The ColE1 Unidirectional Origin Acts as a Polar Replication Fork Pausing Site*

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Co-orientation of replication origins is the most common organization found in nature for multimeric plasmids. Streptococcus pyogenes broad-host-range plasmid pSM19035 and Escherichia coli pPI21 are among the exceptions. pPI21, which is a derivative of pSM19035 and pBR322, has two long inverted repeats, each one containing a potentially active ColE1 unidirectional origin. Analysis of pPI21 replication intermediates (RIs) by two-dimensional agarose gel electrophoresis and electron microscopy revealed the accumulation of a specific RI containing a single internal bubble. The data obtained demonstrated that initiation of DNA replication occurred at a single origin in pPI21. Progression of the replicating fork initiated at either of the two potential origins was transiently stalled at the other inversely oriented silent ColE1 origin of the plasmid. The accumulated RIs, containing an internal bubble, occurred as a series of stereoisomers with different numbers of knots in their replicated portion. These observations provide one of the first functional explanations for the disadvantage of head-to-head plasmid multimers with respect to head-to-tail ones.

Close spacing between potentially active replication origins leads to the inactivation of all but one of them. This phenomenon, known as origin interference, has been confirmed in bacterial (1–3) and eukaryotic plasmids (4–9) as well as in the chromosomes of Saccharomyces cerevisiae (10–14), Schizosaccharomyces pombe (15), Pisum sativum (16), Xenopus (17–20), and mammalian cells (21). Identification of this interference was made possible by using Neutral/neutral (N/N)1 two-dimensional agarose gel electrophoresis to investigate origin activity (4, 22).

Digestion of multimeric forms of pBR322 with restriction endonucleases that cut only once per molecule leads to two different populations of replication intermediates (RIs). The first one generates a “bubble” to “double Y” transition pattern in N/N two-dimensional gels, indicating that the fragment initiates replication from an internal origin. As replication progresses, this bubble grows in a unidirectional fashion until the fork reaches the end of the linearized plasmid. At this point the bubble opens-up and the shape of the RIs changes abruptly from a bubble to a “simple Y”. As the replicating fork goes over the restriction site, it appears at the other end of the fragment and the shape of the RIs changes again to a double Y. The second population generates a simple Y pattern. This pattern results when a DNA fragment is replicated by a single fork traversing the fragment from one end to the other. Detection of this mixture of patterns regardless of the restriction endonuclease that was used was interpreted as an indication that initiation of DNA replication does not occur at all the potential origins of multimeric plasmids in a single replication round (1).

Further experiments, where N/N two-dimensional gels were used to analyze the RIs corresponding to pure populations of pBR322 monomers, dimers, or trimers, demonstrated that initiation of DNA replication in pBR322 occurs indeed only once per molecule (2).

In all these pBR322 multimers the replication origins are co-oriented. This is indeed the most common organization found in nature for multimeric plasmids as well as for chromosomal repeats. The Gram-positive broad host range plasmid pSM19035, originally isolated from Streptococcus pyogenes, is an exception (23). This plasmid contains two long inverted repeated sequences that comprise about 80% of the plasmid. Genetic evidence suggests that the plasmid encoded β-recombinase mediates DNA resolution and the inversion processes that eventually result in its peculiar organization (24). pPI21 (see Fig. 1) is the only stable transformant recovered from Escherichia coli cells when cloning of the pSM19035-derived pDB101 plasmid was attempted in the E. coli vector pBR322. As in the case of its precursor, it also has two long inverted repeated sequences, but it lacks pDB101 DNA sequences, including those coding for β-recombinase (25). The peculiar organization of pPI21 and specifically the fact that it contains two unidirectional replication origins in opposite orientations and no β-recombinase prompted us to investigate how this plasmid replicates in E. coli cells. We anticipated that as in all the other multimeric forms that have been studied so far, only one replication origin would be active in pPI21 per replication round. This was indeed what the results obtained indicated. But surprisingly, we also found that during the replication of pPI21, a specific RI containing a single internal bubble accumulated. This internal bubble spanned precisely between both replication origins. We also found that these accumulated RIs could contain different number of knots within the bubble.

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The abbreviations used are: N/N, neutral/neutral; RI, replication intermediate; N/A, neutral/alkaline; kb, kilobase pair(s). EM, electron microscopy; RFB, replication fork barrier.
Isolation of Plasmid DNA—Cells from overnight cultures were di-

ExPERIMENTAL PROCEDUREs

Bacterial Strains and Culture Medium—The E. coli strain used in this study was DH5αF. Competent cells were transformed with mono-

meric forms of pPI21 as described elsewhere (2). Cells were grown at 37°C in LB medium containing 50 μg/ml ampicillin.

Isolation of Plasmid DNA—Cells from overnight cultures were di-

10 mM Tris-HCl, pH 8.0, and 1 M EDTA, pH 8.0, were added, and the suspension was kept on ice for another 5 min. Cell lysis was achieved by adding 8 ml of lysis buffer (1% Brij-58, 0.4% deoxycholate, 0.063 M EDTA, pH 8.0, and 50 mM Tris-HCl, pH 8.0) and keeping the lysate for 1 h on ice. The lysate was centrifuged at 28,000 × g for 60 min at 4°C to pellet the chromosomal DNA and other bacterial debris. Plasmid DNA was recovered from the supernatant and precipitated by adding 5% volume of 25% polyethylene glycol 6000 and 1.5 mM NaCl in TE (10 mM HCl, pH 8.0, and 1 mM EDTA) and kept overnight at 4°C. The precipitated DNA was pelleted by centrifugation at 6000 × g for 15 min at 4°C, and the pellet was resuspended and incubated in 5 ml of a preheated digestion buffer (100 μg/ml Protease K, 1 mM NaCl, 10 mM Tris-HCl, pH 9.0, 1 mM EDTA, and 0.1% SDS), at 65°C for 30 min. Proteins were extracted twice with 10 mM Tris-HCl, pH 8.0-equilibrated phenol:chloroform:isoamyl alcohol (25:24:1) and once with chloroform:isoamyl alcohol (24:1). The DNA was precipitated with 2.5 volumes of absolute ethanol overnight at −20°C and resuspended in TE buffer. The DNA was digested with restriction endonucleases (Boehringer Mannheim) as recommended by the manu-

facturer in the presence of 100 μg/ml RNase A.

Standard Agarose Gel Electrophoresis—Digest DNA samples were analyzed by standard electrophoresis in agarose gels of different con-

centrations in TBE buffer run at 1–5 V/cm at room temperature. In those cases where the shape of the molecules had to affect migration, electrophoresis was performed as described elsewhere (1–4, 22). The first dimension was in a 0.4% agarose gel electrophoresis (26, 27) was performed as follows. After digestion with the appropriate restriction enzyme, approximately 80 μg of digested DNA was placed in a long thick well of a 0.4% agarose gel electrophoresis and resuspended in TE. The DNA was transferred to BABBSS® nitro-

cellulose-supported membranes (Schleicher & Schuell) in 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) for 16–18 h, and the membranes were baked at 80°C for 2 h. Prehybridization was carried out in 50% formamide, 5× SSC, 5× Denhardt’s solution (100× Denhardt's contains 2% bovine serum albumin, 2% Ficoll, and 2% polyvinylpyrrolidone), 0.1% SDS, and 250 μg/ml sonicated salmon testes DNA at 42°C for 16–18 h. Membranes were hybridized in 50% formamide, 5× SSC, 5× Denhardt's solution, 250 μg/ml sonicated salmon testes DNA, and 10% dextran sulfate with 106 cpm/ml of probe DNA labeled with [32P]dCTP by random priming at 42°C for 24–48 h. After hybridization, the membranes were washed twice for 15 min in 2× SSC and 0.1% SDS at room temperature followed by 2–3 washes in 0.1× SSC and 0.1% SDS at 55°C for 30 min. Exposure of XAR-5 films (Kodak) was carried out at 80°C with two intensifying screens for 1–3 days.

Electron Microscopy—DNA samples enriched for specific molecular species were extracted with phenol, filtered by passage through Sep-

adex LH20, and prepared for electron microscopy by cytochrome c spreading in 50% formamide and carbonate buffer on a water hypo-

phase. The spreading film was picked up with Parlodion-coated copper grids, the DNA was shadowed with platinum/iridium (80:20), and mi-

crographs were recorded using a Phillips EM400 electron microscope (28).

RESULTS

Experimental Model—pPI21 is a 6.9-kb circular plasmid con-

taining two long inverted repeats (Fig. 1). Each repeat is 2.3 kb long, and together they comprise approximately 67% of the molecule. The inverted repeats are separated by two unique segments: a 1.5-kb HindIIIHI-HindIII segment and a 0.5-kb

FIG. 1. Organization and restriction map of pPI21. Thin lines rep-

resent unique DNA sequences. Thick lines represent the two in-

verted repeats.

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**Plasmid Replication with Inversely Oriented Origins**

![Diagram showing Plasmid Replication with Inversely Oriented Origins](image)

**Fig. 2.** Initiation of DNA replication and progression of the replicating fork along the 3.6-kb *PstI-PstII* restriction fragment of pPI21 containing both origins. The map of the restriction fragment is shown at the top. The mass of some important RIs is depicted to the left. Black lines represent replicated DNA, whereas shaded lines represent unreplicated segments.

*PstII-PstII* fragment. Each inverted repeat contains the DNA sequences coding for β-lactamase and a complete potentially active ColE1 replication origin. Only one complete *rop* gene is coded in the plasmid, as indicated in Fig. 1. We have designated ori α the replication origin closer to the sequences coding for the unique *rop* gene. The other origin was called ori β. The two replication origins of pPI21 are 1.4 kb apart.

**Analysis of pPI21 RIs by Two-dimensional Agarose Gel Electrophoresis**—Digestion of pPI21 with *PstI* generates two fragments, a 3.6-kb fragment containing both replication origins and a 3.3-kb fragment that lacks replication origins. The expected shape of the RIs corresponding to the 3.6-kb *PstI-PstII* DNA fragment where initiation occurred only once per molecule at ori α are depicted in Fig. 2. A single initiation event per molecule at ori β would produce identical results. Initiation of DNA synthesis would generate a population of RIs containing a single internal bubble. As replication progresses, this bubble would grow in a unidirectional fashion until the fork reaches the end of the restriction fragment. At this point the mass of the RI would be 1.7 times the mass of the linear unreplicated form. When the replicating fork reaches the end of the fragment, the bubble would open up, and the shape of the RIs would change abruptly from a bubble to a simple Y. This particular simple Y would become accumulated while the replicating fork traverses the other 3.3-kb *PstI-PstII* fragment of pPI21 that lacks replication origins. As the replicating fork re-enters the 3.6-kb fragment at the other end, the shape of the RIs would change again from a simple Y to a double Y (1, 2).

Plasmid DNA was isolated from exponentially growing bacteria, digested with *PstI* and analyzed by *N/N* two-dimensional agarose gel electrophoresis (4). The autoradiogram corresponding to this gel, hybridized with the 0.5-kb *PvuII-PvuII* fragment used as a probe, is shown in Fig. 3. Several prominent spots and the signals expected for RIs were clearly detected above the arc corresponding to linear forms (Fig. 3, right panel, Linears). Two discrete spots occurred on top of this arc of linear forms. The very prominent one to the right (Fig. 3, right panel, 1.0x) corresponded to the unreplicated forms. The weaker one to the left (Fig. 3, right panel, close to 2.0x) was almost twice as big and resulted from partial digestion of the plasmid. A faint bubble signal (Fig. 3, right panel, Bubbles) was observed extending upward as an arc from the 1.0x spot to the upper part of the autoradiogram. A very prominent spot (Fig. 3, right panel, Accumulated-bubble) was clearly seen on top of the bubble arc. Another signal (Fig. 3, right panel, "complex bubbles") was observed to the right of the accumulated bubble. This signal of complex bubbles was not a single spot but a discontinuous arc extending downward. A very faint simple Y arc (Fig. 3, right panel, Simple-Ys) was also detected below the bubble arc. This simple Y arc could be due to a small proportion of dimers in the plasmid population (1, 2). Another signal (Fig. 3, right panel, Double-Ys) emanated from the spot of "accumulated simple Y" upward and tilted to the left. The intensity of this signal became stronger as it moved away from the spot of accumulated simple Y. Finally, another minor spot (Fig. 3, right panel, Broken-Accumulated-Bubble) was detected just below the arc of simple Ys. This spot probably corresponded to breakage at one of the two forks, of the molecules responsible for the spot designated "accumulated bubble." As previously shown (2), breakage at one of the two forks, of a population of RIs containing an internal bubble, generates a secondary pop-

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**Fig. 3.** *N/N* two-dimensional agarose gel electrophoresis of the RIs corresponding to the 3.6-kb *PstI-PstII* restriction fragment of pPI21 containing both origins. After electrophoresis was completed, the DNA in the gels was transferred to a membrane by the Southern protocol and hybridized with the 0.5-kb *PvuII-PvuII* fragment of pPI21, used as a probe. The photograph of a selected autoradiogram is shown to the left with a diagrammatic interpretation to the right. The diagram was prepared after studying different exposures in order to confirm the nature of each signal or spot. For more details see text.
ulation of simple Ys. The arc of simple Ys generated in this way is similar although not identical to the simple Y arc generated by genuine RIs. The results obtained indicated that initiation of DNA replication occurred at a single origin (either ori α or ori β) in pPI21. The genuine RIs generated the signals designated "bubbles," accumulated simple Y and double Ys. These are precisely the two-dimensional gel patterns expected for the RIs diagrammed in Fig. 2. All the signals observed except the one designated "complex bubbles" were standard predictable signals for the RIs corresponding to a specific restriction fragment analyzed by two-dimensional gels (1–4).

The Spot Designated "Accumulated Bubble" Corresponded to a Discrete DNA Species Containing an Internal Bubble That Spanned between Both Origins—To find out the nature of the signal designated "accumulated bubble," two different approaches were taken. First, RIs of pPI21 were digested with two other restriction endonucleases and the resulting restriction fragments of different sizes that still contained both replication origins were analyzed in N/N two-dimensional gels. Because the distance between origins remained constant regardless of the size of the restriction fragments that were analyzed, the main difference between these fragments was the relative size of the accumulated bubble. The RI containing the putative accumulated bubble would be 1.7 times the mass of the linear unreplicated 2.1-kb AlwNI-AlwNI restriction fragment and 1.3 times for the 5.1-kb HindIII-HindIII fragment. And second, electron microscopy (EM) was used to investigate the shape of the molecules contained in DNA samples that were specifically enriched for the accumulated bubbles by preparative agarose gel electrophoresis (26, 27).

Plasmid DNA was isolated from exponentially growing bacteria, digested with AlwNI or HindIII, and analyzed by N/N two-dimensional agarose gel electrophoresis (4). The autoradiogram corresponding to these gels hybridized with the 0.5-kb PvuII-PvuII fragment used as a probe are shown in Fig. 4. The signals corresponding to bubbles, accumulated bubble, simple Ys, accumulated simple Y, and double Ys were clearly detected in both autoradiograms. Their relative positions changed, however, depending on the location of the replication origins and the size of the fragment. Notice that the position of the accumulated bubble along the arc of bubbles changed according to the relative mass of the accumulated bubble in each case. It was located almost at the end of the arc of bubbles in the case of AlwNI (Fig. 4A), it occupied an intermediate position in the case of PstI (Fig. 5), and it was close to the 1.0x linear forms in the case of HindIII (Fig. 4B). These observations strongly suggested that the spot designated "accumulated bubble" corresponded indeed to a discrete pPI21 DNA species containing an internal bubble that spanned between both origins and was accumulated in the cell.

To examine these molecules with EM, a DNA sample digested with PstI was enriched for the signal designated "accumulated bubble" (see "Experimental Procedures"). To confirm that this new DNA sample was enriched indeed for the desired molecular species, an aliquot was analyzed by standard agarose gel electrophoresis run in the same conditions as those used for the second dimension of a N/N two-dimensional gel (see "Experimental Procedures"). Under these conditions, migration of the DNA is significantly influenced by the molecular shape. An aliquot of total DNA digested with PstI was used as a control. After electrophoresis was completed, the DNA was transferred and hybridized with the 0.5-kb PvuII-PvuII fragment of pPI21, used as a probe. The results obtained are shown in Fig. 5. The only prominent band observed in the enriched sample analyzed in lane 1 corresponded to a very slow migrating DNA species, as expected for the accumulated bubble. This species was also present in the total DNA sample digested with PstI analyzed in lane 2, although it was clearly a minor component of this sample.

EM photographs of selected molecules corresponding to the sample analyzed in lane 1 of Fig. 5 are shown in Fig. 6. Out of 286 molecules that were analyzed, 256 (89.5%) contained a single internal bubble (Fig. 6A). The size of the external arms and the bubble corresponded precisely to the sizes expected for the 3.6-kb PstI-PstI fragment containing an internal bubble that spans from ori α to ori β. The remaining 30 molecules (10.4%) were either simple Ys, like that one shown in Fig. 6B, or linears. The simple Ys were likely "broken bubbles," because the three branches of these molecules had different sizes. This is the shape expected for an accumulated bubble broken at one of the two forks (1, 2).

How Did the Accumulated Bubbles Form?—The accumulated bubbles could have formed in two different ways. It could be that initiation of DNA replication in pPI21 occurred at a single origin. When the growing fork that initiated replication at ori α reached ori β, it could be transiently stalled. Pausing of a replicating fork leads to the accumulation of specific RIs that generate a distinct signal on two-dimensional gels (10, 12, 16, 20, 21, 29–33). In pPI21 this pausing would produce a strong signal on top of the bubble arc. Alternatively, it could be that in some plasmid molecules, initiation of DNA replication occurred
used as a probe. The DNA sample run on protocol and hybridized with the 0.5-kb lane 2 was a control and corresponded to the original nonenriched preparation digested with PstI.

A very important observation made in the autoradiograms shown in Figs. 3 and 4 was that in all three cases there was a close spatial association between the signal designated “accumulated bubble” and the signal observed to its right, designated “complex bubbles.” In the autoradiogram corresponding to AluNI (Fig. 4A) it was clearly seen that the complex bubble signal was not continuous but formed by several independent discrete spots that extended downward as an arc. For this reason, it was very important for us to determine unequivocally whether the complex bubbles contained indeed two bubbles.

To investigate the nature of the molecules generating the signal designated “complex bubbles,” DNA that has been digested with PstI was used to prepare several new samples that were enriched for the molecular species migrating between the accumulated bubble and the unreplicated linear forms during the first dimension of the N/N two-dimensional gel shown in Fig. 3 (see “Experimental Procedures”). We confirmed that the new DNA samples were enriched indeed for the species designated “complex bubbles” and looked at them at the EM. All the DNA samples contained predominantly entangled molecules similar or even more complex than that one shown in Fig. 6C. It is worth noting that for the molecules showed in Figs. 6 (A and C), the size of the external arms remained constant.

On the Nature of the Complex Bubbles—If the signal designated “complex bubbles” was due to RIs containing two internal bubbles growing unidirectionally toward each other. When the two growing forks meet, a premature termination event would occur leading to molecules containing a single internal bubble that would accumulate in the cell because these molecules would lack any active replicating fork.

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On the Nature of the Complex Bubbles—If the signal designated “complex bubbles” was due to RIs containing two internal bubbles growing toward each other, the two replicating forks would have to meet somewhere between both origins. To test this possibility we used N/N two-dimensional agarose gel electrophoresis to figure out the shape of the RIs corresponding to the 1.3-kb ApIII-ApIII fragment located between the origins (see Fig. 1). Unfortunately, the results obtained were ambiguous. This was not unexpected because a clear separation of the RIs corresponding to DNA fragments smaller than 1.5–2.0 kb, although possible in some cases, is difficult to achieve in N/N two-dimensional gels (29, 34). To avoid this problem we decided to make a new construct where the unique 0.5-kb PvuII-PvuII restriction fragment of pPI21 was replaced with the unique 1.8-kb EcoRV-PvuII fragment of pBR322. In this new construct, which was named pPI21.1, the distance between ori α and ori β increased to 2.8 kb. E. coli cells were transformed with the new construct, and the corresponding RIs were isolated, digested with PstI, and tested for the presence of the accumulated bubble and complex bubbles in N/N two-dimensional gels. The results obtained confirmed that both signals were still clearly visible, although the shape and the relative position of the complex bubbles changed slightly if compared with their corresponding mobilities in the original pPI21. An autoradiogram of an enriched sample of pPI21.1 digested with PstI and analyzed by N/N two-dimensional gel is shown in the upper part of Fig. 7 (compare this figure with Fig. 3).

Termination of DNA replication was then investigated in the 2.6-kb ApIII-ApIII fragment located between the origins in the new construct. If the signal designated “complex bubbles” was due to RIs containing two internal bubbles growing toward each other, the RIs corresponding to the 2.6-kb ApIII-ApIII fragment would generate a double Y pattern in two-dimensional gels. No detectable signal corresponding to double Ys, indicative for termination events, was observed. The only visible pattern corresponded to a simple Y (data not shown). The observation that no detectable termination occurred between both origins, indicated that the signal designated “complex bubbles” was not the consequence of double initiation.

To confirm this conclusion a DNA sample of pPI21.1 digested with PstI was enriched for the molecules that generated the signals designated “accumulated bubbles” and “complex bubbles” (see “Experimental Procedures”). The enriched DNA sample was then investigated by N/N and N/A two-dimensional gels. In this way we were able to determine precisely the size of the parental and the nascent strands corresponding to the molecules responsible for the accumulated bubble and the complex bubbles. If the molecules that generated the complex bubbles contained two bubbles, their nascent strands would be significantly smaller than the nascent strands of the accumulated bubble. The results obtained are shown in Fig. 7. Notice that the size of the parental as well as the nascent strands of the molecules responsible for the complex bubbles were identical to the size of the parental and the nascent strands corresponding to the accumulated bubble. These results unequivocally demonstrated that the molecules that generated the complex bubbles did not contain two bubbles. They were stereoisomers of the accumulated RI containing an internal bubble. All these molecules analyzed by N/N and N/A two-dimensional gels were generated by digestion of the circular plasmid with a restriction endonuclease. They were not closed circular DNA duplexes and could retain no superhelicity (35). We concluded that the molecules responsible for the signal designated “complex bubbles” were “knotted bubbles”: RIs containing a single internal bubble with different numbers of knots within the bubble. This observation was in agreement with their entangled appearance at the EM (Fig. 6C).

DISCUSSION

The results obtained demonstrated that a specific RI containing an internal bubble accumulated during the replication of pPI21 in E. coli cells. This bubble spanned between the two inversely oriented unidirectional ColE1 replication origins of the plasmid. We concluded that DNA replication initiated at either of the two potential origins of pPI21, but only one origin fired per plasmid. The replicating fork initiated at one origin was transiently stalled at the other nonactive or silent origin,
leading to the accumulation of a specific RI containing an internal bubble. Although interference between ColE1 replication origins appears to occur regardless of origin polarity, pausing of a replicating fork at a silent origin does not take place when both origins are co-oriented (1–3). This observation indicates that the competence of silent ColE1 origins to stall a replicating fork is polar. We also showed that after digestion with several restriction endonucleases, DNA restriction fragments containing the internal bubble occurred as a series of stereoisomers. As superhelicity of naked DNA is sustained only by covalently closed circular DNA duplexes (35), the observation that DNA restriction fragments of pPI21 containing the internal bubble still occurred as a series of stereoisomers, indicates that the accumulated RIs were knotted in their replicated portion. The notion that some of these stereoisomers, specifically those responsible for the signal designated “complex bubbles” in Fig. 3, were indeed knotted bubbles was strengthened by the observation that these knots were only solved by denaturation (Fig. 7) or when digestion with a restriction enzyme introduced a double-stranded cut within the internal bubble itself.

Stalling of Replicating Forks—Replication fork barriers (RFBs) or pausing sites have been identified during the replication of prokaryotic as well as eukaryotic chromosomes (10, 12, 16, 20, 21, 29–33, 36). In the bidirectionally replicated circular chromosome of *E. coli* and *Bacillus subtilis*, the region where the two replicating forks meet is flanked by several polar RFBs. These RFBs are arranged in such a way to form a termination trap about 180° opposite the origin (36, 37). In *E. coli*, these barriers, named ter sites, are 22-base pair sequences that recognize and bind the Tus protein. The ter-Tus complexes seem to arrest replicating forks by inhibiting helicases in an orientation-dependent manner (38). In higher eukaryotes, a conserved specific RFB has been found close to the 3′ end of the rRNA transcription unit (4, 16, 20, 21). Although the nature of this barrier is still unknown, it is speculated that its main function would be to prevent collision between replication and transcription in the case of actively transcribing genes (39). The observation that head-on collision between the T4 bacteriophage DNA replication apparatus and an RNA polymerase transcription complex constitutes an inherent disadvantage (40), clearly supports the aforementioned hypothesis. Stalling of replicating forks due to binding of a protein or protein complexes to specific DNA sequences has been reported also for *oriP* in the Epstein-Barr virus (29, 30) and for centromeric DNA sequences in *S. cerevisiae* (31). Although neither transcription nor the secondary structure of the DNA duplex by itself are responsible for the RFB found at the 3′ end of the rRNA transcription unit in higher eukaryotes (16, 34, 41), experimental evidence indicates that in *vivo* replicating forks pause at (dG-dA)n-(dT-dC)n tracts (42, 43). These tracts are known to favor the formation of triplex DNA (44–46).

We have no indication as to whether the transient stalling of replicating forks in pPI21 is due to protein binding or DNA conformation. It is interesting to note, though, that during initiation of ColE1 DNA replication, the RNAII transcript partially hybridizes with the template DNA; it is subsequently cleaved at specific sites by RNase II and used as a primer for the leading strand synthesis by DNA polymerase I (37, 47, 48). Lagging strand synthesis uses the DNA single-stranded region as a template and terminates specifically at terH, 17 nucleotides upstream from the replication origin (47, 49). Because lagging strand DNA synthesis can be artificially extended beyond terH when the unhybridized portion of RNA II is removed,
it was suggested that the specific arrest of lagging strand synthesis at terH is caused directly by the unhybridized portion of RNAII, which would be ultimately responsible for the unidirectionality of ColE1 replication origins (47). This could also explain the polar pausing of replicating forks at the silent origin we have detected during the replication of pPI21, as depicted in the cartoon shown in Fig. 8A. Another explanation is that the RepA protein bound to the origin at the primosome assembly site (pas) leads to replication fork pausing in a polar-dependent manner (50). This second alternative is schematically shown in the model of Fig. SB. Experiments are currently under way in our laboratory to identify the fine mechanism responsible for the polar replication fork pausing induced by ColE1 replication origins.

Generation of Knotted Bubbles—Covalently closed circular DNA molecules occur in vivo as a series of stereoisomers. Supercoiling is the primary determinant for the distinctive biological features of closed circular DNA (35). The topological constraint of superhelix density is completely eliminated by the introduction of at least one single- or double-stranded break. Either one of these types of breakage allows one strand of the DNA duplex to rotate freely around the other, leading to the complete relaxation of the molecule (35). Knotted circles, on the other hand, are a different type of stereoisomers (51–53). Knots are not untied by introduction of single-stranded breaks and can only be resolved by the complete breakage of the duplex phosphodiester backbone (54). In E. coli, knots are primarily generated by DNA gyrase (51, 55), although topoisomerase I can also produce knots in nicked circular duplex DNA (56). We believe knotting of RIs is infrequent during normal DNA replication mainly because replication is a very fast and dynamic process. In pPI21, however, transient arrest of the replicating fork at the silent origin leads to the accumulation of a specific RI containing an internal bubble. This accumulated RI could be the substrate for DNA gyrase to generate knotted molecules (51, 56). A very important difference between the knotted RIs we have found and nonreplicating knotted circles is that RIs containing a knotted bubble are completely solved by denaturation (see Fig. 7), whereas knotted circles are not (55). Once replication is completed, these knotted RIs would eventually lead to multiply intertwined catenated dimers, which are a common late intermediate in the replication of circular DNA. These are finally decatenated by DNA gyrase or topoisomerase II-related enzymes (57–59).

The lack of palindrome formation during recombinant DNA cloning experiments involving ColE1 plasmids is a well-known paradox (60, 61). Our finding that the disadvantage of head-to-head plasmid multimers with respect to head-to-tail ones is due to pausing of the replicating fork at other inversely oriented silent origins and the consequent formation of knotted bubbles constitutes one of the first functional explanations to solve the aforementioned paradox.

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