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

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**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. © 2000 Wiley-Liss, Inc.

**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-
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 (*Mata his6 lys2Δ201 sst1-3 trp1Δ1 ura3-52*; gift of S. Honigberg) was generated by transforming yeast strain SH521 (*Mata his6 lys2Δ201 sst1-3 ura3-52; 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 alpha-factor synchronization. Cells were synchronized with alpha-factor as follows: JW-1 cells in YEPD medium at 8 × 10⁶/ml were pelleted and resuspended in fresh YEPD medium containing 20 ng/ml alpha-factor and were shaken at 350 rpm for 3 h. For collection of S-phase cells, alpha-factor arrested cells were washed three times with H₂O and released into fresh YEPD medium at 30°C for 30 min. For flow cytometry, 5 × 10⁶ 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 rho⁰-JW1 strain was generated as described [Fox et al., 1991]. DAPI staining verified the lack of mitochondrial DNA in this strain.

Preparation of Semicontact Yeast Cells (Intact Nuclei)

Semicontact 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 KPO₄ (pH 7.5), 0.2% glucose, 0.6 M sorbitol. Oxalyticase (Enzogenetics) was added at 1,000 U/1 × 10⁹ 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 1,000g, 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 1,000g, 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 × 10⁶ cells/μl, frozen slowly above liquid N₂, 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 semicontact 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, spheroplasts frozen without DMSO were poor substrates for replication in Xenopus egg extract.

Immunofluorescence Microscopy

For analysis of nuclear permeability, semicontact cells were diluted with buffer P containing 3% bovine serum albumin (BSA) to a concentration of 3 × 10⁴ 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 semicontact cells, and the mixture was incubated on ice for 1 h. Semicontact 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. Semicontact 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 × 10⁷ G1 or S-phase nuclei were resuspended with 2 µl 5× replication cocktail (150 mM HEPES, pH 7.6, 35 mM MgCl₂, 4 mM TTTT, 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-³²P] 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 × 10⁶ 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 × 10⁷ nuclei were incubated with *Xenopus egg extract* containing 0.5 mM BrdUTP (Sigma) and 20 µCi of [α-³²P] 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 × 10⁶ semintact cells were incubated with 20 µl *Xenopus egg extract* containing 10 µCi of [α-³²P] 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 × 10⁶ nuclei/740 µl egg extract (for detection of ARS1) or 2.2 × 10⁷ 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 electrophoreses 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 semintact 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...
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-32P]-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...
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 (rho0). 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 rho0 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 rho0 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).

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-32P] 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+1) 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 (rho0) were incubated in a Xenopus egg extract and the percent DNA synthesis was determined as in (A). C: Rho0-G1-phase nuclei were incubated in Xenopus egg extract supplemented with [α-32P] dATP and BrdUTP. Total genomic DNA was isolated and centrifuged to equilibrium in a neutral Cs2SO4 gradient, and the counts per minute (cpm) per fraction (squares) collected from the bottom of the tube was determined. The positions of unifilarly 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 Cs2SO4 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).
To confirm that the aphidicolin-sensitive DNA synthesis stimulated by *Xenopus* egg extract is due to semiconservative DNA replication, G1-phase rho<sup>0</sup> nuclei were incubated in *Xenopus* egg extract containing [α<sup>32</sup>P]dATP and BrdUTP. After a 90-min incubation, genomic DNA was purified and subjected to neutral Cs<sub>2</sub>SO<sub>4</sub> 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<sub>2</sub>SO<sub>4</sub> 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.

**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 *NcoI* 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.
Hence, we incubated intact G1-phase yeast nuclei in a *Xenopus* egg extract supplemented with $\alpha^{-32}$P dATP and different 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).

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 1 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 specific sequences within the 2-m origin, as previously demonstrated [Brewer and Gilbert, 1998]. These sequences 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 intermediates that were too short to map 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
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
Initiation of Yeast DNA Replication by Xenopus Egg Extract

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 immunodepleated 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.

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REFERENCES