Regulation of mammalian replication origin usage in *Xenopus* egg extract

Daniela S. Dimitrova and David M. Gilbert*

Department of Biochemistry and Molecular Biology, SUNY Health Science Center, 750 East Adams Street, Syracuse, NY 13210, USA

*Author for correspondence (e-mail: gilbertd@vax.cs.hscsyr.edu)

Accepted 22 July; published on WWW 9 September 1998

**SUMMARY**

*Xenopus* embryos initiate replication at random closely spaced sites until a certain concentration of nuclei is achieved within the embryo, after which fewer, more specific chromosomal sites are utilized as origins. We have examined the relationship between nucleo-cytosolic ratio and origin specification when Chinese hamster ovary (CHO) cell nuclei are introduced into *Xenopus* egg extracts. At concentrations of intact late-G1-phase nuclei that approximate early *Xenopus* embryos, the entire genome was duplicated nearly 4 times faster than in culture, accompanied by a de-localization of initiation sites at the dihydrofolate reductase (DHFR) locus. As the concentration of nuclei was increased, the number of initiation sites per nucleus decreased and initiation at the DHFR locus became localized to the physiologically utilized DHFR origin. Origin specification was optimal at nuclear concentrations that approximate the *Xenopus* mid-blastula transition (MBT). Higher concentrations resulted in an overall inhibition of DNA synthesis. By contrast, with intact early G1-phase nuclei, replication initiated at apparently random sites at all concentrations, despite an identical relationship between nucleo-cytosolic ratio and replicon size. Furthermore, permeabilization of late-G1-phase nuclei, using newly defined conditions that preserve the overall rate of replication, eliminated site-specificity, even at nuclear concentrations optimal for DHFR origin recognition. These data show that both nucleo-cytosolic ratio and nuclear structure play important but independent roles in the regulation of replication origin usage. Nucleo-cytosolic ratio clearly influences the number of replication origins selected. However, titration of cytosolic factors is not sufficient to focus initiation to specific sites. An independent mechanism, effecting changes within G1-phase nuclei, dictates which of many potential initiation sites will function as an origin.

Key words: DNA replication, Cell-cycle, Origin decision point, Mid-blastula transition, *Xenopus* egg extract

**INTRODUCTION**

Pre-blastula *Xenopus* embryos initiate replication at evenly spaced sites distributed at random throughout chromosomes (Hyrien and Mechal, 1993). Similarly, with naked DNA templates or with *Xenopus* sperm nuclei as a substrate, *Xenopus* egg extracts initiate replication within apparently any DNA sequence (Coverley and Laskey, 1994; Gilbert et al., 1995, and references therein). This contrasts sharply with origin-specific initiation of replication observed in cultured fibroblasts (Gilbert, 1998). Recently, it was shown that origin specification in *Xenopus* embryos is first observed after a critical concentration of nuclei is achieved (Hyrien et al., 1995), at or near the mid-blastula transition (MBT). Two different but not mutually exclusive hypotheses have been proposed to explain this transition. One explanation proposes that high concentrations of replication factors in the early embryo allow relaxed origin specification and these factors become titrated as DNA is replicated (Blumenthal et al., 1974; Difflay, 1996; Stillman, 1996). In support of this hypothesis, it was recently reported that high concentrations of sperm chromatin exhibit a lengthened S-phase in *Xenopus* egg extracts (Walter and Newport, 1997). The average rate of replication fork progression was similar at low and high sperm concentrations, implying that fewer origins were utilized at higher nucleo-cytosolic ratios. Since origin mapping was not performed in these experiments, it was not possible to determine whether this reduced replication rate resulted in a more specific selection of origin sites.

An alternative hypothesis proposes that changes in nuclear structure taking place at the MBT focus replication to specific sites (Gilbert et al., 1995; Hyrien et al., 1995). The MBT, which takes place at the 12th cell division, marks the onset of zygotic gene transcription (Kimelman et al., 1987; Newport and Kirschner, 1982a,b) and cell differentiation (Montag et al., 1988), accompanied by changes in nuclear structure that could repress initiation at some sites while facilitating initiation at others. For example, changes in histone (Dimitrov et al., 1993) and nuclear lamina (Benavente et al., 1985; Stick and Hausen, 1985) composition as well as a reduction in nuclear volume (Montag et al., 1988), an increase in the size of supercoiled chromosome loops (Buongiorno et al., 1982), and a change in the distribution of replication proteins to resemble that in somatic nuclei (Leibovitchi et al., 1992), all take place at the MBT.
Direct evidence for a role of nuclear structure in origin specification has come from studies with Chinese hamster ovary (CHO) nuclei introduced into Xenopus egg extracts (Gilbert et al., 1995; Lawlis et al., 1996; Wu and Gilbert, 1996, 1997; Wu et al., 1997). Incubation of intact late-G1-phase nuclei in cytosolic extracts from Xenopus eggs results in the de novo initiation of replication specifically within the CHO dihydrofolate reductase (DHFR) origin locus (Gilbert et al., 1995; Wu et al., 1997). If these same nuclei are washed in non-ionic detergent prior to introduction into extract, replication initiates at apparently random sites distributed throughout a 120 kb region surrounding the DHFR locus (Gilbert et al., 1995). Most strikingly, when metaphase chromosomes are introduced into these same extracts, replication initiates at a novel specific site within the DHFR gene itself (Lawlis et al., 1996). Recognition of this specific site requires topoisomerase II-mediated chromosome condensation. Finally, with intact early G1-phase nuclei as a substrate, replication initiates at apparently random sites (Wu and Gilbert, 1996). This finding allowed for the identification of a distinct G1-phase point (the origin decision point; ODP) at which nuclei experience a transition required for specific recognition of the DHFR origin by Xenopus egg cytosol. Together, these observations suggest that changes in nuclear structure focus initiation to specific genomic regions.

The ability to map a specific replication origin after initiation by Xenopus egg extracts provided us with the opportunity to investigate the relationship between nucleo-cytosolic ratio and origin specification. Here we show that, in Xenopus egg extracts, both nucleo-cytosolic ratio and nuclear structure play important but independent roles in the regulation of replication origin usage. When intact post-ODP CHO nuclei were introduced into Xenopus egg extracts at much lower nucleo-cytosolic ratios than previously attempted, a marked increase in the number of initiation events was observed, coincident with the use of origins not typically utilized by cultured CHO cells. Titration of Xenopus cytosol reduced the number of origins utilized and was necessary for optimal physiologic origin preference with post-ODP nuclei but, was not sufficient to focus replication to the DHFR origin within intact pre-ODP nuclei or permeabilized post-ODP nuclei. Our results are consistent with the presence of a factor(s) in Xenopus egg cytosol that regulates the number of origins fired per genome. However, origin specification requires components of nuclear structure assembled at the G1-phase ODP. We suggest that similar components may be assembled after the MBT in Xenopus embryos.

MATERIALS AND METHODS

Cell culture and synchrony
CHOC 400 is a CHO cell derivative in which a 243 kb segment of DNA containing the DHFR gene has been amplified ~500-fold by stepwise selection in methotrexate (Hamlin et al., 1994). CHOC 400 cells were propagated in Dulbecco’s modified Eagle’s medium (DMEM, Gibco) supplemented with nonessential amino acids and 10% cool calf serum (Sigma) at 37°C in a 5% CO2 atmosphere. Homogeneous populations of cells blocked in metaphase (≥95%) were obtained by mitotic shake off as described (Gilbert et al., 1995), with the following modifications. Mitotic cells were washed once in warm medium and released into G1-phase by plating in fresh medium for 2 hours or 4-6 hours to obtain pre-ODP or post-ODP cells, respectively. Cells were used either fresh or small aliquots were frozen in complete medium supplemented with 10% DMSO and thawed just prior to each experiment.

Preparation of nuclei
Intact nuclei were prepared as described (Wu et al., 1997). Briefly, CHOC 400 cells were resuspended in cold transport buffer at 107 cells/ml, an equal volume of transport buffer containing 150-160 μg/ml digitonin (CalBiochem) was added (to obtain a final concentration of 70-80 μg/ml digitonin) and the cells were incubated for 5 minutes on ice. Permeabilization of the cytosolic membrane was terminated by the addition of one volume of 3% bovine serum albumin in transport buffer and cells were collected by brief centrifugation at 3,000 g. Permeabilized nuclei were prepared following the same procedure, adjusting the final concentration of digitonin, Triton X-100 (Sigma) or Nonidet P40 (Boeringer, Manheim) to the indicated concentrations. Exact concentrations of digitonin and detergents that produce the cell populations described in this report must be determined for each batch of reagent. Cell and nuclear morphology was examined by phase contrast microscopy. Permeabilization of the plasma and nuclear membranes was verified by following the ability to exclude Texas Red-conjugated 1G (150 kDa, Jackson Labs, West Grove, PA) and by staining with 0.1 μg/ml 4′,6-diamidino-2-phenylindole (DAPI), using a Nikon Labophot-2 microscope, as described (Wu et al., 1997).

Analysis of DNA replication in Xenopus egg extracts
Activated Xenopus egg extracts were prepared as described (Chong et al., 1997; Wu et al., 1997) and supplemented with 25 mM creatine phosphate and 50 μg/ml creatine phosphokinase. Rates and extent of DNA replication with CHOC 400 G1 nuclei were evaluated by measuring the amount of acid-precipitable [α-32P]dATP as described (Gilbert et al., 1995; Chong et al., 1997). Specificity of initiation in the DHFR locus was determined by the early labeled fragment hybridization (ELFH) assay as described (Gilbert et al., 1995; Lawlis et al., 1996). Briefly, nuclei were incubated for 90 minutes in a Xenopus egg extract supplemented with 100 μg/ml aphidicolin. Nuclei were then washed free of aphidicolin and the earliest replicating nascent DNA chains were labeled briefly with [α-32P]dATP. 17 unique probes distributed over a 120 kb region that includes the DHFR ori-β were immobilized to nylon filters with an EVENFLOW slot blot apparatus (Laboratory Product Sales, Rochester, NY) and hybridized to the 32P-labeled early replication intermediates. Relative cpm were obtained by phosphorimaging analysis and adjusted for differences in probe size, deoxyadenine content, and hybridization efficiency by normalizing to the corresponding values for parallel hybridizations with labeled replication intermediates from exponentially growing cells. The hybridization probes used in this study have been previously described (Gilbert et al., 1995; Lawlis et al., 1996).

Measurement of replication fork elongation rates
Intact post-ODP nuclei were resuspended at 1,000 or 10,000 nuclei/ml in Xenopus egg extract and allowed to initiate DNA replication in the presence of 100 μg/ml aphidicolin. After 1.5 hours, nuclei were washed three times with ice-cold hypotonic buffer (20 mM Hepes/KOH, pH 7.5, 5 mM KCl, 1.5 mM MgCl2) and resuspended in ice-cold fresh extract containing [α-32P]dATP at their respective nuclear concentrations. Samples were incubated at 21°C and aliquots were stopped at various times by the addition of lysis buffer (50 mM Tris-HCl, pH 7.8, 10 mM EDTA, 0.4 M NaCl, 0.6% SDS) containing 800 μg/ml Proteinase K and 400 μg/ml RNase A. After overnight incubation at 56°C, DNA was extracted five times with phenol and twice with phenol-chloroform and precipitated with 0.8 volumes of isopropanol. DNA pellets were washed with 70% ethanol,
RESULTS

Efficiency of in vitro DNA synthesis

In all of our previous studies, the efficiency of mammalian nuclear DNA replication in *Xenopus* egg extracts was determined at 25,000 nuclei per microliter (150 ng DNA/μl) of egg extract (Gilbert et al., 1995; Lawlis et al., 1996; Wu and Gilbert, 1996, 1997; Wu et al., 1997). At this concentration of nuclei, we rarely observed greater than 30% of the total genomic DNA replicated over a 2-3 hour incubation period. This contrasts sharply to the rate of genome duplication observed when *Xenopus* sperm nuclei are replicated at 1,000 nuclei per microliter (3 ng DNA/μl) in the same preparations of extract (Gilbert et al., 1995). Under these conditions, 100% of the genomic DNA is replicated in less than 1 hour. Two possible explanations for this discrepancy seemed likely. In vivo, *Xenopus* sperm nuclear replication is completed within 20-30 minutes, whereas the length of S-phase in CHO (CHOC 400) cells is approximately 10 hours. Thus, it was possible that the rate of DNA replication is an inherent property of the type of nuclei presented to the extract. For example, initiation of replication could be limited to widely spaced origins within the early replicating, euchromatic fraction of the genome, with the middle and late-replicating fractions of the genome being refractory to initiation. Alternatively, the rate and extent of replication could reflect the titration of limiting factors in the extract at high concentrations of nuclei.

To distinguish between these possibilities, various concentrations of either pre-ODP or post-ODP CHO nuclei were incubated in *Xenopus* egg extract and the rate of genome duplication was determined. CHO (CHOC 400) cells were synchronized by mitotic selection and released into G1-phase for 2 hours (pre-ODP) or 4-6 hours (post-ODP). Intact nuclei were prepared from these cells, incubated in a *Xenopus* egg extract supplemented with [α-32P]dATP at concentrations ranging from 500 to 500,000 nuclei per μl and the percentage of input DNA replicated was determined (Fig. 1A-D). Consistent with previous results, at 25,000 nuclei/μl, less than 30% of the input genomic DNA was replicated over the course of 2 hours. As the concentration of nuclei was reduced to 1,000/μl, the efficiency of replication increased substantially and nearly all genomic DNA was replicated within the same time period. In experiments where nuclei were incubated in *Xenopus* egg extracts supplemented with biotin-11-dUTP, 100% of the nuclei incorporated similar amounts of biotin at all concentrations at or below 25,000/μl (not shown), indicating that the total fraction of input DNA replicated reflects the fraction of the genome replicated in each nucleus. Reducing the concentration to 500 nuclei/μl did not further increase this rate (not shown), suggesting that maximal replication efficiency has been achieved. At concentrations of nuclei above 25,000/μl, the efficiency of replication continued to decrease. At all concentrations of nuclei where significant DNA synthesis was observed, incorporation of [α-32P]dATP was reduced after 1-2 hours and was barely detectable after 3 hours, at which time extracts appear to have lost their capacity to continue synthesizing DNA. Importantly, the same relationship of nucleo-cytosolic ratio to replication rate was maintained whether pre- or post-ODP nuclei served as the substrate for replication (Fig. 1).

Higher efficiency of replication is due to an increased number of synchronously firing replication origins

The experiments described above reveal that the efficiency of replication at 1,000 nuclei/μl of extract (~100% of the genomic DNA replicated in ~3 hours) is greater than that achieved in cultured CHO cells (~10 hour S-phase). The increased efficiency at lower nucleo-cytosolic ratios could be due to: (1) an increased rate of replication fork elongation, (2) an increased number of synchronously firing origins, or (3) a decrease in the number of pause sites for replication that may exist in certain gene clusters (Gerber et al., 1997), or at the boundaries of early- and late-replicating chromosomal domains (Épner et al., 1988; Kitsberg et al., 1993; Selig et al., 1992; Strehl et al., 1997; Tenzen et al., 1997). To distinguish between these possibilities, we measured the elongation rate for nascent DNA strands at 1,000 and 10,000 nuclei/μl extract (Fig. 2). Nuclei were first incubated in a *Xenopus* egg extract supplemented with aphidicolin, which blocks the processive elongation of replication forks, allowing replication bubbles to

Fig. 1. Efficiency of DNA replication at various concentrations of nuclei. Intact nuclei from either pre-ODP (A and C) or post-ODP (B and D) CHOC 400 cells were incubated in *Xenopus* egg cytosol supplemented with [α-32P]dATP at 1,000 (■), 10,000 (●), 25,000 (●), 50,000 (▲), 100,000 (□) and 250,000 (○) nuclei per μl extract. The percentage of input DNA replicated at the indicated times was determined by acid precipitation, assuming a concentration of 50 μM dATP in the extract and 6 pg DNA per nucleus (see Materials and Methods). Shown is the average of two experiments in which several concentrations were analyzed simultaneously and the variation when greater than 10. Additional experiments showed no further increase in replication rate or total fraction replicated from 1,000 to 500 nuclei/μl. In C and D, the first 60 minutes of the reactions shown in A and B are expanded to illuminate the onset of DNA synthesis following a 20 minute lag period (Gilbert et al., 1995; Wu et al., 1997). Similar results were obtained in 4 independent experiments.

resuspended in alkaline loading buffer and separated on a 1% alkaline agarose gel as described (Sambrook et al., 1989).
accumulate with forks arrested close to their sites of initiation. Nuclei were then transferred to a fresh *Xenopus* egg extract lacking aphidicolin and supplemented with \([\alpha-\text{32P}]dATP\). DNA was isolated at various time points and the sizes of nascent \([\alpha-\text{32P}]\)-DNA strands were analyzed by alkaline gel electrophoresis. Results (Fig. 2A and B) demonstrated that labeled nascent strands detected at 1,000 nuclei/µl extract were of the same size and increased in molecular mass at the same rate as those labeled at 10,000 nuclei/µl extract. The calculated rate of elongation, 4.5±1 nts/second falls between similar measurements of replication fork rate that have been made with *Xenopus* sperm chromatin in *Xenopus* egg extracts at 21-23°C (Lu et al., 1998; Mahbubani et al., 1992; Walter and Newport, 1997), and is less than 1/6 the rate observed in cultured mammalian cells at 37°C (~30 nt/second; Brown et al., 1987). Comparison of the intensity of nascent strand labeling at each nuclear concentration indicated that significantly more replication forks were labeled at 1,000 nuclei/µl extract. Since this increase in the number of labeled replication forks at low nucleo-cytosolic ratios was evident from the very earliest time point (30 seconds; Fig. 2A), when replication forks had not traveled far from their sites of initiation (average size ~180 bp), and since the distribution of nascent strand sizes is similar at both concentrations, it is highly unlikely that the decreased labeling at 10,000 nuclei/µl extract is a result of forks that have encountered pause sites. Hence, the data are most consistent with an increase in the number of initiations per nucleus at lower nucleo-cytosolic ratios.

**Increased number of origins fired is accompanied by a disruption of the post-ODP initiation pattern**

The results described above demonstrate that replication of the entire CHO genome can be achieved in *Xenopus* egg extract in a fraction of the time it takes cultured CHO cells, mediated by the initiation of replication at more origins within a shorter period of time. However, these data alone do not demonstrate that *Xenopus* egg extract is eliciting initiation of replication at sites not normally utilized by CHO cells. In CHO nuclei, genome duplication is mediated by a temporally staggered series of clustered initiation events (O’Keefe et al., 1992; Taljanidisz et al., 1989). Thus, an increase in the rate of genome duplication could be effected by a more synchronous firing of these normally staggered initiation events. To more directly assess whether the increased efficiency of replication is mediated, at least in part, by initiation at non-physiological sites, we evaluated the sites of initiation of replication at various concentrations of nuclei using the early labeled fragment hybridization (ELFH) assay (Fig. 3). This assay evaluates the genomic positions of the earliest nascent DNA strands to be synthesized (Gilbert et al., 1995). Nuclei were incubated for 90 minutes in a *Xenopus* egg extract supplemented with aphidicolin. Aphidicolin was then washed away and nascent replication forks were briefly labeled with \([\alpha-\text{32P}]dATP\). The genomic distribution of these short, pulse labeled nascent strands was determined by hybridizing them to 17 unique DNA sequences derived from specific positions encompassing 120 kb of the DHFR locus. In Fig. 3, the relative amounts of hybridization of these labeled strands are plotted vs the map position of each DNA segment, giving a distribution of the relative frequency at which replication initiated near each probe. At 10,000 and 25,000 nuclei/µl extract, most replication forks were arrested by aphidicolin in a region 10-40 kb downstream of the DHFR gene, consistent with all previous origin-mapping results at this locus either after initiation in cultured cells or with intact post-ODP nuclei introduced into *Xenopus* egg extracts (Gilbert et al., 1995; Kobayashi et al., 1998; Lawlis et al., 1996; Wang et al., 1998; Wu and Gilbert, 1996, 1997; Wu et al., 1997, 1998). As the concentration of nuclei was reduced, there was a significant loss in the preference for initiation at the DHFR origin.
indicating that, at low nucleo-cytosolic ratios, a *Xenopus* egg cytosolic factor(s) causes replication to initiate at sites not normally utilized by cultured cells, even within intact post-ODP nuclei. By contrast, no preference for the DHFR origin was detected at any nucleo-cytosolic ratio with pre-ODP nuclei as a substrate. Hence, titration of this factor(s) is not sufficient to limit replication to a single site or a set of preferred sites.

As the concentration of nuclei was increased above 25,000/µl, a decrease in specificity was detected. We interpret this to reflect a decrease in the signal to noise ratio as the amount of DNA synthesis due to bonafide and site-specific initiation of replication decreases relative to other sources of DNA synthesis that would lead to general, de-localized radiolabel of DHFR sequences, such as the elongation of contaminating pre-primed templates (from the <1% contaminating S-phase nuclei) or DNA repair, all of which are negligible at 25,000 nuclei/µl or less (Gilbert et al., 1995, and data not shown). This interpretation is supported by the fact that we observed an overall inhibition of DNA synthesis above 25,000 nuclei/µl. In Fig. 4A we defined the relative specificity of initiation as the average relative early DNA synthesis values for probes B-R, highlighted as a grey line in Fig. 3. We then plotted those values vs the concentration of nuclei in the extract. This curve describes a clear optimal concentration for site-specific initiation of 10,000-25,000 nuclei/µl *Xenopus* egg cytosol. In Fig. 4B the total nanograms of DNA synthesized per µl of extract is plotted vs the concentration of nuclei, using the data from Fig. 1. The two curves are strikingly similar. Fig. 4B is consistent with an excess of some replication factor(s) that becomes limiting between 10,000 and 25,000 nuclei/µl. Fig. 4A is consistent with an activity capable of initiating replication at non-origin sites that also becomes limiting between 10,000 and 25,000 nuclei/µl. As the concentration of nuclei exceeds 25,000/µl, there is a decrease in the total ng of DNA synthesized by the extract (Fig. 4B). This inhibition of DNA synthesis was not accounted for simply by dilution of *Xenopus* egg extract; no significant inhibition of DNA synthesis (at 25,000 nuclei/µl) was observed when extracts were diluted with an amount of buffer equivalent to the volume of nuclei at 100,000/µl (not shown). We conclude that concentrations above 25,000 nuclei/µl lead to an overall inhibition of DNA replication and a loss of detectable origin recognition due to a decrease in the signal to noise ratio in ELFH assays. The decrease in origin recognition at lower concentrations of nuclei is clearly not an artifact of signal:noise ratio. Much less input CHO genomic DNA (containing DHFR-hybridizing DNA sequences) is required to achieve the same number of radiolabeled replication intermediates, due to the greater efficiency of genomic DNA replication at these lower concentrations (Fig. 4C).

It is important to note that pre-ODP nuclei showed the same relationship of replication efficiency vs concentration (Fig. 4B and C) even though recognition of the DHFR origin was not detected at any concentration of nuclei (Fig. 4A). The only

Fig. 3. Titration of a *Xenopus* cytosolic factor(s) that can disrupt the pattern of initiation of replication within post-ODP nuclei. Intact nuclei from either pre-ODP [●] or post-ODP [■] CHOC 400 cells were incubated in *Xenopus* egg cytosol at the indicated concentrations and the sites of initiation of replication were evaluated using the ELFH assay (Materials and Methods). The relative amounts of hybridization of the earliest labeled nascent DNA to each probe from the DHFR locus are plotted against the map position of each probe. The horizontal axis includes diagrams of the genomic region encompassed by these probe, including the positions of the DHFR and 2BE2121 genes (Hamlin et al., 1994). The vertical shaded line highlights the positions of probes B-R, which encompass the primary initiation sites at this locus (Kobayashi et al., 1998) and references therein). It is important to note that since these data express relative values, it is the relative differences between probes and not the area under the curve that should be compared. Shown are the mean of 3 independent experiments and the standard error of the mean, when greater than 1.
detectable difference between pre-ODP and post-ODP nuclei was the specificity of initiation. It appears then, that the factor(s) controlling the number of initiations are independent of those that determine where replication will initiate.

**Origin recognition requires an intact nuclear envelope**

The experiments described above demonstrate that *Xenopus* egg cytosolic factors cannot recognize the DHFR origin within pre-ODP nuclei, even at concentrations of pre-ODP nuclei that are optimal for DHFR origin recognition within post-ODP nuclei. In previous experiments (Gilbert et al., 1995), *Xenopus* egg cytosol also could not recognize the DHFR origin within post-ODP nuclei that were permeabilized with 0.2% Triton X-100 (Triton), suggesting that origin recognition requires some component of the post-ODP nucleus that is lost upon permeabilization. However, Triton permeabilization significantly inhibited the efficiency of DNA replication (Gilbert et al., 1995), raising the possibility that the perceived loss of specificity was due to a reduced signal to noise ratio as was found for high concentrations of intact nuclei (Figs 3 and 4). For this reason, we re-investigated the effects of nuclear membrane permeabilization on the efficiency of replication and specificity of initiation, comparing preparations of nuclei permeabilized with various concentrations of Triton, NP40 and digitonin, in the hopes of finding conditions in which nuclei could be permeabilized without inhibiting DNA synthesis.

We first determined the lowest concentration of each permeabilizing agent that was sufficient to permeabilize 100% of nuclei and we examined the effects of those permeabilizing agents on nuclear morphology (Fig. 5). With digitonin, three distinct and homogeneous populations of nuclei were obtained. At 70-80 μg/ml digitonin, 95-98% of nuclei retained an intact nuclear envelope as determined by the exclusion of labeled IgG. At 350 μg/ml nearly 100% of the nuclei were permeabilized but remained similar in size and morphology to intact nuclei. As the concentration of digitonin was increased to 500 μg/ml, nuclei began to shrink until at 1 mg/ml, virtually all nuclei were significantly smaller than intact nuclei, and became irregular in shape. By contrast, with both Triton and NP40, only one distinct population was obtained, closely resembling the population obtained with 1mg/ml digitonin. In other words, the lowest concentration of Triton and NP40 at which close to 100% of plasma membranes had been permeabilized (0.05%), nearly 100% of nuclei were also permeable, irregularly shaped, and significant nuclear shrinkage was observed. No further nuclear shrinkage or other morphological changes were observed as the concentration of Triton and NP40 was raised to 0.5%, a commonly used concentration for nuclear permeabilization (Kill et al., 1991; Leno et al., 1992; Sleeman et al., 1992).

Next, we examined the efficiency of DNA synthesis with these various nuclear preparations as substrates for replication in *Xenopus* egg extracts (Fig. 6; 10,000 post-ODP nuclei/μl). With the population of digitonin permeabilized nuclei that retained normal morphology (350 μg/ml), or with nuclei permeabilized with 500 μg/ml digitonin, replication proceeded as efficiently as intact nuclei for the first 2 hours of the reaction, after which a reduction in DNA synthesis was observed, resulting in the replication of approximately 2/3 as much input DNA as with intact nuclei (Fig. 6A and D). When nuclei were prepared with 1 mg/ml digitonin, resulting in significant homogeneous nuclear shrinkage (Fig. 5), DNA synthesis by *Xenopus* egg extract was severely inhibited from the earliest stages of the reaction (Fig. 6A and D). Similarly, when nuclei were permeabilized with Triton or NP40 (Fig. 6B,C,E and F), the efficiency of replication was strongly inhibited, either when prepared with 0.05% (~75% inhibition) or 0.5% (90-95% inhibition) detergent. In fact, at concentrations of NP40 or Triton exceeding 0.05%, replication was virtually undetectable. Thus, controlled exposure to digitonin, unlike the non-ionic detergents Triton and NP40, provides a means to permeabilize nuclei while retaining efficient DNA replication.

To evaluate the sites of initiation of replication at the DHFR locus within these various nuclear preparations, nuclei were
prepared from post-ODP CHOC 400 cells with either 0.05% Triton, 0.05% NP40 or 350 μg/ml digitonin. Control nuclei were prepared from the same cells with 75 μg/ml digitonin. 10,000 nuclei/μl were then introduced into Xenopus egg extracts supplemented with aphidicolin for 90 minutes and ELFH assays were performed as in Fig. 3. In all cases, efficient origin recognition was observed with intact control nuclei. Permeabilization of post-ODP nuclei with the non-ionic detergents Triton or NP40 completely eliminated the ability of Xenopus egg cytosol to recognize the DHFR origin. These data confirm and extend previous results using nuclei permeabilized with 0.2% Triton (Gilbert et al., 1995). Importantly, permeabilization with 350 μg/ml digitonin also eliminated origin recognition, even though the efficiency of replication was indistinguishable from that obtained with intact post-ODP nuclei over the course of the 90 minute incubation (Fig. 6D). Since these experiments were carried out at concentrations of nuclei optimal for origin recognition (10,000/μl), they provide further support that nucleo-cytosolic ratio is not sufficient for origin recognition. Specification of the DHFR origin also requires some component(s) of the post-ODP nucleus that is lost upon permeabilization.

**DISCUSSION**

The introduction of mammalian nuclei into Xenopus egg extracts has provided the only efficient system with which to examine the requirements for recognition of a mammalian replication origin. Here we show that the rate of replication and the specific chromosomal sites at which replication initiates are regulated independently in this system. Incubating post-ODP nuclei at concentrations that mimic pre-blastula embryos resulted in the duplication of the entire CHO genome within 3 hours (Fig. 1), nearly four times faster than in culture, yet the replication fork elongation rate (4.5 nt/second) was less than 1/6 that observed in cultured cells. This increased efficiency of replication was mediated by an increased number of initiations per nucleus. Origin-mapping experiments at the CHO DHFR locus demonstrated that the increased number of origins at low nucleo-cytosolic ratios included sites not typically utilized by cultured cells. It is also possible that initiations at physiologically utilized origins, normally staggered throughout a 10 hour S-phase, become more synchronous at lower nucleo-cytosolic ratios. At higher nucleo-cytosolic ratios, genome replication rates were substantially reduced and initiation at the DHFR locus was restricted to the physiologically relevant origin sites. However, when either pre-ODP nuclei or permeabilized post-ODP nuclei were incubated at these higher concentrations, replication initiated at apparently random sites. In particular, pre-ODP nuclei showed the same relationship of nucleo-cytosolic ratio to replication efficiency as post-ODP nuclei. Thus, the titration of embryonic factors by an increasing concentration of nuclei is not sufficient to restrict initiation to physiologically relevant sites. An independent mechanism mediated by components assembled within nuclei at the G1-phase ODP determines which of many potential initiation sites will function as an origin of replication.

It has long been suggested that Xenopus and Drosophila eggs contain a factor that promotes promiscuous DNA
replication and is titrated as cells approach the MBT (Blumenthal et al., 1974; Newport and Kirschner, 1982a,b; Walter and Newport, 1997). We have shown previously that the ability of *Xenopus* eggs to permit the autonomous replication of virtually any microinjected DNA sequence ceases near the MBT (Gilbert et al., 1995). Hyrien and Mechali have shown that initiation of replication becomes focused to the intergenic spacer region of the rDNA locus after the MBT (Hyrien et al., 1995). Recently, Walter and Newport provided evidence for an increase in replicon size as the concentration of *Xenopus* sperm nuclei in egg extract was increased from 1,000 to 10,000 nuclei/μl, concluding that replicon size is controlled by an unidentified factor that becomes limiting at the MBT (Walter and Newport, 1997). The results presented here also support the existence of such a factor. In fact, the optimal concentration for site-specific initiation of replication (Fig. 3) is very close to the concentration of nuclei at the MBT, which takes place at the 12th cell division. Since one *Xenopus* egg contains approximately 0.5 μl of cytosol, the 4,096 nuclei (~6 pg DNA/nucleus for both *Xenopus* and CHO nuclei) at the 12th cell division are at a concentration of approximately 8,000 nuclei/μl of egg cytosol.

What might be the nature of such a factor(s)? Two general models seem likely. One possibility is an essential initiation factor that directly determines the number of sites that will function as origins. One obvious candidate for such a factor is the origin recognition complex (ORC), an evolutionarily

---

**Fig. 6.** Efficiency of DNA replication with permeabilized nuclei. Post-ODP CHOC 400 nuclei were prepared in the presence of various concentrations of either digitonin (A), Triton (B) or NP40 (C) and incubated in a *Xenopus* egg extract at 10,000/μl. The percentage of input DNA replicated at the indicated times was then determined by acid precipitation as in Fig. 1. Nuclei were prepared with (A) 75 μg/ml (■), 350 μg/ml (●), 500 μg/ml (▲) or 1 mg/ml (□) digitonin; (B) 75 μg/ml digitonin (■) and 0.05% (▲) or 0.5% (□) Triton; (C) 75 μg/ml digitonin (■) and 0.05% (▲) or 0.5% (□) NP40. Shown are the results of two experiments, one (A) in which nuclei prepared with various concentrations of digitonin were compared and one (B) in which intact nuclei prepared with 75 μg/ml digitonin were compared to nuclei permeabilized with either Triton or NP40. In D through F the first 90 minutes of the reactions shown in A through C are expanded to illuminate the lag period preceding DNA synthesis. Similar results were obtained in 3 independent experiments.

**Fig. 7.** Permeabilization of nuclei disrupts origin recognition. Post-ODP CHOC 400 nuclei prepared as in Fig. 6 were introduced into a *Xenopus* egg extract supplemented with aphidicolin and the sites of initiation of replication were mapped using the ELFH assay, as in Fig. 3. Shown are the results of three independent experiments after the preparation of nuclei with: (A) 75 μg/ml (■) or 350 μg/ml (●) digitonin; (B) 75 μg/ml digitonin (■) or 0.05% Triton (▲); (C) 75 μg/ml digitonin (■) or 0.05% NP40 (▲). Experiments were done in two groups. One compared various concentrations of digitonin and the second compared intact nuclei prepared in the presence of 75 μg/ml digitonin to nuclei prepared with Triton or NP40. Hence, the data for 75 μg/ml digitonin is the same in B and C. In each panel the standard error of the mean is shown when greater than 1.
conserved, six subunit complex that, in *S. cerevisiae*, is constitutively bound to replication origins (Dutta and Bell, 1997) and appears to determine where replication will initiate (Bielinsky and Gerbi, 1998). The *Xenopus* homologues of ORC proteins are required for the initiation of *Xenopus* sperm replication (Rowles et al., 1996). However, ORC seems an unlikely candidate, since it does not appear to become a limiting factor even at high concentrations of *Xenopus* sperm nuclei (Walter and Newport, 1997). Nevertheless, it remains possible that another unidentified initiation factor directly determines how many pre-formed ORC-DNA complexes initiate DNA replication.

A second possibility is that a nuclear remodeling factor can indirectly influence the number and/or position of initiation sites per nucleus. The MBT is associated with a host of changes in nuclear architecture, from the remodeling of chromatin (Dimitrov et al., 1993) to changes in chromosome loop sizes (Buongiorno et al., 1982; Micheli et al., 1993) and the distribution of replication proteins (Leibovichi et al., 1992). Changes in chromosome architecture have been shown to alter the pattern of initiation in *Xenopus* egg extracts (Lawlis et al., 1996). Supplementing *Xenopus* egg extracts with somatic histone H1 reduces replication efficiency and limits the number of pre-replication complexes formed on sperm chromatin (Lu et al., 1998). Furthermore, when NP40 permeabilized *Xenopus* erythrocyte nuclei were incubated in *Xenopus* egg extract at 2,000 nuclei/µl, somatic histones were rapidly (<15 minutes) replaced by embryonic histones in a nucleoplasmin-dependent manner (Dimitrov and Wolffe, 1996). It is likely that such chromatin remodeling takes place when mammalian nuclei are incubated in *Xenopus* egg extract. Potentially, then, nuclear remodeling and/or histone exchange could lead to an increased number of accessible origin sites at low concentrations of CHO nuclei.

In either case, the ability of *Xenopus* egg cytosol to influence the number of initiations becomes undetectable when the concentration of CHO nuclei approximates the MBT. At these higher concentrations, the pattern of replication reflects properties of the nuclei themselves and a separate mechanism appears to dictate which of the many potential chromosomal sites will function as an origin. Although this mechanism is as yet undefined, our data implicate a nuclear component that is assembled at the ODP and lost upon permeabilization of post-ODP nuclei. Although this mechanism is as yet undefined, our data implicate a nuclear component that is assembled at the ODP and lost upon permeabilization of post-ODP nuclei.

It is tempting to make an analogy between the ODP and the MBT. Both transitions involve the selection of specific origin sites from a much larger set of potential origins and are coincident with architectural changes within nuclei (see Introduction). Although the studies reported here involve the introduction of mammalian nuclei into *Xenopus* egg extracts, our results suggest that two independent processes may also regulate origin usage during *Xenopus* development. The MBT is a sharp transition, marking the onset of transcription (Newport and Kirschner, 1982a,b). The specification of origins appears to be a more gradual transition, after the MBT (Hyrien et al., 1995). We propose that origin specification results from: 1) a titration of egg cytosolic components prior to the MBT, reducing the number of origins fired per nucleus and 2) changes in nuclear structure occurring after the MBT. Such changes may be heterogeneous, taking place at different times and leading to different initiation patterns in different cell lineages. These changes may be similar to those that take place at the ODP in CHO cells.

This work and the salary of D.S.D. were supported by March of Dimes grant #1-FY96-0610 and #1-FY97-0586 to D.M.G.

REFERENCES


