Journal of Cell Science 113, 887-898 (2000)
Printed in Great Britain © The Company of Biologists Limited 2000
JCS1130

Selective activation of pre-replication complexes in vitro at specific sites in mammalian nuclei

Cong-Jun Li, Joseph A. Bogan, Darren A. Natale* and Melvin L. DePamphilis‡

National Institute of Child Health and Human Development, Building 6, Room 416, National Institutes of Health, Bethesda, MD 20892-2753, USA

*Present address: National Center for Biotechnology Information, National Library of Medicine, National Institutes of Health, Bethesda, MD 20894, USA

‡Author for correspondence (e-mail: depamphm@box-d.nih.gov)

Accepted 13 December 1999; published on WWW 14 February 2000

SUMMARY

As the first step in determining whether or not pre-replication complexes are assembled at specific sites along mammalian chromosomes, nuclei from G_1-phase hamster cells were incubated briefly in *Xenopus* egg extract in order to initiate DNA replication. Most of the nascent DNA consisted of RNA-primed DNA chains 0.5 to 2 kb in length, and its origins in the DHFR gene region were mapped using both the early labeled fragment assay and the nascent strand abundance assay. The results revealed three important features of mammalian replication origins. First, *Xenopus* egg extract can selectively activate the same origins of bi-directional replication (e.g. ori-β and β′) that are used by hamster cells in vivo. Previous reports of a broad peak of nascent DNA centered at ori-β/β′ appeared to result from the use of aphidicolin to synchronize nuclei and from prolonged exposure of nuclei to egg extracts. Second, these sites were not present until late G_1-phase of the cell division cycle, and their appearance did not depend on the presence of *Xenopus* Orc proteins. Therefore, hamster pre-replication complexes appear to be assembled at specific chromosomal sites during G_1-phase. Third, selective activation of ori-β in late G_1-nuclei depended on the ratio of *Xenopus* egg extract to nuclei, revealing that epigenetic parameters such as the ratio of initiation factors to DNA substrate could determine the number of origins activated.

Key words: DNA replication, *Xenopus* egg, Hamster nucleus, Eukaryotic replication origin, Pre-replication complex

INTRODUCTION

Initiation of DNA replication in multi-cellular eukaryotes such as frogs, flies and mammals bears striking similarities to this process in single-cell eukaryotes such as yeast. In *Saccharomyces cerevisiae*, pre-replication complexes are assembled at specific DNA sequences thorough the sequential binding of a six protein origin recognition complex (ORC), Cdc6 protein, and a six protein complex consisting of Mcm proteins 2 to 7 (reviewed by DePamphilis, 1999a,b). Homologues for these proteins as well as other proteins involved in the initiation process have been discovered in a variety of multicellular eukaryotes, and several have been shown to be required for DNA replication. Thus, one would expect that pre-replication complexes in multicellular eukaryotes are also assembled at specific genomic sites.

In fact, initiation sites for DNA replication in mammalian cells, like those in the differentiated tissues of frogs and flies, occur at specific genomic loci (reviewed by DePamphilis, 1999a,b). For example, a 200 kb region at the human β-globin gene (Aladjem et al., 1995; Kitsberg et al., 1993) and a 500 kb region at the mouse IgH gene (Ermakova et al., 1999) are each replicated from a single initiation locus. However, while some data suggest that most initiation events occur at specific origins of bi-directional replication (OBRs) located within 0.4 to 2 kb of sequence, other data suggest that initiation events are distributed throughout large regions of 55 kb or more with little preference for one site over another (reviewed by DePamphilis, 1999a,b). Extensive mapping of replication origins at the DHFR and rRNA gene regions (summarized by Kobayashi et al., 1998) as well as a combination of origin mapping and genetic analysis at the URA4 gene region in fission yeast (Dubey et al., 1994) has suggested a solution to this paradox: some replication origins consist of 'initiation zones' that contain one or more primary sites (OBRs) where initiation occurs at a high frequency, as well as secondary sites where initiation occurs at low frequencies (Kobayashi et al., 1998).

The complexity of mammalian replication origins may reflect the fact that DNA contains many potential origins that can be activated in embryos undergoing rapid cell cleavage, but that as development progresses both genetic and epigenetic parameters conspire to repress initiation at some sites while activating it at others (referred to as the 'Jesuit Model'; DePamphilis, 1999a,b). Thus, prior to expression of zygotic genes in *Xenopus*, the absence of DNA transcription, an open chromatin structure, an immature nuclear structure, and a high ratio of initiation proteins to DNA could contribute to activation of large numbers of replication origins. Conversely,

with the onset of zygotic gene expression, the appearance of histone H1, additional nuclear lamins, and possibly other cell constituents could limit initiation to origins with the greatest affinity and the greatest accessibility for pre-replication proteins. This would account for the acquisition of site-specific initiation during *Xenopus* (Hyrien et al., 1995) and *Drosophila* (Sasaki et al., 1999) development.

Further support for this model comes from efforts to identify the requirements for site specific initiation in vitro. When either DNA or chromatin is incubated in *Xenopus* egg extract, it is first organized into nuclei, and then DNA replication begins at many sites 'randomly' distributed throughout the genome. In contrast, when nuclei from G_1-phase hamster cells are used, soluble *Xenopus* proteins initiate DNA replication at or close to the same DNA sites chosen by the hamster cell (Gilbert et al., 1993, 1995a). Site-specificity (but not the ability to initiate replication) is either reduced or lost when nuclei are either permeabilized (Dimitrova and Gilbert, 1998; Gilbert et al., 1995a), or taken from early rather than late G_1-phase cells (Wu and Gilbert, 1997), or taken from SV40 transformed cells (Wu et al., 1998). Therefore, DNA sequence alone does not determine where initiation occurs.

While these results demonstrate that *Xenopus* egg extract can initiate replication non-randomly in mammalian nuclei, the newly synthesized DNA is distributed over a broad region of 80 kb or more with the peak activity centered at the ori-β/ori-β′ locus (~10 kb). This broad peak of newly synthesized DNA could reflect a broad distribution of pre-replication complexes throughout this region, or it could result primarily from the conditions used in these experiments. For example, the hamster nuclei used in these experiments were incubated for 1 to 3 hours in *Xenopus* egg extract containing aphidicolin in order to accumulate initiation events in the absence of DNA synthesis. However, while aphidicolin specifically inhibits replicative DNA polymerases, it does not inhibit synthesis of the first 30 to 40 nucleotides by DNA polymerase-α:DNA primase, the enzyme responsible for initiation of RNA-primed DNA synthesis (Decker et al., 1986), and therefore does not prevent synthesis of short RNA-primed nascent DNA chains in situ (Nethanel and Kaufmann, 1990). This means that replication forks are assembled at replication origins in the presence of aphidicolin, and then held there for an extended period of time before releasing them in order to label nascent DNA at replication origins. Since these conditions can lead to DNA damage and can uncouple DNA unwinding from DNA synthesis (see Discussion), we considered the possibility that the use of aphidicolin to synchronize nuclei at their G_1/S-phase boundary might cause an artifactually broad peak of DNA synthesis centered around a replication origin.

The results presented here demonstrate that the same primary initiation sites used by mammalian nuclei in vivo can be selectively activated by a *Xenopus* egg extract in vitro. The broad peak of initiation activity previously observed in some experiments appears to be an artifact of the experimental conditions. Nevertheless, our results confirm that hamster pre-replication complexes are assembled during G_1-phase, as previously reported (Wu and Gilbert, 1996; Yu et al., 1998), but extend this conclusion to specific chromosomal sites. In addition, selective activation of ori-β in late G_1-nuclei, like activation of DNA synthesis in aphidicolin arrested late G_1-nuclei (Dimitrova and Gilbert, 1998), depended on the ratio of *Xenopus* egg extract to nuclei, revealing that epigenetic parameters such as the ratio of initiation factors to DNA substrate could determine the number of origins activated during animal development.

MATERIALS AND METHODS

Xenopus egg extract

Extracts were prepared from *Xenopus* eggs as previously described (Blow, 1993), except that several of the buffers were modified. Extraction buffer consisted of 50 mM potassium acetate, 50 mM HEPES (pH 7.6), 0.5 mM EDTA, 0.5 mM EGTA, and 0.25 mM dithiothreitol (added just prior to preparation of the extract). Extract dilution buffer was extraction buffer supplemented with 10 μg/ml each of the protease inhibitors aprotinin, leupeptin, and pepstatin. Although various extract preparations differed with respect to their replication activity, extracts made from the same batch of eggs using these modified buffers were 25% to 50% more active compared with extracts prepared using the original extraction buffer (50 mM KCl, 50 mM HEPES (pH 7.6), 5 mM $MgCl_2$, 2 mM dithiothreitol) and extract dilution buffer (50 mM KCl, 50 mM HEPES pH 7.6, 0.4 mM $MgCl_2$, 0.4 mM EGTA, and protease inhibitors as above). Replication activity was increased an additional 50% to 75% if the extract dilution buffer contained 450 mM potassium acetate (estimated final concentration in extract is 80 mM) and 0.1 mM dithiothreitol.

To prepare XlOrc2-depleted extract, anti-XlOrc2 serum was diluted 1:1 with Immunopure(A) IgG binding buffer (Pierce) and then incubated with prewashed Protein A-Sepharose beads (Pharmacia) in a ratio of 2 volumes antiserum to 1 volume beads for 30 minutes at room temperature. The beads were washed three times in IgG binding buffer, and then all of the supernatant removed using a gel-loading, long thin disposable pipette tip. Two volumes of extract were combined with one volume of beads and gently rotated for 30 minutes at 4°C. The beads were removed by centrifugation in an Eppendorf microfuge for 5 minutes at 2,000 rpm, 4°C, to avoid loss of membrane vesicles. The supernatant was then mixed with a fresh aliquot of beads (0.5 volumes), incubated for 30 minutes, and the beads again removed by centrifugation. The supernatant was used for DNA replication.

Hamster nuclei

Nuclei were prepared from CHOC 400 cells, a Chinese hamster ovary cell line with ~1000 copies of the DHFR gene amplicon (Gilbert et al., 1995a; Wu et al., 1997). For G_0-phase cells, 90% confluent monolayers were washed with pre-warmed PBS and then cultured for 48 hours in DMEM supplemented with 0.5% calf serum (dialyzed) but without isoleucine. For G_1-phase cells, cells ~80% confluent were synchronized in mitosis and then released into G_1 (Gilbert et al., 1995a). Origin mapping experiments required 2×10^6 cells. When total DNA synthesis was measured, the DNA in these cells was uniformly prelabeled by culturing them overnight in fresh medium supplemented with 0.1 μCi/ml of [^{3}H]Thd (25 Ci/mmol, Amersham). DNA synthesis time course experiments required 5×10^5 cells per assay.

DNA replication in *Xenopus* egg extract

The indicated number of nuclei were resuspended in 45 μl of a reaction mix (on ice) consisting of 40 μl of *Xenopus* egg low-speed extract, 1 μl 40× ATP regeneration solution, 2 μl [α-^{32}P]dATP, and 2 μl [α-^{32}P]dCTP (10 μCi/μl, 6,000 mCi/mmol, in water, Amersham; Gilbert et al., 1995a; Wu et al., 1997). Total DNA synthesis was measured as acid-precipitable [^{32}P]DNA. Pmoles dATP/nucleus was calculated as (^{32}P-cpm/nucleus)(2 pmoles dATP/total ^{32}P-cpm in reaction). *Xenopus* egg extract contains ~50 μM dATP (Chong et al., 1997), or ~2 pmoles dATP/40 μl extract. For 'early labeled fragment assays', reactions were incubated at 22°C for 40 minutes before stopping DNA synthesis by addition of 200 μl of 20 mM Tris-HCl

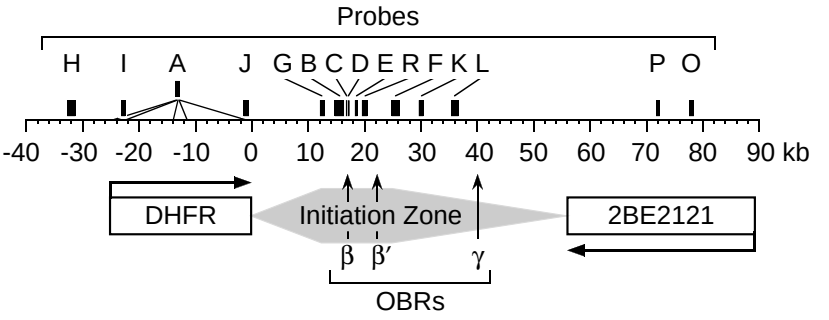

Fig. 1. Fifteen DNA probes (Gilbert et al., 1995a) were located within a ~115 kb locus in the CHO cell genome containing the DHFR and 2BE2121 genes (arrows indicate direction of transcription). These genes are separated by a ~55 kb region containing at least three primary initiation sites (β, β′ and γ; Kobayashi et al., 1998). Results from 2D-gel analyses suggest that initiation events can occur throughout this region (initiation zone), although most of them appear to occur in the 12 kb region containing ori-β and ori-β′ (Dijkwel and Hamlin, 1995; Kalejta et al., 1996).

(pH 7.5), 5 mM EDTA, 0.5% sodium dodecylsulfate (SDS), and 0.1 mg/ml pancreatic RNase A (Sigma). After 30 minutes at room temperature, proteinase K was added to 2 mg/ml and samples were incubated at 45°C over night. DNA was extracted once with an equal volume of phenol and then precipitated by adjusting the sample to 0.3 M sodium acetate (pH 5.6) and three volumes isopropyl alcohol at room temperature. DNA was collected immediately by centrifugation in an Eppendorf centrifuge (4°C, 15 minutes, full speed), resuspended in 0.4 ml 0.6× SSC buffer, sonicated into <2 kb fragments, and then hybridized to membrane bound DNA probes, as described by Gilbert et al. (1995a). Randomly labeled CHOC 400 DNA was prepared by purifying DNA from G_0 cells and then labeling it by nick translation using random primers. RNA-primed DNA was prepared in the absence of RNase treatment by its resistance to λ-exonuclease, as described by Kobayashi et al. (1998).

RESULTS

DNA replication can be initiated in vitro at specific chromosomal sites

Previous studies have identified three primary initiation sites (origins of bi-directional replication, OBRs) within the intergenic region between the DHFR gene and the 2BE2121 gene referred to as ori-β, ori-β′ and ori-γ (Fig. 1; summarized by Kobayashi et al., 1998). To determine whether or not these origins exist prior to S-phase, nuclei from CHOC 400 hamster cells were isolated 4 hours after cells were released from mitosis (2 hours prior to S-phase) and incubated in *Xenopus* egg extract to initiate DNA replication de novo. As previously reported (Gilbert et al., 1995a; Wu and Gilbert, 1996), DNA synthesis began after ~20 minutes of incubation and then continued for at least 40 minutes (Fig. 2A, open circles). The amount of DNA synthesized by 1 hour was equivalent to 10 to 15% of the genome replicated, comparable to previous studies done under identical conditions (Gilbert et al., 1995a; Wu et al., 1997). When the [^{32}P]DNA synthesized during the first 40 minutes of incubation (~20 minutes of DNA synthesis) was fractionated by sucrose gradient sedimentation, ~60% of the newly synthesized DNA was only 0.5 to 2 kb long, and 80% of this was less than 1 kb in length (Fig. 2B), suggesting that it originated primarily from newly activated replication origins. Therefore, total DNA from a 40 minutes incubation was hybridized simultaneously to 15 DNA probes distributed throughout the DHFR gene initiation region (Fig. 1). In this early labeled fragment assay (Vassilev and DePamphilis, 1992), sequences proximal to replication origins give the strongest signal. Since CHOC 400 cells contain ~1000 tandemly integrated copies of a ~250 kb region that includes

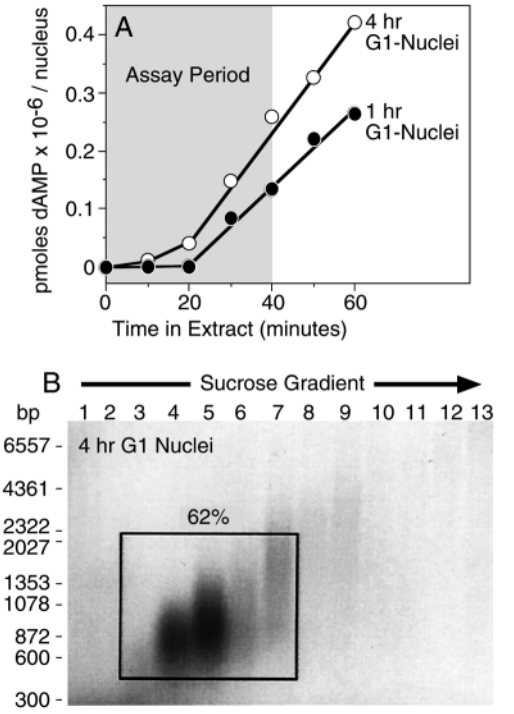

Fig. 2. Induction of DNA synthesis in hamster G_1-nuclei was delayed ~20 minutes and resulted in synthesis of short nascent DNA chains. (A) Nuclei (25,000/μl) from 1 hour (●) or 4 hour (○) G_1-phase CHOC 400 cells (pre-labeled with [^{3}H]Thd) were incubated in *Xenopus* egg extract supplemented with [α-^{32}P]dATP. The ratio of acid precipitable [^{32}P]DNA/[^{3}H]DNA was determined at the times indicated and converted into pmoles of dAMP incorporated per nucleus. (B) Hamster nuclei were isolated 4 hours after release from mitosis and then incubated for 40 minutes in *Xenopus* egg extract. DNA size was determined by heating the sample for 10 minutes at 100°C, placing it in ice for 10 minutes, and then sedimenting it through 10 to 30% sucrose in 10 mM Tris (pH 8.0), 150 mM NaCl and 1 mM EDTA using a Beckman SW50.1 rotor at 45 K rpm, 4°C, for 5 hours. Individual fractions were subjected to electrophoresis in 1.2% agarose containing 50 mM NaOH. [^{32}P]DNA was quantified using a phosphorimager.

the DHFR gene initiation locus, this assay can detect replication origins in these cells without further amplifying the nascent DNA sequences. Results from early labeled fragment assays have been in excellent agreement with results from other assays for mammalian replication origins (summarized by DePamphilis, 1993a,b; Kobayashi et al., 1998).

Several controls were included. The specificity of each probe

(A)

Probe	Map (kb)	Probe (bp)	Exponential Nuclei Blot	c/bp	-Bkg	4 hour G1-Nuclei Blot	c/bp	-Bkg	4h/exp	4h/1h	1 hour G1-Nuclei Blot	c/bp	-Bkg	1h/exp
H	-32	1600		143	124		39	22	0.2	0.2		111	100	0.8
I	-22.5	650		297	278		74	57	0.2	0.3		216	205	0.7
A	-13	643		174	155		57	40	0.3	0.4		108	97	0.6
J	-1	800		392	373		185	168	0.5	0.5		331	320	0.8
G	12.5	685		479	460		349	332	0.7	0.8		436	425	0.9
B	15.5	1532		232	213		145	128	0.6	0.7		206	195	0.9
C	16.5	231		237	218		608	591	2.7	3.1		219	191	0.9
D	17.5	281		255	236		552	535	2.3	2.1		261	250	1.1
E	18.5	380		271	252		286	269	1.1	0.9		311	300	1.2
R	20	887		251	232		200	183	0.8	0.8		246	235	1.0
F	25.5	1250		243	224		95	78	0.3	0.4		196	185	0.8
K	30	700		410	391		356	339	0.9	1.1		321	310	0.8
L	36	1200		322	303		101	84	0.3	0.4		241	230	0.8
P	72	500		521	502		286	269	0.5	0.6		426	415	0.8
O	78	700		225	206		67	50	0.2	0.3		191	180	0.9
λ		649		19			17					11		

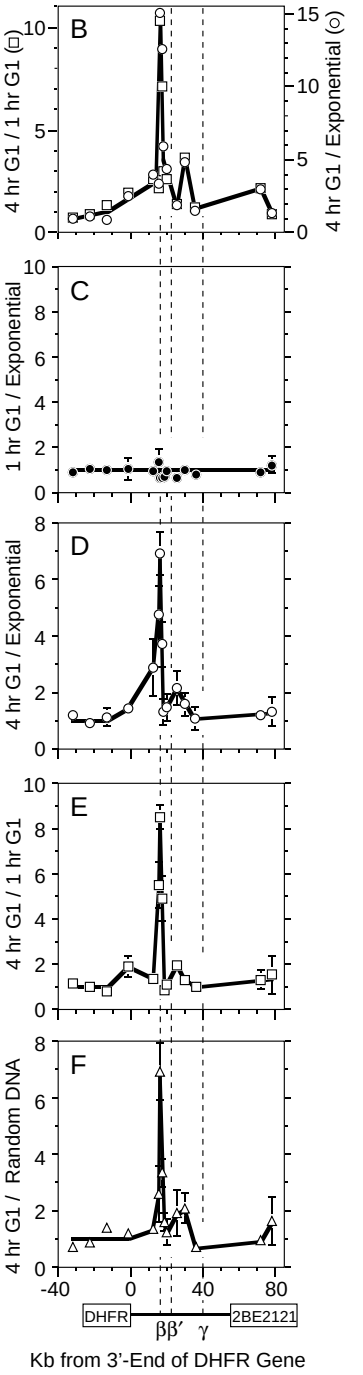

Fig. 3. *Xenopus* egg extract initiated DNA replication at specific sites in nuclei from late, but not early, G_1-phase hamster cells. (A) G_1-phase nuclei were isolated either 1 hour or 4 hours after CHOC 400 cells were released from mitosis and then incubated in a *Xenopus* egg extract supplemented with [α-^{32}P]dATP and [α-^{32}P]dCTP. In addition, nuclei were isolated from exponentially proliferating CHOC 400 cells and subjected to the same protocol. Newly synthesized [^{32}P]DNA was then hybridized to the 15 hamster DNA sequences indicated in Fig. 1, and to a 620 bp fragment of bacteriophage-λ DNA. The amount of [^{32}P]DNA/bp was calculated for each sequence, and the value obtained for λ-DNA (nonspecific hybridization) was then subtracted. These data were then routinely corrected for hybridization variation among the hamster probes in one of three ways. ^{32}P-DNA/bp from 4 hour G_1-nuclei was divided by ^{32}P-DNA/bp from EITHER exponential nuclei, or 1 hour G_1-nuclei, or randomly-labeled CHOC 400 DNA. (B) Data from experiment in A normalized to the mean value for probes H, I and A (the DHFR gene). (C) ^{32}P-DNA/bp hamster DNA from 1 hour G_1-nuclei divided by ^{32}P-DNA/bp from exponential nuclei in four independent experiments. The same result was obtained with 1 hour G_1-nuclei divided by randomly labeled DNA (data not shown). (D to F) ^{32}P-DNA/bp hamster DNA from 4 hour G_1-nuclei divided by the values obtained either from exponential nuclei (○), 1 hour G_1 nuclei (□), or randomly labeled DNA (△). (C to F) The mean ± s.e.m. for four independent experiments.

was confirmed by its ability to anneal with a single restriction endonuclease fragment from CHOC 400 DNA (Gilbert et al., 1995a). Differences in the amount of radioactivity that annealed to each probe due to differences in base composition were eliminated by incorporating both [α-^{32}P]dATP and [α-^{32}P]dCTP during DNA biosynthesis, while differences due to probe size were eliminated by calculating the ratio of [^{32}P]DNA/base pair (bp) for each probe. Differences due to non-specific hybridization were eliminated by subtracting the amount of [^{32}P]DNA/bp that annealed to a bacteriophage-λ DNA fragment from the amount of [^{32}P]DNA/bp that annealed to each hamster DNA probe.

Although the ori-β peak was routinely visible in a simple display of the data (e.g. in Fig. 11B), comparison of results between different preparations of nuclei and egg extract was difficult due to variations in the amount of DNA replication and in the efficiency of hybridization to different probes. Therefore, two additional corrections were applied. First, data were normalized to the DHFR gene by dividing the amount of [^{32}P]DNA/bp that hybridized to each probe by the average amount of [^{32}P]DNA/bp that hybridized to probes H, I and A. Since initiation events have never been detected in this locus by any origin mapping method, it provided a common reference point. Second, the amount of newly synthesized [^{32}P]DNA from 4 hour G_1-phase nuclei that hybridized to each probe was divided by the amount of [^{32}P]DNA from a control sample that hybridized randomly throughout this genomic region.

Three different controls were considered, and each was run concurrently with the experimental sample. First, replication forks were assumed to be randomly distributed throughout the genome in exponentially proliferating CHOC 400 cells, and therefore the ratio of [^{32}P]DNA synthesized in G_1-nuclei to [^{32}P]DNA synthesized in exponentially proliferating nuclei should reveal specific initiation sites. Correction for variation in hybridization using this control generally resulted in a sharp peak of nascent DNA at ori-β and a smaller peak near ori-β′

(Fig. 3B). However, we found that some batches of 'randomly proliferating cells' exhibited a peak at ori-β that could mask the presence or position of specific initiation sites in G_1-nuclei. The size of this peak varied from batch to batch and presumably reflected the fraction of G_1-phase cells present.

The second control was based on a previous report that CHOC 400 nuclei isolated 1 hour after release from mitosis (1 hour G_1-nuclei) initiated replication uniformly throughout the DHFR gene region instead of at specific sites (Dimitrova and Gilbert, 1998; Wu and Gilbert, 1996, 1997). Therefore, the ratio of [^{32}P]DNA synthesized in 4 hour G_1-nuclei to [^{32}P]DNA synthesized in 1 hour G_1-nuclei should also reveal specific initiation sites. As previously reported, 1 hour G_1-nuclei were similar to 4 hour G_1-nuclei in their ability to synthesize DNA (Fig. 2A), but did not initiate replication site-specifically (Fig. 3A,C). Therefore, the ratio of [^{32}P]DNA/bp in 4 hour G_1-nuclei to [^{32}P]DNA/bp in 1 hour G_1-nuclei provided the ideal method for detecting formation of specific initiation sites that arise at later times during G_1-phase and confirmed the presence of a strong initiation site at ori-β (Fig. 3B,E).

Finally, the ability of *Xenopus* egg extract to selectively activate ori-β was confirmed by comparing the ratio of newly synthesized [^{32}P]DNA/bp in 4 hour G_1-nuclei to the hybridization of randomly labeled CHOC 400 [^{32}P]DNA that hybridized to each probe. Again, a strong initiation signal was observed at ori-β (Fig. 3F). Together, these results show that *Xenopus* egg extract can initiate DNA replication at specific sites in hamster nuclei that corresponded to the primary initiation sites mapped in vivo.

DNA replication begins in vitro at the same sites used in vivo

'Nascent strand abundance assays' have been used to quantify the relative number of newly initiated replication bubbles in the DHFR gene region by using competitive PCR to measure the relative abundance of nascent DNA strands with an average length of 0.8 to 1 kb isolated from either synchronized (Kobayashi et al., 1998) or unsynchronized (Pelizon et al., 1996) CHO cells. The results confirmed that ori-β is a primary initiation site contained within a 2 kb locus and identified an additional initiation site (ori-β′) 5 kb downstream (Fig. 1). Comparison of these data with the distribution of ^{32}P-labeled nascent DNA chains of similar length synthesized in hamster G_1-nuclei incubated in *Xenopus* egg extract confirmed that *Xenopus* egg extract initiates DNA replication in late G_1-nuclei at the same ori-β site used by hamster cells in vivo (Fig. 4A,B). Additional studies were then carried out to determine whether or not the [^{32}P]DNA mapped in the early labeled fragment assay represented RNA-primed nascent DNA chains, and whether or not ori-β′ was also activated in vitro.

Previous studies have shown that DNA synthesis in hamster G_1-nuclei incubated in a *Xenopus* egg extract resulted from de novo initiation of DNA replication and not from DNA damage and repair (Gilbert et al., 1995a). Therefore, since most of the Okazaki fragments produced in CHO cells contain RNA primers at their 5′-ends (Burhans et al., 1991), a significant fraction of the newly synthesized [^{32}P]DNA chains produced during incubation of G_1-nuclei in egg extract should also contain RNA at their 5′-ends. The fraction of these chains containing 5′-RNA was determined as the fraction resistant to

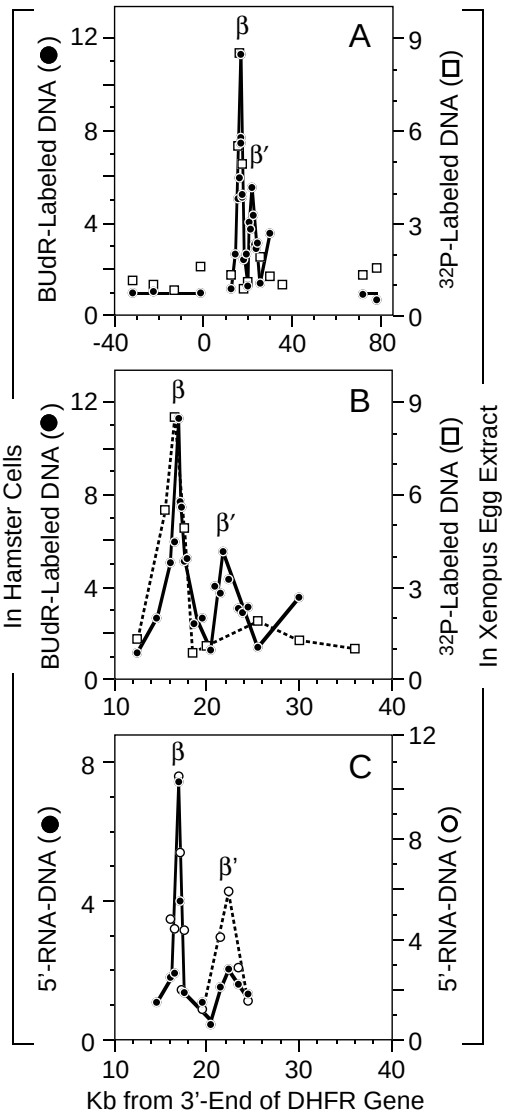

Fig. 4. *Xenopus* egg extract initiated DNA replication in late G_1-nuclei at the same sites activated in vivo by hamster cells (e.g. ori-β and ori-β′). (A,B) Data from Kobayashi et al. (1998) in which competitive PCR was used to measure the relative number of BUdR-labeled nascent DNA chains at different genomic sites produced in vivo by CHO cells (●). These data were compared with the ratio of newly synthesized [^{32}P]DNA produced in 4 hour G_1-nuclei relative to 1 hour G_1-nuclei (Fig. 3C, □). The region from 10 to 40 kb downstream of the 3′-end of the DHFR gene in A is expanded in B. (C) Competitive PCR was used to determine the relative number of RNA-primed nascent DNA chains (5′-RNA-DNA) at different genomic sites produced by 4 hour G_1-nuclei in *Xenopus* egg extract (○). These results were compared to similar data from Kobayashi et al. (1998) using CHO cells in vivo (●).

digestion with λ-exonuclease, as previously described by Kobayashi et al. (1998). λ-Exonuclease degrades 5′-P-DNA chains, but not 5′-P-RNA or 5′-P-RNA-DNA chains (Fig. 5; Bielinsky and Gerbi, 1999). Fractionation of DNA samples by gel electrophoresis before and after treatment with λ-exonuclease confirmed that while all of the internal plasmid DNA control had been degraded, about 70% of the 0.5 to 2 kb [^{32}P]DNA synthesized in hamster G_1-nuclei remained,

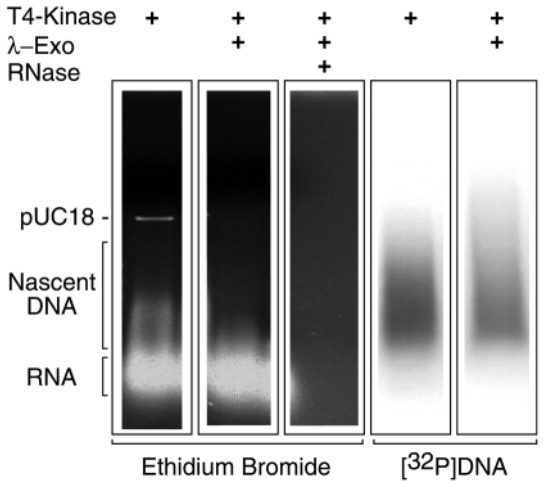

Fig. 5. Newly synthesized DNA in late G_1-nuclei incubated in *Xenopus* egg extract contained 5′-terminal RNA. Nuclei isolated 4 hours after release of CHOC 400 cells from mitosis were incubated for 40 minutes in *Xenopus* egg extract and [^{32}P]DNA 0.5 to 2 kb in length was isolated. DNA was combined with linear, unphosphorylated pUC18 (2.7 kb) and treated with T4-kinase to insure that all 5′-ends were phosphorylated before λ-exonuclease was added to degrade 5′-P-DNA (Kobayashi et al., 1998). One aliquot was then treated with 5 units of RNase I (Epicentre) for 2 hours at 37°C. Samples were fractionated by electrophoresis in 1.2% agarose (TBE buffer). The gel was stained with ethidium bromide, dried and analyzed by a PhosphorImager (Molecular Dynamics).

consistent with initiation of RNA-primed DNA synthesis in hamster G_1-nuclei incubated in a *Xenopus* egg extract (Fig. 5).

These RNA-primed nascent DNA chains were then subjected to competitive PCR in order to quantify the relative abundance of specific sequences. Competitive PCR permits quantification of small amounts of DNA sequences by co-amplifying the target DNA in the presence of known amounts of a competitor DNA that shares the same primer recognition sites. The competitor DNA contains a 20 nucleotide insertion so that its amplified products can be distinguished from those of the target during gel electrophoresis (Fig. 6). Since target and competitor DNA compete for the same PCR primers, they are amplified with the same efficiency. Thus, when the amount of competitor DNA added to the reaction equals the amount of target DNA present, the ratio of amplified competitor to amplified target sequences is 1:1.

Primer set J is located within probe J at the 3′-end of the hamster DHFR gene (Fig. 1) where initiation events have never been detected. Primer sets 6 and 13 are located at the centers of ori-β and ori-β′, respectively (Kobayashi et al., 1998). By selecting competitor concentrations close to the target concentrations, one could ignore the heteroduplexes that formed between target and competitor during the PCR when determining the competitor to target ratios (Kobayashi et al., 1998). The copy number of each probe was determined in an aliquot of the RNA-primed [^{32}P]DNA fraction (nascent DNA, Fig. 6), and in an aliquot of a 1 kb DNA fraction isolated from non-proliferating, serum starved CHOC 400 cells (total DNA, Fig. 6). Nascent DNA from ori-β and ori-β′ was 8- to 9-fold more abundant than nascent DNA from the 3′-end of the DHFR gene (Table 1). When these data were corrected for the experimental variation observed in total DNA using the same three probes, the ratios of ori-β/ori-β′/DHFR gene was 10.7: 5.7: 1 (Table 1). Nine additional PCR primer sets were used (Fig. 4C), and the results confirmed that the same primary initiation sites for DNA replication used by hamster cells in vivo could be selectively activated by *Xenopus* egg extract in vitro.

Site-specificity is independent of *Xenopus* Orc proteins

Depletion of either XlOrc1 or XlOrc2 proteins from *Xenopus*

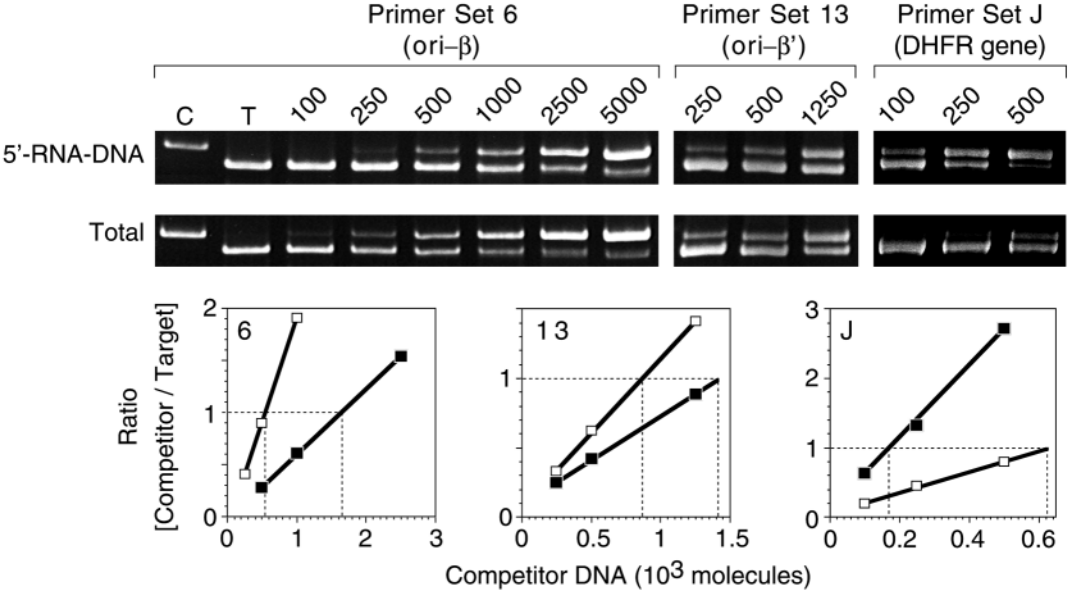

Fig. 6. RNA-primed nascent DNA chains from the DHFR gene initiation zone synthesized by hamster G_1-nuclei in a *Xenopus* egg extract originated predominantly from ori-β and ori-β′. RNA-primed nascent DNA 0.8 kb in length was subjected to competitive PCR using 12 different primer sets, as described by Kobayashi et al. (1998). Primer set 6 was at the center of ori-β, primer set 13 at the center of ori-β′ and primer set J at the 3′-end of the DHFR gene. A fixed amount of target DNA (T) was incubated with increasing amounts of competitor DNA (C). The amplified products were fractionated by gel electrophoresis, stained with ethidium bromide, quantified by densitometry, and the number of copies of competitor DNA (indicated above each gel lane) was plotted against the ratio of the two DNA bands in the gel. The results are summarized in Table 1.

Table 1. Abundance of 5′-RNA-DNA chains in the DHFR gene initiation zone

DNA sample	Primer set		
	6 (ori-β)	13 (ori-β′)	J (DHFR gene)
5′-RNA-DNA	1610	1410	180
Relative to J	9.1	7.9	1
Total DNA	540	870	640
Relative to J	0.84	1.4	1
5′-RNA-DNA/Total DNA	3	1.6	0.28
Relative to J	10.7	5.7	1

Data are from Fig. 6. Numbers are molecules of each primer set in an aliquot of 5′-RNA-DNA chains ~1 kb long from hamster 4 hour G_1-nuclei that have been incubated for 40 minutes in a *Xenopus* egg extract, or in an aliquot of total DNA from G_1-phase CHOC 400 cells.

egg extract has been shown to prevent initiation of DNA replication in either sperm chromatin or plasmid DNA substrates (Carpenter et al., 1996; Coleman et al., 1996; Romanowski et al., 1996; Rowles et al., 1996; Yu et al., 1998), demonstrating that initiation of DNA replication in *Xenopus* egg extract is dependent on a functional interaction of ORC with chromatin. XlOrc2-depleted *Xenopus* egg extract was prepared by extracting it twice with anti-XlOrc2 IgG bound to Sepharose beads. At least 98% of the XlOrc2 protein was removed from the extract as judged by immunoblotting analysis, while mock-depleted extracts retained the bulk of their XlOrc2 (Fig. 7A). As previously reported, depleted extract was no longer able to initiate DNA synthesis in *Xenopus* sperm chromatin (Fig. 7B). However, neither DNA synthesis (Fig. 7B) nor selective activation of ori-β (Fig. 7C) in 4 hour G_1-nuclei was affected by depletion of XlOrc2. Therefore, unlike sperm chromatin, hamster late G_1-nuclei already contained pre-replication complexes, and by analogy with yeast (Bielinsky and Gerbi, 1999), the hamster pre-replication complexes presumably exist at specific chromosomal sites such as ori-β.

Site-specificity is affected by the length of incubation in *Xenopus* egg extract

Previous origin mapping studies from this (Gilbert et al., 1995a, 1993) and other laboratories (Dimitrova and Gilbert, 1998, and references therein) using 4 hour G_1-nuclei observed a broad distribution of newly synthesized DNA that was centered at or close to ori-β/β′, but that extended from the 3′-end of the DHFR gene ~80 kb into the 2BE2121 gene (Fig. 8A, shaded area). In contrast, DNA synthesized in 4 hour G_1-nuclei during the first 40 minutes of its incubation in *Xenopus* egg extract was confined to ori-β and β′ loci (Figs 4B,C, 8A). Probes for ori-γ were not used in these studies.

The experimental protocol in the studies presented here differ significantly from that used in earlier studies. In earlier studies, hamster nuclei were preincubated for 1 to 3 hours in *Xenopus* egg extract containing aphidicolin in order to accumulate initiation events while preventing migration of replication forks away from their origin. Nuclei were then washed free of aphidicolin and nascent DNA was pulse-labeled in a replication cocktail (Wu et al., 1997). In the present study, preincubation

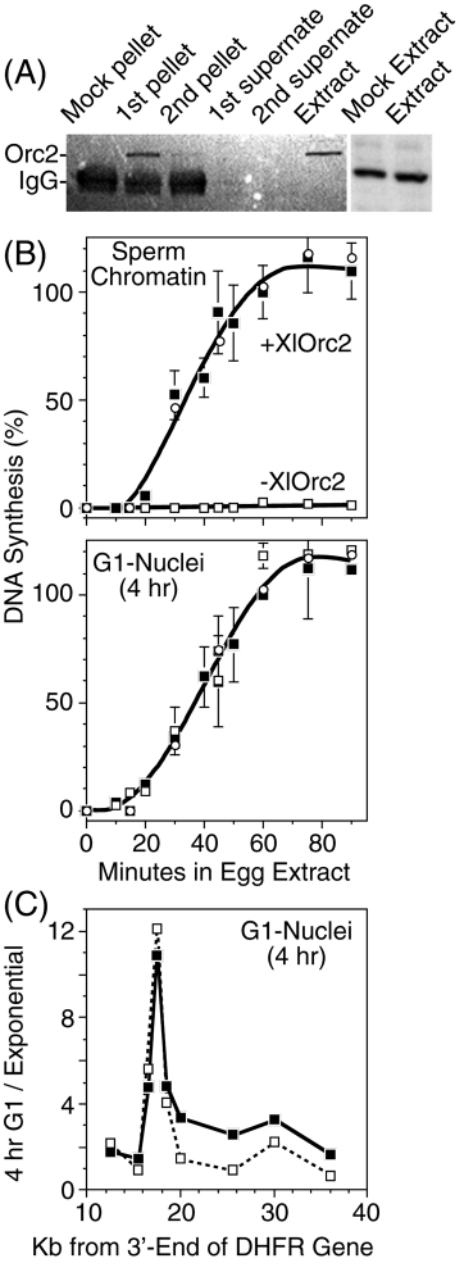

Fig. 7. Site-specificity in hamster late G_1-nuclei is independent of *Xenopus* Orc2 protein. (A) *Xenopus* egg extract (Extract) was depleted of XlOrc2 protein by two successive incubations with anti-XlOrc2 coated beads. Fractions were subjected to SDS gel electrophoresis. XlOrc2 was detected by immunoblotting, and the relative intensities of each XlOrc2 band were quantified by densitometry. Gels are shown for Mock Pellet (beads coated with non-immune serum), 1st and 2nd pellets (beads coated with anti-XlOrc2 serum), 1st and 2nd sup (supernatants from anti-XlOrc2 beads), Extract and Mock Extract (mock depleted egg extract). (B) Either *Xenopus* sperm chromatin or 4 hour hamster G_1-nuclei was incubated in *Xenopus* egg extract (■), or in mock-depleted extract (○), or in XlOrc2-depleted extract (□), and then DNA synthesis was measured as in Fig. 2A. Results were expressed as a percentage of the amount of [^{32}P]DNA detected after 1 hour of incubation. (C) After 40 minutes of incubation in either extract (■), or XlOrc2-depleted extract (□), [^{32}P]DNA synthesized in hamster 4 hour G_1-nuclei was hybridized to DNA probes in the hamster DHFR gene region, as in Fig. 3. Results with mock-depleted extract (not shown) were indistinguishable from those with extract.

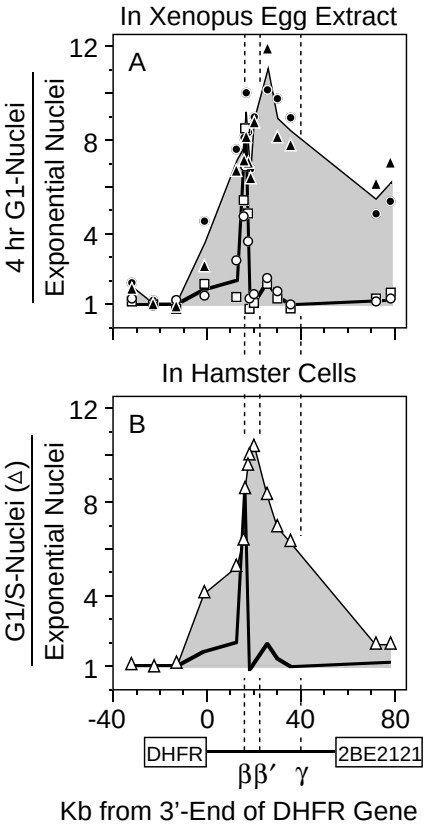

Fig. 8. Aphidicolin synchronization of nuclei resulted in excessively broad peaks in the early labeled fragment assay. (A) Data previously published from this laboratory on incubation of 4 hour G_1-nuclei from CHOC 400 cells in *Xenopus* egg extract using the + aphidicolin protocol (▲, from Fig. 7D in Gilbert et al., 1995) are compared with analogous data presented here using the – aphidicolin protocol (Figs. 3A (○) and 3C (□)). To confirm the reproducibility of the + aphidicolin protocol results, the original experiment was repeated (■). The shaded area is plotted between the two sets of data. (B) Previous data from early labeled fragment assays using CHOC 400 nuclei arrested at their G_1/S-phase boundary and radio-labeled in the same replication cocktail used in the + aphidicolin protocol (shaded area, Δ from Fig. 7B in Gilbert et al., 1995) are compared with 4 hour G_1-nuclei data presented here using the – aphidicolin protocol (solid line).

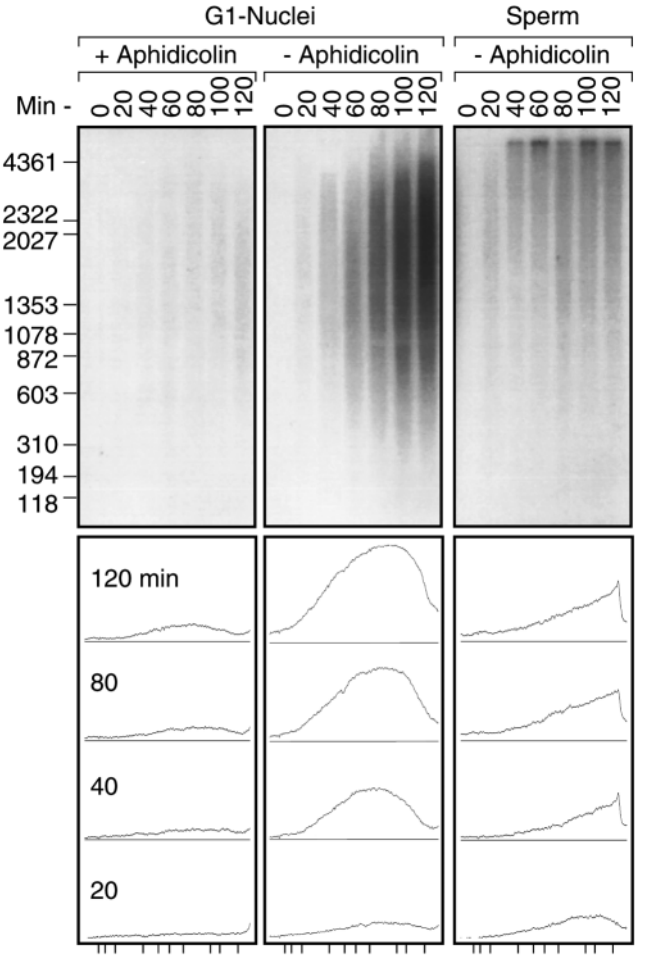

Fig. 9. Prolonged incubation of G_1-nuclei in *Xenopus* egg extract increased initiation events, rather than nascent DNA chain lengths. G_1-nuclei isolated 4 hours after release from mitosis were incubated in *Xenopus* egg extract supplemented with [α-^{32}P]dATP for the times indicated. One reaction also contained 10 μg/ml aphidicolin (Boehringer Mannheim). Another reaction contained *Xenopus* sperm chromatin. [^{32}P]DNA was purified and then fractionated by alkaline gel electrophoresis, as in Fig. 4. The gel was dried and quantified using a PhosphorImager (top panels). Positions of DNA size markers are indicated. The density of selected lanes was determined using NIH Image (bottom panels).

with aphidicolin was omitted, and newly synthesized DNA was labeled immediately as it was synthesized in the egg extract. Therefore, the broad peak observed in earlier studies could have resulted either from replication forks that originated at primary initiation sites (e.g. ori-β and β′) but continued to progress despite the presence of aphidicolin, or from initiation first at primary and later at secondary sites (i.e. non-specific, lower frequency initiation sites).

To test the first possibility, late G_1-nuclei were incubated for up to 2 hours in *Xenopus* egg extract, either with or without aphidicolin present, and then the size and genomic distribution of nascent DNA strands were determined. In the absence of aphidicolin, the amount of DNA synthesized increased 10-fold from 40 minutes to 2 hours, but the length of nascent DNA chains remained limited to 0.5 to 5 kb with a mean length of 1 to 2 kb (Figs 2B, 9). If the initial rate of DNA synthesis reported for CHOC 400 G_1-nuclei synchronized by the '+ aph' protocol (4.5 nts/second; Dimitrova and Gilbert, 1998) was maintained throughout the incubation, then the average length of nascent DNA should have been 5.4 kb after 40 minutes and 10.8 kb after 80 minutes. This limited replication fork migration was not an artifact of our experimental conditions, because DNA synthesized from *Xenopus* sperm chromatin under the same conditions was rapidly extended to 8 kb or longer, as previously reported (Walter and Newport, 1997). Apparently, elongation of nascent DNA requires changes in nuclear structure that have not yet occurred in these G_1-phase nuclei. Therefore, most of the DNA synthesized in late G_1-nuclei resulted from additional initiation events rather than from replication fork migration. Addition of aphidicolin reduced the amount of DNA synthesized by 10-fold at 40 minutes and 20-fold at 2 hour (data not shown). Nevertheless, those DNA polymerases that escaped

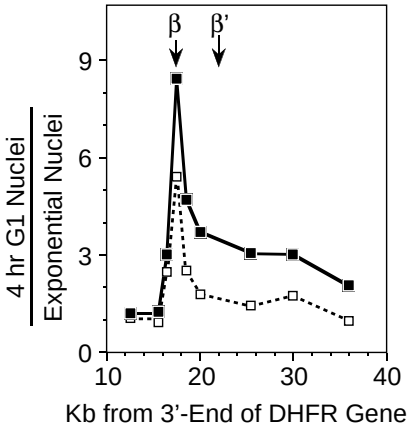

Fig. 10. Prolonged incubation of G_1-nuclei in *Xenopus* egg extract increased initiation events in the ori-β/β′ locus. Nuclei from CHOC 400 cells were isolated 4 hours after release from mitosis and incubated in *Xenopus* egg extract for either 40 (□) minutes (0.15 pmoles dAMP $\times 10^{-6}$ incorporated/nucleus) or 120 (■) minutes (1.5 pmoles dAMP $\times 10^{-6}$ incorporated/nucleus). Newly synthesized [^{32}P]DNA was then hybridized to DNA probes in the DHFR gene region, and the ratios of 4 hour G_1-nuclei/exponential nuclei were calculated, as in Fig. 3.

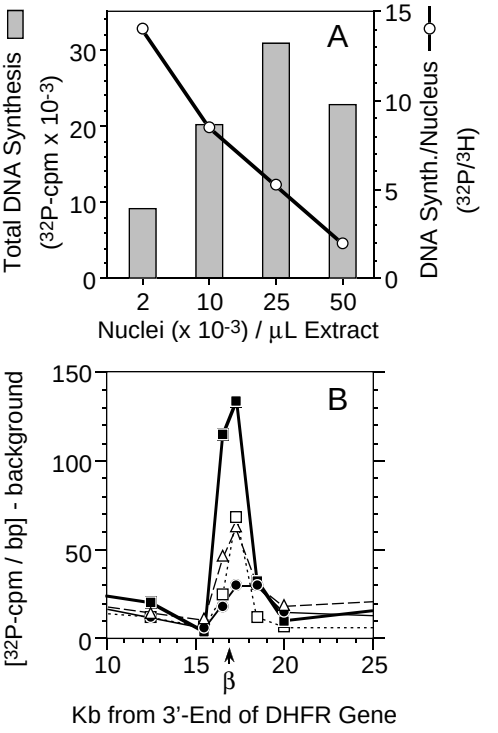

Fig. 11. Increased ratios of *Xenopus* egg extract to G_1-nuclei increased initiation events at secondary sites. (A) Nuclei from CHOC 400 cells containing uniformly labeled [^{3}H]DNA were isolated 4 hours after release from mitosis and incubated for 40 minutes in *Xenopus* egg extract at the ratio of nuclei to extract indicated. Total [^{32}P]DNA synthesis (bars) and the [^{32}P]DNA/[^{3}H]DNA ratio (○) were determined, as in Fig. 2A. (B) Different concentrations of unlabeled nuclei were incubated for 40 minutes in extract supplemented with [α-^{32}P]dATP and [α-^{32}P]dCTP, and [^{32}P]DNA was then hybridized to specific hamster DNA sequences. The ratio of cpm [^{32}P]DNA/bp hamster DNA – cpm [^{32}P]DNA/bp λ-DNA was determined for each hamster DNA probe. Symbols are ● (2,000 nuclei/μl), △ (10,000 nuclei/μl), ■ (25,000 nuclei/μl), □ (50,000 nuclei/μl).

this inhibition traveled no farther than observed in the absence of aphidicolin. Thus, continued DNA synthesis in the presence of aphidicolin could account for some, but not all, of the broad peak of initiation activity.

To determine whether or not prolonged incubation of nuclei in *Xenopus* egg extract (–aph protocol) results in additional initiation events outside the primary initiation sites, incubation times were increased by 20 minute periods from 40 to 120 minutes. Although the amount of DNA synthesized increased 10-fold during this period, the number of initiation events at ori-β and downstream of ori-β increased only 2-fold (Fig. 10), suggesting that the number of initiation events at the ori-β/β′ locus was proportional to the time of incubation. However, the distribution of [^{32}P]DNA was never as broad as observed when late G_1-nuclei were pre-incubated for 2 hours in extract containing aphidicolin (compare Fig. 10 with Fig. 8A). Thus, the number of secondary initiation events appears to increase only marginally with prolonged incubation in *Xenopus* extract, suggesting that the additional DNA synthesis events observed in the + aph protocol resulted from exposure to aphidicolin.

Comparison of the results presented here (–aph protocol) with previous origin mapping studies from our laboratory confirmed this conclusion. G_1/S-phase CHOC 400 nuclei that had been synchronized in vivo using aphidicolin and then incubated in the same replication cocktail used to label replication forks produced by a *Xenopus* egg extract also yielded a broad peak in the early labeled fragment assay (Fig. 8B). Thus, aphidicolin arrested nuclei gave much broader peaks in the early labeled fragment assay than nuclei not exposed to aphidicolin, regardless of the whether or not the nuclei were also exposed to *Xenopus* egg extract.

Site-specificity is affected by the ratio of extract to nuclei

An alternative way to affect the number of initiation sites is by altering the ratio of initiation proteins to DNA substrate. As the concentration of nuclei in the extract was increased, total DNA synthesis increased while the amount of DNA synthesis per nucleus decreased (Fig. 11A). Given the constant rate of fork migration in these extracts (Dimitrova and Gilbert, 1998; Walter and Newport, 1997; data not shown), this was the result of fewer initiation events per nucleus as the ratio of nuclei to extract was increased. Moreover, as the concentration of nuclei in these extracts was increased ~12-fold (from 2,000 to 25,000 nuclei/μl), preference for initiation at ori-β increased ~4-fold (Fig. 11B), revealing that ori-β was selectively activated as the ratio of initiation factors to DNA substrate decreased. At higher nuclei concentrations, both total DNA synthesis and ori-β selectivity decreased, suggesting that as fewer initiation events could occur, other initiation sites were now preferred over ori-β.

DISCUSSION

Selective activation of primary initiation sites in vitro

Previous efforts in this (Gilbert et al., 1993, 1995a) and other (Dimitrova and Gilbert, 1998; Wu and Gilbert, 1996, 1997)

laboratories to identify parameters that determine site-specific initiation in mammalian chromosomes led to the discovery that *Xenopus* egg extract could initiate replication de novo at or close to the same sites used in vivo, if the substrate was nuclei rather than DNA or chromatin. However, these studies routinely observed a broad distribution of newly synthesized DNA that extended from the 3′-end of the DHFR gene into the 2BE2121 gene and was centered at or close to ori-β and β′ (Fig. 8A). While this was consistent with initiation of bi-directional replication at ori-β and ori-β′ followed by arrest of replication forks at various distances from these OBRs, it could also be viewed as a collection of initiation events at many different sites throughout this region (Gilbert et al., 1995a). One potential problem in these studies was the use of aphidicolin to arrest nuclei as they began DNA synthesis and thereby increase the number of initiation events that could be scored (+aph protocol). In the studies presented here, this step was avoided, and newly synthesized DNA was radio-labeled immediately as initiation events occurred in the *Xenopus* extract (−aph protocol).

The results presented here using the −aph protocol demonstrated three important features of mammalian replication origins. First and foremost, *Xenopus* egg extract can selectively activate the same primary initiation sites used by mammalian cells in vivo (e.g. ori-β and ori-β′). Second, as previously reported using the +aph protocol (Wu and Gilbert, 1996), specific initiation sites are not established in mammalian nuclei until late G_1-phase of the cell division cycle (Fig. 3). Third, since these sites can be activated by Orc-depleted *Xenopus* egg extract (Fig. 7), initiation of DNA replication in late G_1-nuclei results from activation of hamster pre-replication complexes that had been assembled in vivo. Similar results were recently reported using the + aph protocol (Yu et al., 1998). Taken together, these results suggest that pre-replication complexes are assembled at specific chromosomal sites during G_1-phase in mammalian cells. In fact, it has recently been shown that OBRs in yeast occur at the same sites where pre-replication complexes are assembled (Bielinsky and Gerbi, 1999). Since the proteins that form pre-replication complexes are highly conserved in the eukaryotic kingdom, one would expect that the same would be true in other eukaryotes as well.

Secondary initiation events

Three parameters have been identified that stimulate additional, secondary initiation events outside of the OBRs: (1) preincubating nuclei in *Xenopus* egg extract in the presence of aphidicolin (+aph protocol, Fig. 8A), (2) extending the incubation of nuclei in extract (−aph protocol, Fig. 10) for up to 2 hours, and (3) increasing the ratio of extract to nuclei (−aph, Fig. 11) and +aph (Dimitrova and Gilbert, 1998). In each case, the lengths of newly synthesized DNA was restricted to a mean of 1 to 2 kb, while the number of DNA chains increased. Similarly, changing the ratio of *Xenopus* egg extract to sperm chromatin changes the frequency of initiation events (Walter and Newport, 1997).

What factors contribute to the appearance of initiation events outside of OBRs? Previous studies have shown that the number of initiation sites used by hamster cells can be increased by holding them at their G_1/S-phase boundary (Laughlin and Taylor, 1979), suggesting that prolonged exposure of DNA to S-phase initiation factors or increased ratios of initiation proteins to DNA increases the frequency of initiation events. *Xenopus* eggs contain at least 10-times greater concentration of initiation factors (e.g. XlOrc proteins) than somatic cells (Tugal et al., 1998), and can remodel chromatin to establish new initiation sites (Dimitrov and Wolffe, 1996; Wangh et al., 1995). Both of these features likely contribute to their ability to activate DNA replication in G_0 phase nuclei.

Prolonged incubation of either *Xenopus* sperm chromatin (Carpenter et al., 1996; Coleman et al., 1996; Rowles et al., 1996; Walter and Newport, 1997, Yu et al., 1998) or hamster mitotic chromosomes (Yu et al., 1998) in *Xenopus* egg extract may also result in DNA synthesis that is not dependent on Orc proteins. Even after removal of 95% to 99% of XlOrc1 and XlOrc2 proteins from *Xenopus* extracts, DNA synthesis remained at 10% to 25% (Carpenter et al., 1996; Rowles et al., 1996; Yu et al., 1998) to as much as 80% (Walter and Newport, 1997) of the level observed after 2 to 3 hours of incubation in mock depleted extracts. Whether or not this DNA synthesis resulted from residual ORC in the depleted extracts was not determined, but the fact that most of it occurred between 1 and 3 hours of incubation indicates that it might have resulted either from DNA damage and repair or from aberrant initiation mechanisms. For example, yeast nuclear extracts can initiate semi-conservative DNA replication in plasmid DNA using DNA helicase II to unwind non-origin sequences in the absence of ORC and Cdc6 proteins, and allow DNA primase-DNA polymerase-α to initiate DNA synthesis (Braguglia et al., 1998). Similarly, SV40 T-antigen, a protein that can interact non-specifically with DNA and initiate DNA unwinding (Wessel et al., 1992), increases the frequency of initiation sites in hamster cell chromosomes (Martin and Oppenheim, 1977), and decreases origin specificity (Wu et al., 1998). Thus, changes in the concentrations of proteins that can either activate or repress initiation sites can determine the number and locations of replication origins. Nevertheless, optimizing the ratio of extract to nuclei was not sufficient to obtain selective activation of OBRs in nuclei synchronized with aphidicolin (Dimitrova and Gilbert, 1998). Moreover, changing this ratio did not induce site specific initiation either in early G_1-nuclei (Dimitrova and Gilbert, 1998; C.-J. Li, unpublished data) or in DNA containing the DHFR gene locus (Gilbert et al., 1995a). The biggest stimulus of secondary initiation events was the use of aphidicolin to synchronize nuclei by arresting them as they enter S-phase.

Aphidicolin induced secondary initiation events

Synchronization of hamster nuclei at their G_1/S-interphase with aphidicolin either in a *Xenopus* egg extract or in cultured cells can lead to the appearance of a broad initiation zone (Fig. 8). This artifact does not result from *Xenopus* Orc proteins creating new initiation sites, because the same results are observed using *Xenopus* extract depleted of Orc proteins (Yu et al., 1998). Therefore, since the broad peak of initiation activity was not observed when the synchronization step with aphidicolin was omitted (Figs 3, 8A), it must result from prolonged arrest of replication forks by aphidicolin. This could occur in three ways. First, some replication forks escape from the aphidicolin block and migrate away from the OBR. They produce nascent DNA chains up to 5 kb long in *Xenopus* activated G_1-nuclei (Fig. 9), allowing cells to be stained with

anti-BrdU antibodies (discussion in Gilbert et al., 1995a). This could account for some, but not all, of the extended radio-labeled nascent DNA around origin loci. Second, prolonged exposure of replicating chromosomes to DNA synthesis inhibitors such as aphidicolin or mimosine results in time dependent accumulation of DNA damage at replication sites (Dinter-Gottlieb and Kaufmann, 1983; Gilbert et al., 1995b; Hughes and Cook, 1996; Kalejta and Hamlin, 1997; Snapka et al., 1991). This could create additional sites where DNA synthesis could occur when these nuclei are subsequently pulse-labeled with radio-labeled DNA precursors. In fact, prolonged incubation of cells in the presence of aphidicolin and other DNA synthesis inhibitors increases the fraction of breaks at fragile sites in human chromosomes (Glover et al., 1984), and stimulates gene amplification at the CHO DHFR gene region thorough DNA breakage (Windle et al., 1991). In addition, the presence of DNA damage may exacerbate the effects described above of exposing nuclei to a powerful S-phase extract. Finally, inhibition of DNA polymerase activity either by aphidicolin or deprivation of nucleotide precursors uncouples DNA unwinding from DNA synthesis. This results in extensively unwound regions of DNA during SV40 T-antigen DNA dependent DNA replication (Bullock et al., 1991; Droge et al., 1985; Snapka et al., 1991), during plasmid DNA replication in a *Xenopus* egg extract (J. Walter and J. Newport, personal communication) and during DNA replication in mammalian cells (Lonn and Lonn, 1988). Thus, when the aphidicolin is removed, DNA synthesis could initiate at many randomly selected sites along the unwound DNA templates (Mechali and Harland, 1982), resulting in the appearance of a broad initiation zone emanating from the OBRs.

If synchronization of nuclei with aphidicolin stimulates secondary initiation events when nuclei begin DNA synthesis, then why did this artifact not show up in nascent strand abundance assays on aphidicolin synchronized hamster cells? Clearly, aphidicolin synchronization did not affect this assay, because the same results were obtained using either synchronized (Kobayashi et al., 1998; see Fig. 4) or unsynchronized CHO cells (Pelizon et al., 1996). In contrast, early labeled fragment assays carried out with aphidicolin synchronized nuclei from either hamster cells (Fig. 8B) or *Xenopus* egg extract (Fig. 8A) produced much broader peaks of origin activity than early labeled fragment assays done on unsynchronized G_1-nuclei activated in *Xenopus* egg extract. The difference is that early labeled fragment assays were carried out using total DNA, whereas nascent strand abundance assays were carried out using purified nascent DNA chains with a mean size of ~1 kb. One kilobase nascent DNA strands arise predominantly from newly initiated replication bubbles, thus excluding longer nascent DNA strands that might arise either from replication forks that escaped the aphidicolin block or from extensive DNA unwinding, as well as shorter DNA fragments that might arise from DNA damage and repair. In fact, failure to purify nascent DNA chains from aphidicolin synchronized cells resulted in failure to detect site specific initiation (Kobayashi et al., 1998). This was not true with unsynchronized cells (Giacca et al., 1997). Furthermore, the more carefully one isolates bona fide replication intermediates, the more clearly one observes site-specific initiation. For example, origin resolution was greater using 5′-RNA-DNA chains than BUdR-labeled DNA (Fig. 4).

The results described in this and in previous publications suggest that secondary initiation sites in mammalian chromosomes result from regions that are particularly sensitive either to DNA damage and repair or to aberrant initiation mechanisms, and the frequency of initiation events that occur outside of the primary initiation sites (OBRs) depend on experimental conditions, such as those described above.

REFERENCES

Aladjem, M. I., Groudine, M., Brody, L. L., Dieken, E. S., Fournier, R. E., Wahl, G. M. and Epner, E. M. (1995). Participation of the human beta-globin locus control region in initiation of DNA replication. *Science* **270**, 815-819.

Bielinsky, A. K. and Gerbi, S. A. (1999). Chromosomal ARS1 has a single leading strand start site. *Mol. Cell* **3**, 477-486.

Blow, J. J. (1993). Preventing re-replication of DNA in a single cell cycle: evidence for a replication licensing factor. *J. Cell Biol.* **122**, 993-1002.

Braguglia, D., Heun, P., Pasero, P., Duncker, B. P. and Gasser, S. M. (1998). Semi-conservative replication in yeast nuclear extracts requires Dna2 helicase and supercoiled template. *J. Mol. Biol.* **281**, 631-649.

Bullock, P. A., Seo, Y. S. and Hurwitz, J. (1991). Initiation of simian virus 40 DNA synthesis in vitro. *Mol. Cell Biol.***11**, 2350-2361.

Burhans, W. C., Vassilev, L. T., Wu, J., Sogo, J. M., Nallaseth, F. S. and DePamphilis, M. L. (1991). Emetine allows identification of origins of mammalian DNA replication by imbalanced DNA synthesis, not through conservative nucleosome segregation. *EMBO J.* **10**, 4351-4360.

Carpenter, P. B., Mueller, P. R. and Dunphy, W. G. (1996). Role for a *Xenopus* Orc2-related protein in controlling DNA replication. *Nature* **379**, 357-360.

Chong, J. P., Thommes, P., Rowles, A., Mahbubani, H. M. and Blow, J. J. (1997). Characterization of the *Xenopus* replication licensing system. *Meth. Enzymol.* **283**, 549-564.

Coleman, T. R., Carpenter, P. B. and Dunphy, W. G. (1996). The *Xenopus* Cdc6 protein is essential for the initiation of a single round of DNA replication in cell-free extracts. *Cell* **87**, 53-63.

Decker, R. S., Yamaguchi, M., Possenti, R. and DePamphilis, M. L. (1986). Initiation of simian virus 40 DNA replication in vitro: aphidicolin causes accumulation of early-replicating intermediates and allows determination of the initial direction of DNA synthesis. *Mol. Cell Biol.***6**, 3815-3825.

DePamphilis, M. L. (1993a). Eukaryotic DNA replication: anatomy of an origin. *Annu. Rev. Biochem.* **62**, 29-63.

DePamphilis, M. L. (1993b). Origins of DNA replication in metazoan chromosomes. *J. Biol.Chem.* **268**, 1-4.

DePamphilis, M. L. (1999a). DNA replication in eukaryotic cells: 1996- 1998. In *Concepts in Eukaryotic DNA Replication* (ed. M. L. DePamphilis), pp. 461-507. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

DePamphilis, M. L. (1999b). Replication origins in metazoan chromosomes: fact or fiction? *BioEssays* **21**, 5-16.

Dijkwel, P. A. and Hamlin, J. L. (1995). The Chinese hamster dihydrofolate reductase origin consists of multiple potential nascent-strand start sites. *Mol. Cell Biol.* **15**, 3023-3031.

Dimitrov, S. and Wolffe, A. P. (1996). Remodeling somatic nuclei in *Xenopus* laevis egg extracts: molecular mechanisms for the selective release of histones H1 and H1(0) from chromatin and the acquisition of transcriptional competence. *EMBO J.* **15**, 5897-5906.

Dimitrova, D. S. and Gilbert, D. M. (1998). Regulation of mammalian replication origin usage in *Xenopus* egg extract. *J. Cell* Sci **111**, 2989-2998.

Dinter-Gottlieb, G. and Kaufmann, G. (1983). Aphidicolin arrest irreversibly impairs replicating simian virus 40 chromosomes. *J. Biol. Chem.* **258**, 3809-3812.

Droge, P., Sogo, J. M. and Stahl, H. (1985). Inhibition of DNA synthesis by aphidicolin induces supercoiling in simian virus 40 replicative intermediates. *EMBO J.* **4**, 3241-3246.

Dubey, D. D., Zhu, J., Carlson, D. L., Sharma, K. and Huberman, J. A. (1994). Three ARS elements contribute to the ura4 replication origin region in the fission yeast, Schizosaccharomyces pombe. *EMBO J.* **13**, 3638-3647.

Ermakova, O. V., Nguyen, L. H., Little, R. D., Chevillard, C., Riblet, R., Ashouian, N., Birshtein, B. K. and Schildkraut, C. L. (1999). Evidence that a single replication fork proceeds from early to late replicating domains in the IgH locus in a non-B cell line. *Mol. Cell* **3**, 321-330.

Giacca, G., Pelizon, C. and Falaschi, A. (1997). Mapping replication origins by quantifying the relative abundance of nascent DNA strands using the competitive polymerase chain reaction. *Methods* **13**, 301-312.

Gilbert, D. M., Miyazawa, H., Nallaseth, F. S., Ortega, J. M., Blow, J. J. and DePamphilis, M. L. (1993). Site-specific initiation of DNA replication in metazoan chromosomes and the role of nuclear organization. *Cold Spring Harb. Symp. Quant. Biol.* **58**, 475-485.

Gilbert, D. M., Miyazawa, H. and DePamphilis, M. L. (1995a). Site-specific initiation of DNA replication in *Xenopus* egg extract requires nuclear structure. *Mol. Cell Biol.***15**, 2942-2954.

Gilbert, D. M., Neilson, A., Miyazawa, H., DePamphilis, M. L. and Burhans, W. C. (1995b). Mimosine arrests DNA synthesis at replication forks by inhibiting deoxyribonucleotide metabolism. *J. Biol.Chem.* **270**, 9597-9606.

Glover, T. W., Berger, C., Coyle, J. and Echo, B. (1984). DNA polymerase alpha inhibition by aphidicolin induces gaps and breaks at common fragile sites in human chromosomes. *Hum. Genet.* **67**, 136-142.

Hughes, T. A. and Cook, P. R. (1996). Mimosine arrests the cell cycle after cells enter S-phase. *Exp. Cell Res.* **222**, 275-280.

Hyrien, O., Maric, C. and Mechali, M. (1995). Transition in specification of embryonic metazoan DNA replication origins. *Science* **270**, 994-997.

Kalejta, R. F. and Hamlin, J. L. (1997). The dual effect of mimosine on DNA replication. *Exp. Cell Res.* **231**, 173-183.

Kalejta, R. F., Lin, H. B., Dijkwel, P. A. and Hamlin, J. L. (1996). Characterizing replication intermediates in the amplified CHO dihydrofolate reductase domain by two novel gel electrophoretic techniques. *Mol. Cell Biol.***16**, 4923-4931.

Kitsberg, D., Selig, S., Keshet, I. and Cedar, H. (1993). Replication structure of the human beta-globin gene domain. *Nature* **366**, 588-590.

Kobayashi, T., Rein, T. and DePamphilis, M. L. (1998). Identification of primary initiation sites for DNA replication in the hamster dihydrofolate reductase gene initiation zone. *Mol. Cell Biol.***18**, 3266-3277.

Laughlin, T. J. and Taylor, J. H. (1979). Initiation of DNA replication in chromosomes of Chinese hamster ovary cells. *Chromosoma* **75**, 19-35.

Lonn, U. and Lonn, S. (1988). Extensive regions of single-stranded DNA in aphidicolin-treated melanoma cells. *Biochemistry* **27**, 566-570.

Martin, R. G. and Oppenheim, A. (1977). Initiation points for DNA replication in nontransformed and simian virus 40-transformed Chinese hamster lung cells. *Cell* **11**, 859-869.

Mechali, M. and Harland, R. M. (1982). DNA synthesis in a cell-free system from *Xenopus* eggs: priming and elongation on single-stranded DNA in vitro. *Cell* **30**, 93-101.

Nethanel, T. and Kaufmann, G. (1990). Two DNA polymerases may be required for synthesis of the lagging DNA strand of simian virus 40. *J. Virol.* **64**, 5912-5918.

Pelizon, C., Diviacco, S., Falaschi, A. and Giacca, M. (1996). High-resolution mapping of the origin of DNA repication in the hamster dihdrofolate reductase gene domain by competitive PCR. *Mol. Cell Biol.* **16**, 5358-5364.

Romanowski, P., Madine, M. A., Rowles, A., Blow, J. J. and Laskey, R. A. (1996). The *Xenopus* origin recognition complex is essential for DNA replication and MCM binding to chromatin. *Curr. Biol.* **6**, 1416-1425.

Rowles, A., Chong, J. P., Brown, L., Howell, M., Evan, G. I. and Blow, J. J. (1996). Interaction between the origin recognition complex and the replication licensing system in *Xenopus*. *Cell* **87**, 287-296.

Sasaki, T., Sawado, T., Yamaguchi, M. and Shinomiya, T. (1999). Specification of regions of DNA replication initiation during embryogenesis in the 65-kilobase DNApolalpha-dE2F locus of Drosophila melanogaster. *Mol. Cell Biol.***19**, 547-555.

Snapka, R. M., Shin, C. G., Permana, P. A. and Strayer, J. (1991). Aphidicolin-induced topological and recombinational events in simian virus 40. *Nucl. Acids Res.* **19**, 5065-5072.

Tugal, T., Zou-Yang, X. H., Gavin, K., Pappin, D., Canas, B., Kobayashi, R., Hunt, T. and Stillman, B. (1998). The Orc4p and Orc5p subunits of the *Xenopus* and human origin recognition complex are related to Orc1p and Cdc6p. *J. Biol. Chem.* **273**, 32421-32429.

Vassilev, L. T. and DePamphilis, M. L. (1992). Guide to identification of origins of DNA replication in eukaryotic cell chromosomes. *Crit. Rev. Biochem. Mol. Biol.* **27**, 445-472.

Walter, J. and Newport, J. W. (1997). Regulation of replicon size in *Xenopus* egg extracts. *Science* **275**, 993-995.

Wangh, L. J., DeGrace, D., Sanchez, J. A., Gold, A., Yeghiazarians, Y., Wiedemann, K. and Daniels, S. (1995). Efficient reactivation of *Xenopus* erythrocyte nuclei in *Xenopus* egg extracts. *J. Cell Sci.* **108**, 2187-2196.

Wessel, R., Ramsperger, U., Stahl, H. and Knippers, R. (1992). The interaction of SV40 large T antigen with unspecific double-stranded DNA: an electron microscopic study. *Virology* **189**, 293-303.

Windle, B., Draper, B. W., Yin, Y. X., O'Gorman, S. and Wahl, G. M. (1991). A central role for chromosome breakage in gene amplification, deletion formation, and amplicon integration. *Genes Dev.* **5**, 160-174.

Wu, J. R. and Gilbert, D. M. (1996). A distinct G_1 step required to specify the Chinese hamster DHFR replication origin. *Science* **271**, 1270-1272.

Wu, J. R. and Gilbert, D. M. (1997). The replication origin decision point is a mitogen-independent, 2- aminopurine-sensitive, G_1-phase event that precedes restriction point control. *Mol. Cell Biol.* **17**, 4312-4321.

Wu, J. R., Yu, G. and Gilbert, D. M. (1997). Origin-specific initiation of mammalian nuclear DNA replication in a *Xenopus* cell-free system. *Methods* **13**, 313-324.

Wu, J. R., Keezer, S. M. and Gilbert, D. M. (1998). Transformation abrogates an early G_1-phase arrest point required for specification of the Chinese hamster DHFR replication origin. *EMBO J.* **17**, 1810-1818.

Yu, G., Wu, J. R. and Gilbert, D. M. (1998). Analysis of mammalian origin specification in ORC-depleted *Xenopus* egg extracts. *Genes Cells* **3**, 709-720.