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Nuclei act as independent and integrated units of replication in a *Xenopus* cell-free DNA replication system

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We have used a novel approach to investigate the control of initiation of replication of sperm nuclei in a *Xenopus* cell-free extract. Nascent DNA was labelled with biotin by supplementing the extract with biotin-11-dUTP, and isolated nuclei were then probed with fluorescein-conjugated streptavidin. Flow cytometry was used to measure the biotin content of individual nuclei and their total DNA content. This showed that incorporation of the biotinylated precursor increases linearly with DNA content. Haploid sperm nuclei replicate fully to reach the diploid DNA content over 2–6 h in the extract. Synthesis stops once the diploid DNA content is reached. Different nuclei enter S phase at different times over >1.5 h, although they share the same cytoplasmic environment. Nuclei reach their maximum rates of synthesis soon after entry into S phase and some replicate fully in <0.5 h, resembling the rates of replication observed in the intact egg. These results indicate that initiations are coordinated within each nucleus such that the nucleus is the fundamental unit of replication in the cell-free system.

Key words: *Xenopus* egg extract/DNA replication/nuclear assembly/biotin-11-dUTP/flow cytometry

Introduction

When eukaryotic DNA replicates, thousands of initiation events must be coordinated in order for the genome to be replicated precisely once per cell cycle. Very little is known about how these events are controlled. There are two key questions: what determines when initiation first occurs in the cell cycle; and what determines the ensuing frequency of initiation? The first question relates to how long the G1 phase lasts, and how the transition into S phase occurs. In this paper we refer to this transition as entry into S phase. The second question relates to the spatial and temporal control of initiation events in the nucleus.

Lohka and Masui (1983, 1984) described a cell-free system derived from *Xenopus* eggs that is able to form apparently normal interphase nuclei from highly condensed sperm nuclei. This system has recently been shown by Blow and Laskey (1986) to initiate and complete DNA replication on nuclei or naked DNA introduced into it. These authors suggested that *in vitro* initiation might result from the ability of the extract to assemble both sperm nuclei and naked DNA into nucleus-like structures. Similar results have been reported by Newport (1987).

Many thousands of sperm nuclei may be replicated together in the common cytoplasmic environment of the egg extract. Although this situation is never encountered by the early *Xenopus* embryo, it can shed light on the way in which the events of S phase are replicated. The aim of this paper is to exploit this feature of the *in vitro* system to investigate the possible relationship between replication and nuclear organization. We demonstrate that individual sperm nuclei replicate rapidly and asynchronously in the *Xenopus* egg extract, indicating that many thousands of initiation events must occur coordinately within individual nuclei, and that nuclei behave as independent units of replication. Whatever control is exerted by the cytoplasm, replication in this system is also controlled at the level of the individual nucleus.

Results

DNA synthesis in nuclei can be quantified using biotin-11-dUTP

Nuclei were incubated in the cell-free extract of *Xenopus* eggs as described by Blow and Laskey (1986), where they initiate and complete DNA replication over a 6-h period. In order to study the replication kinetics of individual nuclei we exploited the observation of Langer *et al.* (1981) that biotin-11-dUTP is a substrate for eukaryotic DNA polymerase α. Sperm nuclei were incubated in the extract with biotin-11-dUTP and probed with either fluorescent-labelled or radiolabelled streptavidin. Figure 1 shows micrographs of re-isolated nuclei labelled with Texas Red–streptavidin and Hoechst 33258. The highly condensed input sperm nuclei shown in Figure 1A stain very poorly with fluorescent streptavidin. After 30 min in the *Xenopus* egg extract (Figure 1B), before DNA synthesis has started, the sperm nuclei have decondensed considerably, but still stain only poorly with streptavidin. However, after 2.5 h in the extract (Figure 1C), when a significant amount of DNA replication has occurred, the nuclei stain strongly with fluorescent streptavidin. The acquisition of streptavidin staining can be prevented by the inclusion of aphidicolin (an inhibitor of DNA polymerase α) in the incubation, as shown in Figure 1D. In parallel experiments DNA was isolated, blotted onto nitrocellulose and probed with [^{125}I]streptavidin. This showed that biotin incorporation was directly proportional to the extent of *in vitro* replication, and was resistant to protease and RNase treatment (data not shown). These results suggest that biotin-11-dUTP and fluorescent streptavidin can be used to monitor the replication of individual nuclei *in vitro*.

Fluorescently labelled nuclei can be analysed by flow cytometry. They are introduced one at a time into a flow chamber, where they are excited by laser light. A series of photomultipliers measures the ensuing fluorescence at different wavelengths. Data relating to the fluorescein fluorescence (for biotin), propidium iodide fluorescence (for total DNA) and light scatter of each nucleus is stored for subsequent analysis. Thus the relationship between biotin content and total DNA content can be investigated.

Figure 2 shows a typical population of nuclei after an incubation of 1.5 h with biotin-11-dUTP, stained for total DNA (Figure 2A) and biotin (Figure 2B). There is marked heterogeneity: some staining strongly with streptavidin, and some hardly at all. Flow cytometry reveals a clear relationship between biotin and total DNA content (Figure 2C). The data are presented in the form of a contour plot showing the distribution of nuclei with respect to total DNA content (horizontal axis) and biotin content

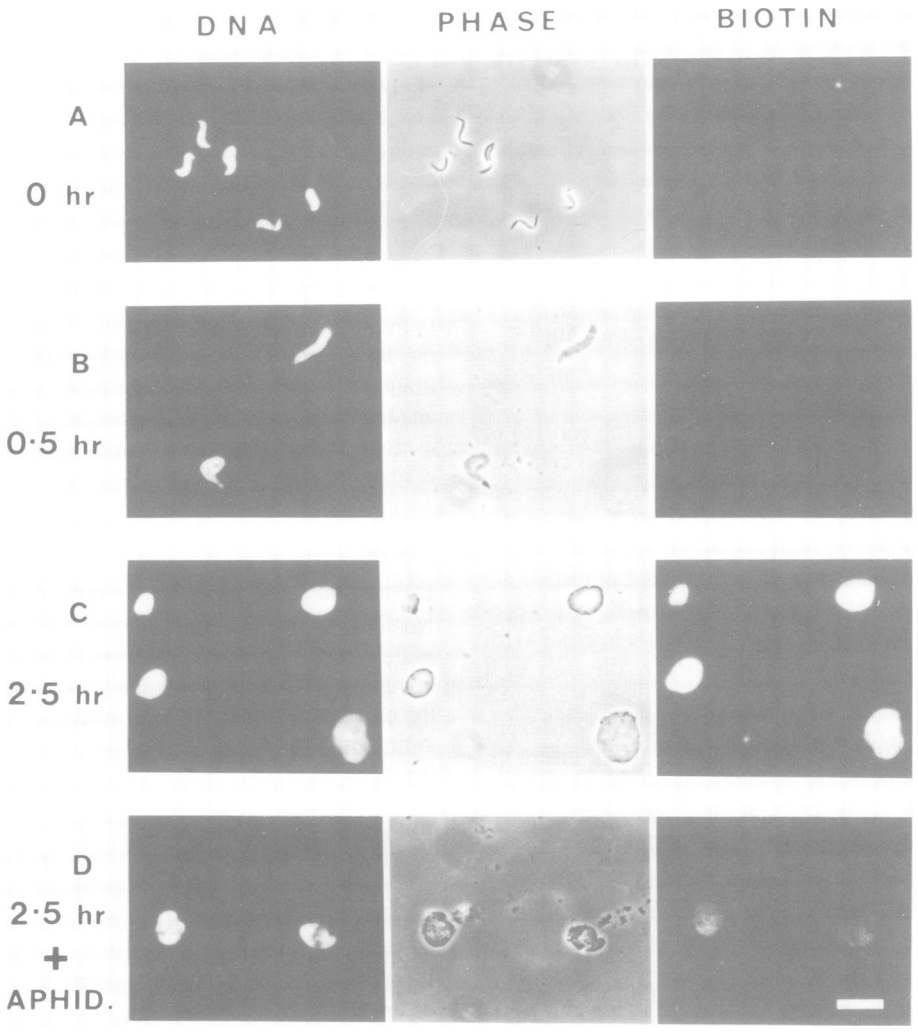

Fig. 1. Incorporation of biotin into nuclei replicated *in vitro* in the presence of biotin-11-dUTP. Sperm nuclei were incubated with 40 μM biotin-11-dUTP in the cell-free replication system for varying lengths of time. They were then isolated and stained with Hoechst 33258 (DNA fluorescence) and Texas Red–streptavidin (biotin fluorescence). Stained nuclei were viewed wet under fluorescence and phase contrast optics. Incubation conditions were: **(A)** 0 h; **(B)** 0.5 h; **(C)** 2.5 h; **(D)** 2.5 h plus 30 μg/ml aphidicolin. Scale bar (for all fields), 20 μm.

(vertical axis). The arrows show the haploid (1N) and diploid (2N) DNA amounts. Virtually all the nuclei fall on a straight line between these two values, demonstrating that the biotin content of an individual nucleus is a direct measure of its DNA replication *in vitro*. The tightness of the straight line between the haploid and diploid populations justifies the separate use of these two parameters to quantify replication kinetics.

Figure 2C also shows some important features of DNA replication *in vitro*. First, most nuclei are either haploid or fully diploid, with only a small proportion in S phase between the two (shown clearly on the cross-sectional view along the horizontal axis). This profile is consistent with individual nuclei replicating fast but asynchronously, rather than as a single cohort. Second, there are no nuclei with DNA or biotin contents greater than diploid. Neither are there haploid nuclei which are labelled with biotin. This shows that no nuclear DNA is replicated more than once *in vitro*. Third, the propidium iodide fluorescence of the highly condensed input sperm nuclei is only ~75% of haploid, due to the limited accessibility of propidium iodide to condensed chromatin (Darzynkiewicz *et al.*, 1977a,b). This can be used to monitor the rate of decondensation of sperm nuclei *in vitro*.

Different nuclei enter S phase at different times in the same extract

Nuclei labelled *in vitro* and displayed as in Figure 2C can be divided into four different subsets: haploid condensed, haploid decondensed, S phase and diploid. The proportions in each subset changes over time (Figure 3). The haploid population, which accounts for all the nuclei at the start of the incubation, falls to represent <20% of all nuclei by 2 h, as nuclei start to replicate. However, nuclei clearly enter S phase at different times over most of this 2-h period. Most nuclei also replicate fast: two thirds of all nuclei that replicate fully have done so by 2 h. The diploid population at 1.5 h is larger than the total number of nuclei that had started replication by 1 h, indicating that some nuclei can replicate fully in <0.5 h. This result is confirmed by pulse-label experiments (see below). Since nuclei replicate both asynchronously and fast, some nuclei may enter S phase after others have replicated fully. In Figure 3 this situation arises between the 1.5 and 2 h time points. As no DNA is re-replicated *in vitro*, this emphasizes the ability of the extract to distinguish replicated from unreplicated DNA. The overall rate of DNA synthesis *in vitro* is more dependent on the rate at which nuclei enter S phase than

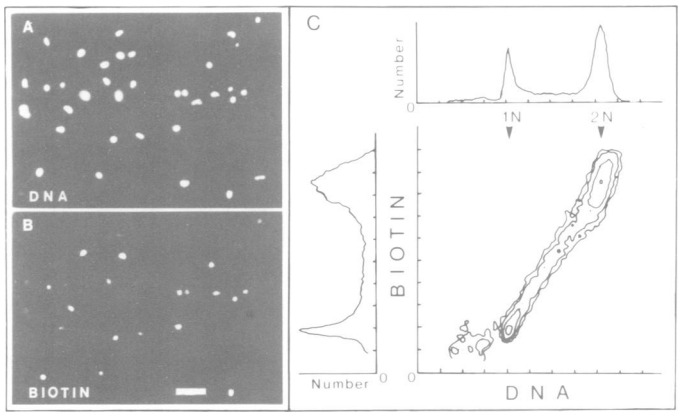

Fig. 2. Relationship between biotin incorporation and DNA content of nuclei labelled *in vitro* with biotin-11-dUTP. Sperm nuclei were incubated for 1.5 h in extract with 8 μM biotin-11-dUTP. Nuclei were then isolated and stained with fluorescent streptavidin and a DNA-binding fluorochrome. **(A)** Nuclei stained with Hoechst 33258 to show total DNA. **(B)** The same field as in **(A)**, but stained with Texas Red–streptavidin to show biotin. Scale bar 50 μm (also for **A**). **(C)** Nuclei were stained with fluorescein–streptavidin and propidium iodide and analysed by flow cytometry to measure the biotin and DNA content of each nucleus. This gives each nucleus a particular position on a graph with two axes representing biotin and DNA content. The distribution of all the nuclei is shown here as a contour plot, representing the density of nuclei in a given area. Contour levels are 3, 8, 30 and 100 in arbitrary units. Standard two-dimensional frequency plots of numbers of nuclei against DNA and against biotin are shown flanking the contour plot. 1N and 2N show the haploid and diploid DNA contents. Nuclei with DNA contents less than haploid largely represent highly condensed sperm nuclei (see data analysis section of Materials and methods). Different fluorochromes are required for flow cytometry to those used in microscopy so that they may be excited by the same light source.

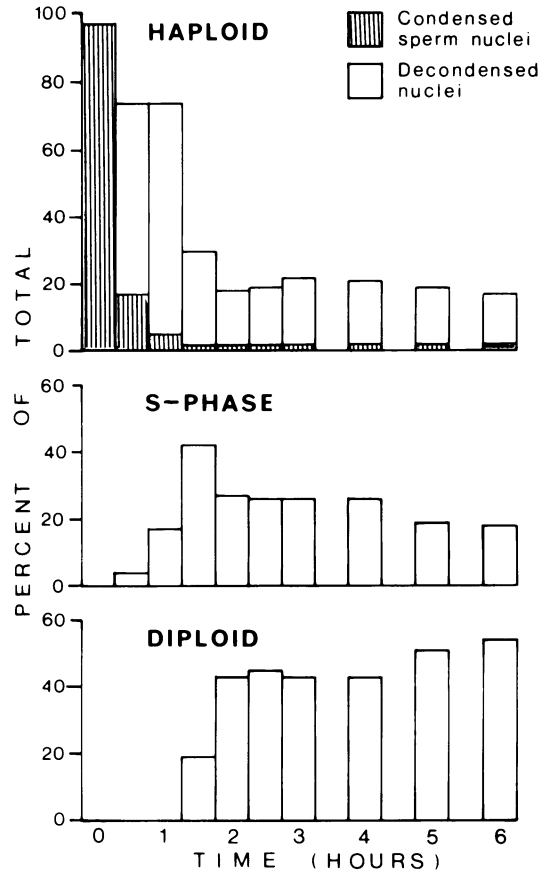

Fig. 3. Time course of replication of nuclei continuously labelled with biotin-11-dUTP. Sperm nuclei were incubated with 8 μM biotin-11-dUTP in the cell-free system, and were isolated for flow cytometric analysis at different times. For each timepoint the number of nuclei in each of four subsets was measured: haploid condensed and haploid decondensed (top panel); S phase (middle panel) and diploid (bottom panel). The numbers are expressed as percentages of all nuclei present. For all timepoints except for 0.5 h, >85% of nuclei fell into one or other of these groups. Most of the remaining nuclei had low DNA contents, and probably represented nuclear fragments. For the 0.5-h timepoint, only 78% of nuclei were classifiable, probably because they are particularly fragile at this stage.

on the time required for individual nuclei to replicate fully. However, some nuclei replicate slowly, resulting in continued synthesis beyond the first 2 h.

Due to their poor staining with propidium iodide, condensed sperm nuclei can be distinguished by flow cytometry (see also Figure 1, panels A and B). They are never observed replicating, and most decondense within 0.5 h. Further, the proportion of nuclei still condensed at 0.5 h (17%) is equal to the number of nuclei still haploid at 6 h. As judged by their light scatter, only nuclei above a certain size enter S phase (data not shown). Thus initiation of DNA replication is dependent on some degree of nuclear formation.

Nuclei reach maximum replication rates soon after entry into S phase

The kinetics of replication can be studied in greater detail by pulse-labelling with biotin-11-dUTP for 0.5 h at different times. This gives eight distinct populations of nuclei, compared with the four with continuous label. The haploid condensed and haploid decondensed populations are the same in both pulse- and continuous-label experiments. However, in pulse-label experiments, the S phase and diploid population can each be divided into three separate subsets depending on their time and rate of replication. These are summarized in Table I; see data analysis in Materials and methods and Figure 5 for details.

Figure 4 shows histograms of the numbers of nuclei in the eight different populations over a 6-h pulse-label experiment. Note that total numbers of nuclei in haploid, diploid and S phase populations are similar to those in the continuous labelling experiment (Figure 3). Figure 4 provides additional information about the rate of replication. In the 1.5-h timepoint, 6% of nuclei were fully replicated during the 0.5-h pulse ('diploid/max biotin' subset). A few of these nuclei are also present in the 2-h timepoint. This clearly demonstrates that some nuclei can replicate fully in <0.5 h, and that replication *in vitro* can approach *in vivo* rates.

The average replication rates of nuclei as they pass from haploid to diploid are shown in Table II. The average nucleus at each timepoint enters S phase at a replication rate of 72–128% of the haploid genome per hour ('S/max biotin' subset). However, nuclei at subsequent stages replicate at lower average rates ('S/part biotin' and 'diploid/part biotin' subsets). The arrival of nuclei in the diploid population over the first 2 h can be accounted for by nuclei continuing replication at initial, or less than initial, rates. Further, some nuclei enter S phase, but stop synthesizing DNA before reaching the diploid DNA content ('S/no biotin' subset). The early appearance of these nuclei (Figure 4) indicates that they are as likely to arise from nuclei which had entered S phase early as from nuclei which had entered late. Thus by their first 0.5 h in S phase, nuclei have reached their maximum replication rates.

These results show how initiation of DNA replication is controlled in the *Xenopus* egg extract. The initiation events in an

Table I. Key to classes of nuclei found in pulse-label experiments plotted in Figure 4

Class	Description
Haploid condensed	Highly condensed sperm nuclei, as shown in Figure 1A.
Haploid decondensed	Decondensed sperm nuclei, as shown in Figure 1B.
Diploid/max biotin	Nuclei fully replicated during the pulse; biotin content therefore the same as diploid nuclei in continuous label experiment (Figure 2C).
Diploid/no biotin	Nuclei fully replicated before the start of the pulse, containing no biotin despite diploid DNA content.
Diploid/part biotin	Nuclei that entered S phase before the pulse, and reached the diploid DNA content during the pulse. Biotin content therefore less than maximum.
S/max biotin	Nuclei that entered S phase during the pulse, but failed to reach the diploid DNA content. Biotin content therefore maximum with respect to DNA content, as S phase nuclei in continuous label experiments (Figure 2C).
S/no biotin	Nuclei that entered S phase before the pulse, but did not replicate at all during the pulse, so contain no biotin.
S/part biotin	Nuclei that entered S phase before the pulse, replicated partially during the pulse, but failed to reach the diploid DNA content. Biotin content therefore less than maximum with respect to DNA content.

See data analysis section of Materials and methods for full details.

individual nucleus are coordinated, so that on entry into S phase an individual nucleus soon reaches its maximal rate of replication. This coordination occurs at the nuclear level as different nuclei enter S phase at different times.

Discussion

Use of biotin-11-dUTP to quantify DNA replication in intact nuclei

We have employed the novel technique of using biotinylated dUTP and fluorescent streptavidin to label nuclear DNA replicated *in vitro* (Langer *et al.*, 1981) in conjunction with flow cytometric analysis. Biotin incorporation into DNA is RNase- and protease-resistant, and is directly proportional to the extent of *in vitro* replication. Flow cytometry shows that the biotin content of individual nuclei increases linearly with their extent of DNA replication, as judged by the total DNA content. Thus biotin labelling and flow cytometry can accurately quantify the extent of replication in whole nuclei.

Agreement of flow cytometry with biochemical analysis of replication

The kinetic data presented here agree well with the biochemical data of Blow and Laskey (1986) who reported that 70–100% of DNA in sperm nuclei was replicated over 6 h under optimal conditions. Flow cytometry shows that after 6 h *in vitro* 60% of the sperm nuclei are completely replicated, and a further 20% are partially replicated. If the propidium iodide fluorescence of the partially replicated nuclei is used to assess extent of replication, and unclassifiable nuclei (<10%) are neglected, this shows replication of 75–80% of the template DNA. Flow cytometry also supports other conclusions drawn by Blow and Laskey from biochemical data. BrdUTP density substitution showed that initiation occurs over a significant period of time, but that no

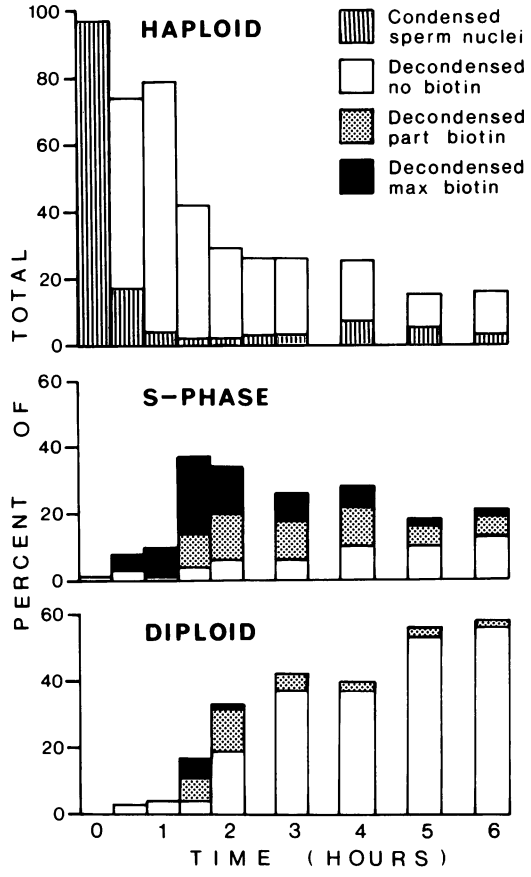

Fig. 4. Time course of replication of nuclei pulse-labelled with biotin-11-dUTP. Sperm nuclei were incubated in the cell-free replication system for different times; 8 μM biotin-11-dUTP was then added, and the incubation allowed to proceed for a further 0.5 h. Nuclei were then isolated and analysed by flow cytometry. The number of nuclei in eight subsets was measured (see Table I for description): haploid condensed and haploid decondensed (top panel); S/max biotin, S/part biotin and S/no biotin (middle panel); diploid/max biotin, diploid/part biotin and diploid/no biotin (bottom panel). The numbers are expressed as percentages of all nuclei present. The presence of early timepoints of small numbers of nuclei with a DNA content greater than haploid, but without biotin, probably represents diploid contaminants of the sperm preparation (Blow and Laskey, 1986).

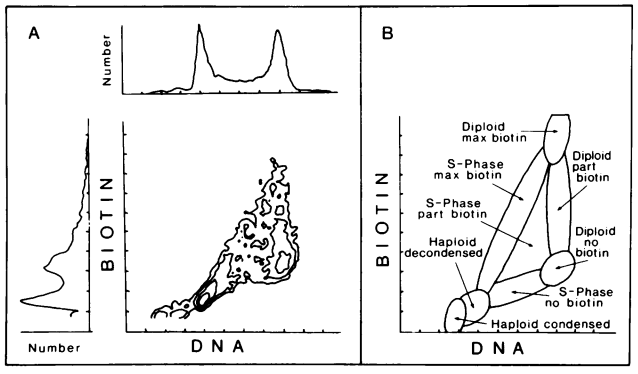

Fig. 5. Sample contour plot of replicating nuclei pulse-labelled with biotin-11-dUTP. **(A)** Sperm nuclei were incubated in the cell-free system for 2 h, with 8 μM biotin-11-dUTP present for the final 0.5 h. Nuclei were then isolated and analysed by flow cytometry. The distribution of nuclei by biotin and DNA content is presented as for Figure 2C. **(B)** Cartoon showing how the contour plot in Figure 5A was subdivided into subsets. Note that because a proportion of the propidium iodide fluorescence bleeds through into the fluorescein channel, the baseline representing no biotin signal runs at an angle of ~30° from the origin through the haploid populations.

Table II. Replication rates of sperm nuclei at different stages of replication, expressed as a percentage of the haploid genome per hour

Subset	Time (h)							
	0.5	1.0	1.5	2.0	3.0	4.0	5.0	6.0
S/max biotin	88	72	128	108	76	100	76	84
S/part biotin	–	50	68	58	44	50	42	38
Diploid/part biotin	–	–	108	78	62	70	66	58

The mean biotin content of nuclei in each subset was measured, and the contribution from propidium iodide bleedthrough subtracted. This figure was then expressed as a percentage of the biotin signal acquired by nuclei replicated fully in the presence of biotin. Since the nuclei in S/max biotin and diploid/part biotin subsets were replicating on average for only half of the pulse, these values were multiplied by two.

DNA is re-replicated. Flow cytometry shows that asynchronous initiation can be accounted for by asynchronous entry of individual nuclei into S phase. Further, no re-replication of nuclei was observed. Instead, haploid nuclei replicate until they reach the diploid DNA content, and then they stop.

Asynchronous entry means that nuclei are independent units of replication

The incubation of many thousands of sperm nuclei together in a common cytoplasmic environment *in vitro* does not correspond to any normal situation in the early *Xenopus* embryo. The asynchronous replication of nuclei *in vitro* contrasts with the synchrony observed in certain multinucleate cells such as *Physarum*. The *in vitro* asynchrony may reflect the inefficiency of processes required for DNA replication (such as chromatin decondensation) which occur much faster *in vivo*, or it may be due to heterogeneity of the sperm nuclei. Whatever its cause, this feature can be exploited to examine the mechanisms by which cytoplasmic factors induce DNA incubated in it to replicate.

After addition of DNA to the extract, there is a lag of 1–2 h, after which synthesis starts abruptly and continues until most of the template DNA has been replicated once (Blow and Laskey, 1986). The data presented here demonstrate that, despite their common environment in the extract, individual nuclei enter S phase at different times over a period of ~1.5 h. Since most nuclei replicate fast (half of all nuclei that enter S phase are diploid by 2 h), the overall rate of replication is mainly dependent on the rate of entry of individual nuclei into S phase. It is possible that this asynchrony reflects heterogeneity of cytoplasmic components in the extract. Although we cannot formally exclude this possibility, asynchrony in both continuous- and pulse-label experiments makes this unlikely, as in the latter case the extract was thoroughly mixed before each timepoint. Therefore DNA in sperm nuclei is not treated homogeneously by the extract, but each nucleus acts as an individual unit of DNA replication.

High initial rates of replication imply coordinated initiation in nuclei

Individual nuclei replicate rapidly in the egg extract. More than 10% of nuclei that replicate fully do so in <0.5 h. This means that the egg extract can reach nuclear replication rates comparable with the intact egg. The average replication rates of nuclei entering S phase are >40% of the genome per 0.5 h pulse. The fork rate of *Xenopus* tissue culture cells is ~8 nucleotides/s (Callan, 1972). With this fork rate, it would require >40 000 synchronous initiations to replicate 40% of the *Xenopus* haploid genome (3×10^9 bp; Dawid, 1967) in 0.5 h. Thus entry into S phase is characterized by a burst of synchronous or near-synchronous initiations in individual nuclei.

On entry into S phase, nuclei have higher average replication rates than nuclei in pulses subsequent to entry. Further, the rate of arrival of nuclei in the diploid subset can be accounted for by nuclei replicating at initial or less than initial rates. Therefore nuclei have reached their maximum replication rates within 0.5 h of entry into S phase. This implies that most, if not all, initiation events in each nucleus occur on entry into S phase.

Flow cytometry shows that DNA in sperm nuclei is not replicated a second time in the *Xenopus* egg extract, consistent with the BrdUTP density substitution profiles presented by Blow and Laskey (1986). The ability of the extract to distinguish replicated from unreplicated DNA is highlighted by some nuclei entering S phase after other nuclei have already reached the full diploid state. If, as suggested above, all initiation events within a single nucleus take place in a burst on entry into S phase, then the distinction between replicated and unreplicated DNA in the egg extract could simply be made at the level of the whole nucleus.

The relationship between DNA replication and nuclear formation

In showing that the nucleus acts as a fundamental unit of replication, this paper adds to the evidence for a relationship between DNA replication and nuclear organization in the *Xenopus* egg extract. (i) With different forms of template DNA, there is a correlation between efficiency of nuclear assembly and efficiency of replication *in vitro* (Blow and Laskey, 1986). (ii) The lag which occurs before replication starts *in vitro* is similar to the time required for nuclei to acquire a complete surrounding envelope (Blow and Laskey, 1986). (iii) There is a strong correlation between extracts that can initiate DNA replication and those that can assemble nuclei. Extracts depleted of membrane components so that they cannot assemble nuclei (Lohka and Masui, 1984), do not initiate DNA replication (Newport, 1987; M.A.Sheehan and J.J. Blow, unpublished results). (iv) In flow cytometry experiments sperm nuclei do not enter S phase until they have attained a critical size. (v) Flow cytometry shows that the egg extract treats nuclei as independent and integrated units of replication.

These results suggest how replication could be controlled *in vitro*. First, nuclei in a common cytoplasmic environment enter S phase at different times, but are never re-replicated. Thus individual nuclei contain important information determining when they will replicate. Second, entry into S phase is characterized by a burst of many synchronous or near-synchronous initiations. Although it may be the cause of asynchrony between nuclei, decondensation of sperm chromatin cannot by itself account for these observations, as it would not produce the observed coordination of initiation. The simplest model that fits all the observations involves accumulation of regulatory components by individual nuclei assembled *in vitro*. Once these components reach a critical threshold level, they activate a signal initiating replication which spreads rapidly throughout the nucleus.

Materials and methods

In vitro replication

Extracts were prepared from activated eggs of *X. laevis* as previously described (Blow and Laskey, 1986). After thawing, 30-μl aliquots were supplemented with 60 mM phosphocreatine (Sigma), 150 μg/ml creatine phosphokinase (Sigma), 6400 demembranated *Xenopus* sperm nuclei (200 ng DNA; prepared as described by Blow and Laskey, 1986) and 8–40 μM biotin-11-dUTP (BRL) or 1 μCi [^{3}H]d-ATP (Amersham). Incubations were performed at 23°C.

Flow cytometry

Preparation and staining of nuclei. For flow cytometric analysis, incubations were stopped by resuspending the extract in 3 ml of fixation buffer (60 mM KCl, 15 mM Hepes–KOH pH 7.4, 15 mM NaCl, 1 mM β-mercaptoethanol) at room temper-

ature. Nuclei were fixed by the addition of 300 μl of 100 mM ethylene glycol-bis(succinimidylsuccinate) (EGS; Pierce) (Miake-Lye and Kirschner, 1985) in dimethyl sulfoxide, stopped after 10 min by the addition of 80 μl of 1 M Tris−HCl pH 7.4. The solution was then underlayered with two sucrose cushions: 200 μl 15% sucrose in Buffer A (60 mM KCl, 15 mM Tris−HCl pH 7.4, 15 mM NaCl, 1 mM β-mercaptoethanol, 0.5 mM spermidine · 3HCl, 0.15 mM spermine · 4HCl) and 200 μl 70% sucrose in Buffer A. Nuclei were spun into the sucrose by centrifugation in a Beckman SW60Ti rotor at 6000 r.p.m. for 10 min at 4°C. Both sucrose cushions were pooled and frozen immediately. In control centrifugations the 70% and 15% cushions received 99% and 1% of *Xenopus* sperm nuclei and 84% and 16% of *Xenopus* erythrocyte nuclei, respectively. The isolated nuclei were stained with 4 μl fluorescein−streptavidin (Amersham) for 1 h at room temperature. Total DNA was stained with 400 μl of a solution of 50 μg/ml propidium iodide and 50 μg/ml RNase. Under these conditions streptavidin and propidium iodide have saturated all their respective binding sites.

Excitation and optical filtration. The nuclei were analysed for DNA and biotin-11-dUTP incorporation simultaneously in the Cambridge MRC custom-built dual laser flow cytometer (Watson, 1980, 1981) which incorporates a modified flow chamber to increase light collection efficiency (Watson, 1985). The Innova 70-5 argon ion laser (Coherent, Palo Alto, CA) was tuned to the 488 nm line at a light power of 100 mW which excites red fluorescence from the propidium iodide/DNA complex and from the fluorescein-tagged streptavidin/biotin complex. The green and red signals were separated by a 580 nm dichoric mirror (Zeiss Ltd) and the respective photodetectors were additionally guarded by a 515−560-nm band pass filter (green) and a 630-nm-long pass filter (red). Forward and 90° light scatter were also collected.

Instrument set up and data collection. The instrument was set up on the red fluorescence channel (DNA) to record the peak from condensed haploid nuclei in channel 300. The high tension voltage of the green fluorescence detector (biotin channel) was then increased to record the short wavelength tail from the red DNA/propidium iodide fluorescence at about channel 50. The signal in the green channel from the propidium-iodide-alone control was due to red fluorescence breaking through the filters at the high voltages used on this photomultiplier. This was an advantage as the instrument could be set up identically for each run by using sperm nuclei (condensed haploid) as a standard for the green channel.

Data collection and processing. The data were collected list-mode on a fast RP07 disc via a dedicated LSI 11/23 and a time-sharing PDP 11/40 computer (all from Digital Equipment Corporation, DEC, USA) after digitization of each signal into peak height, area and width (time of flight through laser beam) within the analogue-to-digital conversion (ADC) range of 1−1024. Processing was carried out in a VAX 8600 computer (DEC) which extracted total DNA (red fluorescence), biotin (green fluorescence) and size from the list-mode data sets. DNA versus biotin, DNA versus size and size versus biotin could then be displayed and analysed on an LSI 11-73 computer. The analysis procedure in the software package included algorithms for positioning eliptical regions to count the numbers of nuclei lying within them (as shown in Figure 3).

Microscopy

Nuclei were prepared for phase and fluorescence microscopy as for the flow cytometry, except that the volume of fixation buffer, EGS solution and Tris were scaled down by a factor of three. Ten microlitres of each of the sucrose cushions were used, and centrifugation was performed in an MSE Centaur 2 at 3500 r.p.m. for 10 min. Nuclei in the pooled sucrose cushions were stained with 0.5 μl Texas Red−streptavidin (Amersham), 4 μl of 100 μg/ml Hoechst 33528 and 20 μg/ml RNase, and were then viewed wet. These fluorochromes are different to those used for flow cytometry as they had to be excited separately for microscopy.

Data analysis

The primary data set concerning propidium iodide fluorescence, fluorescein fluorescence and light scatter for each nucleus was processed using the software described above. The entire population of nuclei from each timepoint was presented on a contour plot similar to the ones shown in Figures 2C and 5A. The position of each nucleus is determined by its propidium iodide fluorescence (horizontal axis) and its fluorescein fluorescence (vertical axis). Subsets of nuclei can then be defined which have particular values of these two parameters. The subsets can then be independently analysed for any of the recorded variables, as in Table II. The histograms in Figures 3 and 4 simply show the proportion of the total population falling into each subset.

The haploid DNA propidium iodide signal was defined using sperm nuclei decondensed in 3 mg/ml polyglutamate and fixed in EGS; this gave the same value as sperm nuclei decondensed in the replication system. The diploid DNA signal is twice this amount. Untreated sperm nuclei are highly condensed and so give a signal lower than haploid. Since virtually all the nuclei fell on a clear straight line between haploid and diploid populations in continuous-label experiments (Figure 2C), calibration of the biotin axis is unnecessary. The full biotin signal is given by the distinct population of diploid nuclei. Propidium iodide bleed-through into the fluorescein channel gave diploid cells without biotin a fluorescein signal 15% of fully biotinylated diploid nuclei (see the 'diploid/no biotin' subset in Figure 5A). In continuous labelling experiments, however, the total amount of fluorescein in the diploid population fell slowly during the incubation, by ~5% over 4 h. This is consistent with dot-blots of DNA labelled with biotin-11-dUTP and [^{3}H]dATP, where the ratio of biotin to ^{3}H fell slowly with time, probably due to biotin being repaired out of DNA in the extract. Higher biotin-11-dUTP concentrations gave a more rapid loss of biotin. In both flow cytometry and dot-blot experiments with replication partially inhibited by aphidicolin, the biotin signal was slightly higher than expected from the extent of replication.

Figure 5A shows a sample contour plot with nuclei pulse-labelled with biotin-11-dUTP from 1 to 1.5 h after addition of nuclei to the extract. The definition of the eight subsets described in Table I is shown in Figure 5B. The 'haploid condensed' and 'haploid decondensed' subsets are the same as in the continuous label experiments. The position of the 'diploid/no biotin' population on the fluorescein baseline can be determined by incubations without added biotin-11-dUTP. The 'S/max biotin' subset is clearly defined in the 1-h timepoint. 'Diploid/max biotin' nuclei are seen as a clear population at 1.5 h at the intersection of the 'S/max biotin' subset line with the diploid propidium iodide line. Evidence that this subset was accurately defined is that the 'diploid/max biotin' population (Figure 4) is equal to the number of nuclei expected to replicate in <0.5 h from Figure 3. The 'diploid/part biotin' subset lies directly between the 'diploid/max biotin' and 'diploid/no biotin' subsets, and the 'S/no biotin' subset between the 'haploid decondensed' and 'diploid/no biotin' subsets. The 'S/part biotin' subset is surrounded by the other seven subsets.

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