Journal of Cellular Biochemistry 80:73–84 (2000)

Initiation of DNA Replication in *Saccharomyces cerevisiae* G1-Phase Nuclei by *Xenopus* Egg Extract

Jia-Rui Wu,[1] Ji-Wu Wang,[1] and David M. Gilbert[2]*

[1]Shanghai Institute of Biochemistry and Shanghai Research Center of Life Science, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 200031, China
[2]Department of Biochemistry and Molecular Biology, SUNY Upstate Medical University, Syracuse, NY 13210

Abstract *Xenopus* egg extracts initiate replication at specific origin sites within mammalian G1-phase nuclei. Similarly, S-phase extracts from *Saccharomyces cerevisiae* initiate DNA replication within yeast nuclei at specific yeast origin sequences. Here we show that *Xenopus* egg extracts can initiate DNA replication within G1-phase yeast nuclei but do not recognize yeast origin sequences. When G1-phase yeast nuclei were introduced into *Xenopus* egg extract, semiconservative, aphidicolin-sensitive DNA synthesis was induced after a brief lag period and was restricted to a single round of replication. The specificity of initiation within the yeast 2 μm plasmid as well as in the vicinity of the chromosomal origin ARS1 was evaluated by neutral two-dimensional gel electrophoresis of replication intermediates. At both locations, replication was found to initiate outside of the ARS element. Manipulation of both cis- and trans-acting elements in the yeast genome before introduction of nuclei into *Xenopus* egg extract may provide a system with which to elucidate the requirements for vertebrate origin recognition. J. Cell. Biochem. 80:73–84, 2000.

Key words: in vitro replication, 2 μm, yeast nuclei, ARS, *Xenopus*

DNA replication in most eukaryotic cells initiates at specific replication origin sites. Although the eukaryotic initiator protein, a heterohexameric complex that binds to eukaryotic origins (ORC), as well as the proteins that regulate once-per-cell-cycle replication of DNA are highly conserved, the sequences that function as origins in different species are highly divergent [Gilbert, 1998]. In *Saccharomyces cerevisiae*, an 11-bp consensus sequence is required for origin activity, in addition to other motifs that are not conserved in sequence but are required and interchangeable between origins [Marahrens and Stillman, 1992; Lin and Kowalski, 1997]. In *Schizosaccharomyces pombe*, origins consist of multiple AT-rich motifs, each of which contribute partially to the activity of the origin and can be replaced with synthetic $(AT)_{50}$ motifs [Okuno et al., 1999]. In metazoan organisms, the structure and sequence composition of replication origins has not yet been defined. Although replication begins within specific chromosomal loci in cultured metazoan cells, no sequence homologies have been identified between metazoan origins, suggesting that the sequences recognized by ORC may be highly degenerate.

Recently, several eukaryotic cell-free systems have been developed that can initiate rep-

Abbreviations used: ARS, autonomously replicating sequence; BrdU, bromodeoxyuridine; CHO, Chinese hamster ovary; 2D, two-dimensional; DAPI, 4′,6-diamidino-2-phenylindole; DHFR, dihydrofolate reductase; 6-DMAP, 6-dimethyl-aminopurine; ODP, origin decision point; ORC, origin recognition complex; pre-RC, prereplication complex.

Grant sponsor: National Institutes of Health; Grant number: GM57233; Grant sponsor: National Natural Science Foundation of China; Grant number: 39770363; Grant sponsors: Scientific Research Foundation for Returned Overseas Chinese Scholars of State Education Commission, Special grant of Chinese Academy of Sciences, Selected grant of Shanghai Branch of Chinese Academy of Sciences; Grant sponsor: Science and Technology Commission of Shanghai Municipality; Grant number: 98XD14015.
*Correspondence to: David M. Gilbert, Department of Biochemistry and Molecular Biology, SUNY Upstate Medical University, 750 East Adams Street, Syracuse, NY 13210. E-mail: gilbertd@mail.upstate.edu
Received 25 May 2000; Accepted 29 June 2000

lication specifically at the same chromosomal origin sites used in vivo. To date, however, origin recognition requires that the substrate be provided in the form of an intact nucleus. A cell-free system in which *S. cerevisiae* G1-phase nuclei are introduced into an extract derived from S-phase yeast cells will replicate approximately 20% of the genome, although there is a high rate of repair DNA synthesis observed in these reactions [Pasero et al., 1997, 1999]. Initiation specifically within yeast origin sequences requires whole nuclei as a substrate; naked DNA is replicated from random start sites [Braguglia et al., 1998]. An analogous system in which mammalian nuclei are introduced into mammalian S-phase extracts has also been developed [Krude et al., 1997; Stoeber et al., 1998; Krude, 1999]. Approximately 4% of genomic DNA within G1-phase mammalian nuclei can be replicated by these extracts. The specificity of initiation in this system with nuclei as a substrate has not been reported, and replication does not initiate with naked DNA as a substrate. By far the most efficient replication system is provided by extracts from *Xenopus* eggs. One hundred percent of *Xenopus* sperm chromatin and 10–20% naked plasmid DNA can be replicated when introduced into a *Xenopus* egg extract [Blow and Laskey, 1986]. Under the appropriate conditions, highly concentrated extracts can replicate up to 100% of naked plasmid DNA [Walter et al., 1998]. Both of these substrates are replicated from random start sites [Hyrien and Mechali, 1992; Mahbubani et al., 1992; Hyrien and Mechali, 1993]. However, when intact G1-phase mammalian nuclei are introduced into *Xenopus* egg extracts, approximately 75% of genomic DNA is replicated and the same origins used in vivo function as start sites in vitro [Gilbert et al., 1995; Wu et al., 1997; Dimitrova and Gilbert, 1998]. Recognition of the physiologically appropriate origin sites in this system requires that nuclei be isolated after a certain point during G1-phase (the ODP). Replication initiates at apparently random sites within nuclei isolated before the ODP [Wu and Gilbert, 1996] or when the post-ODP nuclear envelope is damaged during preparation [Dimitrova and Gilbert, 1998], suggesting that some epigenetic event takes place at the ODP to select which of many potential sites can function as a replication origin.

In *S. cerevisiae* as well, it appears there are many more potential origin sites than are actually used in each cell cycle. Although all ARS elements in *S. cerevisiae* appear to be bound by the ORC [Santocanale and Diffley, 1996], some of these sites are used more frequently than others, whereas some "dormant" origins are not used at all normal growth conditions [Santocanale et al., 1999]. However, when transferred to other locations, or when cloned on plasmids, these same sequences function efficiently as replication origins [Vujcic et al., 1999]. Hence, in yeast nuclei, chromosomal context can dictate which of many potential sites are used as origins and with what frequency. We reasoned that the introduction of yeast nuclei into *Xenopus* egg extracts could provide a system that combines the power of yeast genetics with a highly efficient in vitro replication system, facilitating the identification of epigenetic mechanisms that specify replication origins. Here, we describe conditions in which at least 30% of yeast genomic DNA can be replicated in vitro when G1-phase nuclei are introduced into *Xenopus* egg extracts. We show that efficient semiconservative replication results from de novo initiation of replication within 100% of yeast nuclei. However, unlike with mammalian nuclei as a substrate, neither the replication of 2-μm plasmids within these nuclei nor chromosomal replication near the well-characterized ARS1 origin initiated specifically within the yeast origin sequences. Our results demonstrate that the *Xenopus* in vitro replication system can efficiently initiate replication within yeast nuclei but that pre-RCs assembled at origins within these nuclei are not preferentially recognized by the *Xenopus* egg cytosol. The combination of these two powerful systems, through the immunodepletion of specific proteins in *Xenopus* egg extracts and the manipulation of cis and trans regulators in yeast, may provide a means to determine the components required for origin recognition.

MATERIALS AND METHODS

Yeast Synchronization

S. cerevisiae strain JW-1 (*Mat*a *his*6 *lys*2Δ201 *sst*1-3 *ura*3-52), was generated by transforming yeast strain SH521 (*Mat*a *his*6 *lys*2Δ201 *sst*1-3 *trp*1Δ1 *ura*3-52; gift of S. Honigberg) with plasmid pSc4128 (gift of B. Stillman) and selecting for the restoration of

trp gene on SD-trp plates. Southern blot analysis confirmed that this strain now contained a restored TRP1/ARS1 locus. JW-1 strain lacks the alpha factor protease and is highly sensitive to α-factor synchronization. Cells were synchronized with α-factor as follows: JW-1 cells in YEPD medium at 8×10^6/ml were pelleted and resuspended in fresh YEPD medium containing 20 ng/ml α-factor and were shaken at 350 rpm for 3 h. For collection of S-phase cells, α-factor arrested cells were washed three times with H_2O and released into fresh YEPD medium at 30°C for 30 min. For flow cytometry, 5×10^6 cells were fixed with 50% ethanol for a minimum of 2 h. Cells were pelleted, resuspended with 1 ml 50 mM Na-acetate (pH = 7.2) containing 0.25 mg/ml RNaseA and incubated at 50°C for 1 h. Proteinase K was then added to 1 mg/ml and cells were incubated an additional hour. An equal volume of 50 mM Na-acetate (pH = 7.2) containing 16 μg/ml propidium iodide (Sigma) was added and the cell suspension was incubated at room temperature for 30 min before analysis by flow cytometry. A rho^0-JW1 strain was generated as described [Fox et al., 1991]. DAPI staining verified the lack of mitochondrial DNA in this strain.

Preparation of Semiintact Yeast Cells (Intact Nuclei)

Semiintact yeast cells were prepared as described [Schlenstedt et al., 1993]. Briefly, JW-1 cells were pelleted and resuspended in 100 mM PIPES (pH 9.4) containing 10 mM dithiothreitol, incubated with gentle agitation at 30°C for 10 min and pelleted. Cells were resuspended in YEP medium containing 50 mM KPO_4 (pH 7.5), 0.2% glucose, 0.6 M sorbitol. Oxalyticase (Enzogenetics) was added at 1,000 U/1×10^9 cells. The cell suspension was incubated with gentle agitation at 30°C for 20 min, and spheroplasting was monitored by light microscopy. Spheroplasts were centrifuged 5 min at 1,000*g*, resuspended in YEP medium containing 1% glucose and 0.7 M sorbitol, and incubated at 30°C for 20 min. The spheroplasts were centrifuged 5 min at 1,000*g*, washed twice with ice-cold buffer P (permeabilization buffer: 20 mM Pipes-KOH, pH 6.8, 150 mM K-acetate, 2 mM Mg-acetate, 0.4 M sorbitol), resuspended in ice-cold buffer P containing 10% DMSO at 2.5×10^6 cells/μl, frozen slowly above liquid N_2, and stored at −80°C. Before in vitro replication, spheroplasts were thawed at room temperature (RT), and kept on ice for use (when thawed, spheroplasts become semiintact cells/intact nuclei). One important modification of previous methods was the addition of 10% DMSO to the spheroplasts before freezing. For reasons that we do not understand, spheroblasts frozen without DMSO were poor substrates for replication in *Xenopus* egg extract.

Immunofluorescence Microscopy

For analysis of nuclear permeability, semiintact cells were diluted with buffer P containing 3% bovine serum albumin (BSA) to a concentration of 3×10^4 spheroplasts/μl. A rabbit polyclonal antibody against histone H4 [Lin et al., 1989] (gift of D. Allis) and a mouse monoclonal antibody 414 against nuclear pore epitopes [Kenna et al., 1996] (a gift of D. Goldfarb) were added to 50 μl diluted semiintact cells, and the mixture was incubated on ice for 1 h. Semiintact cells were washed twice for 10 min with ice-cold buffer P containing 3% BSA and then resuspended with 50 μl buffer P containing 3% BSA. Texas Red-conjugated donkey anti-rabbit IgG (Jackson Labs) was added to 3 μg/ml and fluorescein isothiocyanate (FITC)-conjugated donkey anti-mouse IgG (Jackson Labs) was added to 60 μg/ml and incubated on ice for 1 h. Semiintact cells were pelleted and washed with buffer P at RT for 5 min, resuspended in 50 μl buffer P containing 0.5 μg/ml DAPI (Sigma), and incubated at RT for 5 min. Samples were observed with a Nikon Labphot-2 microscope equipped with a 100× Planapo lens and photographed with Kodak Ektachrome P1600 films. Film was scanned with a Nikon scanner and figures were composed with Photoshop software, using only standard brightness and contrast adjustments.

For detection of BrdU-labeled nuclei after incubation in *Xenopus* egg extract supplemented with 0.5 mM BrdUTP, nuclei were washed twice with buffer A (20 mM Pipes-KOH, pH 6.8, 150 mM K-acetate, 5 mM Mg-acetate, 0.25 M sorbitol), and fixed with 50% ethanol at RT for 10 min. Fixed nuclei were spread on a slide and baked onto the slide at 65°C for 5 min. Slides were then incubated with 100% methanol at RT for 10 min, 100% acetone at RT for 30 s,1.5 M HCl at RT for 30 min, then washed and incubated with mouse anti-BrdU antibody (Becton Dickinson) in a humidified chamber at RT for 1 h. The

nuclei were then washed and incubated with FITC- conjugated donkey anti-mouse IgG (Jackson Labs) and 0.5 μg/ml DAPI in a humidified chamber at RT for 1 h. Slides were mounted in Vectashield and viewed by microscopy.

Evaluation of In Vitro DNA Synthesis

To evaluate the ability of permeabilized cells to synthesize DNA in the absence of added cytosol, 2.5×10^7 G1 or S-phase nuclei were resuspended with 2 μl 5× replication cocktail (150 mM HEPES, pH 7.6, 35 mM $MgCl_2$, 4 mM DTT, 500 μM each dTTP, dGTP, and dCTP, 1 mM each CTP, GTP, and UTP, 20 mM ATP, 200 mM creatine phosphate, 100 μg/ml creatine phosphokinase) containing 10 μCi of [α-^{32}P] dATP and incubated at 30°C for the indicated times.

Xenopus egg extract was prepared and supplemented with an ATP-regenerating system before use as described [Chong et al., 1997; Wu et al., 1997]. Most reactions consisted of 6×10^6 nuclei in 20 μl *Xenopus* egg extract at 21°C. Total DNA synthesis was measured by acid precipitation, and the percent replication was calculated as described [Chong et al., 1997; Wu et al., 1997], assuming 0.015 pg DNA/yeast nucleus. For density gradient centrifugation, 1.2×10^7 nuclei were incubated with *Xenopus* egg extract containing 0.5 mM BrdUTP (Sigma) and 20 μCi of [α-^{32}P] dATP at 21°C for 90 min. Nuclei were washed three times with buffer P, then resuspended in lysis buffer (50 mM Tris, pH = 8.0, 10 mM EDTA, 0.4 M NaCl, and 1% sodium dodecyl sulfate) and incubated at 65°C for 60 min. Genomic DNA was then precipitated with isopropanol and resuspended in TE buffer (10 mM Tris, pH = 8.0 and 1 mM EDTA). Neutral pH density gradient centrifugation and alkaline pH density gradient centrifugation were done as described previously [Gilbert et al., 1995]. For analysis of replication intermediates by alkaline gel electrophoresis, 6×10^6 semiintact cells were incubated with 20 μl *Xenopus* egg extract containing 10 μCi of [α-^{32}P] dATP and the indicated concentrations of aphidicolin at 21°C for 90 min. DNA was isolated as for density gradient centrifugation, precipitated with isopropanol, resuspended in 0.4N NaOH and 1mM EDTA at RT for 30 min, and subjected to alkaline gel electrophoresis in a 0.8% agarose gel containing 40 mM NaOH and 1 mM EDTA at 3 V/cm for 24 h. The gel was dried with a 3-mm filter paper tower overnight, and radioactive DNA was detected by Phosphorimaging (Molecular Dynamics).

Two-Dimensional Gel Electrophoresis

2D gel analysis of replication intermediates in asynchronous populations of cultured yeast cells was carried out as described [Wu and Gilbert, 1995]. For detection of replication intermediates generated after in vitro replication, 2×10^8 nuclei/740 μl egg extract (for detection of ARS1) or 2.2×10^7 nuclei /60 μl egg extract (for detection of 2 μm) were incubated in the presence of 3 μg/ml aphidicolin at 21°C for 1 h. DNA was isolated as for gradient centrifugation, resuspended in TE, and digested with either 50 U NcoI (for detection of ARS1) or 50 U SnaBI overnight. Neutral/neutral 2D gel electrophoresis was carried out as described [Brewer and Fangman, 1987]. The gels were transferred to nylon membranes (Hybond N+; Amersham) and hybridized with either the 5-kb NcoI fragment encompassing ARS1 (for detection of ARS1), or with plasmid 82-6B, which contains 1.5 copies of the 2 μm plasmid genome (gift of Dr. Newlon). Images were collected by Phosphorimaging.

RESULTS

Xenopus Egg Extract Stimulates the Initiation of DNA Replication in Yeast G1-Phase Nuclei

With mammalian nuclei as a substrate for replication in *Xenopus* egg extracts, the integrity of the nuclear preparation is critical to maintain both the specificity and the overall efficiency of DNA replication [Gilbert et al., 1995; Wu et al., 1997; Dimitrova and Gilbert, 1998]. To prepare intact yeast nuclei, we adapted a method previously developed for the study of the Golgi apparatus [Baker et al., 1988], which has also been used to reconstitute nuclear import with yeast nuclei in vitro [Schlenstedt et al., 1993]. Yeast cell walls were digested with oxalyticase, and semiintact cells (intact nuclei) were prepared by slowly freezing spheroblasts above liquid nitrogen. After thawing, the integrity of each nuclear preparation was monitored by incubating with fluorescently tagged antibodies against both a yeast nuclear pore antigen present on the surface of nuclei and histone H4, present in the interior of nuclei. Intact nuclei exclude the anti-histone

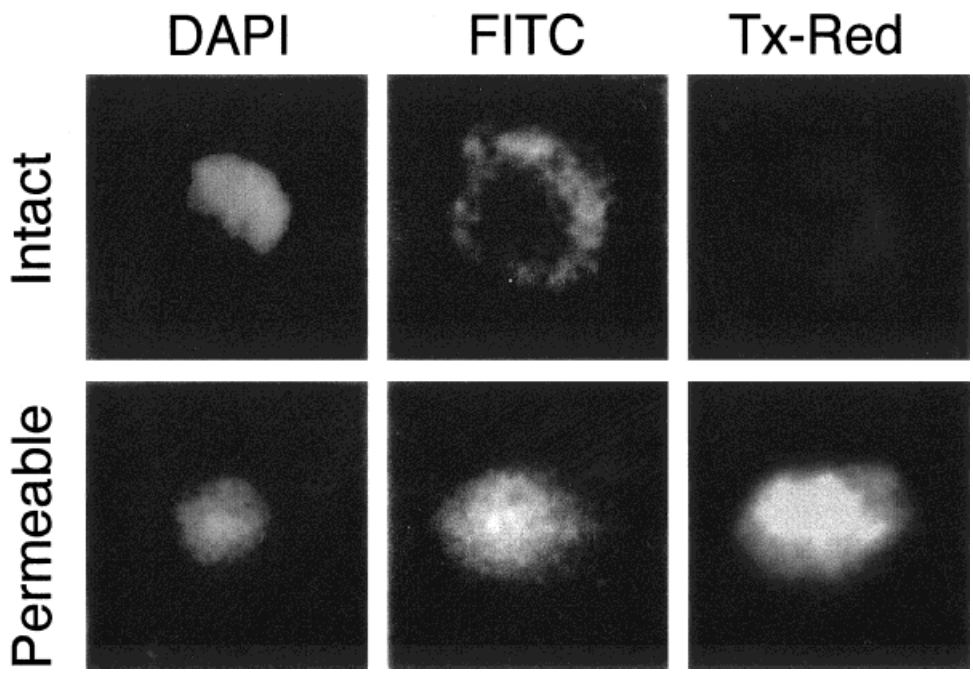

Fig. 1. Assay for intact versus permeabilized nuclei. Intact nuclei were prepared and stained simultaneously with an antibody against a nuclear pore epitope present on the outer surface of the nucleus (MAb414) and an anti-histone H4 antibody, as described in Materials and Methods. DNA was counterstained with DAPI. Aliquots of these nuclei were permeabilized by incubation with 0.02% Triton X-100 on ice for 5 min. Intact nuclei are decorated on the surface with MAb414 but are stained poorly or not at all with the anti-histone H4 antibody. Permeabilized nuclei are stained strongly with both antibodies. Because MAb414 recognizes FG-repeat nucleoporins [Kenna et al., 1996], which are plentiful on both sides of the yeast NPC [Rout et al., 2000], there are also epitopes for MAb414 on the interior surface of the nuclear envelope, which may explain why the nucleus stains more diffusely when permeabilized than when intact.

H4 antibody, which cannot cross an intact nuclear envelope, but are decorated on the surface by the anti-nuclear pore antibody. Nuclei with damaged envelopes stain uniformly with both antibodies (Fig. 1). Typical preparations yielded approximately 80% intact yeast nuclei.

To study initiation of DNA synthesis in vitro, nuclei must be isolated from cells during G1-phase, before the formation of replication forks in vivo. Previous studies [Kuo and Campbell, 1982] have shown that preexisting replication forks within S-phase yeast nuclei can be extended in vitro by enzymes present at those replication forks, whereas G1-phase nuclei are not capable of DNA synthesis under the same conditions. We compared nuclei from cells synchronized in G1 phase (α-factor arrest; Fig. 2A) vs. S-phase (released from α-factor arrest for 30 min; Fig. 2A). When intact G1-phase nuclei were incubated with a replication cocktail containing [α-^{32}P]-dATP, little DNA synthesis was detected in the presence or absence of aphidicolin, an inhibitor of replicative polymerases (Fig. 2B). By contrast, aphidicolin-sensitive DNA synthesis was readily detected with intact S-phase nuclei (Fig. 2B). These results indicate that G1-phase nuclei do not contain significant numbers of preprimed DNA templates.

When intact G1-phase nuclei were introduced into a *Xenopus* egg extract, robust DNA synthesis was observed after a brief lag period (Fig. 3A), indicating that factors from the *Xenopus* egg cytosol can be efficiently imported into yeast nuclei to initiate DNA replication. This DNA replication was aphidicolin sensitive, demonstrating that it was mediated by replicative DNA polymerases (Fig. 3A). However, a measurable component (~10%) of this DNA synthesis was resistant to aphidicolin. Aphidicolin-resistant DNA synthesis could be due to either DNA repair or to mitochondrial DNA synthesis, both of which are mediated by aphidicolin-resistant DNA polymerases. In fact, significant amounts of DNA repair have been observed when yeast G1-phase nuclei are incubated in yeast S-phase extracts [Pasero et al., 1997]. On the other hand, approximately 10% of the total DNA in *S. cerevisiae* consists of

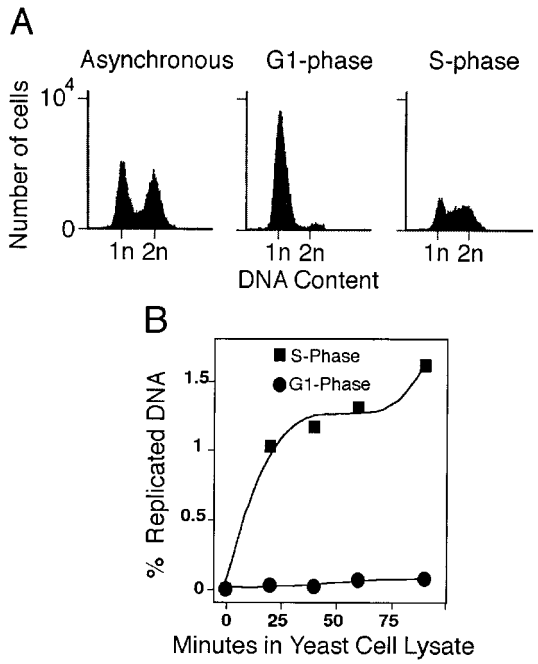

Fig. 2. Evaluation of cell synchrony. **A:** JW-1 cells were collected either during asynchronous growth, after α-factor arrest for 3 h (G1-phase), or after release from α-factor arrest for 30 min (S-phase) and analyzed by flow cytometry. **B:** G1-phase and S-phase nuclei were incubated with a replication cocktail containing [α-^{32}P] dATP and the total counts per minute were incorporated into high-molecular-weight DNA determined by acid precipitation. Parallel reactions with the same nuclear preparations were incubated in the presence of aphidicolin, and the counts per minute incorporated in the presence of aphidicolin were subtracted from that detected in the absence of aphidicolin to reveal the aphidicolin-sensitive counts per minute incorporated, a measure of nuclear replicative DNA synthesis. The percent of total input DNA replicated during the in vitro labeling period was then calculated and plotted as percent replicated DNA.

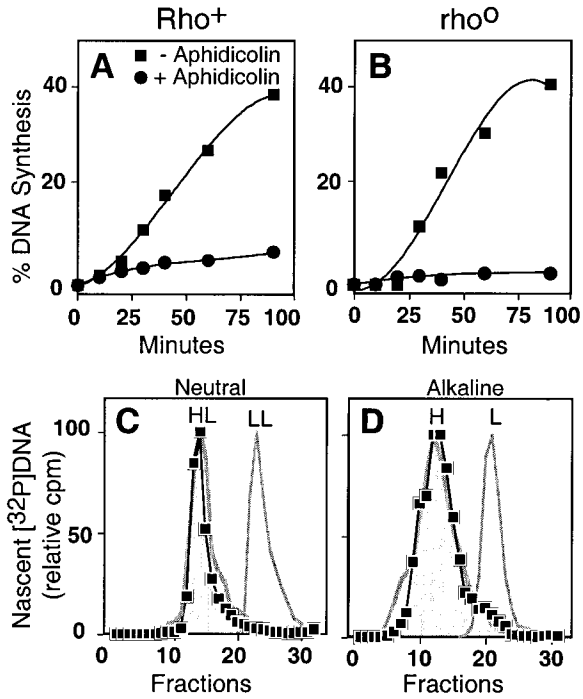

Fig. 3. DNA synthesis in Xenopus egg extract results from semiconservative DNA replication. **A:** Intact G1-phase JW-1 nuclei (Rho$^+$) were incubated in a *Xenopus* egg extract in the presence (circles) and absence (squares) of aphidicolin. The amount of DNA synthesized at each time point was determined by acid precipitation and plotted as a percent of input DNA. **B:** Intact G1-phase nuclei from an isogenic derivative of JW-1 lacking mitochondria (rho^0) were incubated in a *Xenopus* egg extract and the percent DNA synthesis was determined as in (A). **C:** Rho^0-G1-phase nuclei were incubated in *Xenopus* egg extract supplemented with [α-^{32}P] dATP and BrdUTP. Total genomic DNA was isolated and centrifuged to equilibrium in a neutral Cs_2SO_4 gradient, and the counts per minute (cpm) per fraction (squares) collected from the bottom of the tube was determined. The positions of unifiliarly BrdU substituted marker-C^{14}-DNA (HL) and unsubstituted marker-C^{14}-DNA (LL), analyzed in parallel gradients, are indicated as solid gray lines. **D:** Neutral density gradient fractions indicated by the shaded region in (C) were pooled and centrifuged to equilibrium in an alkaline Cs_2SO_4 gradient and the cpm per fraction was determined as in (C). The positions of BrdU substituted marker-C^{14}-DNA (H) and unsubstituted marker-C^{14}-DNA (L), analyzed in parallel gradients, are indicated with a solid gray line as in (D).

mitochondrial DNA [Fox et al., 1991], which is likely to be present in our preparations and could account for the aphidicolin-resistant component. To distinguish between these possibilities, we generated an isogenic yeast strain lacking mitochondrial DNA (rho^0). When intact nuclei from this strain were introduced into *Xenopus* egg extracts, significantly less aphidicolin resistant DNA synthesis was observed (Fig. 3B). Furthermore, the lag period before the onset of DNA synthesis was more pronounced with nuclei from the rho^0 strain. We conclude that the DNA synthesis observed within the first 10–20 min in Figure 3A is largely due to mitochondrial DNA synthesis, whereas nuclear DNA synthesis does not begin until after a 10–20-min lag period, as has been observed with mammalian nuclei [Gilbert et al., 1995; Wu et al., 1997; Dimitrova and Gilbert, 1998]. Because rho^0 strains grow poorly, they are not practical for routine studies. As an alternative, we suggest making all measurements of DNA synthesis in the presence and absence of aphidicolin, plotting only the aphidicolin-sensitive DNA synthesis (the DNA synthesis measured in the presence of aphidicolin subtracted from that observed in the absence of aphidicolin).

To confirm that the aphidicolin-sensitive DNA synthesis stimulated by *Xenopus* egg extract is due to semiconservative DNA replication, G1-phase rho^0 nuclei were incubated in *Xenopus* egg extract containing [α-^{32}P] dATP and BrdUTP. After a 90-min incubation, genomic DNA was purified and subjected to neutral Cs_2So_4 equilibrium gradient centrifugation. Virtually all radiolabeled DNA appeared as a single band of hybrid density (Fig. 3C), consistent with semiconservative DNA replication. Furthermore, a peak consistent with replication of both DNA strands (heavy-heavy DNA) was not observed, demonstrating that no rereplication occurs within the 90-min incubation period. Because hybrid density DNA could, in principle, result from small patches of repair on both DNA strands, the hybrid density fractions were pooled and centrifuged in an alkaline Cs_2So_4 gradient to separate the strands of labeled DNA. Single-stranded DNA synthesized in vitro appeared as a unique band of heavy density (Fig. 3D), confirming that DNA synthesis resulted from semiconservative DNA replication.

A comparison of the properties of DNA synthesis within G1-phase and S-phase nuclei incubated in *Xenopus* egg extract further confirmed that DNA replication stimulated by *Xenopus* egg cytosol results from de novo initiation of replication in the extract and not the extension of preprimed DNA templates. First, DNA synthesis in G1-phase nuclei began after a lag period of approximately 10 min, whereas DNA synthesis in S-phase nuclei began immediately (Fig. 4A,B). Second, DNA synthesis in G1-phase nuclei was inhibited by 6-DMAP (Fig. 4C), which inhibits initiation but not elongation of DNA replication in *Xenopus* egg extract [Blow, 1993; Gilbert et al., 1995]. By contrast, DNA synthesis in S-phase nuclei was largely resistant to 6-DMAP (Fig. 4D). Nearly all G1-phase nuclei were stimulated to enter S-phase in vitro (Fig. 4A), and up to 40% of the total genomic DNA was replicated within 90 min (Figs. 3A and 4B). We conclude that *Xenopus* egg cytosol can efficiently initiate DNA replication within intact G1-phase yeast nuclei.

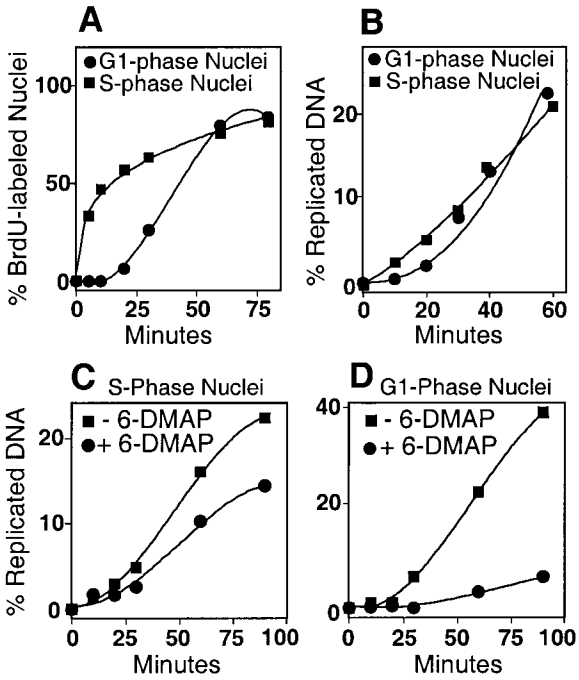

Fig. 4. Properties of DNA synthesis with G1-phase versus S-phase nuclei in *Xenopus* egg extract. **A,B:** Intact JW-1 G1-phase and S-phase nuclei were introduced into *Xenopus* egg extract containing BrdUTP (A) or [α-^{32}P] dATP (B). At the indicated time, aliquots from these reactions were removed, and either the percentage of nuclei that incorporated BrdU (A) or the percentage of genomic DNA replicated (B) was determined as described in Materials and Methods. In (B), the counts per minute (cpm) incorporated in parallel reactions carried out in the presence of aphidicolin were subtracted from the cpm in the absence of aphidicolin and the percentage of total input DNA replicated was calculated from the total aphidicolin-sensitive cpm incorporated, as in Figure 1. **C,D:** Intact JW-1 S-phase (C) and G1-phase (D) nuclei were introduced into *Xenopus* egg extract containing [α-^{32}P] dATP in the presence (circles) or absence (squares) of 6-DMAP. The percentage of input DNA replicated was calculated from the aphidicolin-sensitive cpm incorporated as in (B).

Sites of Initiation of DNA Replication *Xenopus* Egg Extract Are Not Restricted to Yeast Replication Origins

To map the sites of initiation of replication, G1-phase nuclei were incubated in *Xenopus* egg extract and genomic DNA was purified and subjected to neutral-neutral 2D gel electrophoresis analysis. Under these conditions, only a weak Y-arc pattern was detected within a 5-kb *Nco*I fragment encompassing the well-studied yeast origin, ARS1 (not shown). By contrast, a strong replication bubble arc was detected with the same probe when genomic DNA was purified from asynchronous yeast cells cultured in vivo ([Wu and Gilbert, 1995]; also, see Fig. 6C). These results indicate that replication intermediates generated in vitro are more difficult to detect than in vivo. We reasoned that, if we could slow replication fork elongation rates, then we could accumulate replication intermediates close to their sites of initiation.

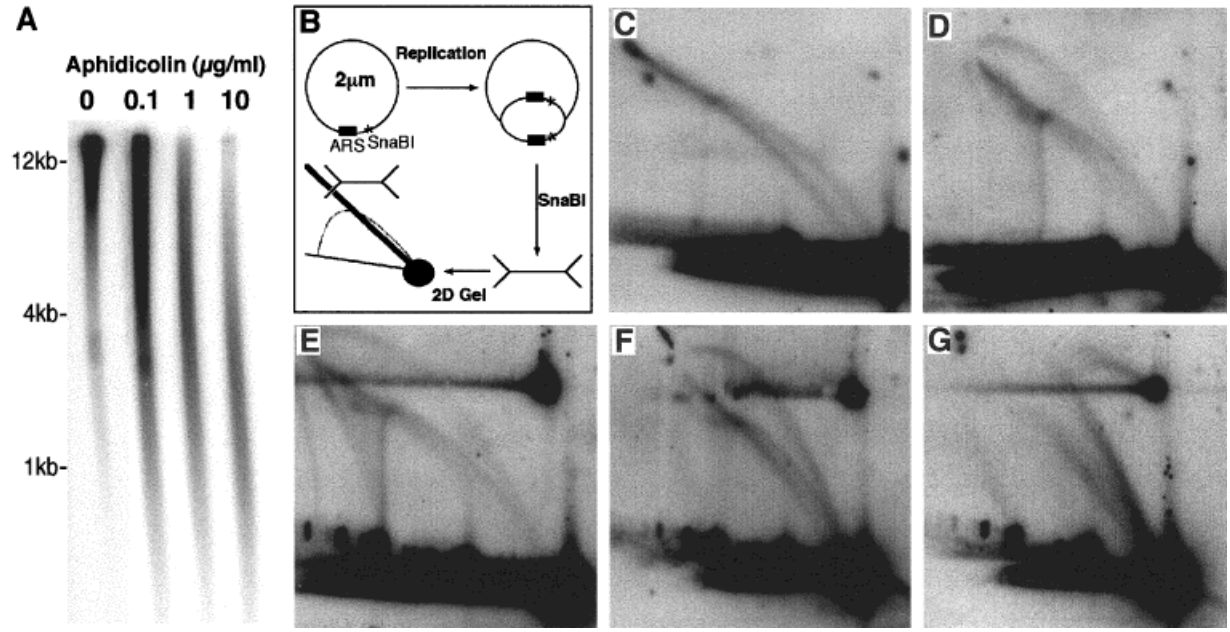

Fig. 5. Analysis of the sites of initiation within the 2-μm plasmid. **A:** Intact G1-phase JW-1 nuclei were incubated in *Xenopus* egg extract containing [α-^{32}P] dATP and the indicated concentrations of aphidicolin for 90 min. Isolated DNA was subjected to alkaline agarose gel electrophoresis. Molecular weight markers are indicated on the left of the gel. **B:** Schematic diagram illustrating the principle of the neutral-neutral 2D-gel assay as it applies to analysis of the 2-μm circle. Replicated DNA molecules are separated in the first dimension by size and in the second dimension by shape. SnaBI digestion of replication intermediates within plasmids that initiate at the unique 2-μm origin produces a double-Y structure that migrates as a sharp spike after 2D-gel analysis as shown in the lower left of (B). **C:** Genomic DNA was isolated from asynchronously growing cultures of JW-1 cells, digested with SnaBI, subjected to a neutral-neutral 2D-gel electrophoresis, and hybridized with a plasmid containing the entire 2-μm sequence. **D:** G1-phase JW-1 nuclei were permeabilized with Triton as in Figure 1 and incubated in a *Xenopus* egg extract containing 3 μg aphidicolin for 60 min. Genomic DNA was purified, digested with SnaBI, and visualized by 2D-gel electrophoresis as in (C). **E–G:** Intact G1-phase JW-1 nuclei were incubated for 60 min in *Xenopus* egg extract supplemented with 1 (**E**), 3 (**F**), or 6 (**G**) μg/ml aphidicolin. 2D gel analysis was carried out as in (C) and (D).

Hence, we incubated intact G1-phase yeast nuclei in a *Xenopus* egg extract supplemented with ^{32}P[dATP] and different concentrations of the elongation inhibitor aphidicolin. The effect of aphidicolin on the size of replication intermediates was then evaluated by alkaline agarose gel electrophoresis. Figure 5 shows that the progression of replication forks was reduced by aphidicolin in a concentration-dependent fashion, offering the potential to control the distance that replication intermediates can travel from their sites of origin.

To test whether aphidicolin could enhance the ability to detect in vitro replication intermediates by 2D gel analysis, we examined the pattern of replication intermediates within the 2-μm plasmid, carried by most strains of *S. cerevisiae*. The 2-μm plasmid provides a convenient replicon to examine, because it is present at 10–20 copies per nucleus and contains a single replication origin [Brewer and Fangman, 1987; Huberman et al., 1987]. One drawback is that this plasmid contains two inverted recognition sites (FRT sites) for the site-specific recombinase FLP. As a result of recombination between FRT sites, the primary sequence of this plasmid exists in two forms in any given cell (A and B form), with sequences between the inverted repeats existing in one of two orientations. Origin mapping studies of circular plasmids are typically performed by digesting the plasmid with an enzyme cutting at a unique site opposite the origin, placing the origin in the center of the resulting restriction fragment. Unfortunately, due to the polymorphic state of the 2-μm plasmid, there is no single restriction site that would cut opposite the origin in both the A and B forms. However, there is a *SnaB* I site that resides next to the ARS sequence in both forms. When a population of 2-μm plasmid molecules containing replication bubbles originating uniquely from the

replication origin are digested with *SnaB* I, these molecules take on a double-Y structure illustrated in Figure 5B. This structure will migrate as a sharp spike in neutral pH 2D gels, diagrammed schematically in Figure 5B. Figure 5C shows a 2D gel analysis of replication intermediates prepared from asynchronously growing yeast cells, digested with *SnaB* I and probed with a 2-μm probe. The hybridization pattern consists almost exclusively of the double-Y spike, demonstrating that, in vivo, replication initiates very close to the *SnaB* I site, as previously demonstrated [Brewer and Fangman, 1987; Huberman et al., 1987].

To evaluate whether replication initiates specifically within the 2-μm origin in vitro, intact G1-phase yeast nuclei were incubated in a *Xenopus* egg extract supplemented with different concentrations of aphidicolin. The aphidicolin concentrations chosen were within the range predicted from Figure 5A to restrict elongation to less than half the size of the 2-μm plasmid. After 60 min, genomic DNA was purified, cut with *SnaB I*, and subjected to 2D gel electrophoresis using a 2-μm plasmid probe. Shown are the results with 1, 3, and 6 μg/ml aphidicolin (Fig. 5E,F,G), which confirm that the use of aphidicolin allows for the detection of in vitro synthesized replication intermediates by 2D gel analysis. Replication intermediates were most easily detected in samples incubated with 3 μg/ml aphidicolin; 6 μg/ml resulted in intermediates that were too short to map by 2D gel electrophoresis (Fig. 5G). Most importantly, although a double-Y arc indicative of initiation near the origin was observed, a clear bubble arc was also detected (Fig. 5E,F), demonstrating that sequences distant from the origin were also used as initiation sites. In fact, this pattern of 2D gel intermediates resembles the pattern obtained when naked plasmid DNA is replicated in *Xenopus* egg extract [Hyrien and Mechali, 1992; Mahbubani et al., 1992], which has been shown to result in the initiation of replication at random sites. Because it has been shown that permeabilization of mammalian nuclei before their introduction into *Xenopus* egg extract leads to a loss in origin specificity [Gilbert et al., 1995; Dimitrova and Gilbert, 1998], aliquots of these same yeast nuclear preparations were also permeabilized with Triton X-100 before in vitro replication (Fig. 1). When the sites of initiation were mapped by 2D gel analysis (Fig. 5D), the pattern of 2-μm replication with permeabilized yeast nuclei was indistinguishable from that with intact nuclei. These results demonstrate that *Xenopus* egg extract does not preferentially recognize the 2-μm replication origin, regardless of the integrity of the yeast nuclear envelope.

To investigate whether *Xenopus* egg extract initiates replication at nonorigin sites within yeast chromosomes, intact G1-phase yeast nuclei were incubated in *Xenopus* egg extract supplemented with 3 μg/ml aphidicolin for 60 min. Genomic DNA was digested with NcoI and subjected to 2-D gel analysis, hybridizing with a probe specific to a 5-kb restriction fragment encompassing the well-studied ARS1 (Fig. 6). Although a prominent replication bubble arc could be observed with replication intermediates prepared from asynchronously growing yeast cells (Fig. 6C), a bubble arc was barely detectable from nuclei incubated in vitro (Fig. 6D). Instead, most replication forks were detected as a Y-arc, indicating that the vast majority of initiation events in vitro took place outside of the NcoI fragment. Because elongation was restricted by the presence of aphidicolin, the initiation events that led to the formation of these replication forks must reside within several kilobases of this NcoI fragment. Since there are no known origins of replication in the nearby flanking sequences, nor any ARS consensus sequences for 9 kb upstream and 7 kb downstream, initiation within the chromosome, as in the 2-μm plasmid, is likely to be taking place at nonorigin sites. The faint bubble arc may represent initiation at low frequency within the ARS or at any site within the 5-kb NcoI fragment. We conclude that initiation within intact yeast nuclei by *Xenopus* egg extract can take place at many sites that do not normally function as origins in living yeast cells.

DISCUSSION

Here, we demonstrate that nuclei from the budding yeast *S. cerevisiae*, synchronized in G1-phase and prepared by methods that preserve the integrity of the nuclear envelope, can serve as an efficient substrate for the initiation of DNA replication in *Xenopus* egg extracts. The DNA synthesis observed is semiconservative, begins after a brief lag period, and is sensitive to aphidicolin and the protein kinase inhibitor 6-DMAP, all consistent with the de

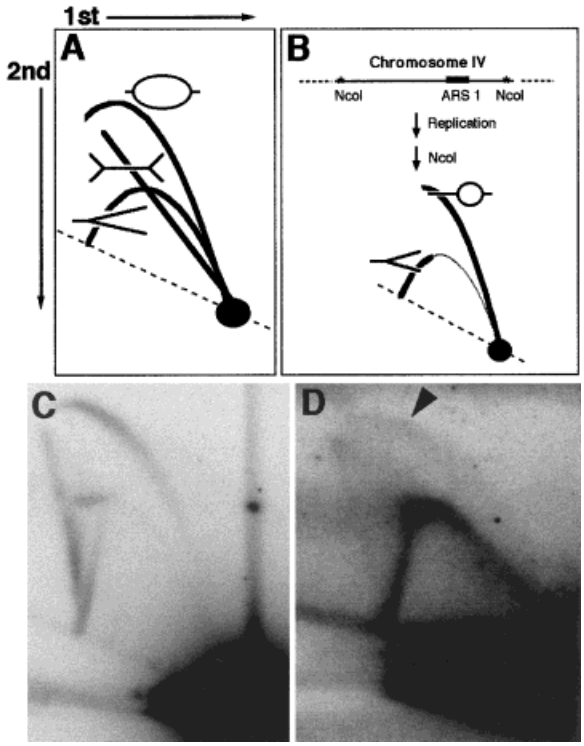

Fig. 6. Analysis of the sites of initiation in the vicinity of the ARS1 chromosomal origin. **A:** Schematic diagram illustrating the general principle of the neutral-neutral 2D-gel assay, showing the theoretical migration patterns of replication bubbles, double Y, and replication fork structures. **B:** Schematic diagram illustrating the principle of the 2D-gel assay, as it applies to analysis of the ARS1 origin. After digestion of genomic DNA containing replication intermediates with NcoI, initiation uniquely within the ARS1 origin should reveal a typical replication bubble structure, collapsing into a fork ark within molecules in which the replication forks have traveled beyond the NcoI sites. **C:** Genomic DNA was isolated from asynchronously growing cultures of JW-1 cells, digested with NcoI, subjected to 2D-gel electrophoresis, and hybridized with a 5-kb NcoI fragment encompassing ARS1. **D:** Intact G1-phase JW-1 nuclei were incubated for 60 min in *Xenopus* egg extract supplemented with 3 μg/ml aphidicolin for 90 min. Genomic DNA was purified, digested with NcoI, and subjected to 2D-gel analysis as in (C). The arrowhead indicates the position of a faint but reproducible bubble arc.

novo initiation of DNA replication in vitro and not DNA repair or the extension of preprimed templates. This demonstrates that initiation factors within *Xenopus* cytosol can be efficiently imported into yeast nuclei and stimulate the assembly of functional replication complexes. Furthermore, because we observe only a single semiconservative round of DNA synthesis (Fig. 3), the mechanisms that prevent reinitiation within one cell cycle are conserved in this heterologous fungal/vertebrate system. Finally, we show that initiation in this system can take place at nonorigin sites. Because pre-RCs are assembled in yeast cells arrested with alpha factor [Diffley et al., 1994; Santocanale and Diffley, 1996], it appears that *Xenopus* egg cytosol cannot preferentially recognize yeast origins even when they are assembled into pre-RCs. However, these same extracts will recognize replication origins within intact mammalian nuclei. Thus, yeast nuclei lack components present in vertebrate nuclei that restrict the sites of initiation of replication to specific origin sites.

This heterologous replication system combines the strength of yeast genetics with a highly efficient *Xenopus* in vitro replication system offering certain advantages over previously described systems. Unlike the use of *Xenopus* sperm or mammalian nuclei as a substrate, the use of yeast nuclei provides the potential to introduce nuclei from strains harboring specific mutations in essential replication proteins to examine the effects of these mutations on the initiation of replication in vitro. A previously described system in which replication is initiated within yeast nuclei by yeast S-phase cytosol has proven quite useful for this type of genetic analysis [Pasero et al., 1997; Pasero et al., 1999]. However, with yeast S-phase cytosolic extracts, only 20% of genomic DNA is replicated, and a large fraction of the DNA synthesis observed is due to DNA repair [Pasero et al., 1997]. In fact, a lag period before the initiation of replication, characteristic of replication systems in which initiation complexes must be assembled, was not observed in yeast S-phase extracts, probably masked by the large amount of DNA repair activity [Pasero et al., 1997]. By contrast, when yeast nuclei are introduced into *Xenopus* egg extract, up to 40% of the yeast genomic DNA can be synthesized in 100% of nuclei within 90 min, and nearly all DNA synthesis results from semiconservative DNA replication.

One disadvantage of this heterologous system is the lack of origin specificity. This lack of origin recognition could be due to an inability of *Xenopus* proteins to recognize yeast ORC and other pre-RC proteins, or it could reflect the fact that fundamental differences exist in the structure of replication origins in yeast versus metazoa [Gilbert, 1998]. An examination of the interaction between *Xenopus* and *S. cerevisiae* replication proteins may help to reveal the

degree of evolutionary conservation of various steps in the initiation of replication. For example, the fact that *Xenopus* egg cytosol initiates replication within yeast nuclei in chromosomal regions that do not contain binding sites for yeast ORC suggests that *Xenopus* ORC molecules can assemble at many sites on the yeast chromosomes. Hence, it will be of interest to determine whether *Xenopus* egg extracts that have been immunodepleted of *Xenopus* ORC proteins can initiate replication within yeast nuclei. If so, this initiation might be focused to sites where yeast ORC is bound. Another potential application of this system is to introduce vertebrate origins into yeast cells (e.g., a YAC containing the CHO DHFR locus), to determine whether the sequences comprising vertebrate origins can assemble within yeast nuclei into the appropriate chromatin structure necessary for recognition by *Xenopus* egg cytosol.

We also describe a useful method to improve the detection of replication intermediates generated in vitro. Our analysis of origin recognition after initiation in *Xenopus* egg extracts was greatly facilitated by the enrichment of replication intermediates by aphidicolin. Other common methods to enrich replication intermediates for 2D-gel analysis include BND-cellulose chromatography [Huberman et al., 1987] and the isolation of DNA bound to the nuclear matrix [Dijkwel et al., 1991]. However, both of these methods require a large amount of DNA as starting material, which may preclude their utility for in vitro studies that generally use smaller numbers of nuclei. Although it is possible to scale up in vitro reactions, including the one describe herein, to make them amenable to BND cellulose and/or nuclear matrix enrichment of replication intermediates, the addition of moderate concentrations of aphidicolin to restrict fork elongation as described here is a more practical approach that may be useful for many in vitro replication systems.

ACKNOWLEDGMENTS

We thank J. Chen for technical assistance, D. Goldfarb and D. Allis for MAb414 and anti-H4 antibodies, C. Newlon for plasmid 82-6B, S. Honigberg for yeast strain SH521, B. Stillman for plasmid pSc4128, and M. Schmitt for critical reading of the manuscript. This work was supported by National Institutes of Health grant GM57233 to D.M.G; and by National Natural Science Foundation of China grant 39770363, Scientific Research Foundation for Returned Overseas Chinese Scholars of State Education Commission, Special grant of Chinese Academy of Sciences, Selected grant of Shanghai Branch of Chinese Academy of Sciences, Science and Technology Commission of Shanghai Municipality grant 98XD14015 to J.-R.W.

REFERENCES

Baker D, Hicke L, Rexach M, Schleyer M, Schekman R. 1988. Reconstitution of SEC gene product-dependent intercompartmental protein transport. Cell 54:335–344.

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

Blow JJ, Laskey RA. 1986. Initiation of DNA replication in nuclei and purified DNA by a cell-free extract of Xenopus eggs. Cell 47:577–587.

Braguglia D, Heun P, Pasero P, Duncker BP, Gasser SM. 1998. Semi-conservative replication in yeast nuclear extracts requires Dna2 helicase and supercoiled template. J Mol Biol 281:631–649.

Brewer BJ, Fangman WL. 1987. The localization of replication origins on ARS plasmids in S. cerevisiae. Cell 51:463–471.

Chong J, Thommes P, Rowles A, Mahbubani H, Blow J. 1997. Characterisation of the Xenopus replication licensing system. Methods Enzymol 283:549–564.

Diffley JF, Cocker JH, Dowell SJ, Rowley A. 1994. Two steps in the assembly of complexes at yeast replication origins in vivo. Cell 78:303–316.

Dijkwel PA, Vaughn JP, Hamlin JL. 1991. Mapping of replication initiation sites in mammalian genomes by two-dimensional gel analysis: stabilization and enrichment of replication intermediates by isolation on the nuclear matrix. Mol Cell Biol 11:3850–3859.

Dimitrova D, Gilbert D. 1998. Regulation of mammalian replication origin usage in Xenopus egg extracts. J Cell Sci 111:2989–2998.

Fox TD, Folley LS, Mulero JJ, McMullin TW, Thorsness PE, Hedin LO, Costanzo MC. 1991. Analysis and manipulation of yeast mitochondrial genes. Methods Enzymol 194:149–165.

Gilbert DM. 1998. Replication origins in yeast vs. metazoa: separation of the haves and the have nots. Curr Opin Genet Dev 8:194–199.

Gilbert DM, Miyazawa H, DePamphilis ML. 1995. Site-specific initiation of DNA replication in Xenopus egg extract requires nuclear structure. Mol Cell Biol 15: 2942–2954.

Huberman JA, Spotila LD, Nawotka KA, El-Assouli SM, Davis LR. 1987. The in vivo replication origin of the yeast 2 μm plasmid. Cell 51:473–481.

Hyrien O, Mechali M. 1992. Plasmid replication in Xenopus eggs and egg extracts: a 2D gel electrophoretic analysis. Nucleic Acids Res 20:1463–1469.

Hyrien O, Mechali M. 1993. Chromosomal replication initiates and terminates at random sequences but at regular intervals in the ribosomal DNA of Xenopus early embryos. EMBO J 12:4511–4520.

Kenna MA, Petranka JG, Reilly JL, Davis LI. 1996. Yeast N1e3p/Nup170p is required for normal stoichiometry of FG nucleoporins within the nuclear pore complex. Mol Cell Biol 16:2025–2036.

Krude T. 1999. Mimosine arrests proliferating human cells before onset of DNA replication in a dose-dependent manner. Exp Cell Res 247:148–159.

Krude T, Jackman M, Pines J, Laskey RA. 1997. Cyclin/Cdk-dependent initiation of DNA replication in a human cell-free system. Cell 88:109–119.

Kuo CL, Campbell JL. 1982. Purification of the cdc8 protein of Saccharomyces cerevisiae by complementation in an aphidicolin-sensitive in vitro DNA replication system. Proc Natl Acad Sci USA 79:4243–4247.

Lin R, Leone JW, Cook RG, Allis CD. 1989. Antibodies specific to acetylated histones document the existence of deposition- and transcription-related histone acetylation in Tetrahymena. J Cell Biol 108:1577–1588.

Lin S, Kowalski D. 1997. Functional equivalency and diversity of cis-acting elements among yeast replication origins. Mol Cell Biol 17:5473–5484.

Mahbubani HM, Paull T, Elder JK, Blow JJ. 1992. DNA replication initiates at multiple sites on plasmid DNA in Xenopus egg extracts. Nucleic Acids Res 20:1457–1462.

Marahrens Y, Stillman B. 1992. A yeast chromosomal origin of DNA replication defined by multiple functional elements. Science 255:817–823.

Okuno Y, Satoh H, Sekiguchi M, Masukata H. 1999. Clustered adenine/thymine stretches are essential for function of a fission yeast replication origin. Mol Cell Biol 19:6699–6709.

Pasero P, Braguglia D, Gasser S. 1997. ORC-dependent and origin-specific initiation of DNA replication at defined foci in isolated yeast nuclei. Genes Dev 11:1504–1518.

Pasero P, Duncker BP, Schwob E, Gasser SM. 1999. A role for the Cdc7 kinase regulatory subunit Dbf4p in the formation of initiation-competent origins of replication. Genes Dev 13:2159–2176.

Rout MP, Aitchison JD, Suprapto A, Hjertaas K, Zhao Y, Chait BT. 2000. The yeast nuclear pore complex: composition, architecture, and transport mechanism. J Cell Biol 148:635–651.

Santocanale C, Diffley J. 1996. ORC- and Cdc6-dependent complexes at active and inactive chromosomal replication origins in *Saccharomyces cerevisiae*. EMBO J 15: 6671–6679.

Santocanale C, Sharma K, Diffley JF. 1999. Activation of dormant origins of DNA replication in budding yeast. Genes Dev 13:2360–2364.

Schlenstedt G, Hurt E, Doye V, Silver PA. 1993. Reconstitution of nuclear protein transport with semi-intact yeast cells. J Cell Biol 123:785–798.

Stoeber K, Mills AD, Kubota Y, Krude T, Romanowski P, Marheineke K, Laskey RA, Williams GH. 1998. Cdc6 protein causes premature entry into S phase in a mammalian cell-free system. EMBO J 17:7219–7229.

Vujcic M, Miller CA, Kowalski D. 1999. Activation of silent replication origins at autonomously replicating sequence elements near the HML locus in budding yeast. Mol Cell Biol 19:6098–6109.

Walter J, Sun L, Newport J. 1998. Regulated chromosomal DNA replication in the absence of a nucleus. Mol Cell 1:519–529.

Wu J-R, Gilbert DM. 1996. A distinct G1 step required to specify the Chinese hamster DHFR replication origin. Science 271:1270–1272.

Wu JR, Gilbert DM. 1995. Rapid DNA preparation for 2D gel analysis of replication intermediates. Nucleic Acids Res 23:3997–3998.

Wu J-W, Yu G, Gilbert DM. 1997. Origin-specific initiation of mammalian nuclear DNA replication in a *Xenopus* cell-free system. Methods 13:313–324.