a stalled fork if the putative residual action of DNA gyrase proved to be key, but it could also be true for partially replicated molecules during unimpaired DNA replication if the latter is negligible (see above).

To find out if the number of knotted bubbles changes at different stages of replication, here we constructed three different plasmids where knotted bubbles could be readily examined after 25, 52 and 81% of replication. Due to DNA stiffness, it is conceivable that small bubbles
won’t be able to accommodate complex knots. DNA curvature increases for knots with large numbers of nodes. For this reason only large bubbles would have the flexibility required to accommodate them. But the plasmids we constructed were rather large (~10 Kb) where the bubble of the smallest 25% replicated was 2.5 Kb. Although this does not eliminate the possibility that bubble size could influence knotting, especially in the case of complex knots, it was previously shown that bubbles of this size could easily accommodate knots with up to 16 nodes (Olavarrieta et al., 2002). The results obtained indicated that the number and complexity of knotted bubbles rose as a function of bubble size.

**RESULTS**

Knotted bubbles are readily detected by two-dimensional (2D) agarose gel electrophoresis in partially replicated plasmids containing a stalled fork after digestion with an enzyme that cuts plasmid DNA only in the unreplicated portion (Olavarrieta et al., 2002; Santamaría et al., 2000; Sogo et al., 1999; Viguera et al., 1996). After digestion with *Pst*I, 2D gel analysis of pBR322 DNA enriched for replication intermediates (RIs) corresponding to monomeric forms led to a very peculiar pattern (Figure 1C). The pattern expected, as predicted by the 2D gel model (Viguera et
al., 1998) was a bubble arc that should switch to a double-Y arc as soon as the replication fork reaches the \textit{PstI} site (Figure 1B). However, not one but several bubble arcs were clearly identified in the autoradiogram (Figure 1C and D). All these bubble arcs started at the 1.0x linear form and vanished abruptly at the position expected for the fork to reach the \textit{PstI} site. These bubble arcs showed increasing electrophoretic mobility during the first dimension and decreasing mobility during the second dimension of the 2D gel system. Such a behaviour was already observed for linear molecules analyzed in 2D gels (Bell & Byers, 1983) as well as for knotted bubbles of relatively high molecular weight (Olavarrieta et al., 2002; Santamaria et al., 2000; Sogo et al., 1999; Viguera et al., 1996). Also, a complete simple-Y arc was absent in the autoradiogram, as expected for the RIs corresponding to monomeric forms (Martín-Parras et al., 1991; Martín-Parras et al., 1992). The series of bubble arcs showed no continuation after the fork has passed the \textit{PstI} site, as a single double-Y arc was detected. We believe this series of additional bubble arcs corresponded to RIs containing a knotted bubble with increasing number of nodes. Knotted bubbles are detected only in linear forms containing an internal bubble (Olavarrieta et al., 2002; Santamaria et al., 1998; Sogo et al., 1999; Viguera et al., 1996) and are expected to dissolve as soon as a
restriction enzyme introduces a double-stranded break within the bubble to generate a double-Y.

pTerE25, pTerE52 and pTerE81 are all derivatives of pBR10 (Figure 2) where the *E. coli* polar replication terminator TerE (Bastia & Mohanty, 1996; Hill *et al.*, 1988) was cloned in its active orientation at three different positions. Progression of the replication fork halts as it encounters a TerE-TUS complex in the proper orientation due to its contra-helicase action (Khatri *et al.*, 1989). We designed these three plasmids so that the replication fork would stop either after 25, 52 or 81% of replication. To confirm the accumulation of RIs replicated to different extents, plasmid DNAs were digested with *Alw*NI and *Sca*I. Comparative amounts of the three digested plasmids were mixed and analyzed in a single 2D gel. Finally, the DNA in the gel was Southern-blotted and hybridized with an appropriate probe. We used the 2D gel computer model (Viguera *et al.*, 1998) to predict the shape and 2D gel patterns expected (Figure 3). In all three cases, blockage of the replication fork at TerE would lead to the accumulation of linear molecules containing an internal bubble that would be 1.25x the mass of non-replicating molecules for pTerE25, 1.52x for pTerE52 and 1.81x for pTerE81. The spots corresponding to these molecules were expected to occur on top of the
bubble arc (Brewer & Fangman, 1987; Friedman & Brewer, 1995; Martín-Parras et al., 1991) at three different positions depending on their corresponding masses (Figure 3). Figure 4 shows a photograph of the autoradiogram obtained together with a diagrammatic interpretation to the right. Unknotted and knotted bubbles were readily detected for all three different plasmids and their relative positions fitted the pattern expected (see Figure 3). The electrophoretic mobility of the “beads-on-a-string” signal corresponding to knotted bubbles, however, differed for the three plasmids. The signal extended downwards and to the right steeply for pTerE25, in a smoother manner for pTerE52 and upwards to the right for pTerE81 (Figure 4). This observation indicated that during the first dimension of the 2D gel system that was run at low voltage in a low percentage agarose gel, all knotted bubbles increased their electrophoretic mobility as DNA knots became more and more complex. But during the second dimension, which was run at high voltage in a high percentage agarose gel, this correlation changed depending on the mass of the molecules. It still held true for pTerE25, but not so for pTerE52 and pTerE81. In the latter case and at least for the left half of the “beads-on-a-string” signal, the electrophoretic mobility actually decreased as node number increased. To better compare the number of knotted bubbles between plasmids, their corresponding DNAs were analyzed in
independent 2D gels. Comparative autoradiogram exposures corresponding to the three plasmids are shown in Figure 5. A densitometric profile of each autoradiogram is shown below. To make comparison more reliable, densitometric profiles were adjusted so that the area corresponding to the signal responsible for unknotted bubbles were made equal for all three different profiles. Then we proceeded to measure the area corresponding to knotted bubbles in each case. Although evident even for the naked eye, densitometric measurements showed clearly that the number and complexity of knotted bubbles increased as a function of bubble size. Only 35% of the bubbles were knotted for pTerE25 where knotted bubbles with up to 9 nodes could be identified. 45% of the bubbles were knotted in the case of pTerE52 and this number jumped to 66% for pTerE81. Knotted bubbles with up to 14 and 22 nodes could be identified for pTerE52 and pTerE81, respectively.

Another very interesting observation that was particularly evident for the autoradiograms corresponding to pTerE52 and pTerE81 was the presence of at least two different and independent families of accumulated intermediates in each case. A higher magnification of the autoradiogram corresponding to pTerE52 together with a diagrammatic interpretation indicating these two families of stereoisomers is shown in Figure 6.
During the second dimension electrophoresis, members of the less abundant family (depicted in gray in the diagram) migrated faster than the most abundant family (depicted in black). Two possible interpretations for these complex signals are discussed below.

**DISCUSSION**

**Knotted bubbles can form during unimpaired DNA replication.** RIs containing knotted bubbles were originally identified in bacterial plasmids containing two inversely oriented unidirectional origins (Viguera et al., 1996). The advancing replication fork halts as soon as it runs-on a second ColE1 origin in the opposite orientation due to the inability of the DnaB helicase to disrupt the RNA 3’ end of a DNA-RNA hybrid (Santamaría et al., 1998). This blockage leads to the accumulation of partially replicated molecules containing an internal bubble. Analysis of these RIs in 2D gels after digestion with enzymes that cut outside the bubble, revealed the presence of a “beads-on-a-string” signal that was assigned to knotted bubbles by neutral-alkaline 2D gels (Viguera et al., 1996) and electron microscopy of RecA-coated molecules (Sogo et al., 1999). It was subsequently shown that knotted bubbles could be readily detected also if replication forks were blocked at a Ter/TUS complex (Olavarrieta et al., 1996).
2002; Santamaría et al., 2000). The electrophoretic mobility of knotted molecules increases as a function of node number (Stasiak et al., 1996). As the trefoil knot - the simplest knot - has three nodes, there is always a gap between the electrophoretic mobility of unknotted and the first knotted form (Olavarrieta et al., 2002; Santamaría et al., 2000; Stasiak et al., 1996; Viguera et al., 1996; Vologodskii et al., 1998). This gap becomes evident when comparing the electrophoretic mobility of molecules with 0, 3, 4, 5, and more nodes. The series of bubble arcs observed in Figure 1C complied with this pattern. This observation together with the fact that the series of bubble arcs showed no continuation in the form of double-Ys, strongly favour they correspond to RIs containing a knot with increasing number of nodes. Knotting has potentially devastating effects (Pieranski et al., 2001), but cells are able to remove DNA knots efficiently. It was recently shown that topo IV alone is responsible for unknotting DNA in E. coli (Deibler et al., 2001).

The number and complexity of knotted bubbles increase as a function of bubble size. Maintenance of (-) supercoiling is essential for the promotion of DNA double helix opening (Crisona et al., 2000). This explains why progression of the replication fork is impeded when both gyrase and topo IV are mutated or inhibited (Hiasa & Marians, 1994;
Hiasa & Marians, 1996; Khodursky et al., 2000; Levine et al., 1998). As ∆Lk diffuses across the replication fork (Champoux & Been, 1980; Peter et al., 1998), its distribution between the unreplicated and replicated portions of the plasmid is a function of the extent of replication. In other words, all the plasmid’s ∆Lk occurs as supercoils at the beginning of replication while most of it takes the form of precatenanes as completion of replication approaches (Peter et al., 1998). Knotted bubbles form when topo IV knot the two daughter duplexes behind the fork (Postow et al., 1999; Sogo et al., 1999). Interestingly, in partially replicated molecules containing a stalled fork, most of the nodes of knotted bubbles have a positive sign (Sogo et al., 1999) indicating that precatenanes were negatively twisted at the time of knot formation (Postow et al., 1999). Experimental evidence showing that the number of knotted bubbles rises as a function of bubble size during unimpaired DNA replication is still missing. But assuming that the topological effects of fork stalling could be negligible (see the Introduction), our finding that in partially replicated molecules the number of knotted bubbles rose as a function of bubble size, would favor the notion that plasmids keep their (-) ∆Lk almost constant from the beginning to the end of the replication process.
On the nature of two different populations of knotted bubbles. Two different families of knotted bubbles were clearly identified for pTerE52 and pTerE81. A similar observation was made for DNA knots made in vitro (Trigueros et al., 2001). The less abundant family we observed for both plasmids could be interpreted in two different ways. They might correspond to unknotted and knotted bubbles carrying a hemiprecatenane. In such a case, the stippled spot marked with a star in the diagrammatic interpretation of Figure 6 would correspond to unknotted bubbles containing a hemiprecatenane. The term hemicatenane was originally used to define a structure arising after homologous pairing of a single-stranded DNA molecule with a duplex, promoted by the RecA protein of *E. coli* (Bianchi et al., 1983; Cunningham et al., 1981; DasGupta et al., 1980) and was later extended to account for linked duplexes where one strand of a duplex is wound around one strand of another duplex (Kmieć & Holloman, 1986; Laurie et al., 1998; Sogo et al., 1986). Evidence for the occurrence of hemicatenanes in vivo was obtained by electron microscopy in psoralen crosslinked replicating SV40 minichromosomes (Laurie et al., 1998; Sogo et al., 1986) and in vitro in plasmid DNA from *Ustilago maydis* (Kmieć & Holloman, 1986). Evidence in vivo was obtained also by 2D agarose gel electrophoresis after inactivation of topoisomerases in SV40 and the yeast 2 µm plasmid (Levac & Moss, 1996) as well as in
pBR322 DNA replicating in *Xenopus* egg extracts (Lucas & Hyrien, 2000). In all these cases, however, it was not possible to determine whether or not the molecules involved were fully replicated. In our case, it would seem more appropriate to call them hemiprecatenanes, as the arms involved were the daughter duplexes of partially replicated molecules (Figure 7). Alternatively, the less abundant family of knotted bubbles observed could correspond to composite knotted bubbles, molecules containing two independent knots. In this case, the stippled spot marked with a star in the diagrammatic interpretation of Figure 6 would correspond to the first element of the most abundant family displaying the simplest trefoil knotted bubble. During the first dimension of the 2D gel system that was run at low voltage in a low percentage agarose gel, the first element of a family of composite knotted bubbles formed by two independent trefoil knots should migrate as a six-noded prime knotted bubble. And this was precisely the case. The observation that a similar case was reported for DNA knots made *in vitro* (Trigueros et al., 2001) further supports this latter interpretation.
EXPERIMENTAL PROCEDURES

Bacterial strains and culture medium. The *E. coli* strain used was DH5αF’. Competent cells were transformed with monomeric forms of the plasmids as described (Olavarrieta et al., 2002; Santamaría et al., 1998; Viguera et al., 1996). Cells were grown at 37°C in LB medium containing 75 mg/ml ampicillin.

Plasmid construction. pBR322 and pBR10-derivatives were used. pBR10 is a derivative of pBR18 (Santamaría et al., 2000) where the 5.8 Kb *EcoRI* fragment of human rDNA was inserted at the unique *AvaI* site of pBR18. The resulting 10.2 Kb plasmid contains a ColE1 unidirectional origin and confers resistance only to ampicillin (Figure 1). To construct pTerE25 two oligos:

5’-CGCGTCTTAGTTACAACATACCTTTAAGAGCT-3’ and
5’-CTTTAAAGTATGGTTGTAACTAAGA-3’ containing the 23 bp that constitutes the *E. coli* TerE terminator (Bastia & Mohanty, 1996; Hill et al., 1988) with a 3’ *SacI* and a 5’ *MluI* tails were annealed to each other and inserted between the unique *SacI* and *MluI* sites of pBR10. To construct pTerE52, another pair of oligos:

5’-TCTTAGTTACAACATACCTTTAATGCA-3’ and
5’-TTTAAGTATGTTGTAACAGATGCA-3’ including the 23 bp that constitutes the *E. coli* TerE terminator (Bastia & Mohanty, 1996; Hill et al., 1988) with two *Nsi*I tails were annealed to each other and inserted at the unique *Nsi*I site of pBR10. Finally, pTer81 was constructed using a third pair of oligos:

5’-AATTCGCTTAGTTACAACATACCTTTAAA-3’ and
5’-AGCTTTAAAAGTATGTTGTAACGATTACG-3’ containing the 23 bp that constitutes the *E. coli* TerE terminator (Bastia & Mohanty, 1996; Hill et al., 1988) with a 3’ *EcoRI* tail and a 5’ *HindIII* tail. These oligos were annealed to each other and inserted between the unique *EcoRI* and *HindIII* sites of pBR10.

### Isolation of plasmid DNA

The isolation of plasmid DNA was performed as described (Martín-Parras *et al.*, 1998; Olavarrieta *et al.*, 2002; Santamaría *et al.*, 1998; Viguera *et al.*, 1996) with two minor modifications: Triton X-100 was used instead of Brij-58 and sodium deoxycholate, and DNA precipitation was performed using isopropanol instead of 95% ethanol.

### Two-dimensional gel electrophoresis and Southern transfer

The first dimension was in a 0.28% agarose gel in TBE buffer at 0.45 V/cm at
room temperature for 69 hours. The lane containing the lambda DNA/HindIII marker sizes was excised, stained with 0.3 µg/ml ethidium bromide and photographed. In the meantime the lanes containing the DNA problem were kept in the dark. The second dimension was in a 0.58 % agarose gel in TBE containing 0.3 µg/ml ethidium bromide at a 90° angle with respect to the first dimension. The dissolved agarose was poured around the excised lane from the first dimension and electrophoresis was at 0.89 V/cm also at room temperature and for 95 hours. Southern transfer was performed as described (Martín-Parras et al., 1998; Olavarrieta et al., 2002; Santamaría et al., 1998; Viguera et al., 1996).

**Non-radioactive hybridization.** Probes were labeled using the Random Primer Fluorescein Kit (NEN Life Sciences Products). Membranes were pre-hybridized in a 20 ml pre-hybridization solution (2x SSPE, 0.5% Blotto, 1% SDS, 10% Dextran Sulphate and 0.5 mgr/ml sonicated and denatured salmon sperm DNA) at 65°C for 4-6 hours. Labeled DNA was added and hybridization lasted 12-16 hours. Then, membranes were successively washed with 2x SSC and 0.1% SDS, 0.5x SSC and 0.1% SDS, 0.1x SSC and 0.1% SDS for 15 minutes each at room temperature except for the last wash that occurred at 65°C. Detection was performed
with an antifluorescein-AP conjugate and CDP-Star, NEN, according to the instructions provided by the manufacturer.

**Densitometry.** Autoradiograms were scanned using a GS-800 BioRad Calibrated Densitometer, and analyzed using the BioRad Quantity One program, version 4.2.2.

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**LEGEND TO FIGURES**

**Figure 1:** Unknotted and knotted bubble arcs formed during unimpaired DNA replication as visualized by 2D agarose gel electrophoresis. **A:** Genetic map of pBR322 showing the relative position of its most relevant features: the ColE1 unidirectional origin, the *E. coli* terminator TerE, the ampicillin and tetracycline resistance genes, the copy-number-control rop gene and the recognition site for *Pst*I. **B:** The RIs of pBR322 after digestion with *Pst*I and the corresponding 2D gel pattern as predicted by the 2D gel computer model (Viguera et al., 1998). **C:** Autoradiogram of the 2D gel. **D:** Diagrammatic interpretation of the signals observed in the autoradiogram. KnB= Knotted Bubbles; UnknB= Unknotted Bubbles; DY= Double-Ys; SY= Simple-Ys; Xr= X-shaped recombinants; BrB= Broken bubbles.

**Figure 2:** Genetic maps of pBR10, pTerE25, pTerE52 and pTerE81 showing the relative position of their most relevant features: the ColE1 unidirectional origin, the *E. coli* terminator TerE, the ampicillin resistance and copy-number-control rop genes and the recognition sites for a number of restriction endonucleases.
**Figure 3:** The RIs of pTerE25, pTerE52 and pTerE81 as predicted by the 2D gel computer model (Viguera et al., 1998). The linear map of each plasmid after digestion with *Alw*NI and *Sca*I is shown on top. The shapes of a selected number of RIs with their corresponding masses appear below. The dashed lines point the location of the Ter site in each case. The expected 2D gel pattern for a mixture of the three plasmids is shown below. The gray spots on top of the bubble arc indicate the signals expected for RIs containing an unknotted bubble in each case.

**Figure 4:** 2D gel autoradiogram corresponding to a mixture of comparative amounts of the three plasmids (pTerE25, pTerE52 and pTerE81) after digestion with *Alw*NI and *Sal*I. The diagram to the right highlights the signals corresponding to unknotted bubbles (on top of the bubble arc) and knotted bubbles.

**Figure 5:** Autoradiograms of 2D gel area where the unknotted and knotted bubbles corresponding to pTerE25 (upper panel), pTerE52 (mid panel) and pTerE81 (lower panel) migrated after digestion with *Alw*NI and *Sal*I. Note the difference in the number and complexity of knotted bubbles. To help visualization of this difference, a densitometric profile of unknotted and knotted bubbles (made using version 1.61 of NIH Image) is shown.
below each autoradiogram. The densitometric profiles were adjusted so that the area corresponding to the signal responsible for unknotted bubbles were made equal.

**Figure 6:** Higher magnification of the area of the 2D gel autoradiogram where pTerE52 unknotted and knotted bubbles migrated. Note the presence of two independent series of “beads-on-a-string” signals. The diagram to the right highlights these two families. The most abundant family is depicted in black and the less abundant in gray. The stippled triangular spot (marked with a star) could correspond to unknotted bubbles containing a hemiprecatenane or to the first element of the family of prime knotted bubbles (for details see text).

**Figure 7:** Cartoons illustrating a portion of an RI displaying a relaxed bubble, a precatenane, a hemiprecatenane, a prime knotted bubble, a prime knotted bubble with a hemiprecatenane, and a composite knotted bubble. Parental strands are depicted in black while nascent strands in red. For the sake of simplicity no attempt was made to keep the length of the daughter duplexes equal. An arrowhead point to a hemicatenane while the insert depicts a hemiprecatenane at a higher magnification.
Figure 1

A: Diagram of pBR322 with markers including PstI, ampR, tetR, ColE1, and rop.

B: Diagram showing various molecular weights in kilobases (kb) as 1.00x, 1.12x, 1.25x, 1.35x, 1.47x, 1.59x, 1.70x, 1.81x, 1.94x, 2.00x, and 2.00x.

C: Gel image showing bands corresponding to the molecular weights.

D: Diagram illustrating the separation of bands in the gel with markers KnB, UnknB, DY, SY, Xr, and BrB.

Figure 1
Figure 2
Figure 3
Figure 4
Knotted = 35%

Knotted = 45%

Knotted = 66%

Figure 5
pTerE52

Figure 6
Figure 7

Replication Intermediates Displaying

- A Relaxed Bubble
- A Hemiprecatenane
- A Prime Knotted Bubble with a Hemiprecatenane
- A Precatenane
- A Prime Knotted Bubble
- A Composite Knotted Bubble