Initiation of DNA Replication in ColE1 Plasmids Containing Multiple Potential Origins of Replication*

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We have investigated the frequency of replication origin usage in bacterial plasmids containing more than one potential origin. *Escherichia coli* recA− cells were selectively transformed with pBR322 monomers, dimers, or trimers. Plasmid DNA was isolated and digested with a restriction enzyme that cut the monomer only once, and the replicative intermediates (RIs) were analyzed by neutral/neutral two-dimensional agarose gel electrophoresis. Evidence for initiation outside the linearized plasmid was found only for oligomers. Moreover, in dimers, the intensity of the signal indicative for external initiation was equivalent to that reflecting internal initiation, whereas it was approximately twice as strong in trimers. To determine whether initiation could occur simultaneously at two origins in a single plasmid, we studied the replication of a neodimer in which both units could be unambiguously distinguished. The results showed that although both origins were equally competent to initiate replication, only one was active per plasmid. These observations strongly suggest that in ColE1 plasmids, replication initiates at a single site even when there are several identical potential origins per plasmid. In addition to the conventional two-dimensional gel patterns, novel specific patterns were observed with intensities that varied from one DNA sample to another. These unique patterns were the result of breakage of the RIs at a replication fork. This type of breakage changes both the mass and shape of RIs. When the entire population of RIs is affected, a new population of molecules is formed that may generate a novel pattern in two-dimensional gels.

The notion that initiation of DNA replication occurs at a single site per plasmid is not novel. The model originally proposed by Jacob et al. (1) for the control of DNA replication and segregation in bacteria assumes that every genetic element constitutes a unit of replication or "replicon." Such a unit could only replicate as a whole. Binding of these elements to a limited number of available sites at the bacterial membrane was suggested as one possible mechanism to regulate the entire population of RIs is affected, a new population of molecules is formed that may generate a novel pattern in two-dimensional gels.

Later, the discovery of low- and high-copy number plasmids led Pritchard (2) to propose a new stochastic model in which the number of initiation events per cell is determined by the relationship between the number of origins and the concentration of some yet undetermined "effectors of initiation." More recently, it has been firmly established that regulation of the initiation of DNA replication in ColE1 plasmids in *utw* is accomplished by the rate of processing and degradation of replicating RNA (3–5). A great deal of experimental information on the control of DNA replication comes from the study of chimeric and heteroplasmid populations (reviewed in Refs. 6 and 7). It was found that two different sets of replication elements may be operationally competent in at least some chimeric plasmids, but whether individual plasmids have single or multiple initiation sites was not assessed (8, 9). A peculiar situation occurs in some *Escherichia coli* plasmids such as F and R6K. The latter, for example, contains not only one, but three different replication origins that are activated in sequence (10, 11). Electron microscopy of replicating RSF1040 plasmids (a derivative of R6K) revealed that although most molecules initiate replication from a single origin, some were observed in which two origins appear to be operating simultaneously (12).

Neutral/neutral two-dimensional agarose gel electrophoresis (13, 14) is frequently used to map the location of replication origins and termini in prokaryotes (15–19) as well as in eukaryotic cells (reviewed in Refs. 14, 20, and 21). This technique allows identification of the mode of replication of any DNA fragment. A piece of DNA replicated passively by a single fork moving from one end to the other generates a "simple Y" pattern in two-dimensional gels. A DNA fragment that is replicated from an internal origin generates a "bubble" pattern. Finally, a DNA fragment replicated by two forks moving in opposite directions generates a "double Y" pattern. In each form of the yeast 2-μm plasmid, where this technique was first tested, every DNA fragment generates a unique pattern, indicating that it replicates always in the same manner (13, 14). In other cases, however, some DNA fragments give rise to more than one pattern, suggesting that they could replicate in several different manners (15–17, 22–30). In most of these cases, a mixture of bubble and simple Y patterns was observed. This mixture was interpreted as an indication that an origin was active within that particular fragment in some cases, whereas the same fragment was replicated passively by a single fork initiated elsewhere in others. It has been suggested, however, that this mixture of bubble and simple Y patterns could be an artifact, the consequence of breaks at a replication fork in those replicative intermediates (RIs) containing an internal bubble (31).

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1The abbreviations used are: RIs, replicative intermediates; kb, kilobase(s); SDS, sodium dodecyl sulfate; ARS, autonomously replicating sequence.
For the multimeric forms of some animal viruses, evidence was obtained suggesting that replication initiates at only some of the potential origins (26, 27, 32, 33). It was not possible to determine, however, whether a few or only one origin is active in each plasmid.

In a previous paper, we described the different patterns obtained when two-dimensional agarose gel electrophoresis is used to analyze the RIs of the unidirectionally replicated plasmid pBR322 (15). The observation of a mixture of patterns regardless of the restriction enzyme used to linearize the plasmid led us to propose that in pBR322, as in the cases of eukaryotic plasmids, not all the potential origins present in multimeric forms initiate replication. To find out how many origins may be active in each individual molecule and to understand the nature of the mixture of patterns sometimes observed in two-dimensional gels, we used this technique to analyze the RIs isolated from E. coli recA- cells that had been selectively transformed with pBR322 monomers, dimers, or trimers as well as a neodimer in which the two units could be unambiguously distinguished.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Culture Medium**—The E. coli strains used in this study were B450 and RYC1000 recA- (kindly provided by F. Moreno, Hospital Ramón, Gajal, Madrid) and DH5αF+, which were transformed with pBR322 monomers or multimeric forms. Cells were grown at 37°C in LB medium containing 50 μg/ml ampicillin and 12.5 μg/ml tetracycline.

**Construction of pBR322 Neodimer**—Monomeric forms of pBR322 were digested with EcoRI and StyI. The resulting 2.9-kb fragment was digested with HincII and ligated to pBR322 neomonomer. Complete pBR322 monomers were digested with PstI, treated with alkaline phosphatase, and ligated to PstI-digested neomonomers at high concentrations to favor the formation of recombinants. The products of this reaction were used to transform E. coli L45αF+ cells that were grown in the presence of both ampicillin and tetracycline. Plasmid DNA from selected colonies were screened for size and checked by restriction enzyme digestion. The 7.3-kb recombinant plasmid obtained was named pBR322 neodimer.

**Isolation of Plasmid DNA**—Plasmid DNA isolation was performed as described by Martin-Parras et al. (15). Cells from overnight cultures were diluted 40-fold in fresh LB medium, grown at 37°C to 4 X 10⁶ cpm/ml probe DNA labeled with [32P]dCTP by random priming at 37 °C for 4 h more. Afterward, the gel lane was washed twice in TBE buffer containing 0.5 μg/ml bovine serum albumin for 2 h on a rocker at room temperature. Restriction enzyme digestion was carried out by placing the gel lane in a sealed bag with ~2 μl of the corresponding restriction enzyme in the presence of 100 μg/ml bovine serum albumin for 75 min. After washing the pellets with 95% ethanol, the DNA was dissolved in TE buffer and stored at 4°C.

**Replication Procedures**—The first dimension was in a 0.4% agarose gel in TBE buffer at 0.6 V/cm at room temperature for 94 h. The DNA containing the DNA/PsfII size markers was excised, stained with 0.5 μg/ml ethidium bromide, and photographed. In the meantime, the lanes containing DNA RIs were kept in the dark. The second dimension was in 1% agarose gel in TBE buffer containing 0.5 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 5 V/cm in a 4°C cold room.

**Restriction Enzyme Digestion In Situ before Second Dimension of Electrophoresis**—The first dimension was done as indicated above. The lane containing the sample DNA was excised and washed briefly with distilled water and then twice with TE buffer for 30 min. It was then incubated twice with 500 μl of the corresponding restriction enzyme buffer for 2 h on a rocker at room temperature. Restriction enzyme digestion was carried out by placing the gel lane in a sealed bag with ~2 μl of the corresponding restriction enzyme solution containing restriction enzyme buffer and 140 units/ml StyI. Digestion lasted for 16 h at 37°C. Then, the lane was washed twice in TBE buffer containing 0.5 μg/ml ethidium bromide for 15 min at room temperature, and the second dimension was carried out as described for standard two-dimensional agarose gel electrophoresis.

**Southern Transfer and Hybridization**—Gels were washed twice for 15 min in 0.05 M HCl and then twice for another 15 min in 0.4 M NaOH containing 1 M NaCl, followed by another 80-min wash in 1 M Tris-HCl (pH 8.0) with 0.015 M NaCl plus 0.015 M NaCl at 55°C. The DNA was transferred to BAW850 nitrocellulose-supported membranes (Schleicher & Schuell) in 10 X SSC (SSC = 0.15 M NaCl plus 0.015 M sodium citrate) for 30 min and the membranes were baked at 80°C. Hybridization was carried out in 50% formamide, 5 X SSC, 5 X Denhardt's solution (100 X Denhardt's solution = 2% bovine serum albumin, 2% Ficoll, and 2% polyvinylpyrrolidone), 0.1% SDS, and 250 μg/ml sonicated salmon testis DNA at 42°C for 16-18 h. Membranes were hybridized in 50% formamide, 5 X SSC, 5 X Denhardt's solution, 250 μg/ml sonicated salmon testis DNA, and 10% dextran sulfate with 10⁶ cpm/ml 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 X SSC and 0.1% SDS at room temperature, followed by two to three washes in 0.1 X 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 1-5 days.

**RESULTS**

In a previous paper (15), we have shown that pBR322 RIs isolated from E. coli RYC1000 cells, when analyzed by two-dimensional agarose gel electrophoresis, generate a mixture of patterns regardless of the restriction enzyme used to linearize the plasmid. Since pBR322 is present as monomers, dimers, and even trimeric forms in these cells (15), the mixture of patterns observed was interpreted as an indication that only some origins were active in the multimers. Those elements of multimeric forms containing silent origins would be...
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Rs from Monomers of pBR322 Do Not Generate Simple Y Pattern—Intact pBR322 isolated from E. coli RYC1000 cells was analyzed by electrophoresis in the first dimension to separate monomers from multimers. The monomeric portion of the lane was excised, and the DNA was digested in situ in the gel with Styl. Subsequently, the second dimension was carried out as previously described. A Southern blot of the resulting gel was probed with radioactively labeled pBR322, and the corresponding autoradiograph is shown in Fig. 1. Restriction enzyme digestion of the DNA in the gel was not complete, and some molecules were not digested to completion. This explains the presence of molecules that ran as supercoiled and open circle forms during the first dimension and as open circle forms during the second dimension. Most molecules, however, were digested and migrated as linear forms during the second dimension with a mass equivalent to 4.3 kb. Since undigested samples contained a variety of molecular populations such as stereoisomers and catenanes, each formed by a series of molecules with different migration properties (data not shown), these molecules generated a smear during the first dimension. After digestion, however, most of these molecules migrated as linear forms of 4.3 kb during the second dimension. This explains the strong signal running horizontally across close to the bottom in the autoradiograph shown in Fig. 1. Superimposed on this smear, two prominent spots were observed. They corresponded to molecules that migrated as open circle and supercoiled forms during the first dimension and as linear forms during the second dimension. The population of Rs ran above the linear forms (13–15). Two different signals were observed. Both corresponded precisely to the patterns described for Styl-digested pBR322 Rs analyzed by standard two-dimensional gel electrophoresis (16). The first began at the spot corresponding to molecules that migrated as open circle forms during the first dimension and as linear forms during the second and extended upright and to the left as a diagonal. This signal ended where molecules with a mass just slightly larger than 4.3 kb had migrated during the first dimension. This is the signal expected for molecules with an internal bubble (15) and is designated “Bubbles” in the diagram of Fig. 1. The second signal started at a retarded position very close to the site where the first signal ended. It also ran upright in a diagonal fashion, but had an inflection at the end. As was previously shown (15), this signal corresponded to double Y forms in which one fork is stalled. No simple Y or any other similar pattern was detected even after very long exposures of the film. This experiment showed that monomeric forms of pBR322 did not generate a simple Y pattern. Another interesting observation in this experiment was that the signal produced by the population of Rs started at the site where monomeric open circle forms had migrated during the first dimension. This means that the undigested Rs observed in this autoradiograph migrated as nicked structures during the first dimension.

Comparison of Two-dimensional Gel Patterns Generated by pBR322 Monomers, Dimers, and Trimers—To confirm the relationship between the simple Y pattern observed in two-dimensional gels and the presence of multimeric forms in the DNA sample (15), we used two-dimensional agarose gel electrophoresis to analyze DNA Rs isolated from bacteria containing exclusively monomers, dimers, or trimers. If initiation of DNA replication occurs at a single site per plasmid, no simple Y signal should be detected in monomers. A 1:1 relationship between simple Y and bubble patterns would occur in dimers, and this relationship would be 2:1 in the case of trimers. A mixture of pBR322 monomers and multimers isolated from the CSH50 recA strain was analyzed by electrophoresis on a standard 0.4% agarose gel. The covalently closed circle forms corresponding to monomers, dimers, and trimers were isolated and used to transform E. coli cells of the recA strain DH5α F. Fig. 2 shows an ethidium bromide-stained 0.4% agarose gel in which undigested (lanes 2, 4, and 6) and EcoRI-digested (lanes 3, 5, and 7) pBR322 minipreps isolated from each of these transformants (monomers in lanes 2 and 3, dimers in lanes 4 and 5, and trimers in lanes 6 and 7) were analyzed. As a control, undigested pBR322 (lane 8) and EcoRI-digested pBR322 (lane 9) corresponding to the original mixture of monomers and multimers were also run. The results obtained showed that each transformant contained

![Fig. 1. Two-dimensional agarose gel electrophoresis of DNA Rs from monomeric forms of pBR322.](http://www.jbc.org/)

Intact plasmid DNA was electrophoresed in the first dimension; the segment of the lane in which monomeric forms had migrated was excised, digested in situ in the gel with Styl, and analyzed in the second dimension as usual. A photograph of the corresponding autoradiograph is shown on top. An interpretation of this autoradiograph is presented below. The positions of X DNA/HindIII size markers in the first dimension are shown on top of the diagram. CCC, covalently closed circles; OC, open circles; L, linear forms.
only one form of pBR322: monomers, dimers, or trimers. Plasmid DNA isolated from each of these transformants was digested with EcoRI, and the RIs were analyzed by two-dimensional agarose gel electrophoresis. The DNA in the gels were transferred to nitrocellulose membranes and hybridized with \[^{32}P\]dCTP-labeled pBR322 (Fig. 3). Four different signals were clearly identified in the sample corresponding to pBR322 monomers (Fig. 3A). The maximally retarded arc arising from the 4.3-kb (1.0X) linear spot corresponded to the so-called bubble pattern. It was generated by a population of molecules containing an internal bubble (15). This arc ended at a retarded position in the gel. Another signal consisted of a straight line extending nearly upright in a diagonal fashion from a site, on the arc of linear forms, with a mass equivalent to 8.7 kb (2.0X), double the mass of linear pBR322 fragments. This signal corresponded to nonreplicative X-shaped recombinant intermediates in which the crossover occurred at different positions along the molecules (15, 22, 35). The third signal that was observed consisted of a straight line that started at a prominent spot above the arc of linear forms and extended upright and to the left in a diagonal fashion towards the signal corresponding to X-shaped recombinant intermediates. The intensity of this signal progressively increased as it approached the signal of X-shaped recombinant intermediates. This signal corresponded to double Y RIs (15). The fourth signal observed started at the 1.0X linear spot and ran below the signal corresponding to molecules containing an internal bubble. This signal also ended at a retarded position where molecules with a relative mass equivalent to ~1.4X would have migrated during the first dimension. The nature of this signal will be discussed later. No complete simple Y pattern was observed for plasmid DNA isolated from *E. coli* cells containing exclusively pBR322 monomers (Fig. 3A). A complete simple Y pattern, however, was clearly identified together with the first three of the aforementioned patterns in the samples isolated from cells containing dimers and trimers (Fig. 3, B and C). This experiment showed that a complete simple Y pattern was generated only by RIs corresponding to multimeric forms of pBR322. Moreover, densitometric analysis of the autoradiographs (data not shown) indicated that the intensity of the simple Y signal was similar to that of the bubble signal for pBR322 dimers, whereas it was clearly the most prominent one for the trimers. This observation strongly supports the notion that initiation of DNA replication occurred at a single origin per plasmid irrespective of the number of potential origins present per plasmid. It is interesting to note that the signal generated by X-shaped recombinants was stronger in the samples corresponding to monomers and trimers. Although the dimer autoradiograph had been exposed for a shorter period, this by itself cannot account for the difference observed. We ignore the cause for this difference.

**Construction of pBR322 Neo-dimer**—To confirm that initiation of DNA replication occurred at a single site per plasmid,
a neodimer was constructed in which the two elements could be distinguished unambiguously. This neodimer contained one complete pBR322 unit and another in which the smaller of the two EcoRI-StyI fragments has been deleted. A map of this neodimer is shown in Fig. 4 (left). An ethidium bromide-stained gel in which plasmid minipreps from pBR322 monomers (lanes 2 and 3) and the neodimer (lanes 4–6) had been electrophoresed is also shown (right). After cleavage with EcoRI, pBR322 monomers migrated as a single band of 4.3 kb (lane 3), whereas the neodimer produced two bands of 4.3 and 2.9 kb (lane 5). After digestion with HindIII, however, this neodimer migrated as a single band of 7.3 kb (lane 6). Fig. 5 shows the population of RIs of the neodimer expected after digestion with EcoRI if only one origin is active per plasmid (it could be either Ori, the replication origin of the complete pBR322 unit, or Ori' that corresponds to the smaller unit of the neodimer) as well as in the theoretical case, in which both origins are simultaneously active. If the only active origin is Ori, the 4.3-kb fragment would generate the classical composite pattern formed by a bubble pattern at the beginning with a switch to a double Y pattern at the end. The 2.9-kb fragment would be replicated by a single fork and would generate a simple Y pattern. The situation would be the opposite if the only active origin is Ori'. Here the larger fragment would be replicated by a single fork generating a simple Y pattern, whereas the smaller fragment would generate a composite pattern. If both origins are simultaneously active, on the other hand, the 2.9-kb fragment would generate the same composite pattern aforementioned, but the 4.3-kb fragment would generate a very peculiar pattern. This would also be a composite pattern in which the first portion would correspond to a standard bubble pattern and the second one to a yet undescribed pattern generated by a population of RIs containing three forks: two corresponding to an internal bubble plus another simple replication fork. This second portion of the signal would start somewhere on the bubble pattern and would run progressively slower as the shape of the RIs becomes more and more complex due to the merging of a branch plus the already existing bubble. It should be noted, however, that this would only occur when both origins fire at the same time. If origin activation is slightly off and Ori' fires first, no unusual pattern would be expected.

Two-dimensional Agarose Gel Electrophoresis of Neodimer's RIs—Plasmid DNA corresponding to the neodimer was isolated, digested with EcoRI, and analyzed by two-dimensional agarose gel electrophoresis. To simplify the interpretation of the autoradiograph, the Southern blot of this gel was hybridized with the smaller of the two EcoRI-StyI fragments of pBR322. In this way, only the 4.3-kb unit of the neodimer was detected. The results revealed the standard composite pattern together with a complete simple Y pattern (Fig. 3D).

No distorted bubble pattern was detected even after long exposures of the film (data not shown). The intensities of the signals corresponding to the simple Y and bubble patterns were similar, suggesting that both origins were used at about the same frequency. This experiment shows that in this neodimer, both origins are equally competent to initiate DNA replication, but only one of them appears to be active per plasmid.

DNA Breakage at Replication Forks May Change Both Mass and Shape of DNA RIs—The signal running below the bubble pattern in Fig. 3A could not have been generated by DNA RIs (15). This signal was probably generated by a secondary population of molecules that derived from the primary RIs. It has been suggested that DNA RIs may be broken at the forks either by single-strand nuclease that might contaminate restriction enzyme preparations or by cumulative shear forces generated during isolation of large DNA molecules (23). The fact that single-stranded breaks are often induced during isolation and purification of DNA samples is well documented (33, 36). These nicks remain unnoticed when double-stranded linear fragments are analyzed in neutral gels and are revealed only when the DNA is denatured and the single-stranded chains are analyzed in alkaline gels. When proteins and RNA are removed, the gaps left between Okazaki fragments upstream of the replicating fork and the nicks generated by topoisomerase I presumably downstream are left unprotected. Therefore, a single-stranded break in any one of the parental strands near replication forks could be easily converted into a double-stranded break.

![Fig. 4. pBR322 neodimer. The genetic map shown to the left corresponds to the neodimer, and the positions of several endonucleases that cut the monomer only once are depicted. The thick line represents the complete unit of the dimer, whereas the thinner line represents the second smaller unit in which the EcoRI-Sty1 fragment had been deleted. Ori indicates the location of the replication origin corresponding to the complete unit. Ori' shows the position of the origin corresponding to the smaller unit. The position and direction of transcription of the genes coding for resistance to ampicillin and tetracycline are also shown. Note that HindIII, SalI, and StyI will cut this neodimer only once. The photograph to the right corresponds to an ethidium bromide-stained agarose gel in which plasmid minipreps corresponding to pBR322 monomers (lanes 2 and 3) and the neodimer (lanes 4–6) were electrophoresed. Lanes 2 and 4 correspond to undigested samples. EcoRI-digested DNAs were run in lanes 3 and 5, and a HindIII-digested sample was run in lane 6. MDNA/HindIII size markers were run in lane 1. bp, base pairs.](http://www.jbc.org/)

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Replication of ColE1 Multimers

When initiation only occurs at Ori

![Diagram showing DNA replication when initiation only occurs at Ori](image)

When initiation only occurs at Ori'

![Diagram showing DNA replication when initiation only occurs at Ori'](image)

When initiation occurs simultaneously at Ori and Ori'

![Diagram showing DNA replication when initiation occurs simultaneously at Ori and Ori'](image)

**FIG. 5. Possible RIs of pBR322 neodimer.** The diagrams shown correspond to the RIs expected for the pBR322 neodimer after digestion with EcoRI if initiation occurs only at Ori, only at Ori', or simultaneously at Ori and Ori'. Stippled lines represent nonreplicated DNA. Solid black lines represent replicated DNA. The relative masses of selected intermediates are indicated. Both populations of intermediates obtained after digestion with EcoRI are shown below their corresponding linear genetic maps. The shaded area over some intermediates indicates those containing three forks: a simple one initiated outside the fragment and two more corresponding to an internal bubble.

To test whether the signal observed below the bubble pattern in Fig. 3A could have been generated due to breakage of the genuine RIs at one fork, we estimated the shape and mass of the broken RIs and their possible migration properties in two-dimensional gels. Fig. 6 depicts the population of pBR322 RIs after digestion with EcoRI when they are selectively broken at the fixed or at the replicating fork. The patterns expected in two-dimensional gels in each case are shown (insets). These patterns were deduced by comparison with those generated by genuine RIs. Note that breakage at the fixed fork would generate two populations of molecules. The first would be similar, although not identical, to the population of RIs that generates a simple Y pattern. The signal produced, however, would return to the arc of linear forms with a mass significantly smaller than 2.0X. In fact, this signal should intersect the arc of linear forms at a mass equivalent to 1.6X (Fig. 6). The second population of molecules would be identical to the population of RIs that generates a simple Y pattern, but the signal produced would end at a retarded position in the gel where molecules with a relative mass of 1.4X would have migrated. The relative number of molecules with the mass and shape of the largest member of this subpopulation would occur more frequently than all the previous ones and would produce a strong dot-like signal at the end of the pattern. Comparison of all the patterns expected if EcoRI-digested RIs containing a bubble are broken at one fork with the autoradiograph shown in Fig. 3A strongly suggests that the signal running below the bubble pattern in Fig. 3A could indeed be caused by either of two subpopulations of broken RIs: the second caused by breakage at the fixed fork and that one generated by breakage at the replicating fork. The prominent spot observed in this autoradiograph at a retarded position where the double Y signal begins reinforced this interpretation. The signal expected for the first subpopulation of molecules generated by breakage at the fixed fork, however, was not observed in this particular sample. This latter signal was readily visualized in another sample that was enriched for RIs by benzoylated naphthoylated DEAE-cellulose chromatography (data not shown).

This type of analysis suggested to us that breakage at one fork of different populations of RIs could generate different patterns. To test this hypothesis, we estimated the shape and possible migration properties of broken RIs digested with two different restriction endonucleases: *PsiI* and *StyI*. The population of *PsiI*-digested RIs selectively broken at the fixed or at the replicating fork is shown in Fig. 7. The same kind of analysis for RIs digested with *StyI* is depicted in Fig. 8. In both cases, the insets represent the patterns expected in two-dimensional gels. For *PsiI*-digested RIs, breakage at the fixed
Broken at the fixed fork

Broken at the replicating fork

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FIG. 6. Prediction of changes in both mass and shape of RIs caused by preferential breakage at forks after digestion with EcoRI. Stippled lines represent nonreplicated DNA. Solid black lines represent replicated DNA. The relative masses of selected intermediates are indicated. The original population of intermediates after linearization is shown below the corresponding linear genetic map and next to its relative mass. The arrows point to the fork where a nick, leading to a double-stranded break, would have occurred. The corresponding transformation in the mass and shape of these molecules is shown to the right. The patterns that would be expected in two-dimensional gels are shown in the insets. Dotted lines represent genuine patterns. Solid black lines represent the new patterns produced by breakage of the RIs at one fork.

fork would generate a population of molecules leading to a simple Y-like pattern. The signal, however, would return to the arc of linear forms with a mass of 1.8X, instead of the 2.0X expected for a conventional simple Y pattern. Breakage of these PstI-digested RIs at the replicating fork would generate a population of molecules leading to a novel pattern in two-dimensional gels. The signal would start as a standard simple Y pattern. At a certain point, however, the shape of the molecules would not change as their mass continues to increase. This would lead the signal to run fairly parallel to the linear forms, but above it. Finally, the last member of this population would occur more frequently than all the previous ones, and this would lead to a strong dot-like signal at the end of the pattern.

For RIs digested with StyI, breakage at the fixed fork would generate two populations of molecules. The first would lead to a very small simple Y-like pattern. The signal, however, would return to the arc of linear forms with a relative mass of 1.3X. The second population of molecules would lead to a pattern identical to a simple Y, but would end at a retarded position in the gel with a mass equivalent to 1.7X. Breakage at the replicating fork, on the other hand, would generate a population of molecules leading to a simple Y-like pattern that would also end at a retarded position in the gel where molecules with a relative mass equivalent to 1.3X would have migrated. As in some previous cases, here also the largest member of this population would occur more frequently than all the others and would produce a strong dot-like signal at the end of the pattern.

The autoradiograph shown in Fig. 9 (upper) corresponds to a two-dimensional agarose gel in which a mixture of pBR322 monomers and multimers was analyzed after digestion with PstI. This particular DNA sample was enriched for RIs by benzoylated naphthoylated DEAE-cellulose chromatography. Fig. 9 (lower) shows a two-dimensional gel in which a population composed exclusively of monomeric forms of pBR322 was analyzed after digestion with StyI. Comparison of the patterns observed with those expected assuming breakage of the RIs at one fork revealed that in both cases, the novel patterns precisely matched the predictions for breakage at either the fixed or the moving fork. The intensity of the novel signals, however, varied from one DNA preparation to another (data not shown). It is interesting to note that the intensity of these novel signals increased in parallel with the conventional signals when the DNA sample was enriched for RIs by benzoylated naphthoylated DEAE-cellulose chromatography (Fig. 9, upper). These observations strongly suggest that preferential breakage of DNA RIs at one fork indeed occurred in a significant number of molecules. The same type of analysis was performed also with pBR322 RIs digested with AlwNI.
Broken at the fixed fork

and PvuII, and the results obtained confirmed that the novel patterns were indeed caused by breakage of the genuine RIs at one fork (data not shown).

DISCUSSION

Initiation of DNA Replication in Bacterial Plasmids Containing More Than One Potential Origin—Multimerization is a major cause of plasmid instability because multimers are maintained at lower copy numbers than monomers and are distributed unevenly throughout the cell population (37). To avoid multimerization, circular molecules such as the E. coli chromosome and some ColE1 plasmids encode specific determinants in which site-specific recombination events efficiently convert multimers to monomers (37, 38). This function, however, does not appear to be universal as several bacterial plasmids and animal viruses are often found as a mixture of monomers and multimeric forms (15, 26, 27, 32, 33). If the probability to initiate DNA replication is the same for every potential origin within a cell (2), multimers will have a replication advantage over monomers simply because they contain more origins. It is not known, however, whether all the potential origins within a multimer are equally competent to initiate replication and if initiation may occur at more than one origin per plasmid. One of the goals of this study was to determine the number of initiation sites per plasmid in pBR322 multimers. The results showed that although all the origins were equally competent to initiate replication, only one appeared to be active per plasmid. It is possible that multiple initiations are specifically avoided in ColE1 plasmids. Alternatively, the probability for a molecule to initiate replication could simply be low, so that multiple initiations would be very unlikely, but not necessarily impossible. The importance of DNA topology for a molecule to initiate replication is well recognized (39-42). The ColE1 RNA I promoter is 100 times more active on a supercoiled molecule than on a relaxed one (43), indicating that ColE1 plasmids probably need to be supercoiled for initiation of DNA replication to occur. Moreover, it has been suggested that a delicate equilibrium between the opposing activities of topoisomerase I and DNA gyrase plays a crucial role in determining the reactivity of a DNA molecule toward initiating DNA replication (44). As the topology of a plasmid is expected to change once replication has initiated (41, 42), a subsequent initiation event would be very unlikely. Multiple sequential initiation is possible, however; and this is the usual way used by some E. coli plasmids to replicate (10, 11, 45). The E. coli chromosome itself is capable of multiple sequential reinitiations under certain circumstances (42). The apparent
Lack of multiple initiations we have observed in pBR322 plasmids carrying more than one potential origin may simply be the consequence of the stochastic nature of initiation of DNA replication (6). In yeast, a 7-kb plasmid carrying two ARS1 sequences located 180° apart can initiate DNA replication simultaneously from both origins in a small but significant number of cases. It may be that multiple initiations could occur in very large oligomeric forms of pBR322. Another way to increase the chance for multiple initiations could be synchronization of DNA replication. These possibilities have not been tested so far. It is also interesting to point out here that although each of the chromosomal tandem rDNA repeats of yeast contains a potential origin, initiation of DNA replication only occurs in 1 out of 5–10 repeats (22, 23, 46). The yeast rDNA ARS element is very weak (47), and this weakness might be sufficient by itself to explain why only 1 in 5–10 of these ARS elements fires in a single S phase. It is also possible, however, that initiation of DNA replication at one ARS may interfere with the activation of other ARS elements located nearby. A minimal physical distance might be required between two adjacent origins for both of them to initiate DNA replication simultaneously.

B. Brewer, unpublished results.

FIG. 8. Prediction of changes in both mass and shape of RIs caused by preferential breakage at forks after digestion with StyI. Stippled lines represent nonreplicated DNA. Solid black lines represent replicated DNA. The relative masses of selected intermediates are indicated. The original population of intermediates after linearization is shown below the corresponding linear genetic map and next to its relative mass. The arrows point to the fork where a nick, leading to a double-stranded break, would have occurred. The corresponding transformation in the mass and shape of these molecules is shown to the right. The patterns that would be expected in two-dimensional gels are shown in the insets. Dotted lines represent genuine patterns. Solid black lines represent the new patterns produced by breakage of the RIs at one fork.

Topology of RIs in Bacterial Plasmids—Our observation that the signal produced by DNA RIs of pBR322 monomers began where nonreplicative open circle forms migrated during the first dimension (Fig. 1) demonstrated that during electrophoresis in the first dimension, the population of undigested RIs observed in this autoradiograph behaved as nicked structures. Since the DNA in this sample was analyzed only after all the proteins associated with the DNA had been removed, this observation does not necessarily indicate that these RIs existed in vivo as nicked structures. It does strongly suggest, however, that if a certain degree of superhelicity was maintained during replication in vivo (48–50), it was due to a protein(s) associated with the DNA. It is well known that during DNA replication, topoisomerase I functions ahead of the fork as a swivelase (42). One of the parental strands is cleaved and rotates around the other to reduce positive supercoiling. The broken strand is then religated, and the enzyme is released. During the process, topoisomerase I secures both ends of the broken strand at the cleavage site. If proteins are removed, however, this nick would become evident, and supercoiling would be lost. We believe that the RIs observed in the autoradiograph shown in Fig. 1 corresponded to the nicked supopulation of RIs in the sample. During the first
The corresponding autoradiographs are shown to the left, with a likely not enough molecules of each kind to generate a detectable pattern. Agarose gel electrophoresis of monomeric forms of pBR322 seen in the autoradiograph simply because there were probably migrated faster and to different extents. They were not be produced by breakage at either fork in a population of RIs that were carefully analyzed since otherwise unnoticed common artifacts such as single-stranded breaks can lead to selective double-stranded breakage of DNA RIs at the forks. As this type of breakage affects both the mass and shape of RIs, this phenomenon could easily lead to misinterpretations of the patterns observed in two-dimensional gels.

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**References**


**FIG. 9. Standard and novel patterns detected in two samples of pBR322 DNA digested with PstI and StyI.** The restriction enzyme used to linearize each plasmid is indicated. Photographs of the corresponding autoradiographs are shown to the left, with a diagrammatic interpretation to the right. The sample digested with PstI was enriched for RIs by column chromatography with benzoylated naphthylated DEAE-cellulose. Stippled lines represent genuine patterns. Solid black lines represent the novel patterns caused by preferential breakage of the RIs at one fork.
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