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Plasmid replication in *Xenopus* eggs and egg extracts: a 2D gel electrophoretic analysis

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ABSTRACT

We have examined the replication patterns of ribosomal DNA plasmids *in vivo* and *in vitro* using *Xenopus* eggs. Plasmids carrying different parts of the *Xenopus* ribosomal DNA sequence were allowed to replicate either *in vitro* in an egg extract or *in vivo* after microinjection into unfertilized eggs. The replication intermediates were analyzed by the 2D gel electrophoretic technique of Brewer and Fangman (1), using original or modified electrophoresis conditions. With standard electrophoresis conditions, the patterns obtained for restriction fragments larger than 5 kb were unreliable because of artefactually distorted Y arcs and unrecognizable bubble arcs. Interpretable patterns could nevertheless be obtained using suitably modified electrophoresis parameters. Under these conditions, replication was found to initiate and terminate at multiple, random locations on each plasmid both *in vivo* and *in vitro*. However, only one or very few of these potential initiation sites are used during the replication of an individual plasmid molecule. We discuss the possible artefacts and misinterpretations that can result when the 2D electrophoresis parameters are not adapted to the size of the fragment examined. We also discuss the relevance of the random replication mode to the mechanisms and the control of DNA replication in eukaryotes.

INTRODUCTION

More than 20 years ago the DNA autoradiographic studies of Huberman and Riggs (2) showed that initiation of eukaryotic DNA replication takes place at discrete sites irregularly spaced along chromosomal DNA molecules. The notion that these sites correspond to specific nucleotide sequences was strongly supported when Stinchcomb *et al.* (3) demonstrated that in yeast some, but not all, restriction fragments derived from chromosomal DNA could confer to circular plasmids the ability to replicate extrachromosomally after transfection into yeast cells. The sequence features of these yeast autonomously replicating sequence (ARS) elements and their functional properties have been reviewed recently (4,5).

Newly developed 2D gel electrophoretic techniques for the analysis of replication-fork-containing restriction fragments have provided definitive proof that at least some of these yeast ARS elements can indeed function as initiation sites for DNA replication on plasmids (1,6) and in their normal chromosomal context (7–10). However it was also found that several ARS elements do not function as origins in their chromosomal context (9–11). Whether this is due to specific features of the chromosomal (as opposed to plasmid) chromatin architecture rather than to a negative coupling effect between multiple ARSs present on the same DNA molecule is still unknown. Furthermore it is not known if every yeast chromosomal origin of DNA replication is an ARS sequence.

The nature of higher eukaryote replication origins is much less clearly defined (for recent reviews see refs. 4,12,13). Several newly developed techniques suggested that specific replication origins exist in the chromosomal DNA of metazoan cells (14–18). However evidence for a relaxed sequence specificity of replication origins has also been reported (19–23). A specific initiation zone has been broadly defined in the amplified DHFR domain of methotrexate-resistant Chinese hamster cells, but the use of different replicon mapping techniques has led to a controversy regarding the sequence specificity of initiation within this zone (18,24). Although the reasons for these conflicting results are unclear, they might indicate that the mechanism of DNA replication in metazoan cells is more complicated than expected (reviewed and discussed in 12,17,25).

Attemps to clone mammalian or avian replication origins by plasmid rescue procedures similar to those employed in yeast have been generally unsuccessful (4,26). A recent and interesting exception showed that any human DNA fragment of sufficient length is able to confer efficient autonomous plasmid replication when inserted into a non replicating, EBV-derived plasmid deleted for the dyad region of the EBV origin (22). 2D gel electrophoretic analysis of the replication intermediates of one such recombinant indicated that replication initiated at multiple locations both on the vector and insert sequences, in contrast to the patterns of fixed origin observed with a vector plasmid containing a complete EBV origin (21).

Studies in the frog *Xenopus laevis* showed that any plasmid molecule injected into unfertilized eggs will replicate under cell cycle control with an efficiency which only depends upon the size of the plasmid, and not on the presence of any special DNA sequence (19,20). These properties are preserved in cell-free extracts prepared from *Xenopus* eggs (27). While these

experiments showed that no particularly elaborate sequence is required for replication in this experimental system, they did not tell where DNA replication is initiated and terminated on an individual plasmid DNA molecule, nor the number of initiation events that can occur on a single plasmid molecule. We have used the 2D gel electrophoretic technique to investigate these questions with plasmids carrying *Xenopus laevis* ribosomal DNA sequences.

The *Xenopus laevis* genes coding for the 40S ribosomal RNA precursor are arranged as a single tandem of an 11−15 kb unit repeated 400 times (Figure 1; reviewed in 28). Since this 4−6 Mbp stretch of DNA is too long to be replicated only from origins located outside of it, we reasoned that if a specific origin can be used in *Xenopus* the ribosomal repeat unit must contain at least one such origin. Indeed observation by electron microscopy of purified ribosomal DNA from *Xenopus* stage 40−50 (larvae) embryos has revealed clusters of microbubbles in the spacer regions of consecutive tandem units (29). In this study we have examined by 2D gel electrophoresis the replication patterns of several different rDNA plasmids, either *in vitro* in an egg extract or *in vivo* after microinjection into unfertilized eggs. The 2D gel patterns show the absence of any specific initiation or termination sites. Whether this reflects a specialized feature of early embryonic DNA replication cycles or is of more general significance is discussed.

We also found that some restriction fragments produced unusual patterns with standard electrophoresis conditions. We show that these artefacts can be avoided by the use of suitably modified electrophoresis conditions, and we discuss the ambiguities and misinterpretations they might lead to if unrecognized.

MATERIALS AND METHODS

Buffers and plasmids

High Salt Barth's (HSB) consisted of 15 mM Tris-acetate (pH 7.6), 110 mM NaCl, 2 mM KCl, 1 mM $MgSO_4$, 0.5mM Na_2HPO_4, 2 mM $NaHCO_3$. Barth's 15 mM Tris-Cl (pH 7.6), 88 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 0.5mM $CaCl_2$. Extract Buffer 10 mM K-Hepes (pH 7.7), 100 mM KCl, 50 mM sucrose, 1mM $MgCl_2$, 0.1 mM $CaCl_2$. TE 10 mM Tris-Cl (pH 8.0), 1 mM EDTA. Ribosomal DNA plasmids pXlr11 and pXlr14 (30) and pXlr101A (31) (Figure 1) were prepared by methods which disfavor retention of adventitious primers (32) and were further purified by banding on an ethidium bromide-CsCl gradient.

Preparation and use of egg extracts

Extracts of activated *Xenopus laevis* eggs capable of performing a single round of replication were prepared using a modification of the method described by Blow and Laskey (27). Eggs were dejellied with 2% cysteine in HSB buffer (pH 7.9), electrically activated in 0.1×Barth's by a single 5 second pulse of 5 $V.cm^{-1}$ (constant current) and incubated in Barth's at 23°C for 15 minutes, during which time degenerated or non activated eggs were discarded. They were then transferred on ice into a 15 ml Corex tube, rinsed 4 times with ice-cold extract buffer and packed by a brief spin at 50 g at 2°C. Excess buffer was removed and the eggs were crushed by a 10 minute centrifugation at 17,000 g in a Sorvall RC5B centrifuge equilibrated at 2°C. The golden coloured cytoplasmic layer was removed and made 250 $\mu g.ml^{-1}$ with respect to cycloheximide, to permit only a single round of DNA replication. The extract was kept on ice and used within 30 minutes.

For *in vitro* replication 100 ml aliquots were incubated at 23°C with 0.5 mg plasmid DNA and 20 mCi of 3000 $Ci.mmol^{-1}$ α^{32}P-dCTP for 95 minutes, a time chosen to obtain a good yield of replication intermediates. The reactions were stopped with an equal volume of Sarkosyl 0.83%, EDTA 66 mM, NaCl 600 mM and incubated for 90 min at 37°C with 150 $\mu g.ml^{-1}$ RNase A. Proteinase K was added to 400 $\mu g.ml^{-1}$ and incubation was continued overnight. DNA was extracted twice with phenol-chloroform, precipitated with ethanol, resuspended in TE buffer and used for restriction enzyme digestion immediately or after storage at −20°C. No changes in the replication intermediates were observed even after a three-week storage at −20°C. 50−200 ng aliquots were digested for 3h at 37°C with 10−40 units restriction enzyme in 200 μl restriction enzyme buffer, precipitated with ammonium acetate and ethanol, resuspended in 15 μl TE and loaded for 2D gel electrophoresis.

Microinjection into *Xenopus* eggs

25−35 unfertilized eggs were injected for each experimental point as described by Méchali and Kearsey (20) with 50 nl of a solution containing 12 ng of plasmid DNA and 0.1 μCi of α^{32}P-dCTP in TE buffer. The eggs were incubated at 22°C for 50−80 minutes, the time at which the first S phase occurs in microinjected unfertilized eggs (20). They were then

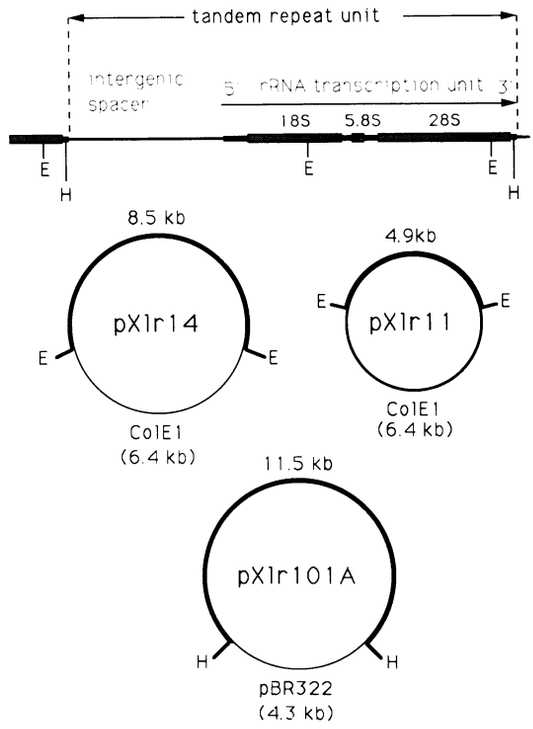

Figure 1. Structure of *X. laevis* ribosomal DNA and of plasmids pXlr11, pXlr14 and pXlr101A. Each rDNA tandem repeat unit comprises a 7.7 kb rRNA transcription unit which is separated from the next by an intergenic spacer of heterogeneous length (2−5 kb). HindIII (H) cuts once and EcoRI (E) cuts twice per repeat unit. Plasmid pXlr101A (31) contains a complete, HindIII-cut rDNA repeat (11.5kb) cloned at the HindIII site of pBR322 (4.3kb). Plasmids pXlr11 and pXlr14 (30) contain either of the two EcoRI fragments (4.9 kb and 8.5 kb) of a rDNA repeat unit cloned at the EcoRI site of the ColE1 vector (6.4 kb).

homogeneised in 20 μl per egg of 50 mM Tris-HCl (pH 8.0), 20 mM EDTA, 1% Sarkosyl, 300mM NaCl. RNase A was added to 100 $\mu g.ml^{-1}$ and the homogenate was incubated for 60–90 min at 37°C. Proteinase K was added to 500 $\mu g.ml^{-1}$ and incubation was continued overnight. Further sample processing was as for the egg extracts.

2D gel electrophoresis

2D gel electrophoresis was as described by Brewer and Fangman (1) except that agarose concentrations, electric field and temperature conditions were eventually modified as noted in the Figure legends. The gels were alkali blotted onto Hybond N^+ nylon membranes (Amersham) which were directly exposed for autoradiography. We found this procedure gave better results than drying the gel prior to autoradiography.

RESULTS

2D gel analysis of replication intermediates

We have used the 2D gel electrophoretic technique of Brewer and Fangman (1) to analyze the replication intermediates of ribosomal plasmid DNA molecules which were replicated and labelled in the continued presence of α^{32}P-dCTP, either *in vivo* in *Xenopus* eggs or *in vitro* in a *Xenopus* egg extract.

In this technique replication-fork-containing restriction fragments are separated from linear restriction fragments by two consecutive electrophoresis in agarose gels (Figure 2). The first electrophoresis is run at $1 V.cm^{-1}$ in a 0.4% agarose gel at room temperature, conditions in which restriction fragments are ideally separated according to size only. A linear fragment migrates as a single band while its replicating intermediates migrate at a slower rate according to their extent of replication. The second dimension is run perpendicular to the first at $6 V.cm^{-1}$ in a 1% agarose gel containing 0.3 $\mu g.ml^{-1}$ ethidium bromide in a 4°C cold room. This second set of conditions maximizes the contribution of shape to migration rate and causes replication intermediates to migrate slower than linear fragments of the same size. Their exact retardation depends on both the number and position of the replication forks they contain. Under optimal conditions each class of replicative intermediates traces a characteristic pattern (Figure 2). This permits the distinction between fragments containing initiation bubbles, fragments being passively replicated by a single fork passing all the way through and fragments replicated by two forks converging toward a termination site. Every restriction fragment will show every type of intermediate if replication initiates and terminates at random positions (Figure 2A). Conversely different fragments will give different patterns in the case of specific initiation or termination (Figure 2 B to D).

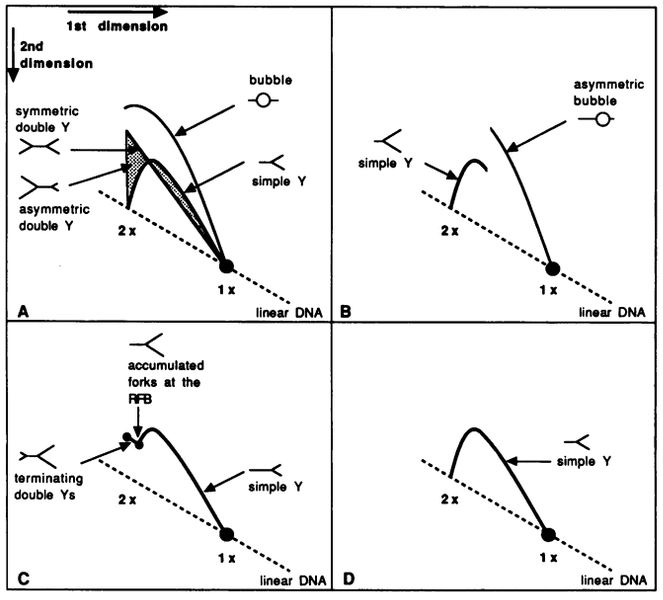

Figure 2. Diagram of idealized 2D gel patterns. The principle of the technique is described in the text. (A) Patterns generated by the three basic forms of replication intermediates. Simple Ys are maximally branched, and therefore maximally retarded in the second dimension, when the molecule is 50% replicated. They start from the arc of linear and they return to it as they approach 100% replication. Bubbles are more branched, and therefore more retarded than simple Ys at all extents of replication; they do not return to the arc of linear as they approach complete replication. Double Ys start from any point on the Y arc and join a nearly vertical line above the 2x point at a position which depends on where on the fragment the two converging forks meet. The collection of double-forked structures differing in extent of replication and relative positions of the two forks are located in a triangle and a thin zone (both shaded grey) delimited by the Y arc and the line of symmetric double Ys. (B) Bubble-to-Y transition pattern produced by a fragment containing an asymetrically located initiation site. (C) Y-to-double Y transition pattern produced by a fragment containing an asymetrically located termination site. A spot of accumulated Ys will be found at the transition point if the termination site acts as a replication fork barrier (RFB). (D) Simple Y pattern produced by a fragment containing neither an initiation nor a termination site.

Replication intermediates of plasmids pXlr11 and pXlr14 *in vitro*

Plasmids pXlr11 and pXlr14 (30; Figure 1) contain either of the two EcoRI fragments (4.9 kb and 8.5 kb) of a rDNA repeat unit

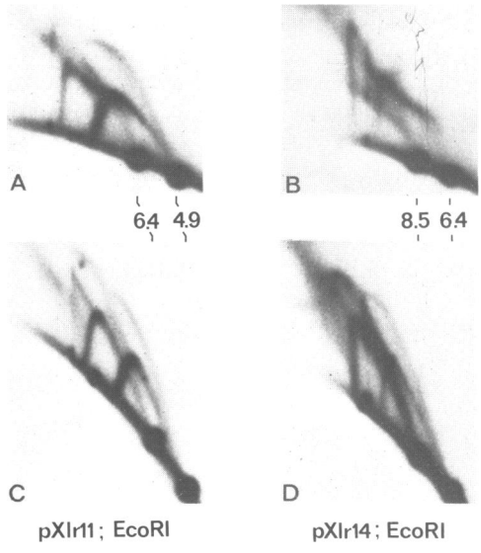

Figure 3. Replication intermediates of pXlr11 and pXlr14 in the egg extract. Each plasmid was replicated in egg extract in the presence of α^{32}P-dCTP; 250 ng of purified DNA were cut with EcoRI and loaded for 2D gel electrophoresis and autoradiography as described in Materials and Methods. The first dimension was in 0.4% agarose at $0.75 V.cm^{-1}$ for 16 h (A and B) or in 0.3% agarose at $1 V.cm^{-1}$ for 19 h (C and D) at room temperature. The second dimension was in 1% agarose at $6.3 V.cm^{-1}$ for 2 h 30 min (A and B) or in 0.6% agarose at $2.25 V.cm^{-1}$ for 17 h (C and D) in a 4°C cold room with 0.3 $\mu g.ml^{-1}$ ethidium bromide and buffer recirculation.

cloned into the EcoRI site of the ColE1 vector (6.4 kb). These two plasmids were incubated in the egg extract in the presence of α^{32}P-dCTP and the labelled replication intermediates were examined after EcoRI digestion and 2D gel electrophoresis. Each panel of Figure 3 shows the replication intermediates of both the vector and the insert fragment of the indicated plasmid.

A complex pattern was obtained after standard 2D gel electrophoresis (Figure 3A and 3B). The 4.9 kb insert of pXlr11 clearly gave a complete Y arc, a complete bubble arc and a triangular smear of termination intermediates (Figure 3A). The pattern produced by the 6.4 kb ColE1 vector was more difficult to interpret. Its general appearance, both in Figure 3A and 3B, is suggestive of an identifiable, though distorted, Y arc, due to the descending branch returning to the diagonal of linears. Plausible termination intermediates are also visible on the left of this descending branch, especially in Figure 3A where they are not obscured by the replication intermediates of a larger fragment. No bubble arc could be discerned out of Figure 3A or 3B for this restriction fragment. However the fuzzy appearance of the ascending branch rising from both 6.4 kb spots suggested that a bubble arc could have been present but insufficiently resolved from the deformed Y arc. The pattern obtained for the 8.5 kb insert of pXlr14 (Figure 3B) was even more perplexing. The figure with a straight ascending branch, a sharp inflection point and a very long, vertically descending branch is probably a very deformed Y arc. Another figure has some shape features of a bubble arc: it rises from the 8.5 kb spot above the diagonal of linears, and does not return to it at complete replication. However it is located beneath, and not above, the presumed Y arc and it does not rise very high above the diagonal of linears.

Because the Y arc deformation increased with the size of the fragment examined, and because the putative bubble arcs were correspondingly less and less retarded during the second electrophoresis, we asked if the patterns observed could be the result of inappropriate electrophoresis conditions for fragments larger than 5 kb. Indeed more typical patterns were observed when other aliquots of the same samples were electrophoresed in lower agarose concentrations and at a lower voltage during the second electrophoresis (Figure 3C and 3D). With these modified electrophoresis conditions, both the vector and the insert fragments of each plasmid gave rise to a complete Y arc, a complete bubble arc and a triangular smear of symmetric and asymmetric termination intermediates. It is important to note that some of these replication intermediates could not be confidently identified when standard electrophoresis conditions were used (Figure 3A and 3B). As indicated in Figure 2 and demonstrated in the Discussion, the presence of the three kinds of replication intermediates for each restriction fragment of each of the plasmids shows that replication initiates and terminates at random positions on these plasmids in the egg extract.

Replication intermediates of plasmids pXlr11 and pXlr14 *in vivo*

In order to check that random initiation and termination of replication is not an artefact of *in vitro* conditions for replication, we also examined the replication of these plasmids *in vivo*. Plasmids pXlr11 and pXlr14 were microinjected into unfertilized eggs and their replication patterns were analysed at the time of the first subsequent S phase (Figure 4). The 2D gel patterns are similar to those obtained when the same plasmids were replicated in the egg extract (Figure 3C and 3D), ruling out loss of any specific initiation factor during extract preparation. The slight differences in the arc shapes are due to the use of different electrophoresis conditions for the first dimension. In Figure 3C

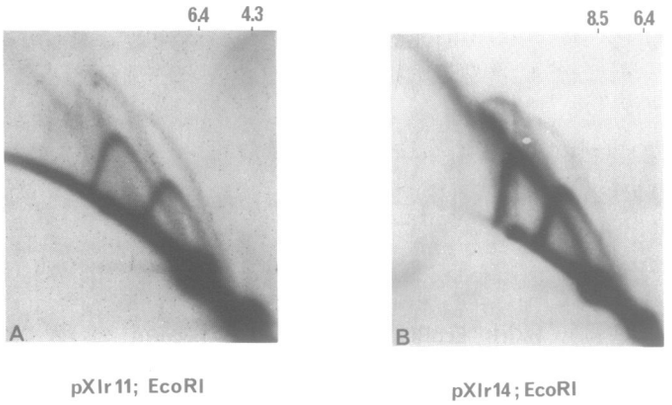

Figure 4. Replication intermediates of pXlr11 and pXlr14 in microinjected eggs. Each plasmid was microinjected with α^{32}P-dCTP; 90–130 ng of purified DNA were cut with EcoRI and loaded for 2D gel electrophoresis and autoradiography as described in Materials and Methods. The first dimension was in 0.3% agarose at 0.45 V.cm^{-1} for 63 h at room temperature. The second dimension was in 0.6% agarose at 2.25 V.cm^{-1} for 19 h 30 min in a 4°C cold room with 0.3 μg.ml^{-1} ethidium bromide and buffer recirculation.

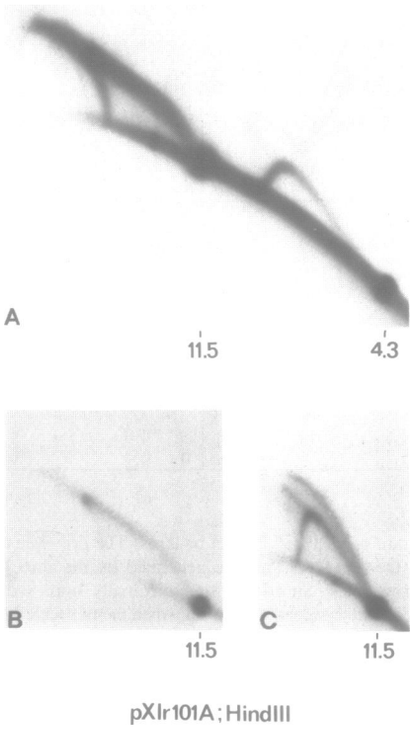

Figure 5. Replication intermediates of pXlr101A in the egg extract. Plasmid pXlr101A was replicated in egg extract in the presence of α^{32}P-dCTP; 250 ng of purified DNA were cut with HindIII and loaded for 2D gel electrophoresis and autoradiography as described in Materials and Methods. The first dimension was in 0.3% agarose at 1.2 V.cm^{-1} for 24 h (A and B) or at 0.35 V.cm^{-1} for 68 h (C) at room temperature. The second dimension was in 0.6% agarose at 1.9V.cm^{-1} for 17 h 30 min in a 4°C cold room (A and B) or at 0.95 V.cm^{-1} for 24 h at room temperature (C) with 0.3 μg.ml^{-1} ethidium bromide and buffer recirculation.

and 3D, the steepness of the Y arc's descending branch markedly increases with increasing size: this indicates that shape can make a significant, size-dependent contribution to the migration rate of Ys during the first electrophoresis, even at the low voltage employed here (1.0 $V.cm^{-1}$). In Figure 4 however, this size-dependent effect, though still observable, was considerably diminished because the voltage was lowered down to 0.45 $V.cm^{-1}$.

Replication intermediates of the complete rDNA repeat plasmid pXlr101A

Plasmids pXlr11 and pXlr14 each contain only a part of the rDNA repeat. Thus a preferential origin of rDNA replication might have been disrupted by the splitting of the repeat unit into two parts. Moreover the absence of a functional transcription unit might have changed the replication pattern of these rDNA sequences. We therefore also examined the replication intermediates of pXlr101A, a plasmid containing a single full repeat unit of *X. laevis* rDNA, including a complete and functional rRNA transcription unit (31), inserted into the HindIII site of pBR322. The plasmid was replicated in an egg extract, cut with HindIII and run on a 2D gel with conditions initially similar to those employed in Figures 2C and 2D.

The pBR322 vector fragment again showed a complete bubble arc, a complete Y arc and a triangular smear of termination intermediates (Figure 5A). However the 11.5 kb rDNA insert showed a very deformed Y arc where partly replicated Ys at the inflection point had a lower first dimension mobility than almost fully replicated intermediates (Figure 5A); under these conditions no bubble arc could be resolved from the thick ascending branch of this Y arc, even at a lower exposure of the autoradiogram (Figure 5B). Nevertheless, the pattern also showed a spike prolonging the ascending branch of the Y arc from the inflection point. Though it resembled a spike of symmetric termination intermediates, its curved shape could also represent the end of a bubble arc. At any rate, many of the replication intermediates of the 11.5 kb fragment migrated in the first dimension more slowly than a 23 (2×11.5) kb linear fragment. This again suggested that the electrophoresis conditions employed, though acceptable for the 4.3 kb fragment (Figure 5A) and very close to those acceptable for the 6.4 kb and 8.5 kb fragments (Figure 3C and 3D), were not appropriate for an 11.5 kb fragment. Indeed when an aliquot of the same sample was electrophoresed at a much reduced voltage in the first dimension and at a reduced voltage and higher temperature in the second dimension (Figure 5C) we could clearly resolve a complete bubble arc, a complete Y arc, and a triangular smear of termination intermediates for this fragment. As discussed later, these overall results again demonstrate random initiation and termination of DNA replication even with a plasmid containing a complete *X. laevis* rDNA repeat. A strictly identical pattern was obtained with pXbr101A, a plasmid containing a complete *X. borealis* rDNA repeat (data not shown).

DISCUSSION

Possible artefacts due to inappropriate electrophoresis conditions

The neutral-neutral 2D gel electrophoretic technique has been successfully used to map origins of DNA replication in yeast (1,6–11) and higher eukaryotes (21,24). The technique relies upon the ability during the first electrophoresis dimension to separate replication intermediates according to mass only, and during the second electrophoresis dimension to make bubbles migrate more slowly than forks of equal mass at all extents of replication. In this study we show that the standard conditions described by Brewer and Fangman (1) are not appropriate when analysing fragments larger than 5 kb (Figure 3). Conditions suitable for fragments up to 8.5 kb were also found inappropriate for a 11.5 kb fragment (Figure 5).

The main features of the artefactual patterns generated by inappropriate electrophoresis conditions are a size-dependent distortion of the Y arc and a change in the relative migration rates of bubbles, Ys and linears. In Figure 3B, the bubbles from the 8.5 kb fragment migrated during the second electrophoresis faster than the corresponding Ys of equal mass at almost all extents of replication, and not very much slower than linears. In Figure 3A and 3B, the bubbles from the 6.4 kb fragment are invisible, presumably because they migrated during the second electrophoresis at the same rate as Ys. In Figure 5A and 5B, the bubble arc from the 11.5 kb fragment is also perfectly superimposed on the ascending branch of the Y arc all along its length. In this case the reason might be that bubbles already migrated slower than Ys of equal mass in the first electrophoresis, causing most bubbles to migrate during the second electrophoresis at the same 2D coordinates as Ys of larger mass. The aberrant position of the Y arc inflection point is indeed consistent with a very excessive contribution of shape to migration rate during the first electrophoresis for the replication intermediates of this fragment.

Linskens and Huberman (33) indicated that the signal generated by a small replication bubble is indistinguishable from the signal generated by a small Y when the origin is located too close to the end of the fragment. We show here that even complete bubble arcs can be missed if the combination of electrophoresis conditions and size of the fragment examined is not chosen very carefully. This can potentially lead to the erroneous conclusion that the restriction fragment examined does not contain initiation sites. It is therefore crucial whenever possible to check that the electrophoresis conditions used for any novel study are able to produce and resolve the 2D gel pattern expected of an identical size fragment with known replicating properties. We have found electrophoresis conditions which extend the size range of fragments that can be analysed by this technique. Conditions suitable for fragments up to 15 kb have also been mentioned elsewhere (21).

Interpretation of the rDNA plasmids replication patterns

When appropriate electrophoresis conditions were used every restriction fragment examined showed the same pattern: a complete bubble arc, a complete Y arc, and a triangular smear of termination intermediates. The different restriction fragments differed only by the relative amounts of each of these three forms, with the ratio of bubbles and double Ys to simple Ys being higher when the fragments examined spanned a larger portion of the plasmid. We (data not shown) and others (J.J. Blow, personal communication) have obtained identical results with non-rDNA plasmids as well. The plasmids used here were prepared by methods which disfavor retention of adventitious primers (32). For this and other reasons extensively discussed elsewhere (27), we are confident that the replication intermediates observed here are due to *de novo* initiation in *Xenopus* rather than elongation of replication intermediates initiated in *E. coli*.

The presence of a complete bubble arc for each restriction fragment examined is compatible with initiation taking place at random positions within each fragment, or with a single initiation site being located by chance at the exact middle of each restriction fragment (assuming bidirectional replication). This second hypothesis is unlikely and is ruled out by three facts : i) Digests with other restriction enzymes gave the same results (data not shown); ii) termination did not occur at a fixed position on the opposite fragment, as judged by the light triangular smear apposed to the descending branch of each Y arc and the presence of a complete Y arc in any case; iii) if the complete bubble arc were produced by initiation at a single point, the labelling density of this arc should increase in proportion to the extent of replication. Since the labelling density of each bubble arc is nearly uniform along its length, it rather appears that replication can initiate along each restriction fragment at a different location on different copies of that fragment.

Termination can also occur at many different locations as indicated by the triangular smears of symmetric and asymmetric termination intermediates. Although the triangular smear might seem very faint for some fragments this conclusion is reinforced by the absence of any visible replication fork barrier which should have resulted in a strong spot of accumulated stalled forks on one of the Y arcs. This result contrasts with the presence of a strong replication fork barrier in yeast rDNA (9,10).

Given random initiation and termination of replication, and assuming progression of opposed forks at equal rates, the chance that one of the two restriction fragments of a plasmid will neither contain the initiation site nor the termination site is low, unless this fragment spans a much smaller part of the plasmid than the other one. Therefore the complete Y arcs observed here (except for the pXlr101A 4.3 kb fragment) cannot result predominantly from single forks passing all the way through the fragment; they are essentially due to the superimposition of incomplete Y arcs of various sizes, resulting from every possible bubble-to-Y (Figure 2B) or Y-to-double-Y (Figure 2C) transition.

Although replication appears to start at many possible sites when one examines a plasmid population, only one or a few such sites must be used to initiate the replication of each individual plasmid molecule. If this were not the case, a large fraction of the replication intermediates would be multibubbled structures and not the predominant bubble and Y arcs which are observed here. However since the ability of this technique to resolve restriction fragments with varying numbers of replication bubbles is unknown, we cannot exclude that early initiation intermediates proceed via a limited cluster of microbubbles which then fuse to form a minibubble. We can only state that any intermediate of this kind is unlikely to occupy a very large portion of the plasmid. All replication intermediates observed in these experiments are consistent with initiation taking place on a very limited zone and replication forks progressing in a bidirectionnal manner. Furthermore the results give no indication of a mechanism in which strand separation may be uncoupled from DNA synthesis, as was proposed by Gaudette and Benbow (34) for chromosomal DNA replication in *Xenopus* embryos.

Implications for eukaryotic DNA replication

Our results confirm that the egg initiation machinery does not need any elaborate sequence to perform its function; they further show that it does not select any preferential (e.g. most easily unwound) sequence on a given plasmid molecule. Thus, in contrast to the situation observed at oriC in *E. coli* (35) and at ARS sequences in yeast (36,37), the ease of DNA unwinding or other sequence-dependent DNA deformation does not appear to be a limiting or regulatory step on the initiation of DNA replication in this system.

The results in *Xenopus* eggs and egg extracts are reminiscent of the study of Calos' group (21,22). These authors showed that any fragment of human DNA of sufficient length can allow an EBV-derived, origin deficient plasmid vector to replicate in human cells (22). 2D gel electrophoretic analysis of one such recombinant showed that replication initiates at multiple locations on the plasmid on both vector and insert fragments (21). A relaxed sequence specificity for replication is therefore not the unique property of *Xenopus* eggs. However the results in these two systems are not exactly similar since fragments of bacterial DNA of comparable size failed to support the replication of the EBV-derived vector in the same cell line (22). No such species specificity was observed in *Xenopus* eggs (19,20).

It is difficult to decide if the use of a single potential origin per plasmid reflects a regulatory process by which a secondary initiation event is prevented on an already initiated DNA molecule, or if it simply is the consequence of a low frequency, stochastic initiation process. Only 1 to 8% of input plasmid molecules had replicated at the time DNA was extracted in these experiments (data not shown). Input DNA must be assembled into interphase nuclei before the initiation of DNA replication can occur, and the efficiency of this process is rather low when purified plasmid DNA is used instead of sperm nuclei as a substrate (38). Furthermore each nucleus thus formed contains several thousands of plasmid molecules, and the percentage of plasmid molecules which undergo replication within each nucleus is not known. The frequency of multiply initiated molecules is consequently expected to be very low if these mutiple initiation events on a single plasmid molecule are independent events. Arguing against this simple explanation however, there is evidence to suggest that multimeric forms of efficiently replicating circular plasmids also initiate replication at only some of the potential origins (1, 39–42). It is possible that this reflects a limitation in the number of supercoil-induced transitions that can occur on a single closed topological domain (43).

It is not clear to which extent these results obtained with plasmids can be extended to the replication of chromosomal DNA in *Xenopus*. The replication of plasmid DNA *in vitro* in *Xenopus* egg extracts or *in vivo* in *Xenopus* eggs does display several significant features of chromosomal replication: it requires a nuclear structure (38) and it falls under cell cycle control (19,20,27). On the other hand, chromosomal replication has a 100% efficiency while plasmid replication efficiency is much lower *in vivo* as well as *in vitro*. It is possible that specific origins of DNA replication can only function in the context of a large chromosomal structure and that they are required to attain the highest replication efficiency necessary for proper chromosome function. Indeed electron microscopy indicated the presence of a specific origin in the intergenic rDNA spacer in many organisms (reviewed in ref. 3) including the rapidly dividing gastrula cells of sea urchin embryos (44) and the cells of *Xenopus* larvae (29). However, a recent 2D gel analysis of the histone genes tandem repeats of early *Drosophila* embryos has provided some evidence for random initiation in chromosomal replicons (23). Another widely proposed explanation is that random initiation is a characteristic of the rapid early embryonic cycles, with progressive specification of more widely spaced origins occuring later in development. Experimental evidence has been reported

for a developmentally regulated change in the density of replication origins in *Drosophila* (45) and the newt *Triturus* (46), although sequence specificity of these replication origins was not investigated in these studies. As mentioned above, examination of this point in rapidly cleaving embryos has provided evidence both for random initiation in *Drosophila* (23) and for specific initiation in sea urchin (44). Together with other studies (21,22), the latter result questions the correlation between differentiation state or S phase length and the sequence specificity of DNA replication initiation in eucaryotes. Further work is required to fully evaluate the biological significance of these two equally documented modes of replicon functioning.

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