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DNA replication initiates at multiple sites on plasmid DNA in Xenopus egg extracts

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ABSTRACT

Cell-free extracts of Xenopus eggs will replicate plasmid DNA molecules under normal cell cycle control. We have used the neutral/neutral 2-D gel technique to map the sites at which DNA replication initiates in this system. Three different plasmids were studied: one containing the Xenopus rDNA repeat, one containing single copy Xenopus genomic DNA, and another containing the yeast 2μm replication origin. 2-D gel profiles show that many potential sites of initiation are present on each plasmid, and are randomly situated at the level of resolution of this technique (500 – 1000bp). Despite the abundance of sites capable of supporting the initiation of replication, pulse-chase experiments suggest that only a single randomly situated initiation event occurs on each DNA molecule. Once initiation has taken place, conventional replication forks appear to move away from this site at a rate of about 10nt/second, similar to the rate observed in vivo.

INTRODUCTION

To replicate their large genomes, eukaryotes initiate thousands of pairs of replication forks at origins distributed along the DNA (1, 2). Factors determining the location of these origins are not well understood. One fruitful approach has been to identify cis-acting DNA sequences that can support the extrachromosomal replication of DNA molecules containing them. These have been identified in budding yeast, and are called Autonomously Replicating Sequences (ARSs) (3). They consist of a highly conserved core region of about 14bp, flanked by up to 200bp of less conserved sequence (4, 5). The core region is absolutely required for ARS activity; the flanking sequence may serve to locally unwind the core region (6), or to provide incomplete matches to the core sequence (7). 2-dimensional gel techniques have determined that ARSs correspond to origins of DNA replication, both when carried on extrachromosomal plasmids (8, 9), or when present on the chromosome (10–12).

The identity of origins of replication in higher eukaryotes is less clear. Cis-acting sequences that confer autonomous replication on extrachromosomal DNA in the somatic cells of metazoans have not been clearly identified (see for example refs. 13 and 14). In one experimental approach, sequences were assayed for their ability to support the replication of a plasmid containing part of the EBV (Epstein-Barr Virus) genome in somatic human cells. The surprising result was that a range of human DNA sequences were capable of supporting autonomous replication of the construct (15, 16). Nevertheless DNA replication appears to initiate at defined places on the chromosomes of somatic cells. The region encompassing the Dihydrofolate Reductase (DHFR) gene in Chinese Hamster ovary cells has been intensively studied. Using a number of techniques, DNA replication has been shown to initiate within particular restriction fragments within this region of the chromosome. However, fine scale mapping of these fragments has yielded conflicting results as to the length of DNA in which initiation can potentially occur (13, 17).

In contrast to the apparent inability of somatic cells to support extrachromosomal replication of nonviral DNA, eggs of the South African clawed toad *Xenopus laevis* will replicate of a wide range of DNA molecules microinjected into them (18, 19). Replication of this exogenous DNA falls under cell cycle control since it is replicated no more than once in each cell cycle (18, 19). A cell-free of Xenopus eggs has been developed that can support in vitro the regulated replication of exogenous DNA (20, 21; reviewed in 22). DNA must be assembled into an interphase nucleus in vitro before the initiation of DNA replication can occur (21, 23–25). DNA undergoes a single round of semiconservative replication during each interphase, and must pass through mitosis before another round of DNA replication can be initiated (20, 26). Like the intact egg, the cell-free extract can support the replication of a range of different plasmid DNAs (20, 27, 23, 25).

The ability of Xenopus eggs to replicate a wide range of DNA molecules means that if specific DNA sequences are required for the initiation of DNA replication in this system, these sequences must be abundant in naturally-occurring DNA.

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However, the actual number of sequences capable of supporting the initiation of replication on these DNA molecules is not known; neither is it known whether certain classes of these potential initiation sites are used preferentially. In this paper we use 2-D gels to map sites of initiation in three different DNA molecules replicating in the Xenopus egg extract. This reveals that on all three plasmids, potential origins are distributed uniformly along the DNA to the limits of resolution of this technique. Despite the abundance of sites capable of supporting the initiation of replication, most individual DNA molecules are apparently replicated from only a single, randomly situated initiation event.

MATERIALS AND METHODS

Preparation and use of the cell-free extract

Interphase extracts were prepared as described (25). 40μl aliquots were incubated with 3−10μg/ml DNA, 250μg/ml cycloheximide, 25mM phosphocreatine and 15μg/ml creatine phosphokinase at 23°C.

Neutral/neutral 2-D gel electrophoresis

Nascent DNA was labelled with 1μCi/μl α^{32}P-dATP from 75−105mins. Labelling was stopped with 200μl (20mM Tris HCl pH 8, 200mM NaCl, 5mM EDTA, 0.5% SDS, 200μg/ml proteinase K), and the incubation continued for 30mins. RNase was added to 2μg/ml for a further 10mins. DNA was extracted twice with phenol, once each with phenol/chloroform and chloroform, and was precipitated with 500μl ethanol. DNA was resuspended in 60μl restriction enzyme buffer, and was digested for 1hr at 37°C. Digested DNA was extracted with phenol/chloroform and ethanol precipitated. The pellet was resuspended and electrophoresed in a 15cm 0.4% TBE agarose gel for 40hr at 12V. The lane was excised, recast in a 15cm 1% TBE agarose gel containing 0.5μg/ml ethidium bromide, and electrophoresed at 120V for 6hr at 4°C. The gel was squashed between glass plates, and was autoradiographed at −70°C using pre-flashed film and intensifying screens.

Densitometry

Autoradiographs were digitised by a linear CCD array scanner using a pixel size of 125μm, and the background optical density (OD) of the film was subtracted. For each autoradiograph, a path along the O-arc was constructed by fitting a spline under tension through guide points specified manually. At equispaced points along the path, pixel values were summed over the width of the arc along lines perpendicular to the path, to give a plot of integrated OD as a function of distance along the arc. This plot was projected onto the first dimension of electrophoresis and transformed to give uniform spacing of OD values as a function of extent of replication. Theoretical curves were constructed using the Stella equation modeler (High Performance Systems Inc., New Hampshire, USA) under the following rules: i) arc intensity is proportional to the number of O-fragments (N) times the extent of replication (R; varying from 0 to 1). ii) Due to the logarithmic migration of DNA in agarose gels, R maps onto the X-axis by the equation $X = \log_{10}(20)/(\log_{10}(20)-1)\times(1-1/\log_{10}(10\times(1+R)))$. iii) For random initiation, N is proportional to 1-R. For initiation from a specific origin, a fragment disappears from the O-arc when one of its forks reaches the end of the fragment.

Measurement of the fork rate

Sperm chromatin was allowed to initiate DNA replication by incubation in egg extract in the presence of 30μg/ml aphidicolin (35). After 2hr, nuclei were then resuspended in Extraction Buffer (50mM KCl, 50mM Hepes KOH, pH 7.5, 5mM $MgCl_2$, 2mM β-mercaptoethanol), pelleted at 1000×g for 2 mins, resuspended in 0.5ml of Extraction Buffer and pelleted again. Washed nuclei were then added to fresh extract containing α^{32}P-dATP, and at various times samples were taken for analysis. Aliquots were digested with Proteinase K and RNase, extracted with phenol and phenol chloroform, and ethanol precipitated. DNA was then electrophoresed on 0.8% alkaline agarose gels.

Pulse-chase experiments

pUCm54 was labelled with 0.2μCi/μl α^{32}P-dATP from 75−80mins. Label was then chased out of the replicative intermediates by the addition of 2.5mM cold dATP, plus 2.5mM $MgCl_2$. At various times after the chase, DNA was isolated as described above and was linearised with Bgl II. DNA was then electrophoresed and autoradiographed as for the first dimension of the 2-D gels.

Calculation of theoretical initiation probabilities

Post-fertilisation cell cycles 2−12 each last 30mins; during this time each fork can replicate 30×60×10 bp = 18kb. In order to ensure complete replication, there must be an initiation event on each 18kb block of the Xenopus genome. If there is an origin every z bp, an 18kb stretch of DNA spans 18000 / z origins. If the probability of initiation at each origin is p, then the probability that initiation does not take place within an 18kb block is $(1-p)^{(18000/z)}$. The Xenopus diploid genome is 6×10^9bp, which comprises about 3×10^5 blocks of 18kb. The probability that at least one origin fires in each of these blocks is $(1-(1-p)^{(18000/z)})^{3\times105}$. For a 99% probability of successfully passing through the cycle, we have $0.99=(1-(1-p)^{(18000/z)})^{3\times105}$. For z=1000 (the maximum permissible from the data presented here), p=0.62, which gives a mean replicon size of approximately 1.6kb. Decreasing potential origin spacing only makes matters worse: for z=10, p=0.01, giving a mean replicon size of 1kb.

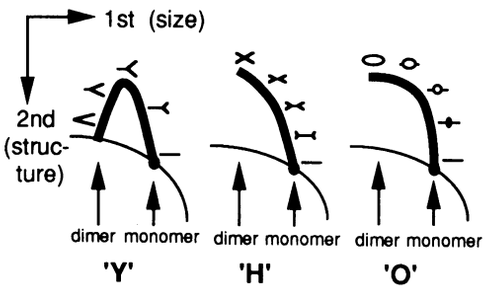

Fig. 1. Schematic diagram of neutral 2-D gel mapping of replication origins. DNA is electrophoresed in the first dimension in an agarose gel under normal conditions, thus separating DNA on the basis of size. Replicative intermediates of a particular restriction fragment range from the unreplicated 'monomer' fragment to a fully replicated 'dimer' twice its size. DNA is then electrophoresed in the second dimension under conditions that separate the DNA largely on the basis of structure. The more branched the DNA, the more it is retarded in the second dimension relative to the arc of linear molecules. Depending on how it is replicated, different restriction fragments will give different patterns: i) fragment replicated by a single fork passing through it ('Y'); ii) fragment replicated by two forks each entering from different sides ('H'); or iii) fragment replicated by forks initiating in the fragment ('O'). Cartoons of the intermediates flank the arcs. For details see (8).

RESULTS

The abundance of potential origins

To map origins of replication in the Xenopus system, we used a variant of the 2-D gel technique of Brewer and Fangman (8). Purified DNA was incubated in Xenopus egg extract, and nascent DNA was pulse-labelled with α^{32}P-dATP. DNA was then isolated and cleaved with restriction enzymes before being separated on 2-D gels as outlined in Figure 1. In the first dimension, fragments are electrophoresed under normal conditions and separate largely by size. Replicative intermediates range from the size of the unreplicated fragment to an intermediate twice its size. In the second dimension, electrophoresis is carried out with a high agarose concentration and a high voltage, separating DNA largely on the basis of structure. The branched nature of replicative intermediates therefore cause them to migrate slower than would linear DNA fragments of the same size. This potentially gives three different patterns, depending on whether the fragment is replicated by a single fork passing all the way through it (Fig 1a, 'Y'), or whether two forks enter it, one from each end (Fig 1a, 'H'), or whether a pair of forks initiate within the fragment (Fig 1a, 'O') (8).

Figure 2a shows a 2-D profile of a fragment of the plasmid pXlr101A, which contains a single copy of the Xenopus rDNA repeat (28). The fragment gives rise to three arcs, corresponding to the three patterns shown in Fig 1. This means that some copies of the fragment were replicated by a single fork passing all the way through it ('Y'), some copies were replicated by two forks terminating in the fragment ('H'), and some copies were replicated by a pair of forks initiating in the fragment ('O'). Of particular importance is the smoothness of the O-arc, since if initiation were to take place at preferred sites, this arc should show marked discontinuities (see below). Fig 2b is a summary of different restriction fragments of pXlr101A that have been analysed by 2-D gels. Each block represents a fragment that gave a 2-D gel pattern similar to that shown in Fig 2a. O-arcs are observed in overlapping fragments throughout the plasmid. No fragments giving different patterns were observed. This means that at the very least, each different restriction fragment must contain a potential origin of replication, and many potential origins must be present on the plasmid.

Other DNA molecules also behave like pXlr101A. Fig 3 shows 2-D gel data from plasmids pUCm54 and pWH5. pUCm54 contains Xenopus genomic DNA from the promoter region of the α-cardiac actin gene; pWH5 is a cloning vector containing the 2μm ARS which functions as an origin of replication in budding yeast (29). The patterns observed with both these plasmids are similar to those seen with pXlr101A, giving composites of smooth O-arcs, Y arcs and H-arcs (Fig 3a and b). These patterns are observed in overlapping fragments throughout each plasmid (Fig 3c and d). No fragments giving different patterns were observed. In particular, no preference for initiation within the 2μm ARS could be observed.

The O-arcs, produced by replication forks initiating within the fragment, were quantified by densitometry. In Fig 4a the densities of six representative O-arcs have been plotted against distance migrated in the first dimension. The X-axis therefore gives a measure of the extent of replication, ranging from no replication (zero) to complete replication of the fragment (one). Arcs appear at their lower end only as they emerge from the monomer spot, and are slightly contaminated at their upper end as they run into

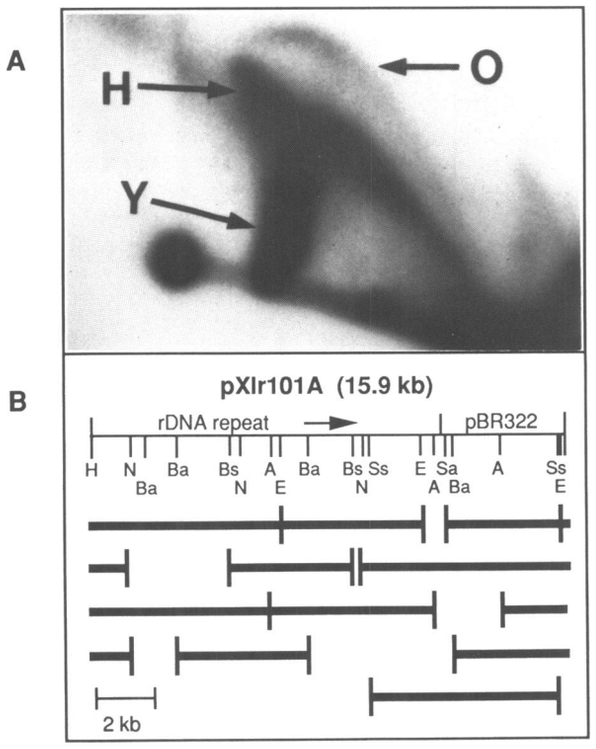

Fig. 2. 2-D gel mapping of pXlr101A replicating in egg extract. (A) The 5.4kb BamH1/Nco I fragment of pXlr101A analysed by 2-D gel electrophoresis. pXlr101A was incubated in egg extract and was pulse-labelled with α^{32}P-dATP. DNA was isolated, cleaved with Bam H1, Nco I and Bgl II, before being separated on 2-D gels and autoradiographed. Arrows show 'Y', 'H' and 'O' forms. (B) Summary of digests performed with pXlr101A yielding 2-D patterns similar to those shown in (A). Each block bounded by a vertical line represents a different restriction fragment. Restriction sites are abbreviated: A, Acc I; Ba, Bam H1; Bg, Bgl II; Bs, Bsu 36; C, Cla I; E, Eco R1; H, Hind III; N, Nco I; P, Pst I; Sa, Sal I; Sm, Sma I; Ss, Ssp I.

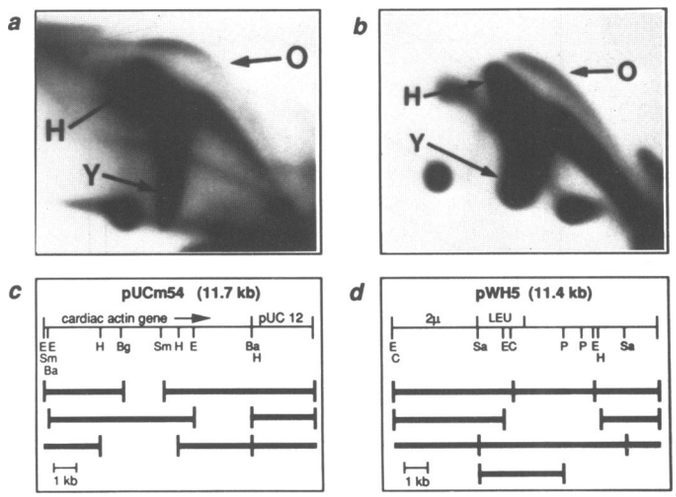

Fig. 3. 2-D gel mapping of pUCm54 and pWH5 replicating in Xenopus egg extract. (A) 2-D gel profile of the 6.5kb Sma I fragment of pUCm54, showing 'Y', 'H' and 'O' arcs. (B) 2-D gel profile of the 4.6 Eco R1 fragment of pWH5, showing 'Y', 'H' and 'O'-arcs. (C) Summary of digests performed with pUCm54 yielding 2-D patterns similar to those shown in (A). Each block bounded by a vertical line represents a different restriction fragment. Restriction enzymes are abbreviated as in Fig 2. (D) Summary of digests performed with pWH5 yielding 2-D patterns similar to those shown in (B).

the end of the H-arc. However, between these regions the O-arc densities follow fairly smooth curves, and all reach their maximum when the fragment is about 50% replicated (migration to 0.65). Fig 4b shows theoretical curves predicted for different numbers of origins within a fragment. Curves for fragments with 20 or less origins (curves II–V) show a series of abrupt drops when forks from each origin reach the end of the fragment. With more than 1000 origins in the fragment (curve I), these discontinuities become smoothed out. The overall shape of curve I is similar to the experimental curves in Fig 4a. However, the experimental curves show some discontinuity at a fine scale. This is probably due to random noise rather than to discontinuities in the signal, since the resolution of individual DNA spots on these gels extends over approximately 5% of the distance between monomer and dimer spots (2–3mm). Further, different autoradiographs of the same fragment show different fluctuations (compare arcs 1 and 2, and arcs 5 and 6). Nevertheless, the smoothness of the experimental curves suggests that discontinuity can be ruled out at the level expected for 5 or fewer possible sites (curves IV and V). Therefore to the limits of resolution of this technique, no preferred sites of initiation can be identified. Since the fragments analysed were about 5kb in size, we can therefore conclude that sites where initiation can potentially occur are spaced no more than 1000bp apart.

The number of initiation events per DNA molecule

Out of all its potential origins, how many are actually used when an individual DNA molecule replicates? If each copy of plasmid DNA were replicated from a single, randomly-situated initiation origin, the maximal density on a 5kb O-arc from a 15kb plasmid should be about 1/6 that of the combined Y- and H-arcs at the top of the Y-arc. In fact the observed O-arcs are several-fold

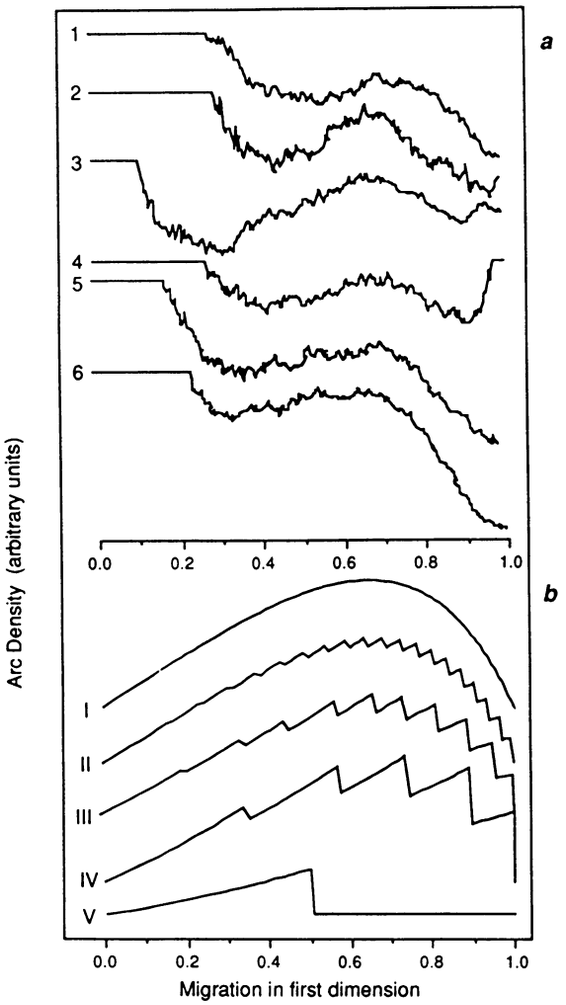

Fig. 4. Densitometry of O-arcs. (A) O-arcs were quantified by densitometry and arc density was plotted on the Y-axis against displacement from the monomer spot in first dimensional electrophoresis as the X-axis (0=monomer, 1=dimer). Different scans are: 1, the 5.4kb BamH1/NcoI fragment of pXlr101A; 2, same fragment as 1 but different autoradiograph; 3, the 6.3kb EcoR1 fragment of pXlr101A; 4, the 3.9kb Sal/Pst fragment of pWH5 (NB this O-arc ran into the 5.1kb Sal fragment near its upper end); 5, the 4.7kb EcoR1 fragment of pWH5; 6, same fragment as 5 but different autoradiograph. (B) Theoretical curves predicted for different number of possible initiation sites for comparison with (A). Sites are equally spaced and used at equal probabilities. Numbers of sites are: I, >1000; II, 20; III, 10; IV, 5; and V, 1.

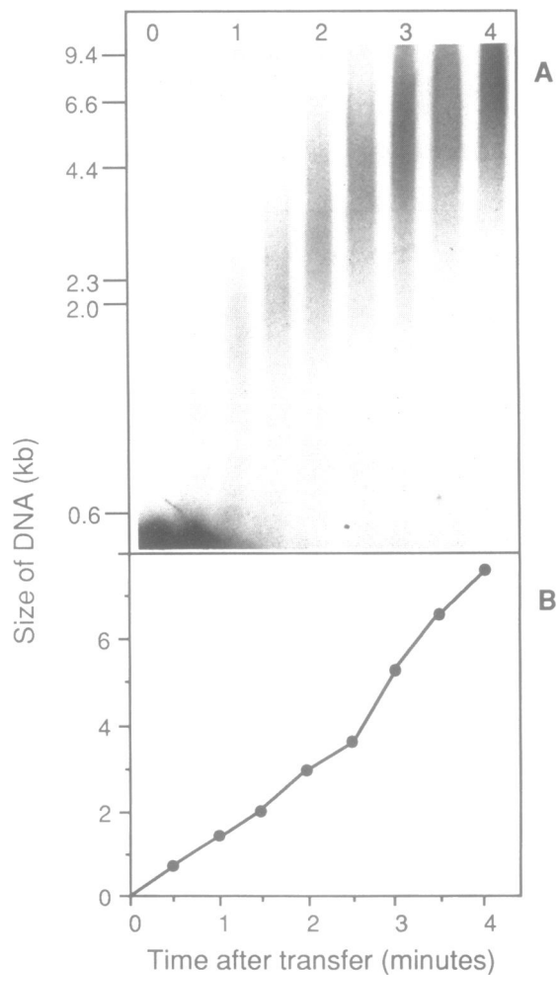

Fig. 5. Measurement of replication fork rate in the cell-free system. (A) Replication forks were allowed to initiate but not elongate in egg extract supplemented with aphidicolin. Nuclei were then transferred to fresh extract without aphidicolin and containing α^{32}P-dATP. At 30 second intervals after transfer, DNA was isolated, run on 0.8% alkaline agarose (strand-separating) gels, and autoradiographed. Numbers above the lanes give the time after transfer in minutes. Size markers in kb are shown. (B) The peak sizes of the nascent DNA fragments from the gel shown in (A) are plotted against time of transfer. The initial slope of this line gives the combined fork rate at the leading and lagging ends of nascent DNA.

less dense than this (Figs 2 and 3), probably due to their reported instability (30). Therefore another method is required in order to determine the number of initiation events that occur on each plasmid. This can be done by measuring how fast replication forks travel in the cell-free system, and then determining the time required for complete replication of a single plasmid molecule. This then gives the number of forks active in replicating a plasmid molecule, and hence the number of initiation events that have occurred on it.

Fig 5 provides a measurement of the rate of replication fork movement. Nascent strands were allowed to initiate, but not elongate, by supplementing egg extract with aphidicolin, an inhibitor of replicative DNA polymerases. Nuclei were then isolated, washed free of aphidicolin, and transferred to fresh extract without aphidicolin. At different times after transfer, DNA was isolated and the nascent DNA strands were analysed on alkaline agarose gels (Fig 5a). After transfer, nascent strands rapidly grow in length, as the stalled forks elongate synchronously. Fig 5b shows the mean strand length plotted against time after transfer. This gives an initial straight line corresponding to a net rate of synthesis per strand of 23nt/sec. Assuming bidirectional replication, this is the net elongation rate at both the leading and lagging ends, and gives a rate of about 11nt/sec for each fork. Four independent measurements of fork rate give a figure of 10 ± 2nt/sec, consistent with the results of Callan (31), who derived a figure of 8nt/sec for the fork rate in Xenopus somatic cells.

Using the fork rate derived from Fig 5, the approximate number of replication forks active on each plasmid DNA molecule can be assessed. In Fig 6a replicating molecules of pUCm54 are pulse labelled with α^{32}P-dATP, and are then chased with cold dATP. At various times after the start of the chase, DNA was isolated, linearised with restriction enzymes, and then electrophoresed on a neutral agarose gel. The replicative intermediates, seen as a smear in lane 1 (end of pulse), are chased into the fully replicated linear position (marked III) over a period of about 15−30mins. Gel scanning, quantified in Fig 6b, shows that the decline in replicative intermediates is biphasic, for the first 15mins showing an initial approximately linear fall with time, which then then tails off. This suggests that most molecules take about 15mins to replicate, with a minority taking approximately twice this time.With a fork rate of 8nt/sec, a single fork could replicate 7.2kb in 15mins. Since pUCm54 is 12kb in size this implies that most copies of the plasmid are replicated by two replication forks, with a minority replicating with perhaps only one.

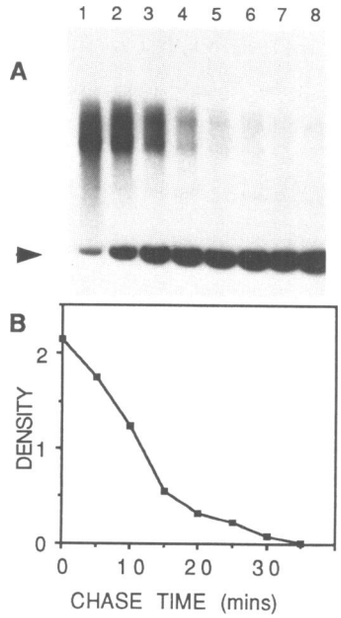

Fig. 6. Pulse-chase of pUCm54 replicating in the extract. (A) pUCm54 was incubated in the cell-free extract and was pulse-labelled with α^{32}P-dATP from 75−95mins. Label was chased out of the replicative intermediates for different periods of time by the addition of cold dATP. DNA was then isolated, linearised with Bgl II, electrophoresed on an agarose gel and autoradiographed. The horizontal arrow shows the position of linear pUCm54. Lanes show the products after the following chase times: Lane 1, 0min; lane 2, 5mins; lane 3, 10mins; lane 4, 15mins; lane 5, 20mins; lane 6, 25mins; lane 7, 30mins; lane 8, 35mins. (B) The gel shown in (A) was scanned, and the total label in the replicative intermediates was integrated and is plotted against the chase time.

DISCUSSION

Xenopus eggs and egg extracts can support the extra-chromosomal replication of a wide range of DNA molecules (18, 19). This suggests that any sequences required for the initiation of DNA replication must be simple enough to occur by chance on many different plasmid DNAs; however it does not indicate how abundant these sites may actually be. We have used neutral/neutral 2-D gel mapping (8) to investigate this point. We find that on three different plasmid DNA molecules, the potential origins of replication are abundant and are all used with similar probability. These three plasmids contain sequences that for different reasons might be expected to be important for origin function: pXlr101A contains a single copy of the Xenopus rDNA repeat, present in the Xenopus chromosome in a large tandem repeat (32); pUCm54 contains the promoter region and start of the Xenopus α-cardiac actin gene; and pWH5 contains the 2μm ARS, which acts as a specific origin of replication in budding yeast. However, none of these three plasmids showed preferential initiation at any particular site. If certain sites were preferentially used, the O-arc densities would be skewed or would show marked discontinuities. However, densitometry of the O-arcs revealed no such discontinuities, and their general shape conformed to that predicted if initiation were to occur with equal probability at all sites. Thus to the limits of resolution of this technique (approximately 500−1000bp) no requirement for initiation at specific sequences could be detected.

Once initiated, replication forks in the cell-free system appear to behave in a manner expected of conventional eukaryotic replication forks. The presence of O- Y- and H- arcs on the 2-D gels is similar to replicative structures observed in yeast and mammalian cells (8, 17). The uniform increase in length of nascent strands seen on alkaline gels is consistent with the conventional model of replication forks progressing at a uniform rate from discrete initiation sites (1, 2). The fork rate derived from the alkaline gels, 10 ± 2nt/sec, is in good agreement with the fork rate observed in Xenopus somatic cells, 8nt/sec (31). Therefore less conventional hypotheses about the mechanism of DNA replication in Xenopus eggs do not seem to be required (33).

Pulse-chase experiments show that most molecules of pUCm54 replicate fully in approximately 15mins, suggesting that no more than 2 active replication forks are responsible for replicating each copy. This suggests that on each DNA molecule, only a single initiation event occurs, which produces two forks progressing

in opposite directions. However, it cannot be ruled out that each initiation event produces only a single unidirectional fork, in which case two initiation events per molecule are required. Nevertheless, the number of initiation sites actually used to replicate each DNA molecule is far lower than the number of sites that could potentially be used.

These results are consistent with the limited electron microscope data concerning replication of plasmid DNA in the intact Xenopus egg (19, 34). Similar results have also been obtained for plasmid DNA microinjected into intact Xenopus eggs (M. Mechali, personal communication). However, it should be emphasized that extrachromosomal replication of plasmid DNA need not directly mimic the way that chromosomal DNA is replicated. Indeed, these results suggest that chromosomal origins of replication must be organised differently to origins on plasmid DNA.

If the distance between two adjacent initiation sites on the chromosome is so large that the DNA between them cannot be fully replicated before the cell enters mitosis, the DNA is likely to be broken as the sister chromatids are pulled apart. In order to prevent such a catastrophe, and with potential origins spaced every 1000bp or less (the maximum consistent with data presented here), the probability of initiation occurring at any one randomly selected origin would have to be high. In order to give a 99% probability that no unreplicated stretches of chromosome were left by the time of mitosis, then the probability of initiation at each potential initiation site in the early Xenopus embryo would have to be about 0.6 (see Materials and Methods for details of the calculation). This would give a mean replicon size of about 1.6kb, which is clearly inconsistent with the observed kinetics of replication, which suggest replicon sizes of 10–20kb (Figs 5 and 6; ref. 21). Increasing the number of potential origins decreases the expected replicon size under these constraints (see Materials and Methods). This implies that chromosomal origins must be controlled in a different manner to the origins used on plasmid DNA. One possibility is that there are stricter requirements for origin function in the chromosome than on extrachromosomal DNA. Similar considerations may apply in budding yeast, where not all origins identified as ARS elements on plasmids may actually function in their chromosomal environment (11, 10). In order to clarify this point, origins operative on Xenopus chromosomes must be studied.

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