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Periodic DNA synthesis in cell-free extracts of *Xenopus* eggs

Christopher J.Hutchison, Richard Cox, Robert S.Drepaul, Miranda Gomperts[1] and Christopher C.Ford

Department of Genetics and Developmental Biology, School of Biological Sciences, University of Sussex, Falmer, Brighton BN1 9QG, UK
[1]Present address: Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge CB2 1QW, UK

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Cell-free extracts prepared from unfertilized eggs of *Xenopus laevis* support DNA synthesis on sperm pronuclei. Continuous labelling studies using [^{3}H]dCTP and pulse labelling studies using [^{32}P]dCTP demonstrate that synthesis occurs in short bursts of 40 min, which are punctuated by periods of 20-40 min during which no synthesis occurs. Density substitution experiments using bromodeoxyuridine demonstrate that this synthesis involves the initiation of replication and reveals that re-initiation events can occur following multiple bursts of replication. The periodic properties of these extracts are sensitive to protein synthesis inhibitors.

Key words: Xenopus laevis/cell-free extracts/sperm pronuclei/pulse labelling

Introduction

A central feature of the eukaryotic cell cycle is the S phase, during which chromosomal DNA is replicated. The mechanisms which regulate DNA replication must ensure that the entire genome is duplicated once only during this period. Several studies have indicated that the central processes controlling DNA replication are firstly a commitment to S phase (Nurse and Bisset, 1981) and secondly the frequency of initiation events within the chromosomal DNA (Edenberg and Huberman, 1975; Hand, 1978; Mechali *et al.*, 1983).

Early *Xenopus* embryos have long been favoured as model systems for the study of the initiation of DNA replication, for several reasons. Firstly, the early cell cycles have no G_1 period (Graham and Morgan, 1966) and consequently do not appear to be rate limited for commitment to S phase (Ford, 1985). Secondly, *Xenopus* eggs contain large stores of enzymes and precursors for DNA synthesis (Woodland and Pestell, 1972; Benbow *et al.*, 1975; Zierler *et al.*, 1985). Thirdly, the large size and reliable source of *Xenopus* eggs makes them ideal for experimental manipulations such as microinjection.

The signals required for the initiation of DNA replication in *Xenopus* eggs are neither species nor phylum specific (Graham *et al.*, 1966; Laskey and Gurdon, 1973; Harland and Laskey, 1980; Smith *et al.*, 1980). In addition, DNA replication on injected templates is tightly coupled to the cell cycle, such that DNA is replicated only once within a single cell cycle, but can undergo a second round of replication in the next cell cycle (Harland and Laskey, 1980). These events do not require specific DNA sequences (Harland and Laskey, 1980; Mechali and Kearsey, 1984). Recent studies have indicated that one of the factors controlling the initiation of DNA replication is maturation promoting factor (MPF). This cell cycle oscillator can drive the nucleus into mitosis (Gerhart *et al.*, 1984; Newport and Kirschner, 1984) and as a consequence the chromatin is effectively withdrawn from signals which initiate DNA replication (Newport and Kirschner, 1984). However, it is not clear from these studies whether MPF is in itself a sufficient signal to promote the reinitiation of DNA replication.

Despite the important findings generated from microinjection experiments, these types of study have serious limitations, particularly in handling large numbers of embryos and in precisely defining the timing of replication events. One way of overcoming these problems would be to develop a cell-free assay which recapitulates many of the cell-cycle-dependent features of DNA replication. Recently, it has been demonstrated that cytochalasin B-treated extracts of *Xenopus* eggs have the capacity to generate sperm pronuclei from demembranated sperm heads (Lohka and Maller, 1985). Previously it had been shown that similar extracts prepared from *Rana* eggs give rise sequentially to chromosome decondensation followed by recondensation, and that aphidicholin-sensitive DNA synthesis occurred during this sequence (Lohka and Masui, 1983). Blow and Laskey (1986) have subsequently demonstrated, by density substitution experiments, that DNA synthesis in *Xenopus* egg extracts is semi-conservative and results from *de novo* initiation events.

In the current study we have investigated the characteristics of DNA replication in *Xenopus* egg extracts, by pulse labelling and density substitution experiments. We report that DNA replication occurs periodically in such extracts, and that reinitiation events on previously replicated molecules can occur. Furthermore, the periodic features of this replication are sensitive to protein synthesis inhibitors.

Results

Pronuclear formation in vitro

Incubation of demembranated sperm heads (Figure 1a,b) with *Xenopus* egg extracts resulted in the formation of pronuclei, as described by Lohka and Maller (1985). Within 20 min most of the sperm nuclei had acquired nuclear membrane structures (Figure 1c), as determined by phase-contrast optics, but their chromatin remained largely condensed, judged by fluorescence staining with DAPI (Figure 1d). During the next 80 min the chromatin dispersed and was accompanied by nuclear swelling (Figure 1e,f). Initially chromatin dispersal was uniform throughout the nucleus (Figure 1f) but at later times, when nuclei became fully swollen, the chromatin appeared to become localized (Figure 1b). Figure 2 illustrates a typical time course of the changes in proportions of nuclei with the pronuclear morphologies described above. The rate at which such changes occurred did vary from extract to extract, the main variation being in the first appearance of partially swollen nuclei.

The capacity to form pronuclei changes with time

Lohka and Masui (1983) have demonstrated that sperm pronuclei generated in cell-free extracts of *Rana* eggs, subsequently undergo

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chromatin condensation and form metaphase-like chromosomes *in vitro*. However, examination by fluorescence microscopy of pronuclei formed in *Xenopus* extracts have failed to provide consistent evidence of changes in chromatin structure resembling chromosome condensation. While in most extracts the chromatin is not uniformly dispersed in nuclei after 80 min (Figure 1), this non-uniformly dispersed state appears to be related to nuclei swelling more rapidly than chromatin disperses. Only in one extract have we observed structures which resemble metaphase chromosomes, while in one other extract nuclei appeared to collapse between 60 and 90 min but subsequently began to re-swell (results not shown).

Although spontaneous chromosome condensation has not been consistently detected during continuous incubation of sperm heads in cytoplasm, chromatin decondensation and pronuclear formation, events which usually follow mitosis, are observed. Presumably the ability to induce chromatin decondensation should disappear as a prerequisite for chromosome condensation. To measure changes in the capacity of the cytoplasm to decondense chromatin and generate pronuclei from sperm heads, egg extracts were incubated at 23°C and demembranated sperm heads were added to separate aliquots at 30-min intervals. Samples were prepared for fluorescence microscopy 30 min after the addition of sperm heads. The results (Figure 3) show that during the first

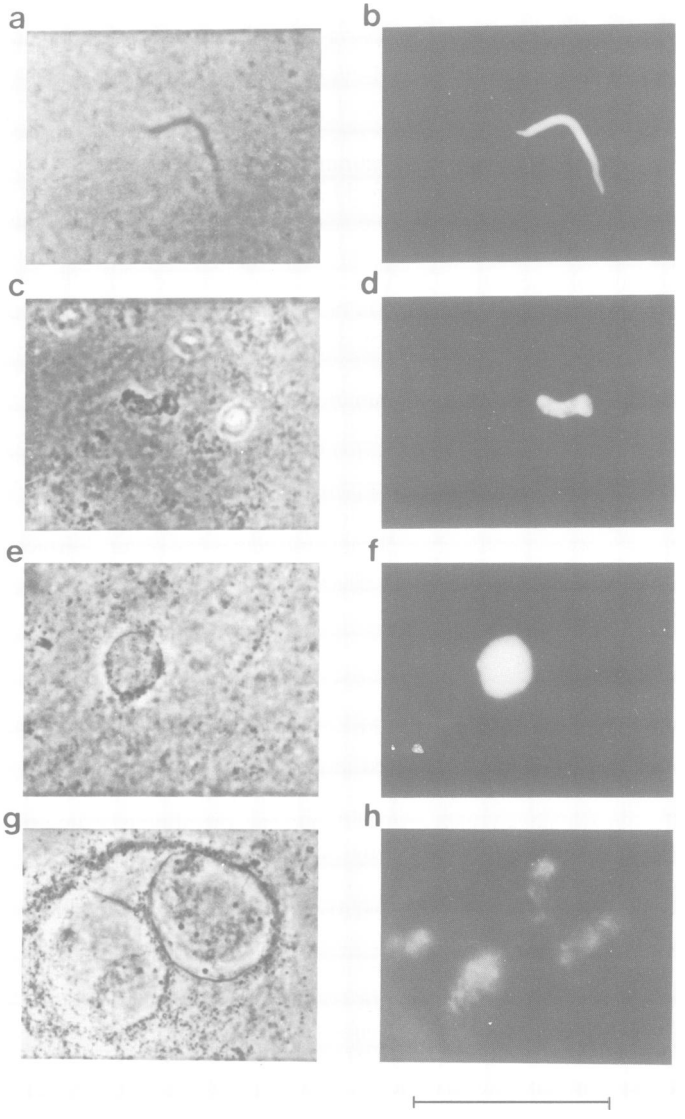

Fig. 1. Changes in the morphology of sperm pronuclei following incubation in egg extracts. Sperm heads were incubated in the extracts for 0 (**a,b**), 20 (**c,d**), 45 (**e,f**) or 90 (**g,h**) min at concentrations of 0.8 × 10^5/100 μl and then prepared for microscopy. **a,c,e** and **g** show micrographs viewed by phase contrast; **b,d,f** and **h** show the same fields viewed by DAPI u.v. fluorescence. Scale bar 25 μm. All fields are at the same magnification.

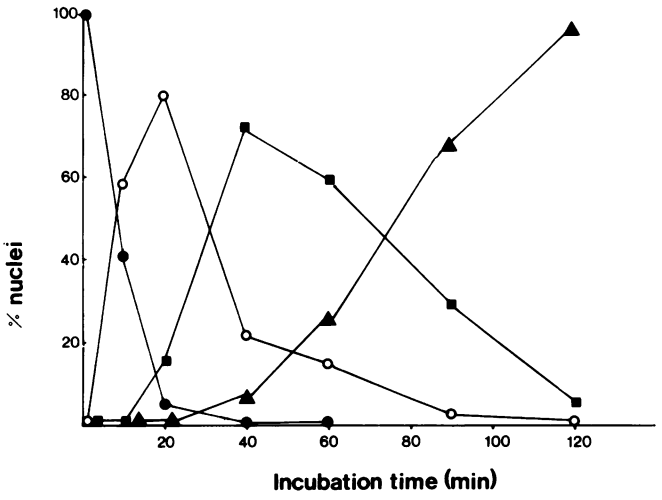

Fig. 2. Time course of the changes in sperm pronuclear morphology in extracts prepared from unfertilized *Xenopus* eggs. 0.8 × 10^5 demembranated sperm heads were added to 100 μl of extract and incubated at 23°C. Samples (4-μl) were prepared for phase and fluorescence microscopy at each time point, 100 nuclei being examined on each slide: ● demembranated sperm heads; ○ nuclei which have acquired membrane structures but remain unswollen; ■ partially swollen nuclei having uniformly distributed chromatin; ▲ fully swollen nuclei having non-uniformly distributed chromatin.

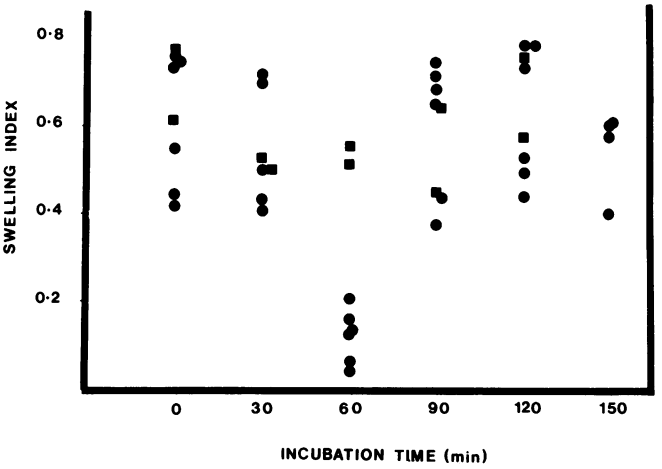

Fig. 3. Time course of changes in the capacity of egg extracts to generate pro-nuclei from demembranated sperm heads. Aliquots of egg extracts (50 μl) were incubated at 23°C, demembranated sperm heads (0.4 × 10^5) were added to separate aliquots at 30-min intervals. Samples were prepared for microscopy 30 min after the addition of sperm heads to the extracts, and examined under phase and fluorescence optics. Nucleic were given an arbitrary value according to size and the degree of chromatin dispersal, i.e. demembranated sperm heads O; nuclei having membranes but remaining unswollen 1; nuclei being partially swollen and having uniformly dispersed chromatin 2; nuclei being fully swollen but having non-uniformly dispersed chromatin 3. One hundred nuclei were scored on each slide, their values were added and then divided by 300 (the maximum possible score for 100 nuclei) to give the swelling index. The figure illustrates results obtained from eight experiments: ● extracts in which marked changes in the capacity to generate pronuclei were observed; ■ extracts in which no changes in the capacity to generate pronuclei were observed.

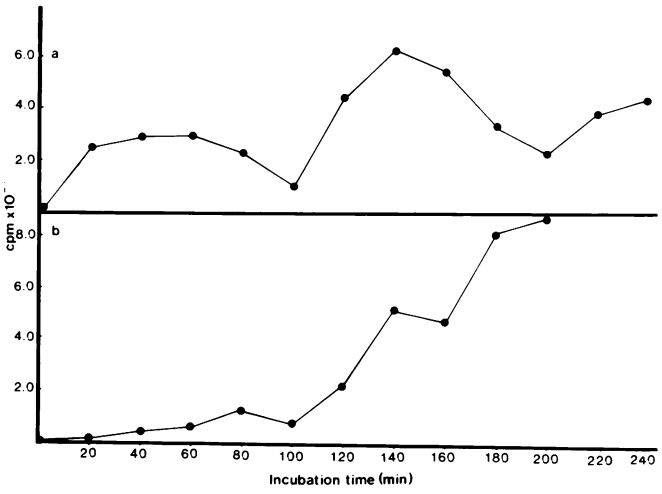

Fig. 4. Time course of the incorporation of [^{3}H]dCTP into acid-insoluble material, following the incubation of [^{3}H]dCTP with egg extracts containing pronuclei. Aliquots (20 μl) of egg extracts, containing 0.8 × 10^5 nuclei and 2 μCi of [^{3}H]dCTP, were incubated at 23°C. Reactions were stopped in individual tubes at 20-min intervals by the addition of pyrophosphate stopping solution (see materials and methods) and the amount of incorporation determined by TCA precipitation. (**a**) the incorporation profile in an extract which gave rise to rapid pro-nuclear formation; (**b**) the incorporation profile in an extract which gave rise to slow pro-nuclear formation.

30 min *in vitro* egg extracts can support a rapid generation of pronuclei from sperm heads. However, demembranated sperm heads which are added to the extracts after 60 min *in vitro* are not converted into pronuclei during the next 30 min but remain largely condensed. Subsequently the capacity to generate pronuclei is recovered. However, we have not observed any significant loss in the capacity to form pronuclei at later times, so that this phenomenon cannot be described as periodic. This result was obtained in six out of eight extracts studied. Over the same time period if cytoplasm was maintained on ice and then transferred to 23°C at the time of addition of sperm heads no variation in the capacity to from pronuclei was observed.

Periodic DNA synthesis in Xenopus egg extracts

The time-related characteristics of DNA synthesis, in egg extracts containing sperm pronuclei, were determined by the incorporation of [^{3}H]dCTP into trichloroacetic acid (TCA)-insoluble material. The results of two such experiments are illustrated in Figure 4 and show several important features of the synthesis observed in these extracts. Incorporation of [^{3}H]dCTP into TCA-insoluble material was first observed between 20 and 40 min after the addition of sperm heads to egg extracts, this being apparently dependent upon the rate of pronuclear formation. For example, in some extracts substantial amounts of incorporation were observed after only 20 min (Figure 4a), whereas in others only small amounts of incorporation were observed at 40 and 60 min (Figure 4b). In general egg extracts which were

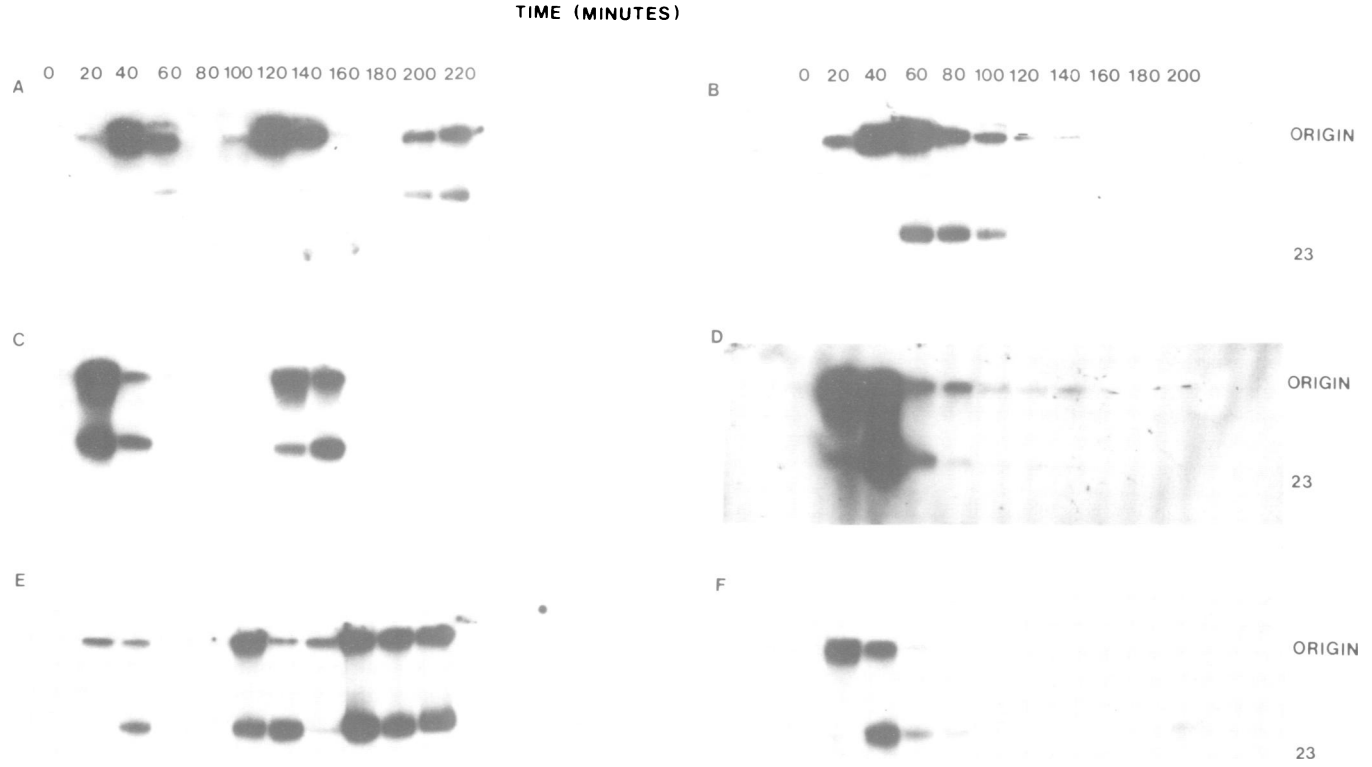

Fig. 5. Pulse labelling of egg extracts containing sperm pronuclei (0.8 × 10^5/100 μl) with [^{32}P]dCTP, in three different extracts. **a,c** and **e** illustrate labelling in the absence of protein synthesis inhibitors, while **b,d** and **f** show corresponding experiments performed in the presence of protein synthesis inhibitors (100 μg/ml cycloheximide in **b** and **f** or 100 μg/ml puromycin in **d**. Each lane contains 15 μl of labelled extract which had been treated with proteinase K (0.5 mg/ml) for 1 h at 37°C. Labelling was started at the times indicated (shown in minutes after incubation of the extract at 23°C) by addition of 2 μCi [^{32}P]dCTP (3 μl) to 40 μl of extract. After 20 min the reaction was terminated by the addition of a buffer containing 5% SDS. The top quarter of each gel is illustrated as no labelled material migrated beyond this point on 0.9% agarose gels. 23 is the position at which a 23-kb *Hin*dIII mol. wt marker migrated on each gel. The upper band did not migrate into the gel and could be removed by phenol extraction of the sample prior to loading. Experiments illustrated in **c** and **d** were performed for 160 min only. Dried gels were exposed to X-ray films for 18 h at −70°C.

able to support rapid pronuclear formation and chromatin decondensation also displayed early incorporation of [^{3}H]dCTP into acid insoluble material. Regardless of the rate of pronuclear formation, all extracts (made in an SW50.1 rotor, in a Beckman L5-65 centrifuge) displayed a period between 60 and 100 min during which no increase in incorporation was observed. This was subsequently followed by a second period of rapid incorporation which again apparently terminated at 140 min. The duration of this second period in which no increase in incorporation was observed varied from extract to extract, but was followed by a third period of rapid incorporation. In several extracts, total incorporation of [^{3}H]dCTP into TCA-insoluble material apparently decreased between 60 and 100 min and also during subsequent periods (140–200 min in Figure 4a). It is not clear what this phenomenon represented, but it would be consistent either with the trapping of precursors within pronuclei at the onset of DNA replication, or with a reduced recovery of DNA at specific times.

One explanation of the results illustrated in Figure 4 would be that DNA synthesis in egg extracts proceeds in bursts, which are interspersed with periods in which no synthesis occurs. To test this possibility, pulse labelling experiments were carried out by adding [^{32}P]dCTP to incubations for 20 min, at successive 20-min intervals. Agarose gels of protease-digested reaction products (Figure 5a,c,e) show that radiolabelled bands of high mol. wt are present at some time intervals, but not at others even though similar amounts of extracted DNA were present in each track, judged by ethidium staining and equal amounts of total radioactivity were present in each incubation. The radioactive bands, both at the origin and in the gel were sensitive to DNAase I digestion.

These results clearly demonstrate that incorporation of [^{32}P]dCTP into high mol. wt (<23 kb) DNA occurs in short bursts, lasting ~40 min, which are punctuated by periods of 20–40 min in which little or no incorporation occurs (Figure 5a,c,e). In contrast, pulse labelling of egg extracts which contained cycloheximide (100 μg/ml) revealed a single period of incorporation lasting between 60 and 120 min (Figure 5b,f). To confirm this finding similar experiments were performed with puromycin (100 μg/ml). Again only a single period of incorporation was observed (Figure 5d).

A comparison of the results illustrated in Figure 5 indicates that the amount of material labelled in the single synthetic period in extracts containing protein synthesis inhibitors is generally greater than the amount of material labelled in any one of the bursts of synthesis in the absence of these inhibitors. Furthermore, estimates of synthesis, made following scintillation counting of material cut from agarose gels, suggest that where DNA synthesis occurs in bursts only 20–30% of the available template is replicated during any one burst (at nuclear concentrations of 0.8×10^5/100 μl of extract). These results suggest that, where periodic DNA synthesis occurs, the extracts transiently lose the capacity for DNA replication before all of the input DNA has been replicated once.

Reinitiation of DNA synthesis can occur on molecules which have replicated once in the same extract

In a recent report, Blow and Laskey (1986) have conclusively demonstrated that the DNA synthesis occurring in *Xenopus* egg extracts, prepared by centrifugation in the presence of cytochalasin B, is semi-conservative and proceeds in a semi-discontinuous manner. Moreover, this replication apparently results from *de novo* initiation events. Previously, Harland and

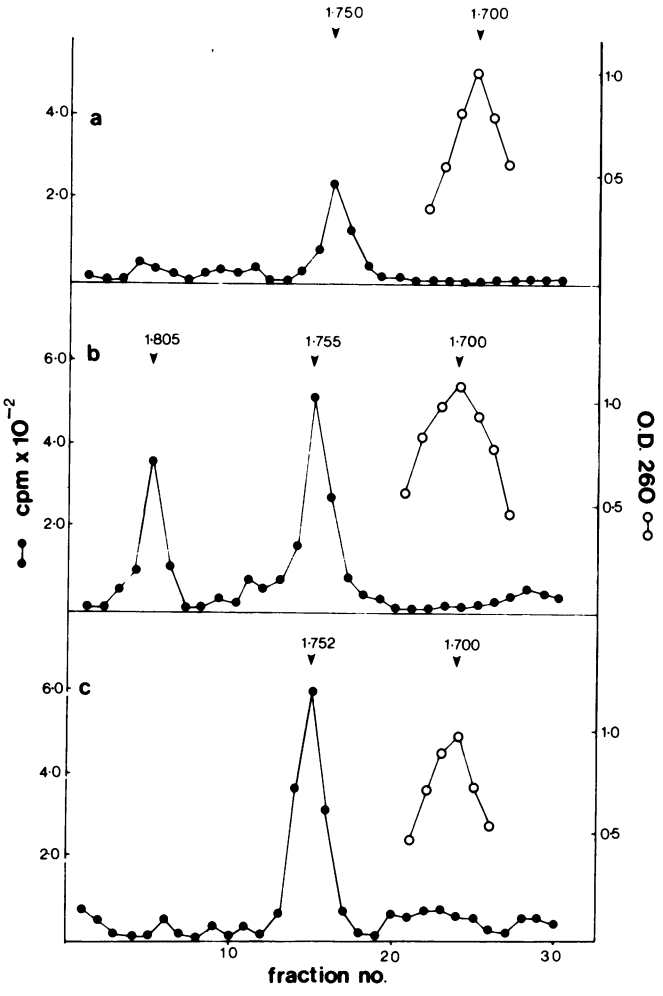

Fig. 6. CsCl density gradient centrifugation of DNA extracted from sperm nuclei replicated in egg extracts which contained [^{32}P]dCTP and BrdUTP. Aliquots (100-μl) of egg extracts containing 0.3×10^4 sperm nuclei, 5 μCi of [^{32}P]dCTP (3000 Ci/mMol) and 0.9 mM BrdUTP were incubated at 23°C for 90 (**a**), 240 (**b**), or 240 min in the presence of 100 μg/ml cycloheximide (**c**). The DNA was extracted and fractionated on CsCl density gradients which contained 160 μl unlabelled calf thymus DNA. The figures above the arrows represent the CsCl densities which were measured by refractive index and normalized with respect to the position of the unsubsituted carrier DNA. Background c.p.m. was determined from gradients formed using extracts which contained BrdUTP and [^{32}P]dCTP but no nuclei. Since no peaks of radioactivity were detectable in the absence of added nuclei this value was subtracted from the data points illustrated in the figure.

Laskey (1980) had shown that re-replication of DNA molecules injected into eggs could occur during incubations of greater length than a single cell cycle, but that reinitiation of replication was blocked in the presence of cycloheximide. To determine the nature of the DNA synthesized in the present system, we labelled egg extracts with [^{32}P]dCTP and the density analogue bromodeoxyuridine (BrdUTP), and examined the synthetic products by CsCl gradient centrifugation.

DNA synthesized during the first 90 min *in vitro* migrated on CsCl gradients to a density of 1.75 g/ml (Figure 6a). This is the expected position of fully substituted heavy–light (HL) DNA and is consistent with semi-conservative replication of this material occurring once. In contrast, DNA synthesized over a period of 240 min, migrated in two peaks, at densities of

Table I. A comparison of the material recovered under the HH and HL regions of CsCl gradients following density substitution experiments

Extract no.	No. of nuclei per 100 μl of extract	% c.p.m. recovered under HL peak	% c.p.m. recovered under HH peak	Total c.p.m. recovered from gradient
1	0.8×10^5	71.2	28.8	8.1×10^3
2	0.8×10^5	97.0	3.0	9.9×10^3
3	0.8×10^5	100.0	0.0	10.1×10^3
4	0.8×10^5	95.0	5.0	9.2×10^3
4	0.3×10^5	77.3	22.7	4.1×10^3
5	0.8×10^5	100.0	0.0	8.4×10^3
5	0.3×10^5	90.1	9.9	3.7×10^3
6	0.3×10^4	60.3	39.7	1.1×10^3
7	0.3×10^4	61.5	38.5	1.3×10^3

A comparison of the radioactive material recovered at the HL and HH peaks of CsCl density gradients, following labelling of 100 μl of egg extracts containing sperm pronuclei with 5 μCi [^{32}P]dCTP and 0.9 mM BrdUTP for 240 min at 23°C. The results for extract 6 are taken from CsCl gradient data shown in Figure 6b.

Table II. The amount of DNA synthesized in 100 μl of egg extract in the presence and absence of BrdUTP

Extract No.	Input DNA (ng)	DNA synthesized in the presence of BrdUTP (ng)	% of input	DNA synthesized in the absence of (BrdUTP (ng)	% of input
1	240	59.5	24.8	163	68.1
2	240	59.3	24.7	178	74.2
4	240	71.7	29.8	194	80.8
5	240	48.8	20.3	(no data)	–
4	90	23.3	26.0	70.3	78.2
5	90	20.9	23.3	(no data)	–
6	9	5.6	62.2	17.3	193.3
7	9	6.0	66.7	18.3	204.0

The amount of DNA synthesized in 100 μl of egg extract was calculated from radioactive material recovered in CsCl density gradients (Ford and Woodland, 1975). For DNA synthesized in the presence of BrdUTP, radioactivity recovered at the HH and HL positions was determined, while for DNA synthesized in the absence of BrdUTP radioactivity recovered at the LL peak was determined. An estimate of the efficiency with which DNA was recovered from CsCl gradients was obtained from the unlabelled carrier DNA.

1.755 g/ml HL and 1.80 g/ml heavy–heavy (HH) DNA respectively (Figure 6b). This result therefore implied that some re-replication had occurred during this period. DNA synthesized over 240 min in the presence of cycloheximide (100 μg/ml), however, migrated in a single peak to a density of 1.752 g/ml HL (Figure 6c). The incorporation observed was completely dependent on the addition of sperm heads. In the absence of sperm heads no peaks of radioactivity were detected on CsCl gradients. These results indicate that reinitiation of newly synthesized DNA does not occur in the presence of protein synthesis inhibitors, but that protein synthesis is apparently not necessary for initial replication. These results are consistent with the patterns of synthesis observed *in vivo* by Harland and Laskey (1980).

Table I illustrates the results obtained from seven experiments in which varying concentrations of sperm heads were added to egg extracts containing [^{32}P]dCTP and BrdUTP, and allowed to replicate for 4 h. It is clear from these data that (i) re-replication of genomic DNA was not demonstrated in all extracts tested, and (ii) that there was considerable variation in the amount of re-replicated DNA between those extracts that did support reinitiation events. It is not clear from our data what this variation resulted from, as periodic synthesis did occur in extracts in which little or no re-replication was detected. The fact that re-replication apparently occurred more efficiently in extracts containing low concentrations of nuclei, presumably resulted from the proportional increase of DNA replicated in the first round, as a percentage of the total available template pool.

The amount of DNA synthesized in egg extracts was calculated from material recovered from CsCl gradients. The precursor pool size in egg extracts was estimated to be 50 μM, with respect to dCTP, by isotope dilution using [^{3}H]dCTP and cold deoxynucleotide triphosphates in a DNA polymerase α assay. Table II shows that for high concentrations of nuclei up to 80% of the template was replicated in 4 h (unsubstituted DNA), whereas at lower starting concentrations of DNA some 200% of the template was replicated. These data strongly support the view proposed by Blow and Laskey (1986) that this synthesis results from efficient initiation of semi-conservative replication.

To analyse the density of the single DNA strands synthesized during the reaction, density-substituted DNA was analysed on neutral CsCl gradients following alkaline denaturation with NaOH. If DNA synthesis was being initiated *de novo*, then the single strands generated fronm alkaline denaturation should have been fully substituted with BrdUTP and would migrate to a single-stranded heavy position. If, however, pre-existing strands were being extended or repaired, then the single strands would migrate between unsubstituted and fully substituted positions depending on the proportion of the molecule that had been newly synthesized. The results of one such analysis, illustrated in Figure 7, indicate that several classes of molecule were generated following alkaline denaturation. The majority of the material recovered from the gradient (57.5%) migrated in a single sharp peak at 1.806 g/ml, indicating the synthesis of large molecules of DNA which were fully substituted with BrdUTP. However, substantial shoulders of radioactivity (29.1%) extended either side of this peak, and presumably represented a population of fully

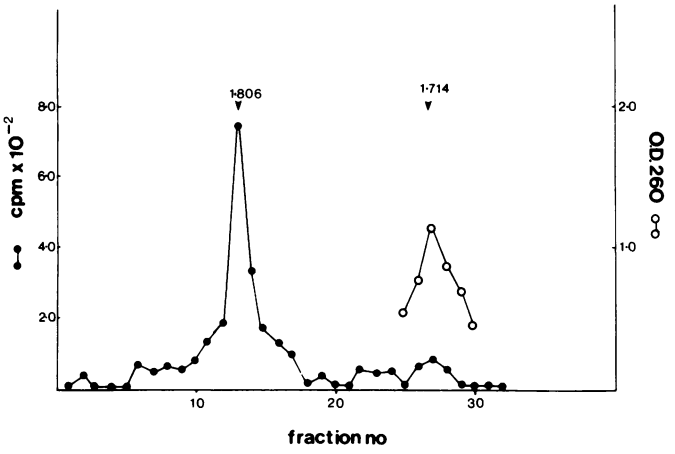

Fig. 7. CsCl density gradient analysis of density-substituted DNA, following denaturation with NaOH. Sperm nuclei (0.3×10^4) were replicated in egg extracts in the presence of 2 μCi [^{32}P]dCTP and 0.9 mM BrdUTP, for 240 min. DNA was extracted, alkali denatured, reneutralized and fractionated on a CsCl density gradient. Figures above the arrows represent the density of CsCl, which was determined by refractive index and normalized to the position of single-stranded carrier DNA. Data points represent c.p.m. minus backgroud. The results in this figure were obtained from a separate extract to the one used to obtain the results of Figure 6. In this extract HL DNA migrated to a density of 1.745 g/ml while HH DNA migrated to a density of 1.79 g/ml.

substituted but shorter DNA molecules. The material migrating close to the position of unsubstituted single strands (1.714 g/ml) represented 13.4% of the acid-insoluble radioactivity recovered from the gradient. This material presumably resulted from repair processes and was only observed on some gradients. These results, together with the estimates of the amount of synthesis and the occurrence of re-replication imply that the majority of the synthesis occurring in these extracts arises from new initiation events.

Discussion

DNA synthesis in extracts from unfertilized *Xenopus* eggs occurs in bursts which last for ~40 min and are interspersed by periods of 20–40 min during which no synthesis occurs. This synthesis is semi-conservative and apparently results from new initiation events, resulting in the replication of a significant proportion of the input of DNA. Furthermore, when multiple bursts of DNA replication are observed, re-replication can occur.

Blow and Laskey (1986) have reported that extracts prepared from parthenogenically activated *Xenopus* eggs can support a maximum of 10 ng of semi-conservative DNA synthesis/μl of extract in 6 h. Using lower starting concentrations of input DNA (2.4 ng/μl as opposed to 15 ng/μl) we have observed the replication of 1 ng of DNA/μl of extract in 4 h. However, whereas our results indicate periodic DNA synthesis in which re-replication can occur, Blow and Laskey (1986) demonstrated a continuous pattern of DNA synthesis and, when using sperm pronuclei, only observed re-replication on recovery of pronuclei and reincubation in fresh extract. These differences may reflect differences in the preparation of the extracts. Whereas we have exclusively used freshly prepared extracts from unfertilized eggs, Blow and Laskey (1986) used frozen extracts prepared from ionophore-activated eggs, which contained added ATP and an energy regenerating system.

In vivo at 23°C, first-round DNA replication occurs at ~20 min post-fertilization and lasts for ~10 min. First mitosis then follows, during which no further replication occurs, and lasts for 20 min. Second-round DNA synthesis resumes at 60 min post-fertilization and lasts for a further 20 min, before terminating at first cleavage (85 min post-fertilization). Third-round synthesis occurs at 95 min post-fertilization and lasts 15–20 min (Miake-Lye *et al.*, 1983). Hence, *in vivo*, the first three rounds of DNA replication take 105 min to complete, each round lasting between 10 and 20 min. *In vitro*, three bursts of DNA synthesis occur in 200 min, each burst lasting ~40 min and being punctuated by periods of 20–40 min in which no DNA replication takes place. Furthermore, *in vivo*, injection of cycloheximide into eggs before first S phase still allows the initiation and termination of the first round of DNA synthesis at the normal time, but completely inhibits the appearance of subsequent rounds of DNA synthesis (Miake-Lye *et al.*, 1983). Similarly, *in vitro* addition of cycloheximide at time zero leads to the appearance of a single period of DNA synthesis, which starts at a similar time but lasts considerably longer than any of the bursts of DNA synthesis seen in the absence of cycloheximide. Hence, in general terms the pattern of DNA synthesis in freshly prepared extracts from unfertilized *Xenopus* eggs, appears to be similar to the patterns of synthesis observed *in vivo*, although the *in vitro* events apparently take twice as long to complete. The similarities of synthesis *in vivo* and *in vitro* extend to the nature of the synthesis as well as its timing.

In vivo, double-stranded DNA templates can only be replicated once within a single S phase. However, in subsequent cell-cycles re-replication can occur (Harland and Laskey, 1980). Hence, in the presence of cycloheximide, DNA synthesized over 5 h *in vivo* migrates on CsCl gradients exclusively to the HL position, whereas DNA synthesized over the same period in the absence of cycloheximide migrates to both the HH and HL positions on CsCl gradients (Harland and Laskey, 1980; Ford *et al.*, 1983; Mechali *et al.*, 1983). *In vitro*, re-replication was observed in five out of seven extracts tested, over a 4-h period in the absence of cycloheximide, but was never observed in the presence of cycloheximide. It is not clear from our results why re-replication does not always occur in extracts which are undergoing periodic DNA synthesis. However, this may be related to events within the pronuclei during periods in which DNA synthesis is not occurring. These events remain as yet undefined.

MPF cycles *in vivo*, the activity appearing just before first mitosis and disappearing before first cleavage, only to reappear before second mitosis and disappear again before second cleavage (Gerhart *et al.*, 1984). This cyclical activity is apparently dependent upon protein synthesis as the appearance of MPF is inhibited by cycloheximide (Gerhart *et al.*, 1984). Newport and Kirschner (1984) have demonstrated that embryos blocked in G_2 by cycloheximide, can be driven transiently into mitosis following the injection of MPF and can subsequently reinitiate DNA synthesis. These types of experiment have led to the suggestion that MPF drives the cell-cycle in early *Xenopus* embryos by periodically withdrawing the chromatin from a cytoplasmic environment which is continuously able to synthesize DNA (Newport and Kirschner, 1984). *In vitro*, we have no evidence that pronuclei consistently enter a mitotic state during the periods in which DNA synthesis is not occurring. However, during the first of these periods we do observe a loss in the capacity to generate pro-nuclei from demembranated sperm heads. It is not clear, however, whether such changes in any way relate to the loss of the capacity to synthesize DNA, as in most extracts the

ability to generate pronuclei is recovered before the capacity to initiate DNA synthesis reappears. What is clear is that the amount of material synthesized in any one burst of DNA replicaton *in vitro*, represents only a fraction of the total available template pool. Hence there is apparently a loss of the capacity to initiate DNA replication before all of the available DNA has been fully replicated. This type of result would be consistent with the termination of 'S' phase by an independent cell-cycle oscillator.

Cell-free extracts from *Xenopus* eggs support periodic DNA synthesis in sperm pronuclei. This synthesis is apparently initiated and terminated in a temporally controlled manner and displays features reminiscent of DNA replication *in vivo*. Several reports have described cell-free extracts which can support pronuclear formation (Lokha and Masui, 1983; Lohka and Maller, 1985; Miake-Lye and Kirschner, 1985) and also efficient DNA replication (Blow and Laskey, 1986). Moreover, pronuclei formed in such extracts can be induced to undergo mitotic events following the addition of MPF (Lohka and Maller, 1985; Miake-Lye and Kischner, 1985). The advantages of the system described above is that periodicities in DNA synthesis occur spontaneously and consequently may provide a more subtle approach to the analysis of cell-cycle oscillators which are presumably responsible for such events.

Materials and methods

Preparation of egg extracts

Extracts from unfertilized *X. laevis* eggs were prepared using the method described by Lohka and Maller (1985). Mature female frogs were stimulated to lay eggs by injecting 700 i.u. human chorionic gonadotrophin (Chorulon, Intervet Laboratories) into their dorsal lymph sacs, 15 h before the eggs were required. Eggs were collected in saline tap water (110 mM NaCl in tap water), to prevent activation, and dejellied in 5 mM dithiothreitol (DTT), 10 mM NaCl, 20 mM Tris−HCl (pH 8.5). Following the removal of their jelly coats, the eggs were rinsed twice in saline tap water and examined. Batches in which >10% of the eggs were necrotic, were discarded. The eggs were then rinsed twice in distilled water and twice in extraction buffer (110 mM KCl, 5 mM $MgCl_2$, 2 mM β-mercaptoethanol, 20 mM Hepes (pH 7.5), 3 μg/ml leupeptin, 0.3 mM phenylmethylsuphonyl fluoride). The eggs were transferred to 5-ml centrifuge tubes and allowed to pack down, excess buffer was removed before centrifugation at 10 000 *g* for 10 min using an SW50.1 rotor (Beckman), in an L5-65 ultracentrifuge. (NB: use of different centrifuges, for example the Beckman L2-65B or the MSE 18, made significant differences to the performance of the egg extracts. This was apparently the result of differences in acceleration time. The L5-65 centrifuge accelerated from 0−10 000 r.p.m. in 25 s). This precedure resulted in a stratified extract which differed slightly from that described by Blow and Laskey (1986). The extract was essentially divided into three fractions: (i) a large insoluble pellet of yolk platelets; (ii) a particulate soluble phase which varied in colour and texture from extract to extract; (iii) a lipid pellicle. All the soluble material between the lipid cap and the yolk pellet was removed and mixed with cytochalasin B to a final concentration of 50 μg/ml. This material was again centrifuged at 10 000 *g* in an SW50.1 rotor for 10 min, in order to remove any further debris. The final extract was stored on ice for use throughout the day.

Preparation of sperm

A single, freshly isolated testis was homogenized gently with a loose-fitting glass homogenizer in 3 ml of Barth X saline. The homogenate was poured into a conical glass centrifuge tube and particulate material was allowed to settle to the bottom of the tube. The sperm suspension was removed and made 10% with respect to both dimethyl sulphoxide (DMSO) and fetal calf serum (FCS), and frozen in 0.5 ml (0.5×10^7 sperm) aliquots in liquid nitrogen. For replication and swelling assays, single 0.5-ml aliquots of sperm were removed from liquid nitrogen and thawed rapidly. The DMSO and FCS was diluted with SUNAP(0.25 M sucrose, 75 mM NaCl, 0.5 mM spermidine, 0.15 mM spermine, Gurdon, 1976) and the sperm recovered by centrifugation at 2500 r.p.m. in an MSE mistral 2L centrifuge. The sperm were resuspended in SUNAP at a concentration of 10^6/200 μl. The sperm were demembranated by the addition of 40 μl of 1 mg/ml lysolecithin to 200 μl of sperm suspension and incubating for 1 min at room temperature. The reaction was terminated by the addition of 1 ml of ice cold SUNAP containing 3 mg/ml BSA. The sperm were again recovered by centrifugation, and resuspended in SUNAP.

In vitro assays

Demembranated sperm, radionucleotides, protein synthesis inhibitors and BrdUTP were added to egg extracts as appropriate, at concentrations such that the total volume of the additions did not exceed 12% of the total volume of extract.

Continuous labelling studies

[^{3}H]dCTP or [^{3}H]dTTP (30 Ci/mMol, Amersham International) was dried under vacuum and resuspended in egg extract to give a final concentration of 10 μCi/100 μl. Demembranated sperm was added to the extract to give final concentrations varying between 0.3×10^5 and 1.0×10^5/100 μl. The extract was then divided into 20-μl aliquots in glass tubes and incubated in a 23°C water bath. Reactions were stopped in duplicate tubes at 20-min intervals by transferring the tubes on to dry ice/ethanol. Frozen samples were stored at −20°C.

Pulse labelling studies

Aliquots (40-μl) of egg extracts, containing 0.8×10^5 sperm heads, were incubated in a 23°C water bath. [^{32}P]dCTP (2 μCi) was added to separate aliquots of egg extracts at 20-min intervals, and incubated with the extracts for 20 min. Reactions were terminated in each aliquot by the addition of 40 μl of a solution containing 80 mM Tris−HCl (pH 8.0), 8 mM EDTA, 0.13% Phosphoric acid, 10% Ficoll, 5% SDS, 0.02% Bromophenol blue. Sample were stored at −20°C.

Density substitution

To 100 μl aliquots of egg extracts were added 5 μCi (0.5 μl) of [^{32}P]dCTP, 5 μl of 20 mM bromodeoxyuridine triphosphate (BrdUTP), and various concentrations of sperm heads in 5 μl, and incubated in a 23°C water bath. Reactions were terminated by the addition of two volumes of 50 mM Tris (pH 8.0), 5 mM EDTA (TE) and placing the samples on dry ice/ethanol. Samples were stored at −20°C.

Determination of pronuclear formation

Demembranated sperm heads were added to egg extracts, at concentrations varying between $0.4 \times 10^5 - 0.8 \times 10^5$/100 μl extract, and incubated at 23°C. Reactions were stopped by pipetting 4 μl of extract plus sperm pronuclei into 4 μl of DAPI mountant [15 mM Pipes (pH 7.2), 80 mM KCl, 5 mM EDTA, 15 mM NaCl, 10 μg/ml DAPI, 3.3% formaldehyde] on a glass slide. The sample was then covered with a glass coverslip, sealed with nail varnish and examined under a Zeiss photomicroscope III using both u.v. fluorescence and phase-contrast optics.

TCA precipitation

Radiolabelled samples were prepared for TCA precipitation by adding 400 μl of ice-cold pyrophosphate solution (0.1 M $Na_4P_2O_7 \cdot 10H_2O$, 1 mM EDTA, 500 μg/ml herring sperm DNA, 1% SDS). Samples were then precipitated with an equal volume of 10% TCA and left on ice for 5 min. Meanwhile, Whatman GF/C 1 filter discs were prepared for filtration by wetting with 0.1 M $Na_4P_2O_7 \cdot 10H_2O$, 0.1 M EDTA, 100 μg/ml dTMP. Samples were filtered through the GF/C 1 filter discs under vacuum and washed three times with 5% TCA and once with ice-cold methanol. The discs were dried, immersed in Beckman HP/b ready solve scintillant and counted in a Beckman LS 1801 liquid scintillation counter.

Agarose gel electrophoresis

Radiolabelled samples in Ficoll/SDS buffers were prepared for gel electrophoresis by digesting with proteinase K (500 μg/ml) for 1 h at 37°C. Samples (30-μl) were then loaded onto 0.9% agarose gels and subjected to a potential difference of 60 V (130 mA). The dye front was allowed to migrate off the end of the gel, in order to remove unbound radionucleotide, and the gel was then rinsed several times in distilled water, before staining in ethidium bromide. Stained gels were dried under vacuum and autoradiographed using pre-flashed X-ray film.

CsCl density centrifugation

Density-labelled samples (300 μl) were prepared for CsCl gradient centrifugation by adding 40 μl of calf thymus DNA (4 mg/ml), 15 μl proteinase K(10 mg/ml) and 36 μl of SDS (10%). The samples were incubated for 1 h at 37°C and then extracted with an equal volume of phenol. Following phenol extraction, material remaining in the aqueous phase was mixed with a 1/10 volume of 3 M sodium acetate and precipitated with two volumes of ethanol (−20°C). Precipatable material was resuspended in TE buffer and loaded directly onto CsCl gradients. CsCl gradients were prepared by adding solid CsCl to water, to give a final volume of 6 ml (with the sample) and a final density of 1.74 g/ml.

Samples were centrifuged for 60 h at 44 000 r.p.m. in a Beckman Ti 75 rotor, and fractions collected and analysed as described by Ford and Woodland (1975).

Alkaline denaturation of density labelled DNA

Density labelled samples, which had been prepared for CsCl gradiency centrifugation, were alkaline denatured by a modification of the procedure described by Smith *et al.* (1980). Briefly, 160 μg of DNA (sample + carrier) resuspended in 200 μl of TE buffer was incubated with 4.7 μl of 5 M NaOH for 5 min. The sample was then neutralized by the addition of 4.7 μl of 5 M HCl and subjected to CsCl density gradient centrifugation as described above.

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