

Replication Origins in *Xenopus* Egg Extract Are 5–15 Kilobases Apart and Are Activated in Clusters That Fire at Different Times

J. Julian Blow,* Peter J. Gillespie,* Dennis Francis,‡ and Dean A. Jackson§||

*Cancer Research Campaign, Chromosome Replication Research Group, Department of Biochemistry, University of Dundee, Dundee DD1 5EH, United Kingdom; †School of Biosciences, Cardiff University, Cardiff CF1 3TL, United Kingdom; ‡University of Manchester Institute of Science and Technology, Department of Biomolecular Sciences, Manchester M60 1QD, United Kingdom; §Sir William Dunn School of Pathology, University of Oxford, Oxford OX1 3RE, United Kingdom

Abstract. When *Xenopus* eggs and egg extracts replicate DNA, replication origins are positioned randomly with respect to DNA sequence. However, a completely random distribution of origins would generate some unacceptably large interorigin distances. We have investigated the distribution of replication origins in *Xenopus* sperm nuclei replicating in *Xenopus* egg extract. Replicating DNA was labeled with [³H]thymidine or bromodeoxyuridine and the geometry of labeled sites on spread DNA was examined. Most origins were spaced 5–15 kb apart. This regular distribution provides an explanation for how complete chromosome replication can be ensured although origins are positioned randomly with respect to DNA sequence. Origins were

grouped into small clusters (typically containing 5–10 replicons) that fired at approximately the same time, with different clusters being activated at different times in S phase. This suggests that a temporal program of origin firing similar to that seen in somatic cells also exists in the *Xenopus* embryo. When the quantity of origin recognition complexes (ORCs) on the chromatin was restricted, the average interorigin distance increased, and the number of origins in each cluster decreased. This suggests that the binding of ORCs to chromatin determines the regular spacing of origins in this system.

Key words: replication origin • *Xenopus* • S phase • ORC • origin clusters

Introduction

A wide variety of DNA molecules can be replicated under apparently normal cell cycle control after introduction into *Xenopus* eggs or egg extracts (Harland and Laskey, 1980; Méchali and Kearsley, 1984; Blow and Laskey, 1986; Newport, 1987; Blow and Sleeman, 1990). This suggests that the establishment of replication origins does not depend on specific DNA sequences. Consistent with this, origin mapping by two-dimensional gel electrophoresis showed that both chromosomal and extra-chromosomal DNA initiate replication randomly with respect to DNA sequence in *Xenopus* eggs or egg extracts (Hyrien and Méchali, 1992, 1993; Mahbubani et al., 1992). Only later in development, when the early embryonic cell cycle lengthens to more closely resemble that of somatic cells, are sites of initiation restricted to specific DNA sequences (Hyrien et al., 1995).

Despite the very large number of initiation sites that could potentially be used by the early *Xenopus* embryo, it is unlikely that they are distributed randomly on chromosomal DNA. The fertilized *Xenopus* egg undergoes 12 syn-

chronous rounds of cell division in ~8 h. With a fork rate of ~10 nt/s (Callan, 1972; Mahbubani et al., 1992), the two replication forks initiated from a single origin could therefore replicate no more than ~25 kb in each 20-min S phase. Hence, of the >200,000 replication origins required to replicate the diploid genome (6,000 Mbp), none can be more than ~25 kb from its neighbor for the genome to be completely replicated. This limitation is absolute because mitotic checkpoints are not operative during these divisions, and early embryos containing unreplicated DNA undergo mitosis and cell division on schedule (Hara et al., 1980; Kimelman et al., 1987). If origins were positioned randomly, there would be a geometric distribution of replicon sizes (interorigin distances), with a long tail of excessively large ones. To ensure with reasonable confidence that no pair of adjacent origins are >25 kb apart would require the majority of them to be very tightly spaced (<1 kb apart), which is not consistent with the observed average spacing of ~10–20 kb (Laskey, 1985; Mahbubani et al., 1992; Hyrien and Méchali, 1993; Hyrien et al., 1995; Walter and Newport, 1997).

In principle, it would be possible to ensure complete replication with randomly positioned origins if new origins could be continuously laid down on unreplicated DNA

Address correspondence to J. Julian Blow, Department of Biochemistry, University of Dundee, MSI/WTB Complex, Dow Street, Dundee DD1 5EH, UK. Tel.: (44) 1382-345797. Fax: (44) 1382-348072. E-mail: j.j.blow@dundee.ac.uk

during S phase. However, this explanation is not consistent with what is known about the assembly of replication origins. Replication origins must fire no more than once in a single cell cycle so that no stretch of DNA is rereplicated. To ensure this, origins are “licensed” by the assembly of Mcm(2–7) proteins onto them in late mitosis and G1, and the licence is only removed as the DNA is replicated (Blow and Laskey, 1988; Chong et al., 1996; Diffley, 1996). In all organisms studied, licensing occurs before S phase, and in the *Xenopus* early embryo, it occurs only in late mitosis before nuclear envelope assembly (Blow and Laskey, 1988; Chong et al., 1995). These observations argue strongly against models invoking origin assembly during S phase.

Alternatively, the early *Xenopus* embryo could overcome the “random completion problem” by assembling replication origins at regular intervals along chromosomal DNA. Several observations are consistent with this proposal. First, the rate of DNA synthesis is fairly uniform during S phase (Blow and Watson, 1987), inconsistent with origins being placed at random. Second, small circular DNA molecules (<10–15 kb) replicating in *Xenopus* egg extract appear to use only a single origin (Mahbubani et al., 1992; Lucas et al., 2000), while the density of replication bubbles on chromosomal rDNA suggested only a single replication bubble per 10–15 kb repeat (Hyrien and Méchali, 1993). Third, *Xenopus* origin recognition complex (XORC),¹ which is required for the establishment of functional replication origins, saturates sperm chromatin at about one copy per 8–15 kb (Rowles et al., 1996, 1999; Walter and Newport, 1997). When the quantity of the XORC on chromatin was restricted, the rate of replication decreased (Rowles et al., 1999). These results suggest that origin spacing is in some way regulated in the early *Xenopus* embryo.

In this paper, we use two different DNA fiber techniques to examine the distribution of replication origins on sperm nuclei replicating in *Xenopus* egg extract. We show that clusters of replication origins fire at different times throughout S phase. Within these clusters, replication origins are fairly regularly spaced at 5–15 kb, close to the figure for XORC saturation. This spacing of origins, which must occur without regard to DNA sequence, provides a potential resolution of the “random completion problem.”

Materials and Methods

Preparation of Labeled Nuclei

Metaphase-arrested *Xenopus* egg extracts and demembrated *Xenopus* sperm nuclei were prepared and stored frozen as described (Chong et al., 1997). Extracts immunodepleted with anti-XOrcl antibodies were prepared as described (Rowles et al., 1996; Chong et al., 1997). For use, 20- μ l aliquots of extract were supplemented with 60,000 sperm nuclei (~10 ng DNA/ μ l extract), 100 μ g/ml cycloheximide, 25 mM phosphocreatine, 15 μ g/ml creatine phosphokinase, 0.3 mM CaCl₂, and either 1 μ Ci [α -³²P]dATP (3,000 Ci/mmol; Amersham Pharmacia Biotech), 100 μ Ci [methyl,1'2'-³H]dTTP (90–130 Ci/mmol; Amersham Pharmacia), or 400 μ M BrdU triphosphate (BrdUTP), and incubated at 23°C for various periods. To synchronize nuclear assembly in immunodepleted extracts (see

¹Abbreviations used in this paper: BrdUTP, BrdU triphosphate; DIRVISH, direct visual hybridization; DMAP, dimethylaminopurine; ORC, origin recognition complex; XORC, *Xenopus* ORC.

Fig. 9), 3 vol of 6-dimethylaminopurine (6-DMAP) treated extract (which is incapable of assembling licensed replication origins; Blow, 1993) were added after an initial 25-min incubation (Chong et al., 1997). Samples for TCA precipitation were taken as described (Chong et al., 1997). For isolation of chromatin, extract was diluted in 1 ml Nuclear Isolation Buffer (50 mM KCl, 50 mM Hepes-KOH, pH 7.6, 5 mM MgCl₂, 2 mM DTT, 0.5 mM spermine 3HCl, 0.25 mM spermidine 4HCl, 1 μ g/ml aprotinin, 1 μ g/ml leupeptin, 1 μ g/ml pepstatin; Chong et al., 1997) at 4°C supplemented with 10 μ g/ml aphidicolin and then underlayered with the same buffer containing 10% sucrose. The chromatin was pelleted at 2,100 *g* in a swinging bucket centrifuge for 5 min at 4°C. Overlying buffer was then carefully removed, and the nuclear pellet was resuspended in 100 μ l Nuclear Isolation Buffer supplemented with 10 μ g/ml aphidicolin and 10% sucrose and then immediately frozen in cardice.

DNA Fiber Autoradiography

Nuclei labeled with ³H were thawed and 20- μ l aliquots were mixed with 20- μ l droplets of sodium phosphate buffer (pH 6.8) on subbed microscope slides. The nuclei were lysed and autoradiography slides were prepared by using established methods (Van't Hof, 1975). In brief, a drop of trypsin (2 mg/ml in 0.15 M sodium chloride, 0.015 M sodium citrate, 0.5 M disodium phosphate, pH 6.8) was added to the nuclear suspension on a subbed slide in a Petri dish containing a strip of moistened filter paper and incubated (15 min, 37°C). 20 μ l 2 mM EDTA was then added (3 min, 37°C) followed by 20 μ l 1% (wt/vol) sarkosyl (Sigma-Aldrich) in 2 mM EDTA at 37°C. The Petri dish was tilted to allow the lysate to run down the slide slowly, and the slides were then dried at 37°C and placed overnight in formalin fumes at room temperature. Subsequently, the slides were rinsed in 5% TCA (twice for 10 min) and 100% ethanol (twice for 10 min). The slides were then dipped in 1:1 Ilford K2 nuclear research emulsion and exposed for 2, 4, or 6 mo (Kidd et al., 1987).

Analysis of ³H-labeled Tracks

Autoradiography slides were viewed by bright field microscopy using a ZEISS Axioskop microscope with 20 \times and 100 \times oil immersion objectives. Fibers suitable for measurement were selected by the following criteria: (a) each fiber consisted of >4 tracks lying on an unambiguous direct line; (b) the width of each track was <2 μ m, which pilot exposures had suggested was the maximum width of a single DNA fiber after a 6-mo exposure; (c) each selected fiber did not cross other fibers and showed no sign of branching along its entire length; (d) the grain density along each track appeared uniform and continuous (this requirement may have eliminated almost fully replicated fibers where sections of the two sister strands had completely separated and hence had half the grain density of tracks where both sister strands still lay together); and (e) the local grain background was low enough that it was highly unlikely to contribute single grains lying along the course of the fiber. When appropriate tracks for measurement were identified, they were projected via a charge-couple device camera onto a monitor. Measurements were performed using a calibrated ruler marked off at 0.2- μ m intervals. For each fiber, consecutive measurements were recorded of track length, gap length, and center-to-center distance. A “gap” was defined as a distance of >0.2 μ m (the approximate diameter of a silver grain) where there was no part of a silver grain across the entire width of the track. Where a track consisted of only a single grain, this fact was also recorded. Removal of all measurements involving single-grain tracks from the accumulated statistics did not significantly effect the results.

DNA Fibers for Modified DIRVISH

DNA fibers were prepared and sites containing BrdU were visualized following direct visual hybridization (DIRVISH), by a modification of the protocol described previously (Parra and Windle, 1993; Jackson and Pombo, 1998). In brief, 2- μ l aliquots of nuclei labeled with BrdU were diluted in PBS, and 10³ nuclei in 2- μ l were spotted onto cleaned glass slides and lysed with 5 μ l 0.5% SDS, 200 mM Tris-HCl, pH 7.4, 50 mM EDTA. After 10 min at 20°C, slides were tilted (15°C to horizontal), allowing a stream of DNA to run slowly down the slide, and were then air dried and fixed in methanol/acetic acid (3:1). Spreads containing fewer BrdU-labeled nuclei were prepared by diluting the labeled samples 3- and 10-fold with unlabeled HeLa cells resuspended in PBS (10⁶ cells/ml) before spreading. This allowed replicons from a single labeled cell to be identified. After immunolabeling, samples were selected so that labeled tracks could be assigned to individual fibers with certainty. Spreading was per-

formed under conditions that produce DNA fibers with an extension of 2.59 ± 0.24 kb/ μ m (Jackson and Pombo, 1998). DNA fibers containing BrdU were labeled after standard procedures (Jackson and Pombo, 1998). Spreads were incubated with 2 M HCl (1 h, 20°C) to denature DNA, washed three times in 0.1 M borate and PBS+ (PBS containing 1% BSA, 0.1% Tween), and incubated (30 min, 20°C) with 5% donkey serum in PBS+. Slides were then incubated (1 h, 20°C) with a mouse anti-BrdU IgG (20 μ g/ml; Caltag), washed five times in PBS+, and incubated (1 h, 20°C) with donkey anti-mouse IgG conjugated with Cy3 (1:750 dilution; Jackson ImmunoResearch Laboratories). Finally, samples were washed five times in PBS+, two times in PBS, and mounted in Vectashield (Vector Laboratories). Photographs were taken using a ZEISS Axiophot microscope fitted with standard filter sets (ZEISS sets 02, 09, and 15), using Titermax 400 black and white film. Distance measurements were made from projected images (10 \times magnification of negative) using a magnifying eyepiece calibrated to 0.1 mm, which allowed measurement to 0.2 μ m. The microscope output was calibrated by standard procedures using an England finder.

Analysis of BrdU-labeled Tracks

Samples used to measure origin spacing were selected as follows. For early origins, clusters of labeled sites were chosen in DNA fibers that were <25% labeled (i.e., ~50% labeled within the cluster and unlabeled in surrounding DNA, and where DNA replicated from early sites was ~40–50% of total). The fibers from this class of replicon were clearly similar in length within clusters, suggesting that their synthesis is activated synchronously. Tracks were rejected that were likely to arise from fusion of opposing forks from two origins activated close together (i.e., those greater than two times cluster average), while tracks needed to be a minimum of ~1 μ m to be convincingly identified. Later origins were selected as being short replicons activated close to labeled patches measuring >30 kb in fibers that were ~50% labeled throughout.

Computer Modeling of Different Origin Distributions

A C program was written to model the consequences of either regularly spaced or randomly distributed origins on a 100-Mb segment of chromosomal DNA (approximating a single *Xenopus* chromosome). Regularly spaced origins were located at a distance of 4–14 kb from their nearest neighbors, with a parabolic distribution and an average spacing of 9 kb. Randomly spaced origins were located at a minimum of 100 bp from their nearest neighbors (to account for the physical size of the origin proteins), also with an average spacing of 9 kb. 10–60-kb blocks were then randomly chosen to organize origins into clusters. Clusters were activated in eight waves, each wave occurring every 125 s. Every 10 s unreplicated origins within an active cluster were given a chance to fire with a probability of 0.1. Forks moved at 10 nt/s. Every 5 min, results were sampled and center-to-center distances were recorded. The C source code for this program is available on request.

Results

DNA Fiber Autoradiography on Replicating Sperm Pronuclei

We first adapted the classic technique of DNA fiber autoradiography to allow us to investigate the distribution of replication origins on chromosomal DNA replicating in *Xenopus* egg extracts. In DNA fiber autoradiography, replicating DNA is labeled with [³H]dTTP and the DNA is then spread on glass slides that are coated with a photographic emulsion. After an appropriate exposure, the slides are developed, revealing a track of silver grains along sections of labeled DNA. Several different features of the way replication occurs can be deduced from the pattern, size, and distribution of tracks. To apply this technique to replication of *Xenopus* sperm nuclei in *Xenopus* egg extracts, we first determined the replication kinetics in vitro (Fig. 1 A). Consistent with previous reports (Blow and Laskey, 1986; Blow and Watson, 1987; Newport, 1987;

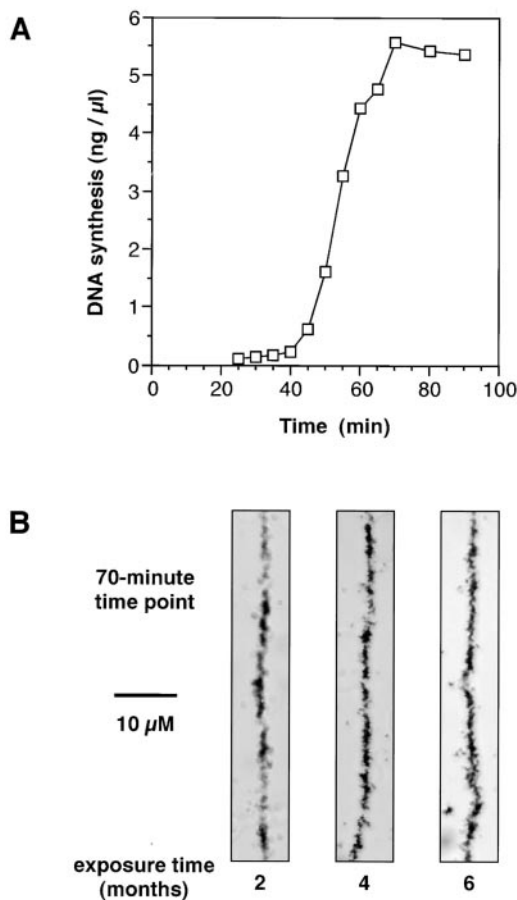


Figure 1. Establishing conditions for DNA fiber autoradiography using *Xenopus* egg extracts. (A) *Xenopus* sperm nuclei were incubated in *Xenopus* egg extract supplemented with [α -³²P]dATP. At the indicated times, aliquots were taken and DNA synthesis was assessed by TCA precipitation. (B) *Xenopus* sperm nuclei were incubated for 70 min in *Xenopus* egg extract supplemented with [³H]dTTP. DNA was isolated, spread on glass slides, and coated with photographic emulsion. After 2, 4, or 6 mo, slides were developed and examined by light microscopy for the continuity of fiber labeling. Bar, 10 μ m.

Blow and Sleeman, 1990), there was an initial lag period of ~40 min, during which time replication origins were assembled on DNA, and nuclei competent to support replication were produced. After this lag, S phase began abruptly and lasted ~25 min. During this time, chromosomal DNA is precisely duplicated (Blow and Laskey, 1986; Blow and Watson, 1987). The S phase length in vitro was similar to that reported in vivo (15–20 min; Graham, 1966), and most of the nuclei in these types of extract start to replicate within ~5 min of each other (data not shown). Therefore, we believe that the replication occurring in vitro is a good model of what occurs as a consequence of a natural fertilization in vivo.

Since replication origins are expected to be very closely spaced in the early *Xenopus* embryo, we needed to find labeling conditions that would allow complete tracks of replicated DNA to be identified unambiguously. We supplemented egg extract with 50 μ M [³H]dTTP at high specific activity. Since the extract contains 50 μ M each of the

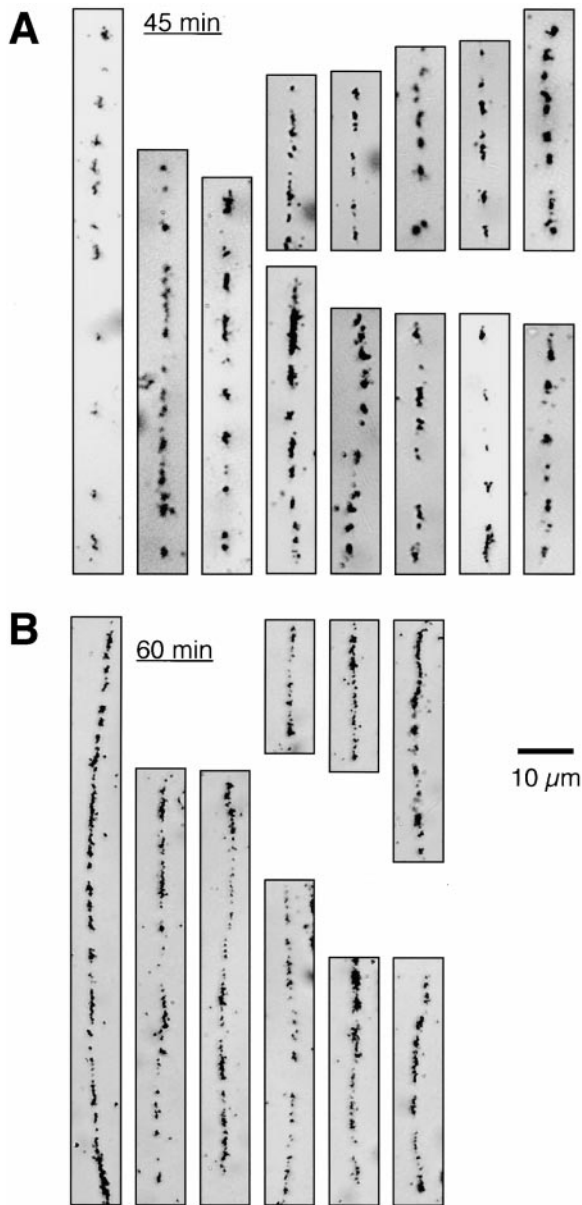


Figure 2. Representative examples of fiber autoradiography samples from early and late S phase. *Xenopus* sperm nuclei were incubated in *Xenopus* egg extract supplemented with [^3H]dTTP for either (A) 45 min or (B) 60 min. DNA was isolated, spread on glass slides, and coated with photographic emulsion. After 6 mo, slides were developed and examined by light microscopy. Bar, 10 μm .

dNTPs (Blow and Laskey, 1986), this should mean that $\sim 50\%$ of all dTTP incorporated into DNA will be radiolabeled. We then incubated sperm nuclei in this extract for 70 min, by which time DNA replication should be largely complete (Fig. 1 A). The DNA was then spread on glass slides, which were then coated with photographic emulsion, and exposed for 2, 4, or 6 mo before being developed. The resultant density of silver grains along individual tracks was assessed by light microscopy (Fig. 1 B). Although a 2-mo exposure is commonly used to obtain good tracks from DNA replicating in somatic cells, we needed to ensure track continuity because of the anticipated small replicon size in the *Xenopus* system. Essentially complete

tracks of nascent DNA were seen after a 2-mo exposure, with the tracks becoming predictably denser after 4- and 6-mo exposures. In all exposures, the density of silver grains varied along the length of individual tracks, consistent with changes in the adenosine + thymidine content of the DNA and randomness in the conversion of silver grains by radioactive decay. Using a 6-mo exposure, most tracks $< 20 \mu\text{m}$ in size were effectively complete (where $1 \mu\text{m}$ DNA = 3 kb; Rogers, 1973; Van't Hof, 1975).

Therefore, we repeated the same labeling protocol, but instead isolated DNA after incubations of 45, 50, 60, or 70 min. The DNA was then spread and exposed for 6 mo before being developed and analyzed by light microscopy. Preliminary inspection of the slides showed a clear increase in the quantity and length of labeled tracks from 45 to 70 min. At the 45-min time point the majority of tracks were short ($< 3 \mu\text{m}$), extending in arrays over tens or hundreds of kilobases (Fig. 2 A). A small number of longer tracks were also seen. At later times, a more complex picture was observed (Fig. 2 B). In addition to the presence of many fairly long tracks (10–50 μm), we also saw numerous small tracks resembling those seen in the 45-min sample. Most DNA molecules from the later time points contained a mixture of both short and long tracks (Fig. 2 B). This suggests that many replication origins initiate only late in S phase, as is observed in other eukaryotic cells.

To quantify the spacing between adjacent replication origins, we measured the distance between the centers of adjacent tracks (center-to-center distances). Since different replication forks are extended at similar rates, this gives a good estimate of the origin-to-origin distance. To minimize subjective bias, a set of strict criteria was used to select fibers for measurement (see Materials and Methods). For the 45-min time point, center-to-center distances for 59 individual DNA fibers (total length 4,468 μm) containing 840 tracks were measured. The distribution of center-to-center distances is shown in Fig. 3 A. Most of the center-to-center distances were in the range 1.5–4.5 μm (~ 5 –15 kb), with a peak at 2.6 μm (~ 8 kb). Some much larger center-to-center distances were also recorded ($> 10 \mu\text{m}$), which were associated with large unlabeled gaps in the fibers. To quantify these stretches of unreplicated DNA, measurements were made of the length of unlabeled DNA between the labeled tracks ("gap length"; Fig. 3 B). This revealed a spread of distances, with a mean of 3.6 μm and a long tail with many tracks separated by gaps of $> 8 \mu\text{m}$ (~ 24 kb). There is a limit to the length of gap this protocol can detect, as confidence in the continuity of the (unlabeled) DNA fiber declines with distance, so these measurements are expected to significantly underrepresent longer gaps. With a fork rate of ~ 10 nt/s in this system (Callan, 1972; Mahbubani et al., 1992), origins spaced > 25 kb (8 μm) apart would be unable to replicate all the intervening DNA in an S phase of 20 min. Therefore, the DNA between these distantly spaced origins must contain origins that fire later in S phase if replication is to be completed on schedule.

The same set of measurements was performed on DNA later in S phase at 60 and 70 min (Fig. 3, C–F). Fig. 3, C and E, show center-to-center distances for the 60- and 70-min time points. Despite the DNA fibers being at a later stage of replication (67% replicated at 60 min and 81% at

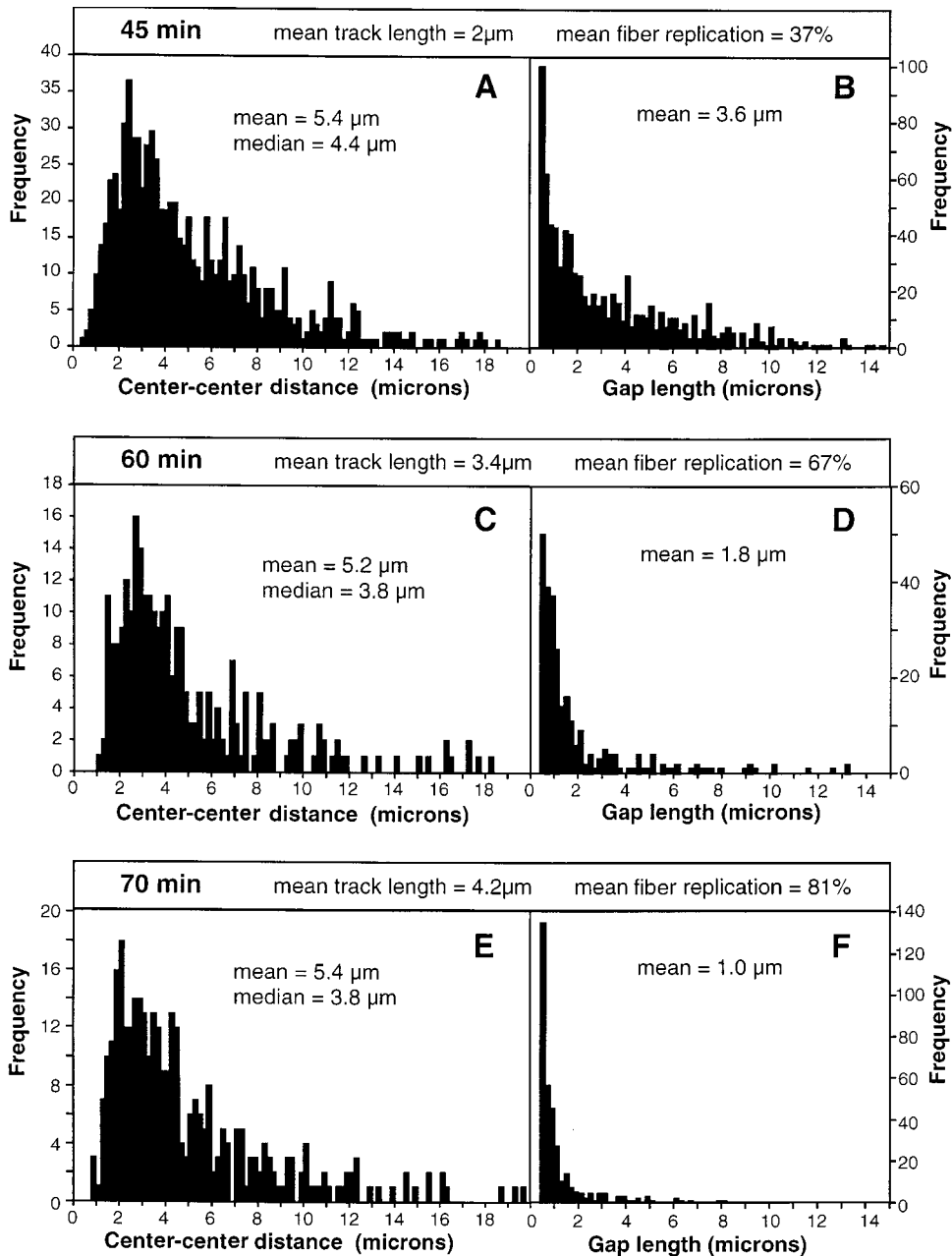


Figure 3. Center-to-center distances and gap length of fiber autoradiography samples from different stages of S phase. *Xenopus* sperm nuclei were incubated in *Xenopus* egg extract supplemented with [^3H]dTTP for either (A and B) 45 min, (C and D) 60 min, or (E and F) 70 min. DNA was isolated, processed for fiber autoradiography, and exposed for 6 mo. After development, fibers were examined by light microscopy to measure the center-to-center distance between adjacent tracks (A, C, and E), and to measure the length of the gaps between adjacent tracks (B, D, and F).

70 min, compared with 37% at 45 min), the distribution of center-to-center distances was very similar to the distribution seen at 45 min. Most of the center-to-center distances were in the range 1.5–4.5 μm (\sim 5–15 kb), with a peak at 2.2–2.6 μm (7–8 kb). However, in contrast to the 45-min time point, the large center-to-center distances in the 60- and 70-min time points were mainly associated with very long tracks rather than large gaps. This is clearly seen in the gap distribution profiles (Fig. 3, D and F) showing that longer gaps ($>$ 4 μm) were much less abundant than they were at 45 min. Nevertheless, at 60 min a few long gaps $>$ 4 μm remained, suggesting that even at this late time, some origins are still to fire. The striking similarity of the center-to-center distances in the 45-, 60-, and 70-min time points suggests that this degree of regularity in spacing is a general feature of all replication origins in this system.

Many adjacent tracks, particularly those that were

closely spaced, appeared to be of similar lengths, implying that they fired at about the same time (Fig. 2). This appeared to be true of both early- and late-replicating DNA, suggesting that there were synchronous clusters of origins firing at different times in S phase. A similar pattern of clustered replication origins has been seen in somatic cells (Hand, 1978; Van't Hof et al., 1978). We quantified this local synchrony by comparing the length of adjacent tracks in the 45-min sample, as shown in Fig. 4. When both tracks were less than \sim 4 μm , there was a reasonable correlation between their lengths, as evidenced by most track pairs lying close to the diagonal in the figure. Statistical analysis (ignoring tracks that consisted of only a single silver grain) gave a positive correlation of 0.16 between adjacent tracks (highly significantly different from random, $P < 0.0001$). For longer tracks, which are likely to have been formed by the fusion of tracks from more than one replication origin,

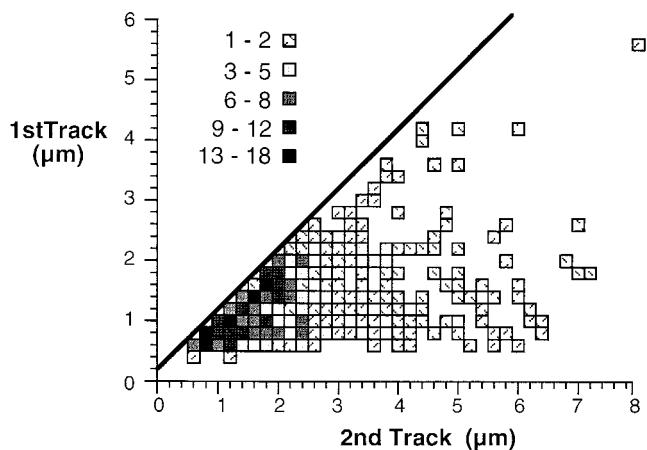


Figure 4. Correlation between the length of adjacent tracks observed by fiber autoradiography. From the 45-min sample set described in the legend to Fig. 3, the lengths of adjacent tracks were correlated. For each adjacent pair of tracks, the larger track length is given on the horizontal axis, the shorter track length is shown on the vertical axis. Track pairs where one or more of the tracks were either >8 kb or represented by only a single silver grain have been omitted.

the synchrony declined. Initiation events are therefore locally synchronized in this system, consistent with the existence of clusters of origins that fire at different stages of S phase. However, because this analysis is restricted to rather short fibers ($\sim 75 \mu\text{m}$ average) in a population of fibers prepared from nuclei at slightly different stages of S phase, it is not possible to assess the degree of initiation synchrony throughout individual nuclei.

Examination of Track Distribution over Longer Distances by BrdU Labeling

To look at longer stretches of DNA, we used a modification of an alternative technique called DIRVISH (Parra and Windle, 1993; Jackson and Pombo, 1998), involving BrdU labeling and visualization of labeled DNA from individual nuclei. DNA from sperm nuclei replicating in *Xenopus* egg extracts was labeled with BrdU, and nuclei were then isolated before being lysed in situ and spread on glass slides. Sites of incorporation on spread DNA were analyzed by indirect immunofluorescence using anti-BrdU antibodies. The DNA is stretched more than in standard techniques, giving $1 \mu\text{m} = 2.6$ kb (Jackson and Pombo, 1998), but gives straighter fibers. Fig. 5 A shows a low power view of a single spread nucleus at a late stage of S phase. The intensely labeled part of the spread contains a mixture of DNA bundles and single fibers, while a skirt of discrete fibers surrounds this DNA-rich zone, commonly spreading a further 1–2 mm. Single DNA fibers are not evident at this low magnification (Fig. 5 A). However, at higher magnification (Fig. 5 B), individual DNA fibers can be identified with ease and the extent of replication established. In this particular example of a nucleus late in S phase it is obvious that most DNA fibers are fully replicated. The continuity of the tracks is fairly good, though not quite as complete as the 6-mo exposure using ^3H (Fig. 1 B). Fig. 5 C shows a detail from a single nucleus at a very early stage of S phase which contained thousands of labeled tracks of similar length. The synchrony of initiation in

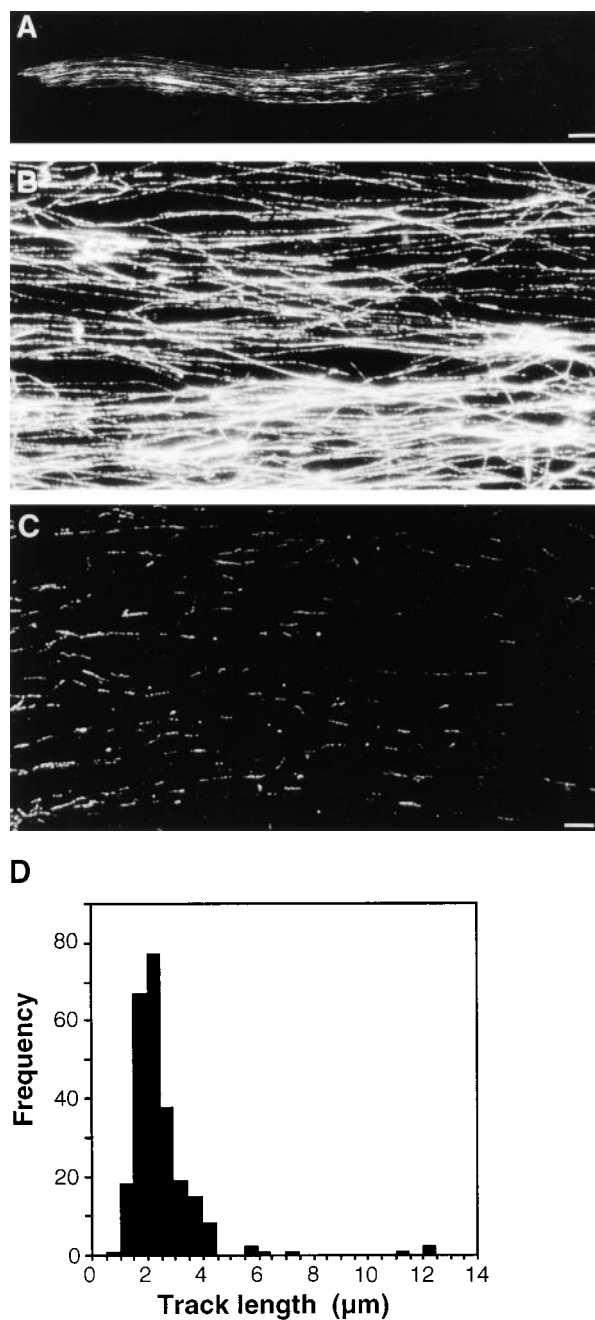


Figure 5. Visualization of replicated DNA in individual nuclei by DIRVISH. *Xenopus* sperm nuclei were incubated in *Xenopus* egg extract supplemented with BrdUTP, and after various times nuclei were isolated. Aliquots were then supplemented with SDS, spread over a glass surface and processed for anti-BrdU immunofluorescence. (A) Low power image of a single nucleus incubated for 60 min in egg extract that has almost completed S phase. (B) Higher magnification of part of the nucleus shown in A, showing DNA bundles and individual fibers. (C) Part of a nucleus incubated for 45 min in egg extract, showing many short tracks of replicated DNA. (D) Distribution of the track lengths of the sample shown in C. Bars: (A) $30 \mu\text{m}$; (B and C) $3 \mu\text{m}$.

this sample was assessed by measuring the length of these tracks, and their distribution is shown in Fig. 5 D. There is a tight distribution of track lengths of $\sim 2 \mu\text{m}$ (~ 5 kb), indicating that all the tracks derived from origins initiated synchronously ~ 4 min previously.

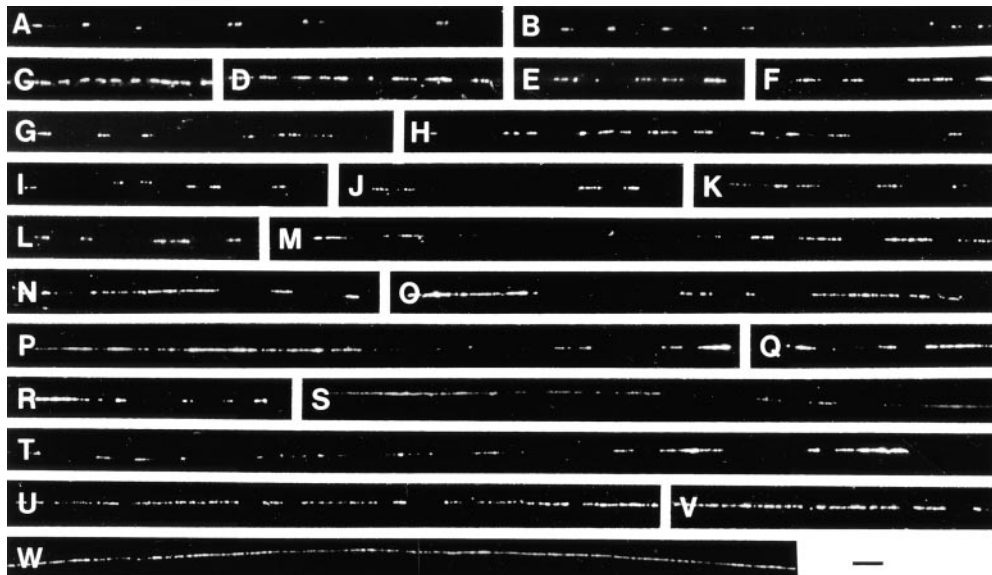


Figure 6. Representative examples of DIRVISH samples from different stages of S phase. *Xenopus* sperm nuclei were incubated in *Xenopus* egg extract supplemented with BrdUTP for either (A and B) 40 min, (C–L) 45 min, (M–T) 50 min, or (U–W) 60 min. Nuclei were isolated, supplemented with SDS, spread over a glass surface, and processed for anti-BrdU immunofluorescence. Bar, 3 μ m.

A gallery of immunolabeled fibers from nuclei at different stages through S phase is shown in Fig. 6. Sperm nuclei entered S phase after \sim 40 min in egg extract (Fig. 1 A), and at this time a few labeled fibers with very short labeled patches were seen (Fig. 6, A and B). Many more nuclei en-

tered S phase in the following 5 min (Fig. 6, C–L). At this stage, local groups of origins were clearly activated together to give clusters of similarly sized labeled patches. Each cluster contained an average of 7.3 origins (1,159 labeled tracks in 159 clusters). By 50 min (i.e., within 10 min of entering S phase), the fusion of adjacent forks from clusters of early-firing origins gave extended patches labeled from multiple origins (Fig. 6, M–T). Once fusion has occurred, it is no longer possible to assign sites of initiation. However these long tracks frequently lay adjacent to clusters of newly activated labeled tracks (Fig. 6, N–T), clearly demonstrating that initiation occurs in asynchronous clusters. Each of the late-firing clusters, where short tracks were observed adjacent to fused tracks of $>$ 30 kb, contained an average of 4.4 origins (323 labeled tracks in 73 clusters). Late in S phase, after 60 min incubation, very long stretches of replicated DNA were apparent as a consequence of extensive track fusion (Fig. 6, U–W).

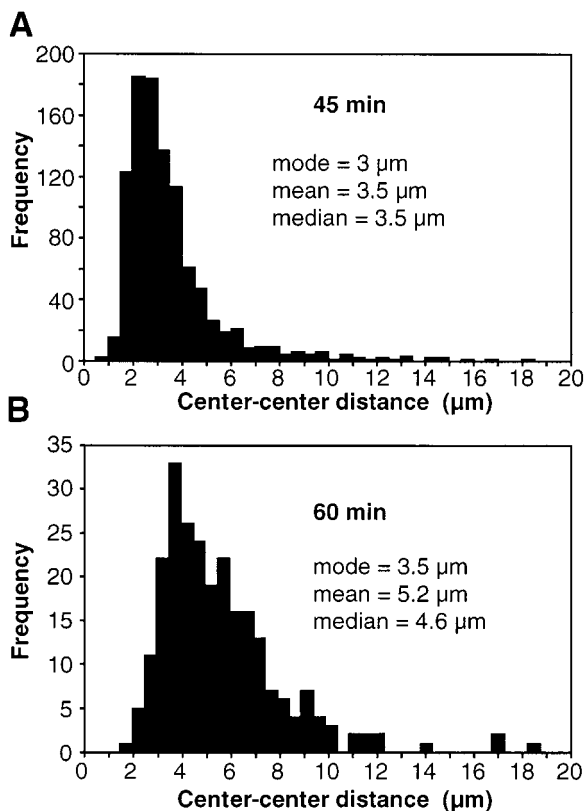


Figure 7. Center-to-center distances of DIRVISH samples from early and late S phase. *Xenopus* sperm nuclei were incubated in *Xenopus* egg extract supplemented with BrdUTP for either (A) 45 min or (B) 60 min. Nuclei were isolated, supplemented with SDS, spread over a glass surface, and processed for anti-BrdU immunofluorescence. Fibers were examined by immunofluorescence microscopy to measure the center-to-center distance between adjacent tracks.

We next determined the average center-to-center distance of tracks revealed by the modified DIRVISH protocol. From the early S phase samples (40–45 min), 1,000 center-to-center intervals were measured from 3,800 μ m DNA (9.8 Mb). As shown in Fig. 7 A, this gave a distribution with a sharp peak at \sim 3 μ m (8 kb) that closely resembled the peak obtained by fiber autoradiography at 2.6 μ m (Fig. 3 A). The distribution obtained by DIRVISH had fewer larger center-to-center distances $>$ 6 μ m than was obtained by fiber autoradiography, most likely because of the greater degree of synchrony between the DIRVISH samples selected for measurement (see Materials and Methods). To investigate the clustering of late-firing origins, 250 center-to-center distances from origins activated within mid/late S phase nuclei were measured, where short tracks were observed adjacent to tracks of at least 30 kb. As shown in Fig. 7 B, this gave a very similar profile to the previous ones (Fig. 3, A and C, and Fig. 7 A). A peak was seen at 3.5 μ m (\sim 9 kb), with an average center-to-center distance of 5.2 μ m (13.5 kb), apparently spaced a little more unevenly than origins activated at the onset of S phase.

Another advantage of DIRVISH spreads is the ability to analyze long contiguous stretches of DNA. In Fig. 8 three such contigs, each containing \sim 1 Mb DNA, are shown. This long-range view of the arrangement of repli-

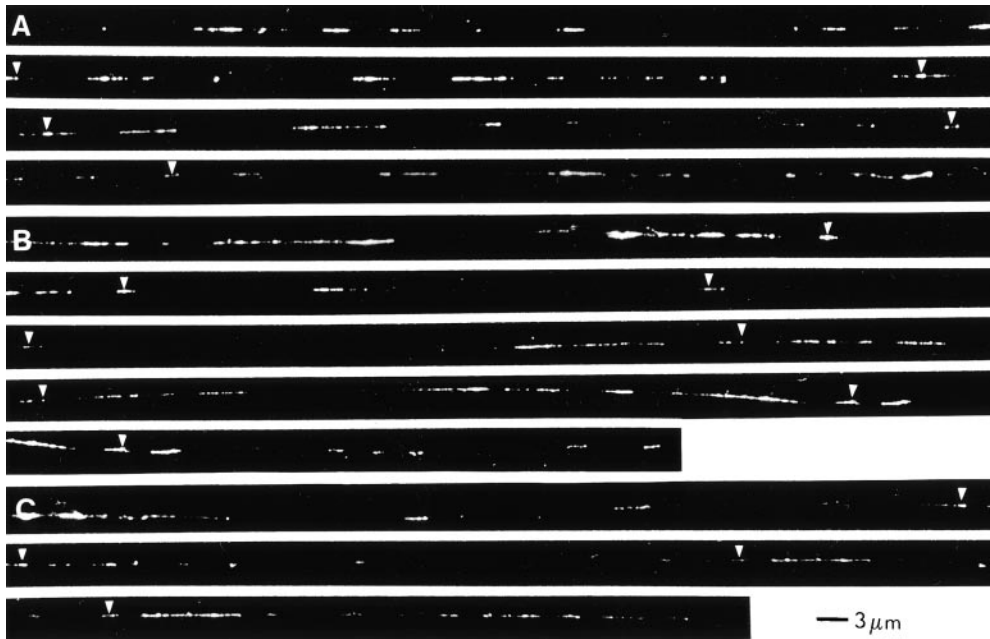


Figure 8. Long fibers observed by DIRVISH. *Xenopus* sperm nuclei were incubated in *Xenopus* egg extract supplemented with BrdUTP for 50 min. Nuclei were isolated, supplemented with SDS, spread over a glass surface, and processed for anti-BrdU immunofluorescence. A, B, and C show three contiguous fibers, with arrowheads marking sites of overlap. Bar, 3 μm .

cation in DNA fibers from in vitro-assembled nuclei confirms that synthesis is activated at different times within S phase. In these examples, where synthesis is $\sim 50\%$ complete, it is clear that some regions covering 50–150 kb are heavily labeled, although adjacent sites have only just begun synthesis. The clustered nature of the short tracks is also evident from these pictures. Many large gaps between the clusters are evident, ranging in size 12 μm (~ 30 kb)–38 μm (~ 100 kb), suggesting that further initiation events are still needed for replication to be completed.

Effect of Limiting XORC on Origin Distribution

Previous work has shown that when *Xenopus* sperm are incubated in *Xenopus* egg extract, the chromatin becomes saturated with ~ 1 copy of XORC every 8–15 kb (Rowles et al., 1996, 1999; Walter and Newport, 1997). Limiting the quantity of XORC on the DNA reduces the overall rate of replication (Rowles et al., 1999). These results would be consistent with the idea that the regular deposition of XORC every ~ 5 –15 kb could account for the regular origin distribution we have observed. To investigate this idea further, we established an assay system where replication kinetics and labeled fibers could be analyzed on nuclei replicating in the presence of different quantities of XORC. *Xenopus* egg extracts were immunodepleted with antibodies specific for XORC1 (Rowles et al., 1996) (which deplete the entire XORC complex), or with nonimmune antibodies. Sperm nuclei were incubated for 25 min in mixtures of these extracts, or in different quantities of undepleted extract, and chromatin was then isolated and immunoblotted for XORC1 (Fig. 9 A). In undepleted or nonimmune-depleted extracts, the chromatin became saturated with XORC1. However, when the nonimmune-depleted extract was diluted 6- or 18-fold in XORC1-depleted extract, the quantity of XORC1 assembled onto the chromatin was lowered. Immunodepletion of extracts disturbs nuclear assembly and hence makes replication less synchronous. To overcome this problem, immunodepleted extracts were allowed to assemble chromatin for 25 min

and were then supplemented with 6-DMAP-treated extract, which can support nuclear assembly and the elongation stage of DNA replication but which cannot support the assembly of functional replication origins (Blow, 1993). Analysis of subsequent DNA replication (Fig. 9 B) showed that the maximal replication rate was approximately in proportion to the quantity of XORC1 assembled onto the DNA. Replicating nuclei were also isolated and analyzed by DIRVISH (Fig. 9 C). As the quantity of XORC on the DNA declined, there was an increase in the average spacing of replication origins, and a reduction in the number of origins found in each cluster. An increase in the number of isolated tracks (cluster size of 1) also occurred as XORC was limited. There was a striking parallel between “origin density” (origins per cluster \div mean origin spacing), replication rate, and XORC1 loading. These results are consistent with each origin being specified by the binding of a single XORC molecule to sites roughly spaced along the DNA.

Discussion

We have investigated the timing and distribution of initiation events on *Xenopus* sperm nuclei replicating in *Xenopus* egg extract. This reveals that origins are spaced at 5–15-kb intervals. This regular spacing can ensure complete genome replication, while replication origins are not defined by primary DNA sequence.

Overall Structure of S Phase in *Xenopus* Egg Extract

We show that in the *Xenopus* cell-free system, different replication origins fire at different times during S phase. Origins are grouped into clusters that initiate almost synchronously, while different clusters fire at different times. Early-firing clusters appear to contain slightly more origins (average of 7.3) than late-firing clusters (average of 4.4). Within each cluster, most origins are spaced 5–15 kb apart with a fairly sharp peak at 8–9 kb. The average cluster therefore covers ~ 50 kb DNA. With a fork rate of 10

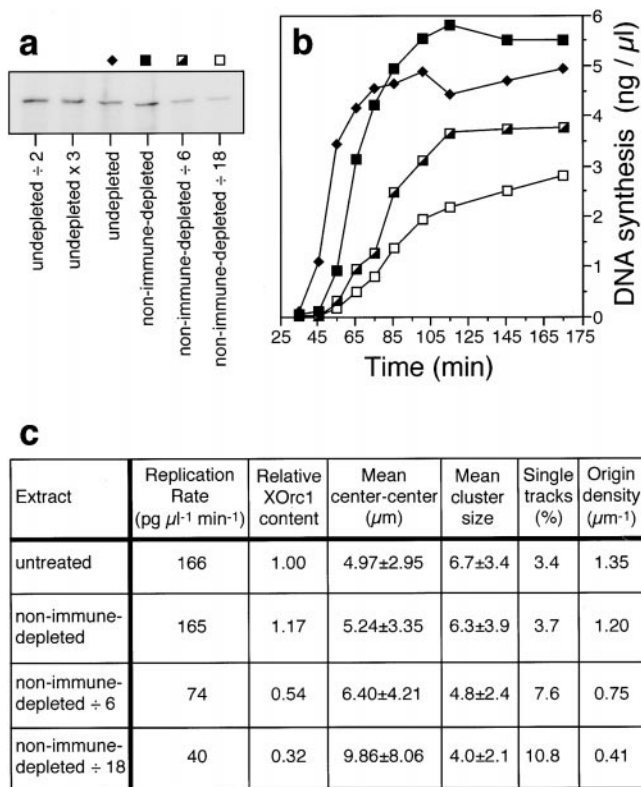


Figure 9. Effect of limiting XORC on origin distribution. *Xenopus* egg extracts were immunodepleted with anti-XOrc1 antibodies or with nonimmune antibodies. Aliquots of *Xenopus* sperm nuclei (400 ng DNA) were incubated in either 40 μl untreated extract, 40 μl nonimmune-depleted extract, 40 μl of a 5:1 ratio of XOrc1-depleted extract plus nonimmune-depleted extract (non-immune-depleted extract \div 6), 40 μl of a 17:1 ratio of XOrc1-depleted extract plus nonimmune-depleted extract (non-immune-depleted extract \div 18), 20 μl untreated extract (untreated extract \div 2), or 120 μl untreated extract (untreated extract \times 3). (A) After 25 min, chromatin was isolated and immunoblotted for XOrc1. (B and C) After 25 min, a further 3 vol of 6-DMAP-treated extract were added to drive synchronous nuclear assembly without allowing further origin assembly. (B) [α - ^{32}P]dATP was added to the extract and total DNA synthesis at the indicated times was determined by TCA precipitation. (C) BrdUTP was added to the extract, and at 55 min (untreated extract) or 65 min (depleted extracts) nuclei were isolated and DNA was analyzed by DIRVISH. The mean center-to-center distance and mean number of origins in each cluster were determined. “Origin density” (origins per cluster \div mean origin spacing) is also shown.

nt/s (Callan, 1972; Mahbubani et al., 1992) a pair of forks can replicate \sim 1.2 kb/min, meaning that a typical cluster will be fully replicated within 4–12 min out of an S phase of \sim 20 min. The large gaps that are left between early firing clusters are replicated by initiation of further origin clusters later in S phase.

This pattern is similar to that found in the somatic cells of metazoans and plants, where small clusters of origins fire at different stages of S phase (Callan, 1972; Hand, 1975, 1978; Van’t Hof et al., 1978; Jackson and Pombo, 1998). However, the replicon size in the *Xenopus* system (5–15 kb) is significantly smaller than the replicon size in mammalian cells (50–150 kb), and the firing of all the different clusters in *Xenopus* is compressed into a much

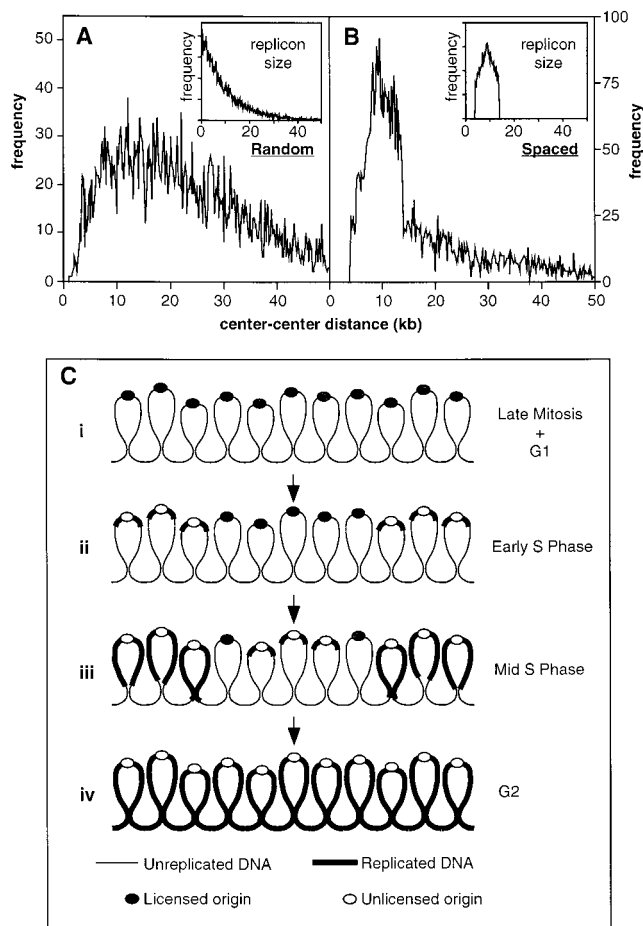


Figure 10. Spacing of replication origins in the early *Xenopus* embryo. (A and B) Results generated by a computer program written to simulate the S phase kinetics of sperm nuclei replicating in *Xenopus* egg extracts. Replication origins were either positioned at random, with a mean replicon size of 9 kb (A), or were set up with a parabolic distribution over 4–14 kb and a mean replicon size of 9 kb (B). The insets show the replicon sizes obtained. Both sets of origins were then activated in asynchronous clusters (parameters based on the kinetic data presented in this paper, see Materials and Methods). The center-to-center distances of nascent DNA tracks after 15 min of S phase are shown in the main graphs. (C) Model of replication origin use in the *Xenopus* system. A small segment of chromosomal DNA (\sim 100 kb) is shown looped for convenience. (i) In late mitosis licensed origins are assembled at \sim 9-kb intervals. (ii) In early S phase, two clusters initiate synchronously. As the origins are replicated, they convert to the unlicensed state. (iii) During later parts of S phase, other clusters of origins initiate replication synchronously. (iv) By G2, all DNA has replicated and all origins have converted to the unlicensed state.

shorter time period. Our results make an interesting contrast to data obtained by electron microscopy of replicating DNA in the early *Drosophila* embryo (Blumenthal et al., 1973). Similar to the *Xenopus* system, replication origins were regularly spaced in the early *Drosophila* embryo (Blumenthal et al., 1973). However, all origins appeared to initiate very early in the *Drosophila* S phase, and origin spacing was regular enough that where one or more origins had failed to fire, interorigin distances representing multiples of the basic value were clearly seen. The spacing

between origins in *Xenopus* was not this precise, falling within a broad 5–15-kb range, and interorigin distances representing multiples of the value were not evident. These different replication strategies may reflect the different lengths of time available to complete S phase: 3.5 min in the early *Drosophila* embryo, 20 min in the early *Xenopus* embryo, and 7–12 h in mammalian somatic cells.

Resolution of the “Random Completion Problem”

No special DNA sequences are required for DNA to replicate in *Xenopus* eggs or egg extracts (Harland and Laskey, 1980; Méchali and Kearsley, 1984; Blow and Laskey, 1986; Newport, 1987; Blow and Sleeman, 1990), with replication origins being positioned randomly with respect to DNA sequence (Mahbubani et al., 1992; Hyrien and Méchali, 1992, 1993). However, if replication origins were placed on DNA completely at random, there would be a high probability that at least one pair of adjacent origins would lie >25 kb apart, and so would not be able to replicate all the intervening DNA in the time available (the “random completion problem”) (Laskey, 1985; Mahbubani et al., 1992; Hyrien and Méchali, 1993). Fig. 10 A shows results obtained with a computer simulation of randomly spaced origins on a 100-Mb stretch of DNA (approximating a single *Xenopus* chromosome), with a mean replicon size of 9 kb. The inset shows that this gives the expected geometric distribution of replicon sizes. Origins were grouped into clusters, which were fired asynchronously in an artificial S phase using the kinetic parameters derived in this paper (see Materials and Methods for details). The main graph in Fig. 10 A shows the broad distribution of center-to-center distances that are seen after 15 min of S phase with randomly positioned origins. Due to the presence of some very large replicons, the DNA takes a long time to replicate fully (80.2 ± 9.9 min). The results obtained with randomly positioned origins therefore do not match our observed data.

In contrast, the restriction of origins to sites spaced 5–15 kb apart provides a simple resolution to the “random completion problem,” while still allowing origins to be positioned randomly with respect to DNA sequence. All the potential origin sites can be utilized without creating the very small replicons that predominate if origins are positioned completely at random. This situation is modeled in Fig. 10 B. A parabolic distribution of origins spaced 4–14 kb apart was established (Fig. 10 B, inset), giving a mean replicon size of 9 kb. These origins were then grouped into clusters, which were fired asynchronously during an artificial S phase, using exactly the same parameters as were used for Fig. 10 A. The resultant distribution of center-to-center distances closely matches the distributions we have established by fiber autoradiography and DIRVISH, with a predominant peak at 5–15 kb (Figs. 3 and 7). The spaced origins gave an S phase length of 29.4 ± 1.4 min, similar to that obtained *in vitro* (Fig. 1).

The use of spaced origins implies that XORC is spaced on sperm chromatin in the *Xenopus* system. Although XORC binds with high affinity to unlicensed chromatin, sperm nuclei incubated in *Xenopus* egg extract become saturated with 1 copy of XORC per 8–15 kb (Rowles et al., 1996, 1999; Walter and Newport, 1997). When the quantity

of XORC is limited, the replication rate drops in approximate proportion to the quantity of XORC on the chromatin, and this is reflected in both an increase in center-to-center distance and a decrease in cluster size (Rowles et al., 1999; and this paper).

Fig. 10 C shows a cartoon depicting our conclusions. XORC binding sites are spaced 5–15 kb on the DNA, and XORC fills these sites during late mitosis. These origins become licensed for replication, and this state persists throughout G1 (Fig. 10 C, i). At the start of S phase, clusters of origins fire synchronously to give a burst of initiation events (Fig. 10 C, ii). As each origin initiates, it converts to the unlicensed state so that the origin will not fire again. The forks initiated within each cluster replicate the intervening DNA and terminate as they encounter one another (Fig. 10 C, iii). As this is occurring, further clusters of licensed origins fire synchronously to replicate the DNA within the unreplicated gaps, and this process continues until all the DNA has been replicated (Fig. 10 C, iv).

While this paper was under revision, a paper was published by Herrick et al. (2000) describing an analysis of origin distribution in the *Xenopus* system, using a DNA spreading technique called “molecular combing” and a fluorescence-based detection system for newly replicated DNA. The authors concluded that replication origins are randomly distributed in the *Xenopus* system. We believe that this conclusion is incorrect. The fluorescence-based detection system used by Herrick et al. has a significantly lower spatial resolution than is given by fiber autoradiography, and prevents the unambiguous identification of replicons at the lower end of the range that we observe. Even with this lower spatial resolution, the center-to-center data presented by Herrick et al. much more closely resemble the distribution predicted for regularly spaced origins (Fig. 10 B) than randomly spaced origins (Fig. 10 A). Herrick et al. also failed to note any origin clustering, which has important implications for the interpretation of their data. Moreover, Herrick et al. provide no explanation for the XORC binding data or for the “random completion problem.” We are therefore confident that our description of a regular 5–15-kb origin distribution is correct and can provide an explanation for the establishment of replication origins in the early *Xenopus* embryo that is consistent with all currently available data.

Mechanism of Origin Spacing

It is currently unclear how the spacing of replication origins is achieved in this system, although it appears to be mediated by restricting the binding of XORC to chromatin. Two general types of mechanism can be envisaged. In the first, specific sites on chromatin where XORC can bind would be present only once every ~5–15 kb. Since origins are positioned randomly with respect to DNA sequence, these XORC binding sites would have to be determined by epigenetic factors such as higher order chromatin structure. One possible candidate for a repeating epigenetic structure with this sort of spacing is the organization of DNA into chromosomal loops (Cook, 1991). The size of these loops shows some correlation with the average replicon size as they both increase during *Xenopus* development (Buongiorno-Nardelli et al., 1982). However, certain

observations suggest that loop size may not in general determine origin position (for review see Hyrien et al., 1997).

In another model to account for regular origin spacing, origins can be assembled at any point on the chromatin, but an "exclusion zone" is created around each origin, preventing another origin from being assembled too close to it. This exclusion zone could be created by the propagation of an altered chromatin configuration that represses XORC binding. Data consistent with this sort of model have recently been presented by Lucas et al. (2000), who observed that small plasmid molecules replicating in the *Xenopus* system had only a single replication bubble. At present it is unclear which of these types of models is correct. Further fractionation of the proteins required to establish replication origins on *Xenopus* sperm chromatin (Gillespie and Blow, 2000) may allow us to understand more about the mechanisms involved.

We would like to thank Alan Prescott for help with microscopy and Anne Donaldson for comments on the manuscript.

This work was funded by the Cancer Research Campaign (grants SP2385/0101 and SP1967/1001).

Submitted: 12 October 2000

Revised: 16 November 2000

Accepted: 17 November 2000

References

- Blow, J.J. 1993. Preventing re-replication of DNA in a single cell cycle: evidence for a replication licensing factor. *J. Cell Biol.* 122:993–1002.
- Blow, J.J., and R.A. Laskey. 1986. Initiation of DNA replication in nuclei and purified DNA by a cell-free extract of *Xenopus* eggs. *Cell.* 47:577–587.
- Blow, J.J., and R.A. Laskey. 1988. A role for the nuclear envelope in controlling DNA replication within the cell cycle. *Nature.* 332:546–548.
- Blow, J.J., and A.M. Sleeman. 1990. Replication of purified DNA in *Xenopus* egg extract is dependent on nuclear assembly. *J. Cell Sci.* 95:383–391.
- Blow, J.J., and J.V. Watson. 1987. Nuclei act as independent and integrated units of replication in a *Xenopus* cell-free system. *EMBO (Eur. Mol. Biol. Organ.) J.* 6:1997–2002.
- Blumenthal, A.B., H.J. Kriegstein, and D.S. Hogness. 1973. The units of DNA replication in *Drosophila melanogaster* chromosomes. *Cold Spring Harb. Symp. Quant. Biol.* 38:205–223.
- Buongiorno-Nardelli, M., G. Micheli, M.T. Carri, and M. Marilley. 1982. A relationship between replicon size and supercoiled loop domains in the eukaryotic genome. *Nature.* 298:100–102.
- Callan, H.G. 1972. Replication of DNA in the chromosomes of eukaryotes. *Proc. R. Soc. Lond. B Biol. Sci.* 181:19–41.
- Chong, J.P.J., P. Thömmes, and J.J. Blow. 1996. The role of MCM/P1 proteins in the licensing of DNA replication. *Trends Biochem. Sci.* 21:102–106.
- Chong, J.P.J., M.H. Mahubani, C.Y. Khoo, and J.J. Blow. 1995. Purification of an Mcm-containing complex as a component of the DNA replication licensing system. *Nature.* 375:418–421.
- Chong, J.P.J., P. Thömmes, A. Rowles, H.M. Mahubani, and J.J. Blow. 1997. Characterization of the *Xenopus* replication licensing system. *Methods Enzymol.* 283:549–564.
- Cook, P.R. 1991. The nucleoskeleton and the topology of replication. *Cell.* 66:627–635.
- Diffley, J.F. 1996. Once and only once upon a time: specifying and regulating origins of DNA replication in eukaryotic cells. *Genes Dev.* 10:2819–2830.
- Gillespie, P.J., and J.J. Blow. 2000. Nucleoplasmin-mediated chromatin remodeling is required for *Xenopus* sperm nuclei to become licensed for DNA replication. *Nucleic Acids Res.* 28:472–480.
- Graham, C.F. 1966. The regulation of DNA synthesis and mitosis in multinucleate frog eggs. *J. Cell Sci.* 1:363–374.
- Hand, R. 1975. Regulation of DNA replication on subchromosomal units of mammalian cells. *J. Cell Biol.* 64:89–97.
- Hand, R. 1978. Eucaryotic DNA: organization of the genome for replication. *Cell.* 15:317–325.
- Hara, K., P. Tydeman, and M. Kirschner. 1980. A cytoplasmic clock with the same period as the division cycle in *Xenopus* eggs. *Proc. Natl. Acad. Sci. USA.* 77:462–466.
- Harland, R.M., and R.A. Laskey. 1980. Regulated DNA replication of DNA microinjected into eggs of *Xenopus laevis*. *Cell.* 21:761–771.
- Herrick, J., P. Stanislawski, O. Hyrien, and A. Bensimon. 2000. Replication fork density increases during DNA synthesis in *X. laevis* egg extracts. *J. Mol. Biol.* 300:1133–1142.
- Hyrien, O., and M. Méchali. 1992. Plasmid replication in *Xenopus* eggs and egg extracts: a 2D gel electrophoretic analysis. *Nucleic Acids Res.* 20:1463–1469.
- Hyrien, O., and M. Méchali. 1993. Chromosomal replication initiates and terminates at random sequences but at regular intervals in the ribosomal DNA of *Xenopus* early embryos. *EMBO (Eur. Mol. Biol. Organ.) J.* 12:4511–4520.
- Hyrien, O., C. Maric, and M. Méchali. 1995. Transition in specification of embryonic metazoan DNA replication origins. *Science.* 270:994–997.
- Hyrien, O., C. Maric, and I. Lucas. 1997. Role of nuclear architecture in the initiation of eukaryotic DNA replication. *Biochimie.* 79:541–548.
- Jackson, D.A., and A. Pombo. 1998. Replicon clusters are stable units of chromosome structure: evidence that nuclear organization contributes to the efficient activation and propagation of S phase in human cells. *J. Cell Biol.* 140:1285–1295.
- Kidd, A.D., D. Francis, and M.D. Bennett. 1987. Replicon size, mean rate of DNA replication and the duration of the cell cycle and its component phases in eight monocotyledons of contrasting DNA C values. *Ann. Bot.* 59:603–609.
- Kimelman, D., M. Kirschner, and T. Scherson. 1987. The events of the mid-blastula transition in *Xenopus* are regulated by changes in the cell cycle. *Cell.* 48:399–407.
- Laskey, R.A. 1985. Chromosome replication in early development of *Xenopus laevis*. *J. Embryol. Exp. Morphol.* 89:285–296.
- Lucas, I., M. Chevrier-Miller, J.M. Sogo, and O. Hyrien. 2000. Mechanisms ensuring rapid and complete DNA replication despite random initiation in *Xenopus* early embryos. *J. Mol. Biol.* 296:769–786.
- Mahubani, H.M., T. Paull, J.K. Elder, and J.J. Blow. 1992. DNA replication initiates at multiple sites on plasmid DNA in *Xenopus* egg extracts. *Nucleic Acids Res.* 20:1457–1462.
- Méchali, M., and S. Kearsley. 1984. Lack of specific sequence requirement for DNA replication in *Xenopus* eggs compared with high sequence specificity in yeast. *Cell.* 38:55–64.
- Newport, J. 1987. Nuclear reconstitution in vitro: stages of assembly around protein-free DNA. *Cell.* 48:205–217.
- Parra, I., and B. Windle. 1993. High resolution visual mapping of stretched DNA by fluorescent hybridization. *Nat. Genet.* 5:17–21.
- Rogers, A.W. 1973. Techniques of Autoradiography. Elsevier, London. 372 pp.
- Rowles, A., J.P. Chong, L. Brown, M. Howell, G.I. Evan, and J.J. Blow. 1996. Interaction between the origin recognition complex and the replication licensing system in *Xenopus*. *Cell.* 87:287–296.
- Rowles, A., S. Tada, and J.J. Blow. 1999. Changes in association of the *Xenopus* origin recognition complex with chromatin on licensing of replication origins. *J. Cell Sci.* 112:2011–2018.
- Van't Hof, J. 1975. DNA fiber replication in chromosomes of a higher plant (*Pisum sativum*). *Exp. Cell Res.* 93:95–104.
- Van't Hof, J., A. Kuniyuki, and C.A. Bjerknes. 1978. The size and number of replicon families of chromosomal DNA of *Arabidopsis thaliana*. *Chromosoma.* 68:269–285.
- Walter, J., and J.W. Newport. 1997. Regulation of replicon size in *Xenopus* egg extracts. *Science.* 275:993–995.