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The migration behaviour of DNA replicative intermediates containing an internal bubble analyzed by two-dimensional agarose gel electrophoresis

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ABSTRACT

Initiation of DNA replication in higher eukaryotes is still a matter of controversy. Some evidence suggests it occurs at specific sites. Data obtained using two-dimensional (2D) agarose gel electrophoresis, however, led to the notion that it may occur at random in broad zones. This hypothesis is primarily based on the observation that several contiguous DNA fragments generate a mixture of the so-called 'bubble' and 'simple Y' patterns in Neutral/neutral 2D gels. The interpretation that this mixture of hybridisation patterns is indicative for random initiation of DNA synthesis relies on the assumption that replicative intermediates (RIs) containing an internal bubble where initiation occurred at different relative positions, generate co-migrating signals. The latter, however, is still to be proven. We investigated this problem by analysing together, in the same 2D gel, populations of pBR322 RIs that were digested with different restriction endonucleases that cut the monomer only once at different locations. DNA synthesis begins at a specific site in pBR322 and progresses in a uni-directional manner. Thus, the main difference between these sets of RIs was the relative position of the origin. The results obtained clearly showed that populations of RIs containing an internal bubble where initiation occurred at different relative positions do not generate signals that co-migrate all-the-way in 2D gels. Despite this observation, however, our results support the notion that random initiation is indeed responsible for the peculiar 'bubble' signal observed in the case of several metazoan eukaryotes.

INTRODUCTION

In *Escherichia coli*, initiation of DNA replication has been extensively analysed both *in vivo* and *in vitro* (1). Two essential elements are required to initiate DNA synthesis: a *cis*-acting element, the origin of replication (OriC); and a *trans*-acting element, the origin activator (dnaA). DnaA binds to four 9-mers at OriC leading to DNA melting at three 13-mers at the left edge of OriC. Then, the dnaB helicase loads in and further melts the duplex for replication of the entire template. Requirement of *cis*- and *trans*-acting elements is also a common feature for initiation of DNA replication in many eukaryotic viruses (2, 3). Simian-Virus 40 (SV40), Epstein–Barr Virus (EBV) and Bovine Papillomavirus (BPV), all show a similar mechanism for the initiation of DNA replication. In *Saccharomyces cerevisiae*, initiation of DNA synthesis also occurs at specific sites (4, 5, 6). The yeast *cis*-acting elements are called autonomously replicating sequences (ARSs), because they confer the capability to replicate autonomously to the plasmids containing them (7). Different ARSs share several structural features. They are 100–200 bp long A+T-rich sequences containing a highly conserved 11-bp core consensus sequence. This ARS core consensus sequence (ACS) is essential for ARS function and it is the binding site for a specific multiprotein complex, ORC, which stands for 'Origin Recognition Complex' (8, 9). By analogy with the mechanism of initiation of DNA replication at the *E.coli* origin, it is thought that binding of ORC to the ACS leads to a localised unwinding of the duplex DNA somewhere in the nearby. Unwinding would be facilitated by the high A+T content of the DNA and would facilitate loading of the enzymes engaged in DNA synthesis (10).

Contrary to the situation in prokaryotes and the budding yeast where it is firmly established that DNA synthesis starts at specific sites, initiation of DNA replication in higher eukaryotes is still a matter of controversy. Several different techniques were developed and used to map the location of the initiation site for DNA replication in the dihydrofolate reductase (dhfr) domain of the Chinese hamster. DNA synthesis at each fork occurs in a continuous manner on the leading strand and in a semi-discontinuous manner (*via* the synthesis of Okazaki fragments) on the lagging strand (1). As a consequence, a switch in the polarity of leading and lagging strands exists at each origin of bi-directional replication (OBR). Two different techniques were developed and used to identify a bias in the hybridisation signal of nascent DNA to single-stranded DNA templates representing unique segments throughout the dhfr domain (11, 12). The results obtained coincide in the identification of a discrete OBR approximately 17 kb downstream the 3′ end of the dhfr gene. A third technique was designed to measure the quantities and sizes of nascent strands using the polymerase chain reaction (PCR) to amplify the signal from nascent bromodeoxyuridine (BrdUrd)-substituted DNA from single copy loci in exponentially growing cells. When this technique was used to analyse the dhfr domain, a discrete OBR was identified again approximately

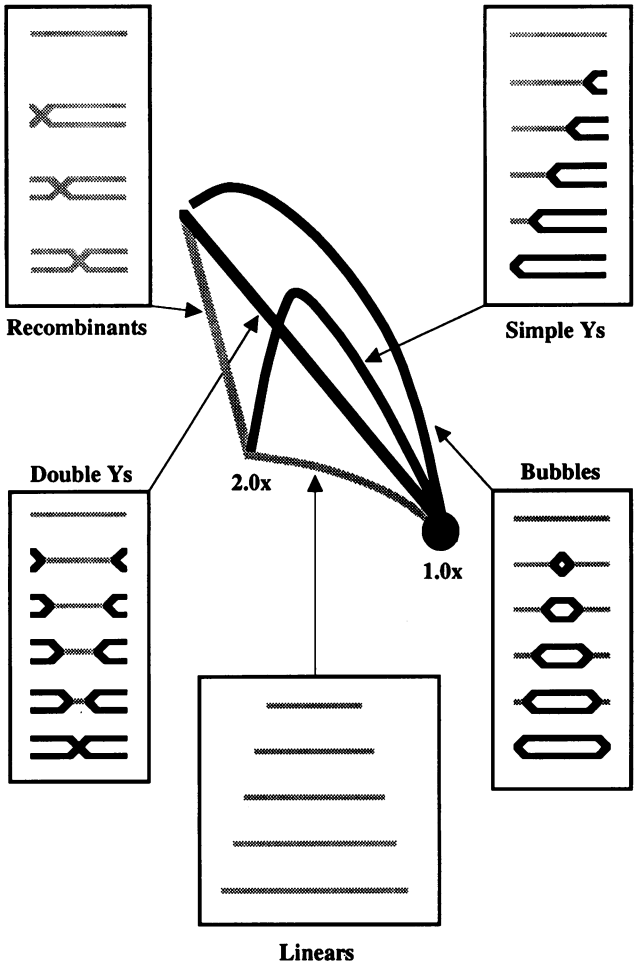

Figure 1. Cartoons of different populations of DNA molecules and their migration patterns in 2D gels. The first dimension, from left to right, separates molecules in proportion to their mass; the second dimension, from top to bottom, maximises the effect of retardation caused by the shape of each individual molecule. Stippled lines indicate non-replicating and unreplicated DNA; solid black lines indicate replicated DNA.

15–20 kb downstream the 3′ end of the dhfr gene (13). When two-dimensional agarose gel electrophoresis (2D gels) was used to analyse the RIs from this same region, however, no discrete origin for bi-directional replication was found. On the contrary, the results obtained led the authors to suggest that initiation occurs at random throughout a region spanning more than 50 kb (14, 15). This conclusion was reached after finding that several contiguous restriction fragments generate a mixture of the so-called 'simple Y' and 'bubble' patterns in 2D gels. Supporting the hypothesis for random initiation in higher eukaryotes, it was found that substitution of the dyad region of an EBV-derived plasmid by almost any large enough (12 kb or more) human DNA fragment, promotes autonomous replication of the recombinant plasmid in human cells (16). 2D gels led to the notion that replication initiates at multiple locations in at least one of these plasmids (17). Again, this conclusion was reached after finding that several contiguous restriction fragments generate a mixture of a complete 'bubble' and 'simple Y' patterns. The same mixture of hybridisation signals is observed in *Xenopus laevis* eggs microinjected with a number of specific plasmids (18, 19). These results support previous findings showing that almost any DNA molecule injected in *Xenopus* eggs replicate with an efficiency that depends only on the size of the molecule (20). In early embryos of *Drosophila melanogaster*, 2D gels also indicate that in the tandem repeats of histone genes, replication initiates at multiple locations on the repeating units (21). In all the latter cases where 2D gels were used to investigate the mode of replication of specific DNA fragments, the idea for random initiation comes from the observation that several contiguous DNA fragments generate a mixture of a complete 'bubble' and 'simple Y' signals. The interpretation that this mixture of signals is indicative for random initiation of DNA synthesis relies on the assumption that RIs containing an internal bubble where initiation occurred at different locations generate co-migrating signals in 2D gels. Replication of a DNA fragment by a single fork initiated elsewhere leads to a 'simple Y' pattern in 2D gels (Fig. 1). An active origin asymmetrically located at one end of the fragment generates a composite signal that starts as a 'bubble' but is soon converted into a 'simple Y'. As the location of the initiation site moves from the end toward the centre of the fragment, the 'bubble' signal becomes longer and the 'simple Y' shortens as it starts at higher masses (4, 22). It was suggested that bi-directional random initiation of DNA replication generates a mixture of these populations of RIs, that altogether are responsible for the complete 'bubble' and 'simple Y' signals observed in the autoradiograms (14, 17, 18, 19, 21). If this hypothesis is correct, in those cases where DNA is not labelled *in vivo*, the signal generated by RIs containing an internal bubble should be stronger at low masses and its intensity should progressively decrease as the mass of the RIs increases (Fig. 2). This would be so because no matter where initiation takes place along the fragment, all low mass RIs would contain an internal bubble. As replication progresses, however, those RIs where the origin is located asymmetrically within the fragment, would progressively lead to 'simple Y' patterns. The only population of RIs containing an internal bubble that would generate a complete signal (up to 2.0×) would be that one where initiation occurred precisely at the centre of the fragment. Surprisingly, in several cases where a mixture of a complete 'bubble' and 'simple Y' signals was found and interpreted as indicative for random initiation, the relative intensity of the signal corresponding to RIs containing an internal bubble appears stronger and sharper at high masses (14, 17, 21). This observation suggests that the peculiar 'bubble-like' signal observed in higher eukaryotes might indicate something different from random initiation. Alternatively, it could be that the discrete 'bubble' signals generated by different populations of RIs where initiation occurred at different relative positions, do not co-migrate all-the-way in 2D gels. In such a case, the shape and intensity of the resulting 'bubble' signal would probably differ from that one generated in other systems where initiation occurs at a specific site.

The principal aim of the present work was to test whether different populations of RIs containing an internal bubble where initiation occurred at different relative positions, generate co-migrating signals in 2D gels.

MATERIALS AND METHODS

Bacterial strains and culture medium

The *E.coli* strain used in this study was DH5αF′. Competent cells were transformed with pBR322 dimeric forms as described elsewhere (23). Cells were grown at 37°C in LB medium containing 50 μg/ml ampicillin and/or 12.5 μg/ml tetracycline.

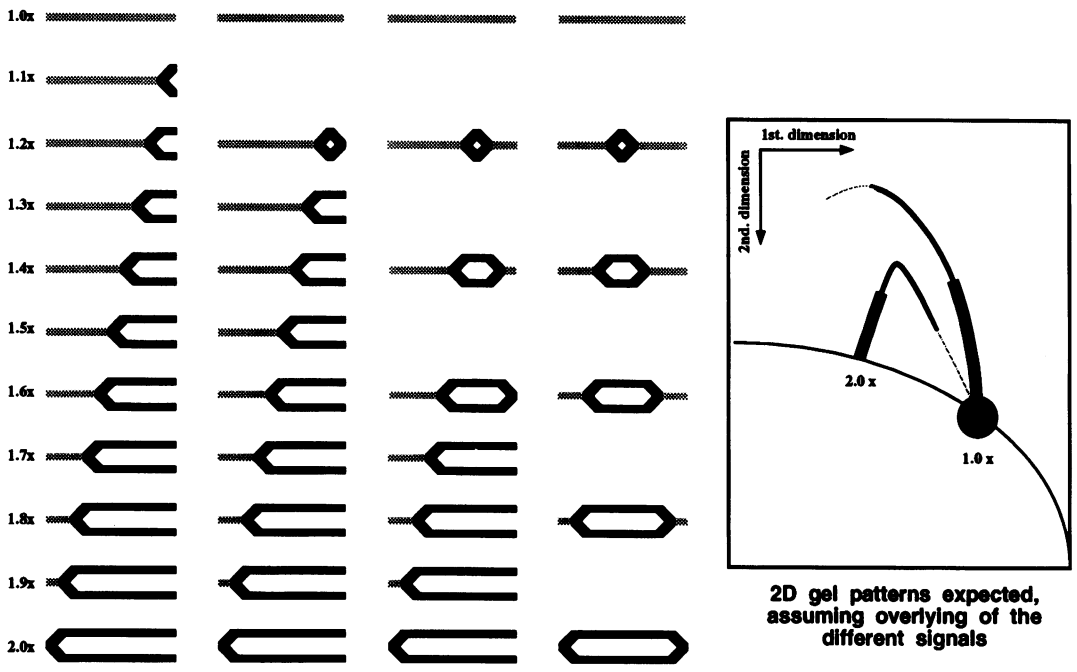

Figure 2. Populations of DNA RIs where initiation occurs at different relative positions. At the far left, RIs generated by the progression throughout the fragment of a single fork initiated elsewhere. The relative mass of each RI is indicated. At the far right, RIs generated by two forks growing bidirectionally from an origin of replication located precisely at the centre of the fragment. The two intermediate populations are generated by two forks moving bidirectionally from origins that are asymmetrically located within the fragment. The cartoon at the right illustrates the 2D gel patterns expected for a mixture of all these populations of RIs assuming that those containing an internal bubble generate co-migrating signals regardless of the relative position of the bubble along the molecule. Stippled lines indicate unreplicated segments, while solid black lines indicate segments that have already replicated.

Isolation of plasmid DNA

Cells from overnight cultures were diluted 40-fold into fresh LB medium, grown at 37°C to exponential phase (A_{600} = 0.4–0.6), quickly chilled and centrifuged. Cells were washed with 20 ml of STE buffer (0.1M NaCl, 10mM Tris–HCl {pH 8.0} and 1mM EDTA {pH 8.0}), harvested by centrifugation and resuspended in 5 ml of 25% sucrose and 0.25M Tris–HCl {pH 8.0}. Lysozyme (10 mg/ml) and RNase A (0.1 mg/ml) were added and the suspension was maintained on ice for 5 minutes. Afterwards 2 ml of 0.25M EDTA {pH 8.0} were added and the suspension was kept on ice for another 5 minutes. Cell lysis was achieved by adding 8 ml of lysis buffer (1% Brij-58, 0.4% sodium deoxycholate, 0.063M EDTA {pH 8.0} and 50mM Tris–HCl {pH 8.0}) and keeping the lysate for 1 hour on ice. The lysate was centrifuged at 26,000 g for 60 minutes at 4°C to pellet the chromosomal DNA and other bacterial debris. Plasmid DNA was recovered from the supernatant and precipitated by adding 2/3 volume of 25% polyethylene glycol 6000 and 1.5M NaCl in TE and kept overnight at 4°C. The precipitated DNA was pelleted by centrifugation at 6,000 g for 15 minutes at 4°C and the pellet resuspended and incubated in 5 ml of a preheated digestion buffer (100 μg/ml Proteinase K in 1M NaCl, 10mM Tris–HCl {pH 9.0}, 1mM EDTA and 0.1% sodium dodecyl sulphate {SDS}), at 65°C for 30 minutes. Proteins were extracted twice with 10mM 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 in 0.3M Na Acetate with 2.5 volumes of absolute ethanol overnight at −20°C and resuspended in TE (10mM HCl {pH 8.0} and 1mM EDTA). The DNA was digested with restriction endonucleases (Boehringer Mannheim) as recommended by the manufacturer in the presence of 100 μg/ml RNase A and 100 U/ml RNase T1.

DNA digestion and analysis by two-dimensional agarose gel electrophoresis

The DNA was digested with the indicated restriction endonucleases, mixed two by two, and analysed by 2D agarose gel electrophoresis (4, 24). The first dimension was in a 0.4% agarose gel in TBE buffer at 0:6 V/cm and room temperature for 34 hours. The lane containing the lambda DNA/*Hin*dIII marker sizes 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 a 1% agarose gel in TBE 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 the electrophoresis was at 5 V/cm in a 4°C cold room.

Southern transfer and hybridisation

Gels were washed twice for 15 minutes in 0.05M HCl, then twice for another 15 minutes in 0.4M NaOH containing 1M NaCl followed by another 60 minutes wash in 1M Tris-HCl {pH 8.0} with 1.5M NaCl. The DNA was transferred to BAS85® Nitrocellulose supported membranes (Schleicher and Schuell, Inc.) in 10×SSC (SSC is 0.15M NaCl plus 0.015M sodium citrate) for 16–18 hours and the membranes were baked at 80°C for 2 hours. 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),

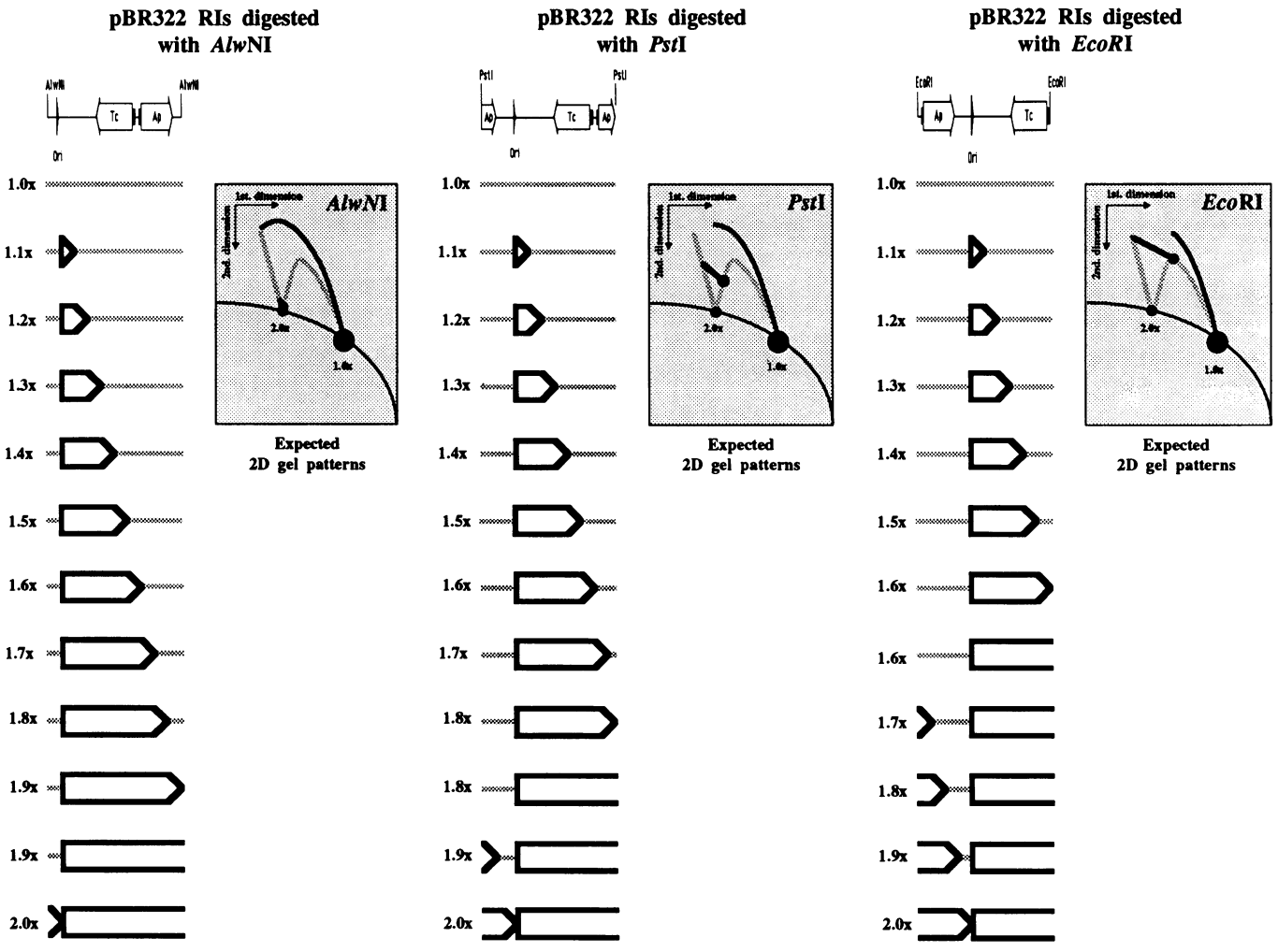

Figure 3. Progression of the replication fork along the genome of pBR322 as expected after digestion with three different restriction endonucleases that cut only once per monomer. The restriction enzyme used is indicated at the top of each panel following by the resulting linear map and the population of RIs from mass 1.0× to 2.0×. Stippled lines represent unreplicated segments, while solid black lines represent replicated segments. At the right of each panel, a cartoon illustrates the 2D gel patterns expected for each population of RIs. The patterns corresponding to non replicative recombination intermediates and simple branched forms ('simple Ys') are indicated by stippled lines.

0.1% SDS, and 250 μg/ml sonicated salmon testes DNA at 42°C for 16–18 hours. Membranes were hybridised in 50% formamide, 5×SSC, 5×Denhardt's solution, 250 μg/ml sonicated salmon testes DNA and 10% dextran sulphate with 10^6 cpm/ml of probe DNA labelled with ^{32}P-dCTP by random priming, at 42°C for 24–48 hours. After hybridisation, the membranes were washed twice for 15 minutes 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 minutes. Exposure of XAR-5 films (Kodak) was carried out at −80°C with two intensifying screens for 1–3 days.

RESULTS

One of the great advantages of 2D agarose gel electrophoresis is its capability to distinguish between DNA molecules with the same mass but different shapes (4). Populations of nonlinear DNA molecules containing an internal bubble ('bubbles'), a simple branch ('simple Y') or a double branch ('double Y') generate discrete patterns in 2D gels that are easily distinguishable from each other and different from the pattern generated by linear molecules (Fig. 1). Even more interesting is the observation that recombinant DNA intermediates where the cross-over occurred at different relative positions migrate differently in 2D gels (4, 22, 25). This property has been exploited to map the replication terminus in pBR322 (24) and recombination hot-spots in *S.cerevisiae* (26). Therefore, for recombination intermediates, the relative position of the cross-over determines the migration behaviour of the molecule. This is similar to the case of bent DNA linear molecules where the relative location of the bent locus affects the migration behaviour of the fragment (27). As the centre of bending approaches the centre of the fragment, its electrophoretic mobility decreases. Actually, the circular permutation method developed to map the location of the centre of bending is precisely based on this principle (28). As for 'simple Y' RIs, we have recently shown that for *Pst*I and *Eco*RI digested pBR322 RIs, the genuine population of 'simple Y' forms and a secondary one generated by breakage at one fork of those RIs containing an internal bubble, migrate differently in 2D gels (23). Therefore, it is likely that molecules of the same mass containing

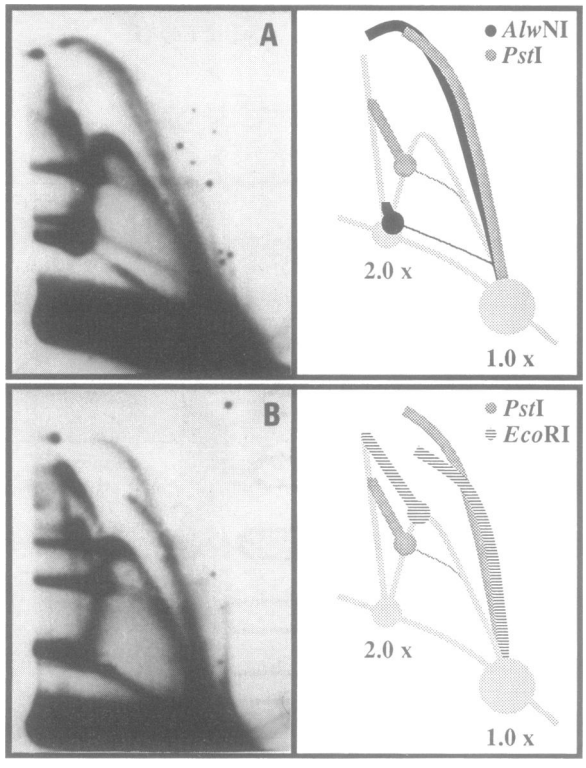

Figure 4. The autoradiograms to the left shows the hybridisation patterns generated by mixtures of two different populations of dimeric forms of pBR322 analysed by 2D agarose gel electrophoresis. Their corresponding diagrammatic interpretations are shown to the right. In (**A**) the mixture corresponds to *Alw*NI and *Pst*I digested RIs. In (**B**) the mixture corresponds to *Pst*I and *Eco*RI digested RIs. In the diagrammatic interpretation of the autoradiograms, the patterns corresponding to each one of the three different populations of RIs are drawn distinctly to facilitate its recognition. Solid black lines represent *Alw*NI digested RIs; stippled lines represent *Pst*I digested RIs; and horizontally hatched lines represent *Eco*RI digested RIs. The thin lines indicate the secondary patterns caused by breakage of those RIs containing an internal bubble at one fork (23).

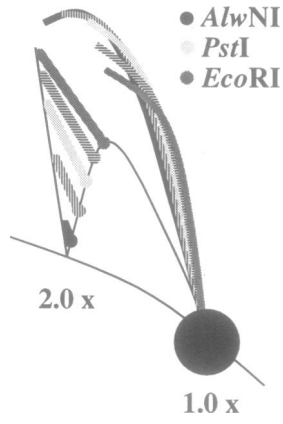

Figure 5. Diagrammatic interpretation of the autoradiograms shown in Fig. 4 A & B superimposed. The patterns corresponding to each one of the different populations of RIs are drawn distinctly to facilitate its recognition. Solid black lines represent *Alw*NI digested RIs; lightly stippled lines represent *Pst*I digested RIs and heavily stippled lines represent *Eco*RI digested RIs. Vertically and horizontally hatched lines represent the patterns expected for RIs digested with restriction enzymes that cut only once per monomer between those previously mentioned.

an identical internal bubble located at different relative positions also could migrate to different extents. If this is so, populations of DNA RIs where initiation occurred at different relative positions would not generate co-migrating signals. To test this hypothesis, we analysed together in the same 2D gel, populations of pBR322 RIs that had been digested with different restriction endonucleases that cut only once per monomer. The main difference between these sets of RIs was the relative position of the origin (Fig. 3). The patterns expected for each of these populations is already known (24). What is still ignored is whether the signals generated by these different sets of RIs containing an internal bubble, co-migrate. To simplify the interpretation of the autoradiograms, we studied the RIs derived from dimeric forms of pBR322. It is known that in pBR322 as well as in some eukaryotic viruses, initiation of DNA replication occurs at a single origin irrespective of the number of potential origins present per plasmid (23, 24, 29). Consequently, when a dimer is digested with a restriction enzyme that cuts only once per monomer, two different types of molecules are released. One contains the active origin and the other is replicated by a single fork that moves throughout the fragment from one end to the other. The latter generates a 'simple Y' signal that constitutes a useful reference in the autoradiograms. We investigated first a mixture of *Alw*NI and *Pst*I digested pBR322 RIs. In a separate gel we studied a mixture of *Pst*I and *Eco*RI digested RIs. The populations of RIs expected after digestion with *Alw*NI, *Pst*I and *Eco*RI are depicted separately in Fig. 3. Note that for each particular mass up to 1.6×, when all three populations are still represented by RIs containing an internal bubble, the only difference is the relative position of the bubble along the fragments. The autoradiograms obtained are shown in Fig. 4 A & B, together with their corresponding diagrammatic interpretations. For *Alw*NI and *Pst*I (Fig. 4A), the signals corresponding to RIs containing an internal bubble co-migrated at low masses. The two signals were clearly distinguished from each other in the intermediate zone and they co-migrated again at the end. The latter occurred because in those RIs containing an internal 'bubble', as one of the forks approaches the end of the fragment the corresponding electrophoretic mobility increases (4, 22). In the intermediate zone, the *Pst*I signal migrated slightly above the signal corresponding to *Alw*NI. This occurred because in the intermediate mass range, the bubble is more centred in *Pst*I as compared to *Alw*NI digested RIs. As the *Pst*I 'bubble' signal ended at a lower mass, the electrophoretic mobility of the last RIs increased and the latter portion of the signal produced co-migrated with the signal corresponding to *Alw*NI. The situation was slightly different when RIs digested with *Pst*I and *Eco*RI were analysed together (Fig. 4B). Here both signals were indistinguishable at low masses. At the end of the *Eco*RI signal, however, they were clearly distinguished from each other. Again, this occurred because the last RIs digested with *Eco*RI increased their mobility at a lower mass than those digested with *Pst*I. A diagram of the three signals observed is shown in Fig. 5 and will be discussed later.

DISCUSSION

The results obtained in the present work clearly showed that different populations of DNA RIs containing an internal bubble, where initiation occurred at different relative positions, do not generate signals that co-migrate all-the-way in 2D gels. Despite

this observation, however, our results support the notion that random initiation is indeed responsible for the 'bubble-like' signal observed in the case of several metazoan eukaryotes (14, 17, 18, 19, 21). When the three 'bubble' arcs generated by pBR322 RIs that were digested with *Alw*NI, *Pst*I and *Eco*RI are superimposed and the 'bubble' arcs that would be generated after digestion with restriction enzymes that cut between the enzymes used in this study are drawn by extrapolation (Fig. 5), it becomes evident that co-migration of the different signals varies from 1.0× to 2.0×. Therefore, if a DNA sample contains a mixture of different populations of RIs where initiation occurred at random, the shape and intensity along the 'bubble' signal generated by this mixture of RIs would differ from that one generated by RIs where initiation occurred at a specific site. The results obtained in the present report led us to predict that in the case of random initiation, the 'bubble-like' signal would be strong and sharp toward the 2.0× end (where the different 'bubble' signals are expected to co-migrate), becoming broader and weaker as the mass of the RIs diminishes. These are precisely the main characteristics of the peculiar 'bubble' arc observed in the dhfr domain of Chinese hamster cells (14), human cells transfected with derivatives of EBV containing human DNA sequences (17), *Xenopus* eggs microinjected with various plasmids (18, 19) and *Drosophila* embryos (21).

In summary, we have shown that random initiation of DNA replication is not expected to generate a discrete simple signal corresponding to a complete 'bubble' arc in 2D gels. This observation led us to explain the puzzling difference in the shape and intensity of the 'bubble' arc that was observed between systems where initiation occurs at a specific site (4, 6, 22, 23, 24, 29) and metazoan eukaryotes, where initiation appears to occur at random in broad zones (14, 17, 18, 19, 21). It would be very interesting to know whether the 'bubble-like' signal generated in higher eukaryotes co-migrates with the 'bubble' arc generated by a population of RIs of the same size where initiation is known to occur bi-directionally from a specific site.

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