Replication occurs at discrete foci spaced throughout nuclei replicating in vitro

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Summary

Demembranated Xenopus sperm nuclei were induced to replicate synchronously in a low-speed supernatant (LSS) of Xenopus eggs by preincubation in a high-speed supernatant (HSS). DNA replication was observed by incorporation of [α-32P]dATP, BrdUTP or biotin-dUTP.

Biotin-dUTP incorporation, visualised with fluorescent streptavidin, reveals a striking pattern of replication foci throughout replicating nuclei. We show that this represents a precursor to the bright uniform fluorescence seen later. Confocal microscopic analysis of nuclei fixed early in replication reveals that these foci of DNA replication number about 100–300 for each nucleus and probably represent the replicon clusters already described for tissue culture cells. Foci are evenly distributed throughout the nuclei and are not concentrated at or near the nuclear envelope.

Complete replication of each nucleus occurs in an average time of only one hour in this system. Hence we calculate that there must be at least 300–1000 replication forks together in each cluster. Furthermore, pulse labelling at later times in the period of replication reveals a similar pattern of foci indicating that replication forks remain tightly clustered in groups of at least 300 throughout the period of DNA replication.

Key words: DNA replication, replicon clusters, Xenopus egg extract, nuclear assembly, confocal microscopy, sites of initiation of DNA synthesis, sites of elongation of DNA synthesis.

Introduction

Eukaryotic DNA synthesis involves initiation at multiple sites. The spacing between consecutive initiations has been studied by fibre autoradiography in which DNA is labelled during a radioactive pulse and visualized by autoradiographic silver tracks (reviewed by Edenberg and Huberman, 1975; Hand, 1978). The results of such studies reveal that there are around 100 000 initiations in a mammalian nucleus spaced at average intervals of between 50 000 and 300 000 base pairs (bp).

There is growing evidence of the importance of nuclear structure in the regulation of DNA replication. First, DNA replication in cell-free systems from Xenopus eggs shows a striking correlation with the efficiency of nuclear assembly (Blow and Laskey, 1986; Blow and Watson, 1987; Newport, 1987; Sheehan et al. 1988). Each nucleus in a cell-free extract behaves as an integrated and independent unit of replication (Blow and Watson, 1987), and procedures that inhibit assembly of the nuclear envelope also inhibit replication (Sheehan et al. 1988). Second, studies of the nuclear matrix (reviewed by Verheijen et al. 1988) and replication-associated proteins have suggested that replication occurs at fixed sites within the nucleus (Nakamura et al. 1984, for polymerase α; Miyachi et al. 1978; Takasaki et al. 1981; Celis and Celis, 1985a,b; Bravo and McDonald-Bravo, 1985, for PCNA; Hutchison and Kill, 1989, for both PCNA and polymerase α). Early studies on the nuclear matrix were challenged as possible artefacts induced by the high salt concentrations used in their purification. However, more recent studies using agarose beads have avoided this (Jackson and Cook, 1986a,b,c) and in particular have also shown that a substantial proportion of the aphidicolin-sensitive DNA polymerases is immobilized on a skeletal structure.

We used a cell-free system from Xenopus eggs that initiates and completes DNA replication in vitro (Blow and Laskey, 1986). DNA replication in this system was monitored by studying the incorporation of biotinylated dUTP as revealed by fluorescent streptavidin binding (Blow and Watson, 1987). We observed a striking pattern of discrete foci of replication, similar to patterns seen for cultured cells in vitro (Nakamura et al. 1986; Bravo and McDonald-Bravo, 1987; de Bruyn Kops and Knipe, 1988; Nakayasu and Berezney, 1989). A scanning laser
microscope was used to analyse the distribution of foci in synchronously replicating nuclei in the cell-free system. We calculate that each focus must contain at least 300 replication forks and that they remain similarly clustered throughout the period of DNA replication.

Materials and methods

Replication of nuclei in vitro

Extracts were prepared from activated eggs of *Xenopus laevis* as either a low-speed supernatant (LSS) (Blow and Laskey, 1986) or a high-speed supernatant (HSS) (referred to as the soluble fraction by Sheehan et al. 1988). The HSS lacks the larger particulate matter (e.g. membrane vesicles) and so is unable to form nuclear envelopes around chromatin.

Incubation of demembranated *Xenopus* sperm nuclei in LSS was performed as described before (Blow and Laskey, 1986). Alternatively, nuclei were preincubated in HSS before LSS and supplemented with 60 mM-phosphocreatine (Sigma), 150 μg ml⁻¹ creatine phosphokinase (Sigma). Usually 8–12 ng ml⁻¹ demembranated sperm nuclei were added and preincubated at 22°C for 1 h. During this period the sperm chromatin decondensed. LSS, thawed and supplemented with energy regenerator as before, was added at a ratio of 3:1 (LSS:HSS) and incubation proceeded at 22°C. Replication was detected by continuous or pulse labelling with either 20 μM-5-biotin-11-deoxyuridine triphosphate (biotin-11-dUTP, Enzo) or 20 μM-5-biotin-19-deoxyuridine triphosphate (biotin-19-dUTP, Calbiochem), 500 μM-5-bromodeoxyuridine triphosphate (BrdUMP, Sigma), or 200 μCi ml⁻¹ deoxyadenosine-5'-[α-³²P]triphosphate (³²P)dATP, Amersham).

Radiolabelling

For measurement of [³²P]dATP incorporation duplicate samples were dried onto Whatman GF/C filter papers, precipitated with trichloroacetic acid (TCA), washed with ethanol, dried and counted in Optiscint (LKB Pharmacia).

Quantification of DNA replication was based on a dATP pool size of 50 μM (Blow and Laskey, 1986) and hence the percentage of [³²P]dATP incorporated ×0.654 yields DNA replicated as ng μl⁻¹ extract.

Microscopy

For microscopic detection of biotin-dUTP incorporation samples containing nuclei were diluted and fixed with ethylene-glycolb(isuccinimidylsuccinate) (EGB) (Blow and Watson, 1987) before overlaying onto 1 ml of 30% sucrose in buffer A (Blow and Watson, 1987), beneath which was a polylysine-coated coverslip. Nuclei were spun onto the coverslips at 3000 revs min⁻¹ for 10 min using a Sorvall RC-5B rotor and Sorvall RC-5B centrifuge.

After centrifugation most of the supernatant was sucked off and the coverslip removed to a buffer A wash in a multwell dish. For the detection of biotinylated derivatives the coverslip was transferred to a second well containing 500 μl buffer A with 1 μl of either fluorescein- or Texas Red-conjugated streptavidin (Amersham) and 10 μl of either 20 μg ml⁻¹ Hoechst 33258 or 12.5 μg ml⁻¹ propidium iodide (PI, Sigma) and 12.5 μg ml⁻¹ RNase A (Sigma). For confocal microscopy fluorescein-streptavidin (green fluorescence) and PI (red fluorescence) were preferred. After incubating with the fluorochromes for 30 min the coverslip was washed in fresh buffer A and distilled water before being mounted in 90% glycerol, 1% DABCO, in phosphate-buffered saline (PBS).

Nuclei were viewed on either an ordinary fluorescence microscope (Nikon Optiphot with Episcopic—Fluorescence attachment EF-D) or a Biorad MRC 500 confocal microscope.

Numerical analysis of confocal photomicrographs

In order to estimate the number of replicon clusters in a nucleus a confocal series of optical sections through the nucleus was made (as in Fig. 3, see below). Since three-dimensional reconstruction software was not available spots of fluorescence representing the replicon clusters were copied onto acetate sheets, the sections reassembled and the significant spots counted. This technique meant that it was also possible to separate replicon clusters that had only vertical displacement.

Measurement of the distance between the centres of adjacent replicon clusters was performed on enlargements of confocal photomicrographs, only the bright (in focus) spots were used and the measurement was made between nearest neighbours from 103 foci in five nuclei. (This method can be used only as an approximate guide to nearest neighbour distance; sometimes out-of-focus foci may be nearest neighbours and so the accuracy of this data is limited by the high resolution of confocal microscopy.)

Results

DNA is replicated at discrete clustered sites in the nucleus

When demembranated sperm nuclei are incubated in a low-speed supernatant (LSS) of activated *Xenopus* eggs chromatin decondensation and nuclear envelope reformation occur (Lokha and Masui, 1983, 1984; Blow and Laskey, 1986; Sheehan et al. 1988). Subsequently, a single, complete round of DNA replication is visualised by either BrdU or biotin-dUTP incorporation (Blow and Laskey, 1986; Blow and Watson, 1987). Nuclei within this type of incubation behave independently, so that each nucleus takes approximately 60 min to replicate its DNA, but the start of replication is asynchronous, so that replication of all nuclei may not be complete until 4–6 h (Blow and Watson, 1987). Fig. 1 shows the results of labelling newly replicated DNA by incorporation of biotinylated dUTP (present from the start of the incubation) followed by staining with fluorescent streptavidin. In addition to brightly stained and unstained nuclei (Fig. 1B), some show discrete punctate staining (Fig. 1E). The appearance of punctate staining is reproducible and can be seen in a small and variable proportion of the nuclei at any time when replication is detectable in the incubation. Note that the bulk DNA (visualised by Hoechst 33258, Fig. 1A and D; propidium iodide, see below Fig. 5D; or chromomycin, not shown) does not show a punctate pattern, and that a punctate distribution for bulk DNA has never been seen in replicating nuclei.

Punctate nuclei can also be seen in nuclei that have incorporated BrdU, detected using a mouse anti-BrdU antibody followed by a fluorescent rabbit anti-mouse IgG antibody (data not shown), as also reported for embryonic fibroblast nuclei in culture (Nakamura et al. 1986).

With both methods, the proportion of overall brightly staining nuclei increases with incubation time. However,
Fig. 1. Microscopy of demembranated *Xenopus* sperm nuclei replicating in a *Xenopus* activated egg LSS for 1.5 h. Replication is visualised by staining the incorporated biotin-dUTP with fluorescent streptavidin. Biotin-dUTP was present at 20 µM from the start of the incubation. Fig. 1A and B shows a group of nuclei at low magnification, either Hoechst 33258 stained (A) or fluorescent streptavidin stained (B). At higher magnification some nuclei show a punctate pattern of biotin-dUTP incorporation (E) and can be compared with the phase-contrast image (C), or with the total DNA stained with Hoechst 33258 (D).

the asynchrony of replication and the low percentage of nuclei that show the punctate pattern leave open the possibility that punctate nuclei represent a few aberrantly replicating nuclei. Therefore we have modified the incubation procedure to obtain synchronous initiation of replication.

**Replication in the extract can be synchronised to distinguish different stages**

It has been shown previously that incubating demembranated *Xenopus* sperm nuclei in a high-speed supernatant (HSS) of *Xenopus* egg extract will decondense the chromatin without nuclear envelope re-formation or DNA replication (Lokha and Masui, 1984; Sheehan et al. 1988) or low-speed supernatant (LSS, shown here) results in nuclear envelope re-formation and DNA replication.

Fig. 2 presents the results obtained from nuclei pre-incubated for 1 h in HSS at 22°C before addition of LSS at a ratio of 3:1 (LSS:HSS), together with [³²P]dATP and biotin-dUTP. Samples were taken at various times and assayed by TCA precipitation or streptavidin staining followed by fluorescence microscopy. [³²P]dATP incorporation was converted to ng DNA replicated per µl incubation (see Materials and methods), whilst the biotin-dUTP was scored according to the fluorescent pattern of the nuclei (unstained, pale, clearly punctate or bright fluorescence when viewed under a 100X objective lens). (N.B. close examination of bright nuclei frequently reveals some level of punctate appearance so it has been necessary to adopt an arbitrary boundary for this classification.)

Fig. 2A shows that essentially all the nuclei added replicated between 30 and 90 min after addition of the low-speed supernatant (2.2 ng µl⁻¹ replicated compared to an estimated DNA input of 2.1 ng µl⁻¹). There was no significant change in the amount of DNA replicated over the next 60-min period. This contrasts sharply with asynchronous replication in LSS alone, where significant replication can still be occurring 4 h after the addition of LSS to the nuclei (Blow and Watson, 1987).

When nuclei are pre-incubated in HSS, the earliest time-point that shows incorporation of [³²P]dATP (Fig. 2A) or biotinylated dUTP (Fig. 2B) is 30 min after the addition of LSS. Fig. 2B shows that 75% of the nuclei have initiated replication by this time and that 93% have initiated by 45 min. Most of the remaining 7% fail to replicate. Intermediate time-points from other

**Foci of DNA replication**
experiments (not shown) confirm that under these conditions, initiation is nearly synchronous.

**Replication sites are clustered at the earliest stages detectable**

Fig. 2A shows that no detectable DNA has replicated at 15 min and that only 7.1% of the input DNA has replicated at 30 min. Nevertheless, Fig. 2B shows that 75% of the nuclei have initiated replication at 30 min in the same incubation and that 54% of all nuclei show a clear punctate pattern at this early time as exemplified in Fig. 3. There is no evidence of patterning in the bulk DNA distribution within the nucleus, however (Figs 1A,D, 3 and 5D), so the punctate pattern represents the distribution of only nascent DNA, not the bulk DNA.

We cannot distinguish between initiation at clustered sites and initiation at diffuse sites followed by immediate clustering of replication forks. Nevertheless, it is clear that organization into clustered replication foci is an early event.

**Replication clusters are intermediates in complete replication**

Fig. 2B shows that the punctate pattern of fluorescence occurs in 54% of nuclei at 30 min, yet just 15 min later this value has dropped to 17%. In contrast the percentage showing bright fluorescence rose from 21% to 77% in the same samples. The percentage of nuclei that finally show bright fluorescence at 150 min is 94%, at which time the input DNA has completely doubled (Fig. 2A). Therefore, the punctate pattern seen in 54% of the nuclei at 30 min does not appear to represent aberrantly replicating nuclei but to represent an intermediate stage in complete replication of most nuclei.

**Replication sites remain clustered throughout replication**

The data presented so far are from continuously labelled experiments where the fluorescent and radioactive precursors were added at the time of LSS addition. These experiments suggest that early replication events are clustered and that later the whole nucleus becomes fully labelled. However, as explained below, each bright focus must contain at least 300–1000 replication forks, so later stages of replication could proceed in two ways. Either forks could diverge from these clusters throughout the nucleus to replicate the remaining DNA, or alternatively replication forks could remain clustered throughout replication. With the former hypothesis, elongation would show a diffuse fluorescent staining pattern whilst the latter should show replication occurring in a similar clustered manner to initiation. These alternatives were addressed by pulse-labelling studies.

Fig. 4 presents data from a population of near-synchronously replicating nuclei (as in Fig. 2), which were pulse labelled for 5 min with biotin–dUTP and [α-32P]dATP immediately before fixation. This shows a high percentage of punctate nuclei (22–55%) throughout S-phase. Asynchronous initiation within each nucleus is not sufficient to explain the high percentage with a punctate appearance late in S-phase for two reasons. First, significant incorporation between discrete foci is not seen in unenhanced confocal images of pulse-labelled nuclei (see below); and second, a similar punctate patterning persists until the last few minutes of replication. These data therefore indicate that elongation occurs also at clustered foci.

**Replication sites are distributed uniformly throughout the nucleus, not concentrated at the membrane**

Figs 3 and 5 show nuclei either fixed early in the replication cycle with biotin–dUTP added at the time of LSS addition and fixed 35 min later (Fig. 3), or pulsed with biotin–dUTP for 5 min at 60 min after LSS addition (Fig. 5A, B and C). The figures show optical sections through nuclei taken at 1 μm (Fig. 3) or 0.5 μm (Fig. 5).
Fig. 3. Normal fluorescence and confocal microscopy of nuclei fixed at 30–35 min incubation in the LSS. Biotin–dUTP was present from the start, and was revealed by fluorescent streptavidin staining. The fluorescence microscope reveals nuclei showing a punctate pattern of biotin–dUTP incorporation (C and F). This pattern does not coincide with either bulk DNA distribution (B and E, Hoechst 33258 staining) or structures seen under phase-contrast (A and D). Series of optical sections taken through two nuclei at 1 μm intervals on the confocal microscope showing that the foci of biotin–dUTP incorporation are evenly distributed throughout the nuclei (G and H).

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Fig. 4. Nuclei replicating as in Fig. 2 were pulse-labelled with biotin-dUTP for 5 min at various times immediately prior to fixing. Many nuclei show a punctate pattern of incorporation throughout the period of DNA replication. This indicates that replication is occurring at foci throughout the whole period of DNA replication.

intervals using a confocal microscope. From these and other series (not shown) it can be seen that the punctate pattern is distributed throughout the nuclei. The fluorescent spots appear to be as numerous and as closely packed throughout the nucleus as at the nuclear envelope. Furthermore, even at late times (Fig. 5) DNA synthesis occurs almost exclusively at foci. The background nuclear fluorescence seen on the ordinary fluorescent microscope (Fig. 3C and F) is not seen in unenhanced images on the confocal microscope, suggesting that it is the result of out-of-focus flare, the problem that the confocal microscope is designed to overcome.

Blow and Watson (1987) estimated that *Xenopus* sperm nuclei would require at least $5 \times 10^4$ initiations per haploid genome in order to complete replication in one hour even if they all initiate synchronously (within a single nucleus). However, it is clear from the micrographs shown here (Figs 1, 3 and 5) that there are not $5 \times 10^4$ fluorescent foci. Three-dimensional reconstructions of serial sections indicate between 100 and 300 fluorescent foci per nucleus. This number is similar to the average of 126 shown for BrdU-labelled tissue culture cells (Nakamura et al. 1986), even though the time of replication is very different in these two systems.

Measurements were made from much enlarged photographs of confocal sections to estimate the distance between adjacent fluorescent foci. The mean nearest neighbour distance is 0.86 μm (average of 103 measurements). This must be regarded as an approximation, since the resolving power of the confocal microscope would exclude nearer foci that are in another focal plane.

Discussion

*Most nuclei show clustered foci of DNA replication*

In order to study the temporal relationship between DNA replication and nuclear organisation, we developed a protocol that results in synchronised rapid DNA replication *in vitro*. Using this protocol input DNA doubles within one hour and 75% of all nuclei enter replication within a 15 min period (Figs 2A and B). In addition Fig. 4 shows that nuclei stop replicating in broad synchrony.

Biotin–dUTP labelling of nuclei synchronised in this way illustrates two points. First, the high percentage of punctate nuclei seen at the 30 min time-point shows a direct precursor relationship to the high percentage of completely bright nuclei seen at 45 min (Fig. 2B). Therefore, the punctate appearance of newly incorporated biotin–dUTP represents normal replication in this system. Second, since these punctate nuclei were fixed before replication proceeded sufficiently to show only a bright appearance, the punctate pattern reflects the localisation of early replication; that is, the sites of initiation.

Replication is not preferentially associated with the nuclear envelope

A striking feature of the cell-free replication system from *Xenopus* eggs is the requirement for a nuclear membrane for initiation of DNA replication (Lokha and Masui, 1984; Blow and Laskey, 1986; Newport, 1987; Hutchinson et al. 1987). One possible explanation for this would be a requirement for membrane attachment of DNA for initiation of replication. This would be consistent with the numerous reports that DNA replication occurs on a fixed matrix, scaffold, skeleton or cage (Berezney and Coffey, 1975; Dijkwel et al. 1979; Pardoll et al. 1980; McCreedy et al. 1980; Berezney and Buchholtz, 1981). However, these studies would be equally compatible with the distribution of the fixed sites throughout the nucleus rather than just at the membrane. Indeed, Huberman et al. (1973) argued that replication occurs throughout the nucleus rather than at the nuclear envelope, on the basis of autoradiography of $[^3H]$thymidine incorporation. The increased spatial resolution of the scanning confocal microscope and biotinylated precursors establish unequivocally that all detectable stages of replication occur at sites distributed throughout the nucleus and not localised at the membrane.

**Replication forks stay clustered throughout the replication cycle**

Figs 3 and 4 show that the number, size and distribution of replication foci remain roughly constant throughout the time taken to replicate a nucleus.

The number of major foci estimated from three-dimensional reconstructions of optical sections (e.g. Fig. 5) is 100–300 foci per nucleus. In contrast, at least 50 000 initiations ($10^6$ replication forks) would be necessary to replicate $3 \times 10^9$ bp of DNA in 1 h at the *in vivo* fork movement rate of about 800 bp s$^{-1}$ (Callan, 1972) even when initiation was entirely synchronous. This means that there must be at least 330, and possibly more than 1000, replication forks in each bright cluster.

The persistence of a similar pattern of replication foci throughout the period of replication without a raised level of background incorporation indicates that forks also...
Fig. 5. Confocal microscopy of nuclei pulse-labelled with biotin–dUTP for 5 min immediately before fixation late in the replication period (65 min after LSS addition). Many nuclei show a punctate pattern (A–C, fluorescent streptavidin stained; A and B, four sections through single nuclei; C, one section through two nuclei; D, four sections through three nuclei PI-stained for total DNA). This suggests that at a time when >80% of nuclei would show a totally bright fluorescence in continuous label (Fig. 2B) DNA replication is still occurring at foci similar in number and distribution to those seen at initiation (Fig. 3). Bar, 10 μm.
remain tightly clustered instead of dispersing to replicate the surrounding DNA. It appears to us that this could be achieved in two ways: either chromatin fibres must be arranged in bundles of at least 300 parallel DNA molecules, so that forks can proceed along them uniformly in a concerted fashion; or, alternatively, the forks could remain immobilized at fixed sites, with DNA being spooled through from the surrounding area, as has been suggested by many groups in studies of the nuclear matrix, cage or skeleton (reviewed by Verheijen et al. 1988).

The first of these possibilities predicts the organisation of the interphase nucleus into many bundles of parallel fibres, which have not been observed in diploid (or haploid) nuclei to our knowledge and which we regard as unlikely. The second possibility appears more likely to us and we think that these observations refocus attention on models of immobilised replication complexes and dynamic DNA loops.

Mullinger and Johnson (1983, 1987) have shown that artificially induced premature condensation of replicating chromosomes produces segmented chains of clustered chromatin fibres. Our data suggest that the haploid Xenopus sperm nucleus would have an average of 10 replication foci per chromosome, and so could be the structural basis for the segmentation of chromosomes seen by Mullinger and Johnson. We also suggest that the foci might provide the physical basis for observations that different large regions of animal chromosomes replicate at specific times in the cell cycle (Hand, 1978).

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References


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