Chromosome Architecture Can Dictate Site-specific Initiation of DNA Replication in *Xenopus* Egg Extracts

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Abstract. *Xenopus* egg extracts initiate DNA replication specifically at the dihydrofolate reductase (DHFR) origin locus with intact nuclei from late G1-phase CHO cells as a substrate, but at nonspecific sites when purified DNA is assembled by the extract into an embryonic nuclear structure. Here we show that late G1-phase CHO nuclei can be cycled through an in vitro *Xenopus* egg mitosis, resulting in the assembly of an embryonic nuclear envelope around G1-phase chromatin. Surprisingly, replication within these chimeric nuclei initiated at a novel specific site in the 5′ region of the DHFR structural gene that does not function as an origin in cultured CHO cells. Preferential initiation at this unusual site required topoisomerase II–mediated chromosome condensation during mitosis. Nuclear envelope breakdown and reassembly in the absence of chromosome condensation resulted in nonspecific initiation. Introduction of condensed chromosomes from metaphase-arrested CHO cells directly into *Xenopus* egg extracts was sufficient to elicit assembly of chimeric nuclei and preferential initiation at this same site. These results demonstrate clearly that chromosome architecture can determine the sites of initiation of replication in *Xenopus* egg extracts, supporting the hypothesis that patterns of initiation in vertebrate cells are established by higher order features of chromosome structure.

CENTRAL to understanding how animal cells regulate DNA replication is to understand the nature of their origins of replication. Considerable progress has been made in our understanding of replication origins in animal viruses and single-celled eukaryotic organisms. However, in higher eukaryotes, origins of replication have been reproducibly identified only within the context of a cellular chromosome (7, 10). Although replication initiates within defined chromosomal loci in cultured cells, in most cases, cloned DNA templates reintroduced into those cells are replicated poorly or not at all. In the few cases where replication of cloned DNA templates in cultured cells has been achieved (24, 26), or whenever bare DNA has been introduced into *Xenopus* eggs or egg extracts (for review see 10), virtually any DNA sequence will function as an origin of replication. Furthermore, studies with rapid early cleavage-stage embryos of *Xenopus* (20) and *Drosophila* (31) have shown that replication initiates at random sites in their cellular chromosomes. In *Xenopus* embryos, it has been shown that the selection of specific chromosomal sites as replication origins does not occur until the blastula stage of development (21), coincident with a loss in the ability to replicate microinjected plasmid DNA molecules (16). A number of changes in nuclear structure have been documented to occur at this time during *Xenopus* development (6, 27), and it has been suggested that these changes could restrict the initiation of replication to specific sites in differentiated cells (16). Thus, the hypothesis has been proposed that origins of replication in multicellular organisms are established by features of nuclear structure, rather than by direct interaction of initiation factors with their specific cognate DNA sequence motifs (10, 16).

Even within the chromosomes of cultured mammalian cells, replication does not initiate within 100–200 bp of a singular DNA sequence motif as it does in *Saccharomyces cerevisiae*, but it initiates throughout regions that may be as large as several tens of kilobases. This has been most extensively examined in the vicinity of the CHO dihydrofolate reductase (DHFR)[1] gene (see Fig. 2 *C*; for review see 17). Using a variety of techniques that quantify nascent strand size or polarity, most initiation activity would appear to originate from an origin of bidirectional replica-

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1. *Abbreviations used in this paper*: DAPI, 4′,6-diamidino-2-phenylindole; DHFR, dihydrofolate reductase; 6-DMAP, 6-dimethylaminopurine; ELFH, early labeled fragment hybridization; MAR, matrix attachment region; OBR, origin of bidirectional replication; ODP, origin decision point; pre-CX, precondensation chromosome; pre-RC, prereplication complex; SAR, scaffold attachment region.

tion (OBR) located ~17 kb downstream of the DHFR gene (ori-β, see Fig. 2 *C*) and/or a second OBR ~20 kb further downstream (ori-γ, see Fig. 2 *C*) (11, 17). However, physical mapping of replication intermediates using neutral/neutral and neutral/alkaline two-dimensional gel electrophoresis has detected the presence of initiation sites throughout the region between ori-β and ori-γ and, to a lesser extent, extending outside of this region, but never within the DHFR structural gene (14). A nearly identical pattern of initiation has been observed at both the single-copy DHFR locus in CHO cells and in a derivative cell line (CHOC 400) in which ~243 kb of DNA containing the DHFR gene has been amplified to ~1,000 copies per cell (17). Similar patterns of initiation have been detected in the vicinity of other mammalian replication origins (25, 32, 39). The simplest interpretation of these results is that replication can initiate at any site within a defined region of the chromosome. The frequency of initiation at any particular site within this region is still a matter of debate. Several attempts to develop a functional assay that can specifically recognize the DHFR origin sequences in cloned DNA templates have failed (8, 9). Thus, it appears that origins of replication in mammalian cells are rather loosely defined sequences that are recognized only in the context of a cellular chromosome.

To address more directly the role of chromosomal and nuclear context in the selection of replication origins, we have been taking advantage of the ability of cell-free extracts from *Xenopus* eggs to initiate replication within various DNA-containing substrates (e.g., naked DNA, chromatin, and whole cell nuclei). We have found that the sites at which replication will initiate within the CHO DHFR locus are dependent upon the nature of the substrate presented to the extract (16, 37). When late G1-phase CHOC 400 cells were gently permeabilized with digitonin, preserving the integrity of the nuclear membrane (intact nuclei), *Xenopus* egg extracts initiated replication with a pattern indistinguishable from the pattern seen in cultured CHO cells. These results demonstrated clearly that, with the appropriate substrate, replication in *Xenopus* egg extracts accurately recapitulates the events that occur upon entry of mammalian nuclei into a normal S phase. If these same nuclei were washed with nonionic detergent or hypotonic buffer, or if purified DNA templates from the DHFR locus were introduced as a substrate, replication initiated at nonspecific sites, demonstrating that origin specificity requires CHO nuclear structure. In addition, when intact nuclei were prepared from CHOC 400 cells synchronized at different times during G1 phase, *Xenopus* egg extracts initiated replication at nonspecific sites in early G1-phase nuclei, allowing the identification of a discrete point in the cell cycle at which nuclei undergo a transition that specifies the DHFR origin (the origin decision point [ODP]) (37). Thus, the CHO DHFR origin is selected by a component of nuclear structure that is assembled during G1 phase. This selection process may be similar to the specification of origins that occurs at the blastula stage of *Xenopus* development (21).

In this study we have begun a dissection of the late G1-phase (post-ODP) nucleus using the ability of metaphase-arrested *Xenopus* egg extracts to dissassemble isolated nuclei. By first taking nuclei apart, and then stimulating extracts to proceed into interphase, we have successfully cycled post-ODP CHOC 400 nuclei through a *Xenopus* mitosis, resulting in the assembly of a *Xenopus* egg nuclear envelope around CHO G1-phase chromatin. Surprisingly, when replication was initiated within these chimeric nuclei, neither the origin-specific initiation pattern preprogrammed within post-ODP CHO nuclei nor the nonspecific pattern of initiation expected from nuclei assembled in *Xenopus* egg prevailed. Instead, a dramatic shift in origin preference was observed, to a site that does not normally function as an origin in cultured CHO cells. Since the events of chromosome condensation and nuclear envelope breakdown can be uncoupled in these extracts by either high concentrations of substrate nuclei or in the presence of topoisomerase II inhibitors, we were able to demonstrate that recognition of this novel site required chromosome condensation during mitosis. Furthermore, when condensed metaphase CHOC 400 chromosomes were introduced into *Xenopus* egg extracts, replication initiated at this same unusual site. We conclude that chromosome architecture is one component of nuclear structure that is capable of dictating the sites of initiation of replication. Since chromosome architecture is restructured during the early G1 phase of each cell cycle, these results provide a framework for understanding how replication origins may be established in mammalian cells.

Materials and Methods

Cell Culture and Permeabilization

CHOC 400 cells were maintained in DME supplemented with nonessential amino acids and 5% FCS at 37°C. Cells were synchronized in metaphase with a completely reversible 4-h exposure to 0.05 μg/ml nocodazole, as described in Gilbert et al. (16). Synchronized cells were collected by shake off and were either used directly (metaphase cells) or plated to fresh medium at 37°C for 4 h (post-ODP). Cells were permeabilized with digitonin as described in Gilbert et al. (16). 2.5×10^6 nuclei per ml were stained by adding 1 μg/ml 4′,6-diamidino-2-phenylinodole (DAPI) and 150 μg/ml affinity-purified, Texas red–labeled IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) and observed directly. Photographs were taken with a Labophot-2 (Nikon Inc., Garden City, NY) using a ×100 plan lens (NA = 1.25) and composed using Photoshop software (Adobe Systems, Inc., Mountain View, CA) using only standard brightness and contrast adjustments.

DNA Synthesis in Xenopus Egg Extracts

Activated and unactivated (metaphase-arrested) *Xenopus* egg extracts were prepared as described (4). Each batch of activated extract was first tested for the efficiency of replication with G1-phase nuclei and for the specificity of initiation, and each batch of metaphase-arrested extract was tested for its ability to elicit nuclear membrane breakdown and chromosome condensation. With activated extracts, variation is seen in the length of the lag period (10–30 min) before initiation and in the total amount of input DNA replicated (5–30%). Some, but not all, batches of extract that are poor at initiating replication within G1-phase nuclei can also give poor initiation specificity. With metaphase-arrested extracts, nuclear membrane breakdown typically took 60 min. However, with some extract batches, complete breakdown took 90–120 min. Similarly, with some extracts, it was not sufficient to simply add 0.3 mM calcium chloride after nuclear membrane breakdown to observe complete reassembly of chromatin into pseudonuclei. Some batches of extract required the addition of an equal volume of fresh activated extract simultaneous with calcium. Cs_2SO_4 density gradient centrifugation, measurement of the fraction of input DNA replicated (by acid precipitation), and the early labeled fragment hybridization (ELFH) assay have been described (16). For experiments with VP16, after nuclear membrane breakdown was complete (60 min),

samples were diluted with 20 vol of cold transport buffer (16) and centrifuged at 3,000 *g* for 10 s, and the pelleted chromatin was resuspended at 50,000 nuclei per μl of fresh activated extract. Stock solutions of 30 mM 6-dimethylaminopurine (6-DMAP; Sigma Chemical Co., St. Louis, MO) were stored at −20°C in water and 50 mM VP16 (Sigma Chemical Co.) at −20°C in DMSO.

DNA Probes. Details of the construction of the probes shown as shaded boxes (see Fig. 2 *C*) have been described (16). Probes shown as black boxes were prepared as follows: probe T is a HindIII/PstI fragment encompassing the promoter and first exon of the DHFR gene. Probe N is a BamHI/HindIII fragment from within the second intron of the DHFR gene. Probe S is a HindIII fragment from the fourth exon of the DHFR gene, excised from plasmid pB61H1 (22), which was generously supplied as a gift from L. Chasin (Columbia University, New York). All three of these probes were cloned into the polylinker of pBluescript II SK (+). Probe Q is a HinfI/PvuII fragment encompassing the matrix attachment region (MAR) (13), cloned into the SmaI site of pGEM-7 and generously supplied as a gift from J. Hamlin (University of Virginia, Charlottesville).

Results

Cycling G1-Phase CHOC 400 Nuclei through a Xenopus Egg Mitosis

Extracts prepared from unactivated *Xenopus* eggs are naturally arrested in metaphase II of meiosis (33) and have been shown to disassemble nuclei from exponentially growing rat liver cells, HeLa cells, and chicken erythrocytes in a process that mimics the normal series of events during mitosis (2, 29). The first step is the phosphorylation and consequent solubilization of nuclear lamin proteins. As the chromatin becomes released from the nuclear lamina, chromosomes are condensed, and the nuclear membrane is broken down into membrane vesicles. We reasoned that, if we could stimulate these extracts to enter S phase after the disassembly of post-ODP CHOC 400 nuclei, it would result in the assembly of a *Xenopus* egg nuclear structure around the CHOC 400 G1-phase chromatin. This would give us the opportunity to determine which initiation pattern would prevail, the origin-specific pattern predetermined within CHOC 400 nuclei, or the nonspecific pattern created when nuclei are assembled in *Xenopus* egg extracts.

We first confirmed that intact nuclei from G1-phase CHOC 400 cells could be disassembled in the metaphase-arrested *Xenopus* extract. CHOC 400 cells were synchronized in mitosis by shake off and allowed to proceed 4 h into G1 phase (post-ODP) by plating into fresh prewarmed medium. Intact nuclei were prepared from G1-phase cells by permeabilization with digitonin and introduced into an extract prepared from metaphase-arrested *Xenopus* eggs. For most preparations of metaphase-arrested egg extract, nuclei incubated at concentrations up to 50,000 nuclei per μl of extract were efficiently converted to a sea of metaphase chromosomes within 60 min (Fig. 1 *A*; *60 Min*). Central to the success of this approach was the ability to drive these extracts back into interphase, to reform *Xenopus* egg-like "pseudonuclei" around the CHO chromatin, and to initiate DNA replication. Ca^{++} is the trigger that naturally releases *Xenopus* eggs from the metaphase II block upon fertilization (33). We found that the addition of Ca^{++}, after nuclear membrane breakdown is complete, successfully drove the extract back into interphase, causing the reassembly of a nuclear membrane around the CHO chromatin within 60 min (Fig. 1 *A*).

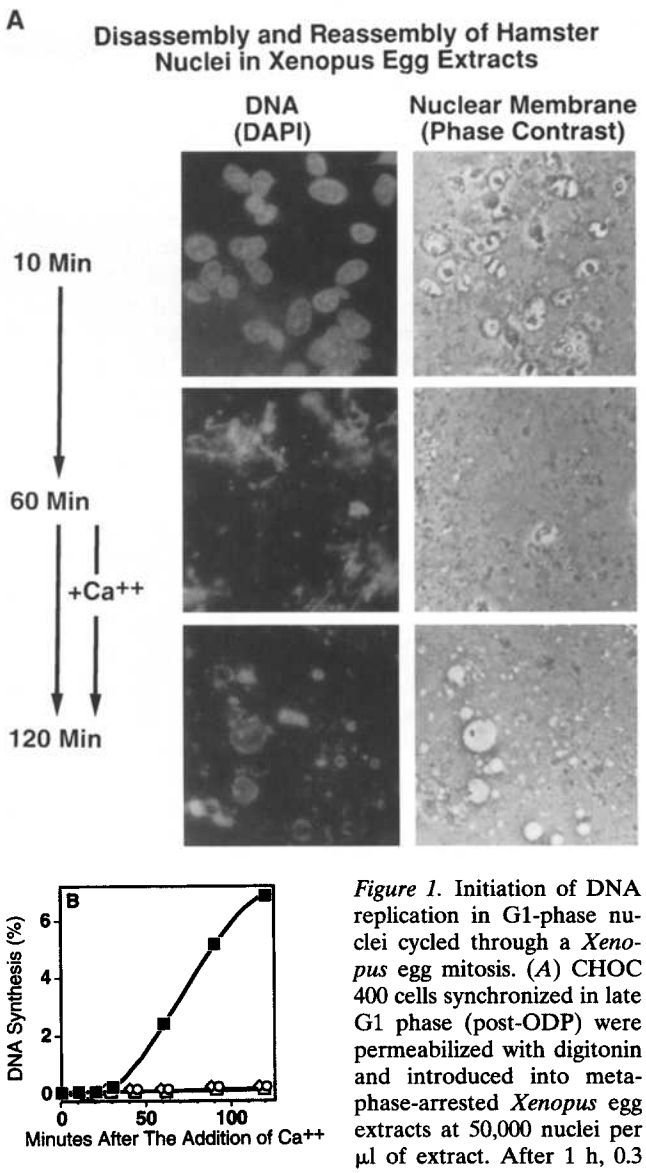


Figure 1. Initiation of DNA replication in G1-phase nuclei cycled through a *Xenopus* egg mitosis. (*A*) CHOC 400 cells synchronized in late G1 phase (post-ODP) were permeabilized with digitonin and introduced into metaphase-arrested *Xenopus* egg extracts at 50,000 nuclei per μl of extract. After 1 h, 0.3 mM calcium chloride was added to activate the extract. Aliquots were removed for photography at the indicated times. DNA was observed by staining with DAPI. Nuclear membrane reformation was monitored by phase-contrast microscopy. (*B*) At the time of addition of calcium chloride, extracts were supplemented with [α-^{32}P]dATP alone (■), or [α-^{32}P]dATP in the absence of added Ca^{++} (○), in the presence of 6-DMAP (□), or in the presence of aphidicolin (◇). At the indicated times, aliquots were removed and the amount of DNA synthesis was quantified by acid precipitation and expressed as a percentage of the input DNA.

To verify that DNA replication could initiate within these chimeric nuclei, DNA synthesis was measured in these same extracts by monitoring the incorporation of [α-^{32}P]dATP into acid-precipitable nucleic acids. DNA synthesis was found to begin after a delay of 40–60 min after the addition of Ca^{++} (Fig. 1 *B*), coincident with the assembly of chromatin into nuclei. This is consistent with a requirement for the completed assembly of a nuclear membrane before initiation of replication as has been shown for the replication of *Xenopus* sperm chromatin (5). Several properties of the DNA synthesis in chimeric nuclei

were consistent with its resulting from de novo initiation of chromosomal DNA replication. First, DNA repair, mitochondrial DNA synthesis, and the extension of preexisting replication forks do not require the assembly of a nuclear membrane and begin without a delay in *Xenopus* egg extract (16). Second, DNA synthesis in chimeric nuclei was sensitive to aphidicolin, an inhibitor of replicative polymerases that does not inhibit repair DNA polymerase β or mitochondrial DNA polymerase γ (16). Third, DNA synthesis was sensitive to the protein kinase inhibitor 6-DMAP, which inhibits replication initiation but not the elongation of preprimed DNA templates (Fig. 1 *B*) (4, 16, 37). Finally, DNA synthesis required the addition of Ca^{++} to the extract, demonstrating that it resulted from cell cycle–regulated, S-phase–dependent DNA replication (Fig. 1 *B*).

With Chimeric Nuclei as Substrate, Xenopus Egg Extract Initiates Replication within the 5′ End of the DHFR Gene

The sites of initiation of DNA replication at the DHFR locus were mapped within chimeric nuclei using the ELFH assay (Fig. 2). This recently developed assay quantifies the distribution of nascent DNA at various positions within the DHFR locus shortly after the initiation of DNA synthesis (16, 37). The ELFH assay has allowed us to assess the specificity of initiation more rapidly and with smaller DNA preparations than was possible with prior methodologies. G1-phase CHOC 400 nuclei were introduced into metaphase-arrested *Xenopus* egg extract for 60 min, a sufficient time to allow nuclear membrane breakdown and chromosome condensation in all nuclei. Extracts were stimulated to enter S phase by the addition of Ca^{++}, and then incubated in the presence of aphidicolin for an additional 2 h to allow reformation of nuclei, initiation of replication, and the accumulation of newly formed replication forks arrested close to their sites of initiation by aphidicolin (Fig. 2; *Cycled*). As a control, aliquots of the same G1-phase CHOC 400 nuclei were incubated in the same extract in parallel, except that Ca^{++} was added from the beginning (along with aphidicolin) to prevent nuclear membrane breakdown (Fig. 2; *Not Cycled*). Nuclei were then washed free of aphidicolin, nascent DNA chains were labeled briefly with [α-^{32}P]dATP, and the resulting ^{32}P-DNA chains were hybridized to 15 unique probes distributed over a 130-kb region that included the DHFR ori-β (Fig. 2 *C*). To control for differences in probe size, deoxyadenosine content of the segments analyzed (which would influence specific activity), and the hybridization efficiency of genomic DNA to each probe, replication intermediates were also labeled in nuclei from exponentially growing CHOC 400 cells (which serve as a pool of replication forks distributed randomly throughout the DHFR locus), and then hybridized to these same probes (Fig. 2 *A*). The cpm hybridized to each probe with the earliest labeled nascent DNA was then corrected for this variation by calculating the ratio of ^{32}P-DNA per probe with synchronized nuclei to the corresponding value for exponentially proliferating nuclei (16, 37).

Consistent with our previously reported results (16, 37) with intact post-ODP nuclei that were not cycled (Fig. 2 *B*), replication initiated within the ori-β initiation locus at sites distributed in a pattern indistinguishable from that of cultured cells (The ELFH pattern for cultured cells is shown in references 16 and 37 and Fig. 7 *B*). Results with the ELFH assay are consistent with all previous origin-mapping data at this locus that, taken together, suggest a broad region of initiation activity, with the potential for one or more preferred sites within that region (see Introduction). The degree to which ELFH results reflect highly preferred and localized sites of initiation of replication vs a delocalized set of equally preferred sites depends on the efficacy with which aphidicolin has arrested replication forks close to their sites of initiation before pulse labeling nascent DNA. Thus, the ELFH assay does not resolve the debate concerning details of the physiology of replication initiation that more cumbersome techniques have yet to resolve. Instead, it provides a means to rapidly evaluate the specificity of replication initiation, a process that may take place with an imprecision that precludes higher resolution.

In chimeric cycled nuclei, replication also initiated site specifically. However, the preferred site(s) for initiation of DNA synthesis in these nuclei mapped to the 5′ end of the DHFR structural gene, in a region that is refractory to initiation in cultured CHO cells (Fig. 2). To simplify discussion, we have designated this region of the chromosome ori-δ, even though it does not normally function as an origin of replication. We will refer to the chromosomal region encompassing the initiation zone detected in cultured cells as ori-β (ignoring, for simplicity, the possible existence of ori-γ). We have previously shown that *Xenopus* egg extract has no preference for the initiation of replication within DNA sequences encompassing the ori-δ region when naked DNA is introduced as a substrate (16), indicating that it is a unique combination of the assembly of a *Xenopus* nuclear envelope around condensed CHO G1-phase chromosomes that has revealed this novel origin.

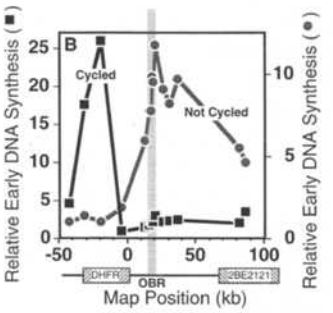
Recognition of ori-δ Requires Chromosome Condensation

In the course of these experiments, we observed that different preparations of *Xenopus* egg extract differed in their capacity to condense chromosomes during the disassembly reaction. With the same concentration of input nuclei, extracts that condensed chromosomes to a greater extent also showed a greater preference for initiation at ori-δ after extract activation. To address this relationship between chromosome condensation and site-specific initiation of replication more directly, different concentrations of nuclei were incubated in the same metaphase-arrested *Xenopus* egg extract preparation, and the degree of chromosome condensation during nuclear disassembly was related to the specificity of replication initiation after extract activation (Fig. 3). To ensure that the replication capacity of the extracts was not compromised by the increasing concentration of nuclei, all reactions were adjusted to the same concentration of nuclei by dilution with fresh activated extract after nuclear membrane breakdown was complete. Under these conditions, the efficiency of DNA synthesis after activation was identical in all reactions (Fig. 3 *A*).

At the lowest concentration (25,000 nuclei per μl extract), G1-phase nuclei were converted into condensed

A

Map Position (kb)	Probe	Probe Size (bp)	Exponential Raw Data	Exponential Relative cpm	Cycled Raw Data	Cycled Relative cpm	Cycled Corrected cpm	Cycled Rel. Early DNA Syn.	Not Cycled Raw Data	Not Cycled Relative cpm	Not Cycled Corrected cpm	Not Cycled Rel. Early DNA Syn.
-43	H	1600		7.03		13.29	1.89	4.59		3.97	0.56	1.00
-32	I	650		2.59		18.81	7.26	17.62		2.00	0.77	1.37
-20	A	643		1.76		18.87	10.70	25.95		1.00	0.57	1.00
- 5	J	800		2.74		1.13	0.41	1.00		2.93	1.07	1.90
12.5	G	685		3.06		1.90	0.62	1.50		10.31	3.37	5.97
15.5	B	1532		6.14		4.40	0.72	1.74		27.02	4.40	7.80
16.5	C	231		1.00		1.00	1.00	2.43		4.37	4.37	7.74
17.5	D	281		1.10		1.12	1.01	2.45		6.11	5.54	9.81
18.5	E	380		1.76		1.61	0.92	2.22		9.42	5.36	9.50
20	R	887		3.89		4.89	1.26	3.05		25.80	6.63	11.75
25.5	F	1250		6.60		5.94	0.90	2.19		33.71	5.11	9.05
30	K	700		3.61		3.44	0.95	2.31		16.70	4.62	8.19
36	L	1200		6.99		7.23	1.03	2.51		38.28	5.48	9.71
81	P	500		3.00		2.58	0.86	2.08		9.35	3.12	5.53
86	O	700		5.86		8.46	1.44	3.50		15.37	2.62	4.65
N/A	λ	649		.32		.28	N/A	N/A		0.30	N/A	N/A



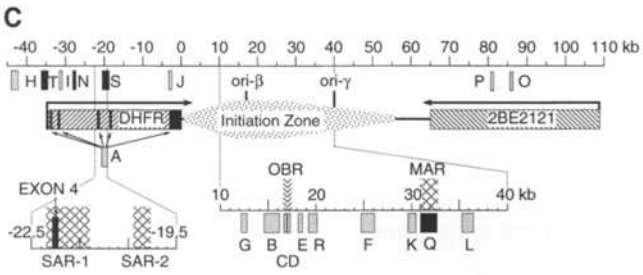


Figure 2. With cycled nuclei as a substrate, *Xenopus* egg extract initiated replication in the 5′ region of the DHFR structural gene. (*A*) G1-phase CHOC 400 nuclei (50,000 per μl of extract) were disassembled and reassembled as shown in Fig. 1, and aphidicolin was added at the same time as calcium chloride (*Cycled*). As a control, the same G1-phase nuclei were also incubated in the same extract supplemented with calcium chloride and aphidicolin from the beginning, to activate the extract and to prevent nuclear disassembly (*Not Cycled*). 2 h after the addition of calcium, nuclei were washed free of aphidicolin, and the earliest replicating nascent DNA chains were labeled briefly with [α-^{32}P]dATP. 15 unique probes (*C*) distributed over a 130-kb region that includes the DHFR ori-β were immobilized using a slot blot apparatus and hybridized to the ^{32}P-labeled early replication intermediates as described (16). Relative cpm were obtained by phosphorimaging analysis (*Relative cpm*). To correct for variation between probes as a result of differences in probe size, deoxyadenine content, and hybridization efficiency, labeled nascent DNA from exponentially proliferating CHOC 400 cells was hybridized to the same probes. The relative cpm for each probe with either G1-phase or M-phase early replication intermediates was divided by the corresponding value for exponentially proliferating nuclei (*Corrected cpm*). These values were then normalized by adjusting the lowest corrected value to 1.00 (*Rel. Early DNA Syn.*). Probe λ is a segment of bacteriophage λ DNA included in each experiment to evaluate the degree of nonspecific hybridization. (*B*) The Relative Early DNA Synthesis values (defined in *A*) for early replication intermediates in nuclei that were either *Cycled* (■) or *Not Cycled* (●) were plotted vs the map positions of each probe. The horizontal axis includes a diagram to orient the DHFR and 2BE2121 transcription units. The vertical shaded line shows the position of the previously mapped OBR (see Introduction). Similar results were obtained in 10 independent experiments. (*C*) The positions of the 15 probes H through O (*shaded boxes*) used to map the sites of earliest DNA replication are shown in relation to the DHFR and 2BE2121 transcription units (direction of transcription indicated with arrow). The A probe is a DHFR cDNA, consisting of exons 1–5 (totaling 488 nt) and 155 nt of exon 6, and is positioned at the center of the DHFR gene to represent its average position. Probes T, N, S, and Q (*black boxes*) are additional probes used to obtain the data shown in Fig. 7. Also shown are the positions of ori-β and ori-γ, as well as the initiation zone mapped by two-dimensional gel electrophoresis (see Introduction). The ori-β region is expanded to show the position of the OBR, as it has been most precisely defined, and the MAR located between ori-β and ori-γ (13). The DNA near the fourth DHFR exon is expanded to show the positions of two closely linked SARs (22).

chromosomes, and subsequent replication initiated within ori-δ, consistent with the results shown in Fig. 2. When these same nuclei were disassembled at 100,000 nuclei per μl extract, it was more difficult to observe individual chromosomes, and a significant amount of the chromatin derived from individual nuclei remained in bundles. Under these conditions, specific initiation within the ori-δ locus was reduced. Finally, at 160,000 nuclei per μl, condensation of chromatin was limited to globular regions within nuclei, and the ability to discern individual chromosomes was almost completely inhibited, although nuclear membrane breakdown was complete. Subsequent initiation of DNA replication showed very little preference for the ori-δ locus. Higher concentrations of nuclei (250,000 per μl) prevented nuclear membrane breakdown and resulted in preferential initiation within the ori-β locus after stimula-

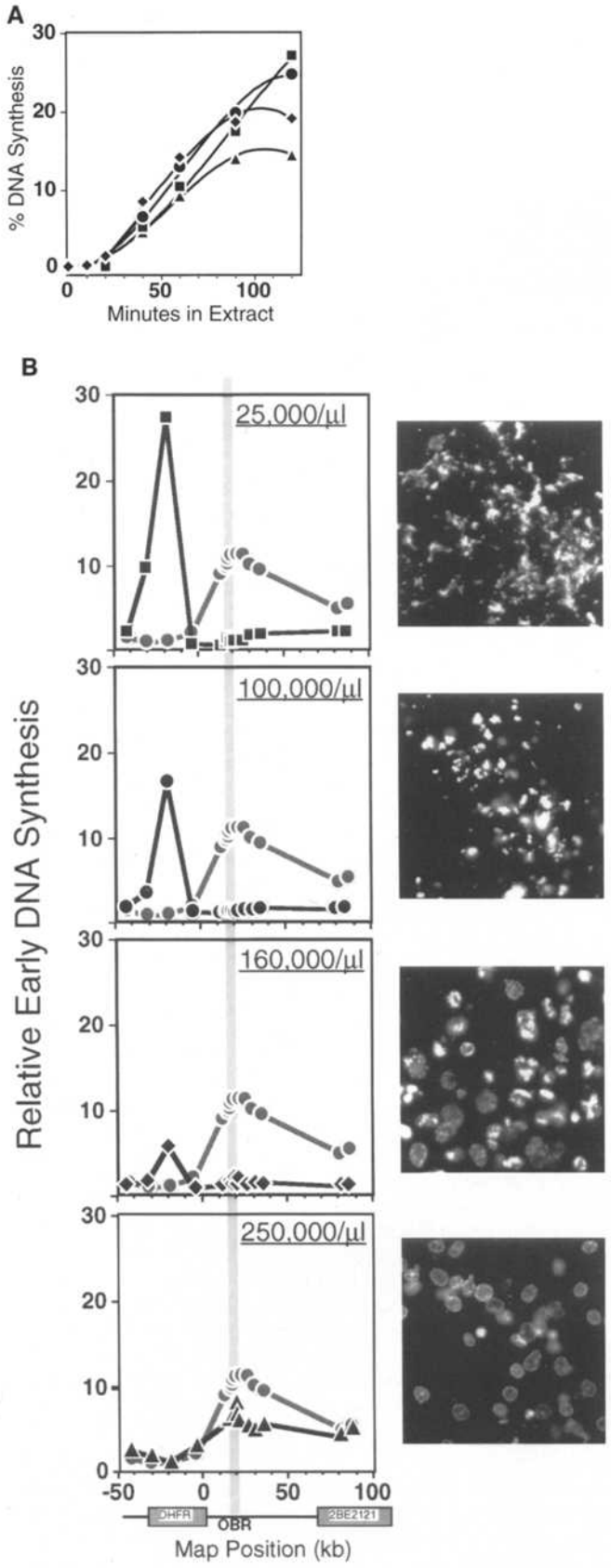


Figure 3. The relative preference for the ori-δ locus correlates with the degree of chromosome condensation. G1-phase CHOC 400 nuclei were incubated in a metaphase-arrested *Xenopus* egg extract at 25,000 (■), 100,000 (●), 160,000 (◆), and 250,000 (▲) nuclei per μl of extract for 60 min. (*A*) Calcium chloride was added, nuclei from all samples were adjusted to 12,500 per μl extract with fresh activated *Xenopus* egg extract supplemented with [α-^{32}P]dATP, and the fraction of input DNA replicated was quantified by acid precipitation as in Fig. 1. (*B*) Nuclei were stained with DAPI as in Fig. 1 and photographed (*right panels*). Calcium chloride was then added, and nuclei from all samples were diluted to 12,500 per μl extract (as in *A*) with fresh activated *Xenopus* egg extract supplemented with aphidicolin. 2 h later, the sites of initiation of replication were mapped (symbols as in *A*) by the ELFH assay as shown in Fig. 2. As a control, the same nuclei were incubated at 25,000 nuclei per μl extract supplemented with calcium chloride and aphidicolin from the beginning to activate the extract and to prevent nuclear disassembly (●). Total hybridization to all 15 probes was similar whether specificity was detected or not, indicating that the ELFH pattern reflects the relative distribution of the same number of aphidicolin-arrested early replication forks in all cases (see text). Axes are labeled as in Fig. 2. Similar results were obtained in two independent experiments.

tion of replication. These data (Fig. 3 *B*) demonstrate that nuclear envelope breakdown is not sufficient to establish ori-δ as a preferred replication start site and strongly implicate chromosome condensation as a necessary prerequisite.

Lack of specificity observed with high concentrations of nuclei was most likely due to initiation of replication at sites distributed throughout the region examined by these probes, rather than to specific initiation at other sites. Since the total amount of hybridization to the various probes was similar whether specificity was detected or not, an equal number of aphidicolin-arrested early replication forks must have been present within the region covered by the probes in both cases. If replication forks had been arrested close to a specific site that is not near the probes used in these experiments, then the total hybridization to these probes would be significantly reduced. Thus, as was the case when intact early G1-phase (pre-ODP) nuclei were introduced into *Xenopus* egg extracts containing aphidicolin (37), the inhibition of chromosome condensation when post-ODP nuclei were cycled through a *Xenopus* egg mitosis resulted in the accumulation of aphidicolin-arrested early replication forks at sites distributed throughout the DHFR locus. Although we cannot conclude that initiation under these conditions occurs at random sites, we refer to this as nonspecific initiation.

Chromosome condensation has been shown to involve at least two separate steps. The first is the local condensation of the 30-nm fibers, which may be mediated by the phosphorylation of histones H1 and H3 (2, 29). With rat liver nuclei incubated in metaphase-arrested *Xenopus* egg extract, this step is completed rapidly, before the visible condensation of chromosomes (29). The second step is the radial coiling of the metaphase scaffold axis to achieve an additional 10-fold compaction (30). This step has been shown to require the activity of topoisomerase II, a major component of the metaphase chromosome scaffold (2, 29). In particular, topoisomerase II inhibitors added to *Xenopus* egg extracts have been shown to inhibit the gross rearrangement of chromosomes during the coiling step, but not the phosphorylation of histones H1 or H3, lamin solubilization, or nuclear membrane breakdown (29). Similarly,

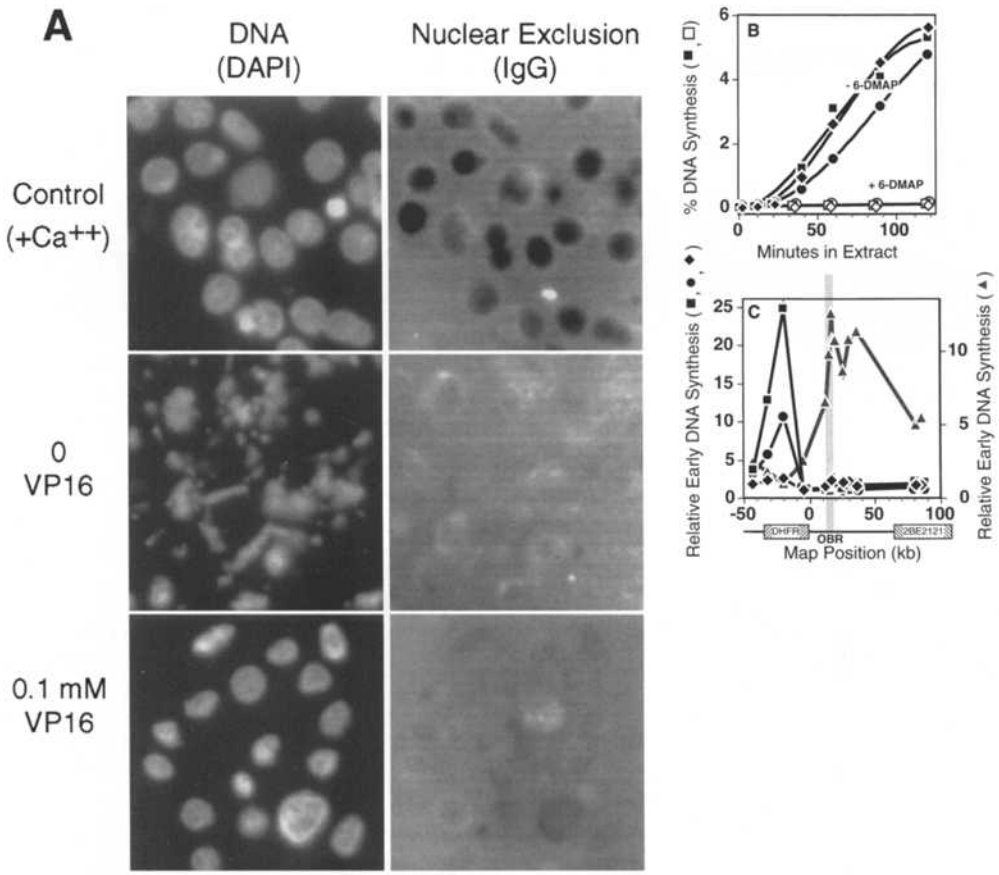


Figure 4. Recognition of ori-δ by *Xenopus* egg cytosol requires topoisomerase II–mediated chromosome condensation. (*A*) G1-phase CHOC 400 nuclei (50,000 per μl extract) were incubated in a metaphase-arrested *Xenopus* egg extract in the absence (*0 VP16*) or presence (*0.1 mM VP16*) of VP16. As a control, the same nuclei were incubated in extract supplemented with calcium chloride to prevent nuclear disassembly (*Control* [*+Ca++*]). After 1 h, aliquots were removed for observation. DNA was observed by staining with DAPI. Nuclear membrane formation was monitored by the exclusion of Texas red–labeled affinity-purified IgG. (*B*) G1-phase nuclei were disassembled in metaphase-arrested *Xenopus* egg extract in the absence (■) or presence of 0.03 mM (●) or 0.1 mM (◆) VP16 as shown in *A*. After 1 h, samples were washed free of VP16 (Materials and Methods) and resuspended in fresh activated extract, in either the presence (*open symbols*) or absence (*filled symbols*) of 6-DMAP. (*C*) In aliquots of the samples shown in *B*, aphidicolin was added at the time of extract activation, and 2 h later, the sites of initiation of replication were mapped by the ELFH assay as shown in Fig. 2. As a control, the same nuclei were incubated at 25,000 nuclei per μl extract supplemented with calcium chloride and aphidicolin from the beginning to activate the extract and to prevent nuclear disassembly (▲). Axes are labeled as in Fig. 2. Similar results were obtained in two independent experiments.

immunodepletion of topoisomerase II from metaphase-arrested *Xenopus* egg extracts inhibits the condensation of chicken erythrocyte chromatin, resulting in the formation of precondensation chromosomes (pre-CXs) consisting of clusters of swollen chromatids in which the coiling of the metaphase scaffold has been inhibited (2). The spherical particles observed within nuclei incubated in metaphase-arrested extracts at high concentrations (Fig. 3; *160,000/μl*) closely resemble the appearance of pre-CXs.

To determine whether the coiling of the metaphase scaffold was necessary for recognition of ori-δ, G1-phase CHOC 400 nuclei were incubated in metaphase-arrested *Xenopus* egg extract in the presence of various concentrations of the topoisomerase II inhibitor VP16. We found that 0.1 mM VP16 was sufficient to prevent chromosome condensation but still allowed nuclear membrane breakdown (Fig. 4 *A*). Lower concentrations of VP16 resulted in variable and intermediate degrees of chromosome condensation, including the appearance of pre-CXs in some nuclei (not shown). Direct activation of extracts after nuclear membrane breakdown in the presence of VP16 led to variable (up to 80%) reduction in the efficiency of DNA synthesis (not shown). This inhibition could be due to the stabilization of covalent DNA–topoisomerase II complexes by VP16, an otherwise unstable state that has been shown to be reversible upon removal of the inhibitor (23, 34). To alleviate the inhibition of DNA replication after nuclear membrane breakdown in the presence of VP16, chromatin was washed and transferred to a fresh activated extract. As shown in Fig. 4 *B*, the efficiency of DNA synthesis was nearly identical whether or not the inhibitor was present during nuclear membrane breakdown. The sites of initiation of replication were then evaluated by the ELFH assay (Fig. 4 *C*). Results of these experiments demonstrated that specific recognition of ori-δ was prevented by the presence of VP16 in a dose-dependent manner. These results are consistent with those of Fig. 3 and strongly suggest that the topoisomerase II–mediated step of chromosome formation creates an architecture that reveals ori-δ as a preferred replication start site recognized by *Xenopus* egg cytosolic factors.

DNA Replication in CHOC 400 Metaphase Chromosomes by Xenopus Egg Extract Initiates at ori-δ

The experiments described above demonstrate that the topoisomerase II–mediated coiling of chromosomes during

mitosis is required to create a unique nuclear structure that leads to initiation of replication at ori-δ. If replication in *Xenopus* egg extracts initiates at sites determined by structural elements of the substrate, as has been proposed (16), then the chromosome architecture adopted during metaphase should be sufficient to elicit the novel origin choice. To test this hypothesis, we introduced metaphase chromosomes from CHOC 400 cells directly into activated *Xenopus* egg extracts.

CHOC 400 cells were synchronized in metaphase by brief (4-h) exposure to nocodazole and collected by shake off. Half of these cells were allowed to proceed 4 h into G1 phase (post-ODP) by plating into fresh pre-warmed medium. Both metaphase and G1-phase populations were permeabilized with digitonin and introduced into an extract prepared from activated *Xenopus* eggs. The formation of a *Xenopus* nuclear envelope around metaphase chromosomes was monitored by the ability to exclude fluorescently labeled IgG (Fig. 5 *A*). Digitonin permeabilization left the G1-phase nuclear membrane intact, whereas permeabilized metaphase cells contained bundles of condensed chromosomes lacking a nuclear membrane. *Xenopus* egg extract stimulated the assembly of a nuclear membrane around these metaphase chromosomes and the simultaneous decondensation of chromatin. Virtually all chromosomes were assembled into nuclei within 60 min (Fig. 5 *A*). DNA synthesis was measured in these same extracts by monitoring the incorporation of [α-^{32}P]dATP into acid-precipitable nucleic acids (Fig. 5 *B*). DNA synthesis in G1-phase nuclei began after a typical 20-min lag period (16, 37), whereas in metaphase chromosomes, DNA synthesis did not begin until 40–60 min, consistent with a requirement for the completed assembly of a nuclear membrane before initiation of replication (5). In addition, DNA synthesis in metaphase chromosomes was sensitive to aphidicolin and 6-DMAP (Fig. 5 *B*), all properties consistent with its resulting from the initiation of DNA replication. Finally, DNA synthesis with metaphase chromosomes as a substrate was semiconservative. When extracts were substituted with [α-^{32}P]dATP and 5′-bromodeoxyuridine triphosphate to increase the density of ^{32}P-labeled nascent DNA, and the nascent DNA was centrifuged to equilibrium in a neutral density gradient, virtually all of the ^{32}P-DNA appeared as a single band of hybrid density (not shown). Taken together, the DNA synthesis stimulated by *Xenopus* egg cytosol with metaphase chromosomes as a substrate appears to result from the initiation of DNA replication.

The sites of initiation of replication within both metaphase chromosomes and G1-phase nuclei were mapped by the ELFH Assay. Permeabilized metaphase and G1-phase CHOC 400 cells were incubated in *Xenopus* egg extract for 2 h in the presence of aphidicolin. Nuclei were then washed free of aphidicolin, and the positions of arrested replication forks were analyzed by ELFH (Fig. 6). Results revealed that, in nuclei assembled in *Xenopus* egg extracts around CHO metaphase chromosomes, replication initiated at or near the same ori-δ locus that was recognized when G1-phase nuclei were cycled through a *Xenopus* egg mitosis. Thus, the structure of metaphase chromosomes, not the act of passing through a *Xenopus* egg mitosis, is sufficient to establish this site as an origin of replication.

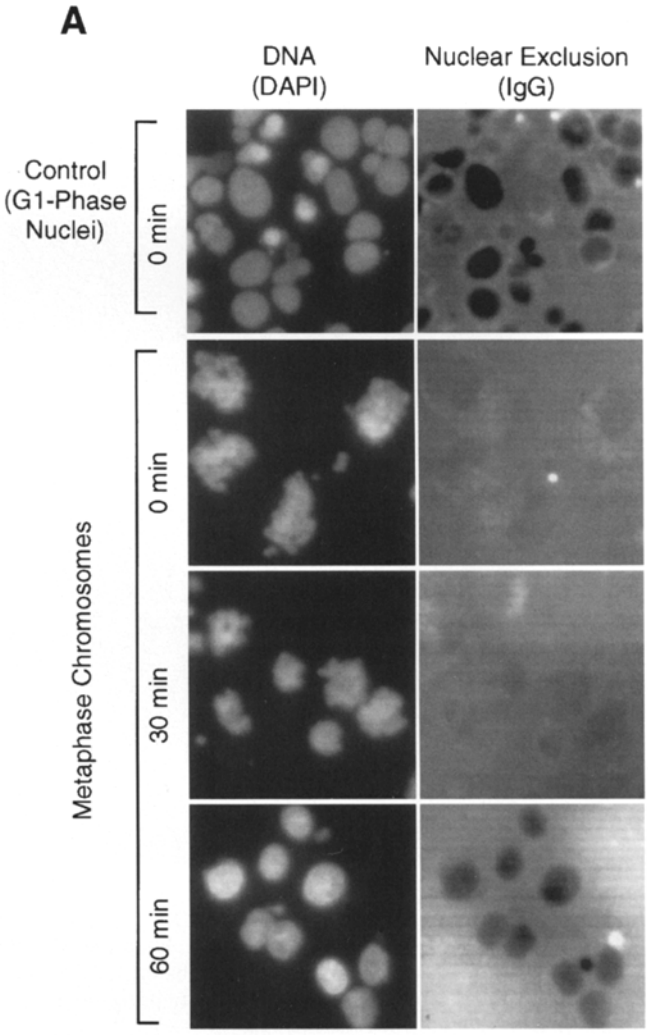


Figure 5. Assembly of CHO metaphase chromosomes into nuclei and the initiation of DNA replication by *Xenopus* egg extract. (*A*) CHOC 400 cells synchronized in either G1 phase or M phase were permeabilized with digitonin and introduced into activated *Xenopus* egg extract. At the indicated times, aliquots were removed. DNA was observed by staining with DAPI. Nuclear membrane formation was monitored by the exclusion of Texas red–labeled affinity-purified IgG. (*B*) Either G1-phase (●) or M-phase (■) cells were permeabilized as in *A* and introduced into *Xenopus* egg extract supplemented with [α-^{32}P]dATP in either the presence (*open symbols*) or absence (*closed symbols*) of 3 mM 6-DMAP. At the indicated times, aliquots were removed, and the amount of DNA synthesis was quantified by acid precipitation as in Fig. 1.

Early Replication Forks Derived from the ori-δ Locus Are Distributed throughout a 20-kb Region Centered between DHFR Exons 3 and 4

In various experiments, the intensity of hybridization of early replication intermediates to probe I was less than A

A

Map Position (kb)	Probe	Probe Size (bp)	Exponential Raw Data	Exponential Relative cpm	Metaphase Raw Data	Metaphase Relative cpm	Metaphase Corrected cpm	Metaphase Rel. Early DNA Syn.	G1-Phase (Post-ODP) Raw Data	G1-Phase (Post-ODP) Relative cpm	G1-Phase (Post-ODP) Corrected cpm	G1-Phase (Post-ODP) Rel. Early DNA Syn.
-43	H	1600		11.63		11.35	0.98	2.36		6.33	0.54	1.51
-32	I	650		3.62		10.14	2.80	6.76		1.31	0.36	1.00
-20	A	643		2.62		10.00	3.82	9.21		1.00	0.38	1.06
- 5	J	800		4.32		1.94	0.45	1.09		2.13	0.49	1.37
12.5	G	685		3.48		2.17	0.62	1.50		11.97	3.44	9.53
15.5	B	1532		9.01		4.75	0.53	1.27		32.15	3.57	9.88
16.5	C	231		1.00		1.00	1.00	2.41		5.84	5.84	16.17
17.5	D	281		1.32		1.05	0.80	1.93		6.08	4.62	12.81
18.5	E	380		1.89		1.36	0.72	1.75		8.43	4.47	12.38
20	R	887		4.78		3.42	0.71	1.72		19.68	4.11	11.40
25.5	F	1250		6.08		5.58	0.92	2.22		25.02	4.12	11.41
30	K	700		4.08		2.94	0.72	1.74		15.25	3.74	10.37
36	L	1200		11.75		6.19	0.53	1.27		27.57	2.35	6.50
81	P	500		5.19		2.18	0.42	1.01		7.36	1.42	3.93
86	O	700		11.15		4.62	0.41	1.00		15.32	1.37	3.81
N/A	λ	649		0.25		.25	N/A	N/A		0.30	N/A	N/A

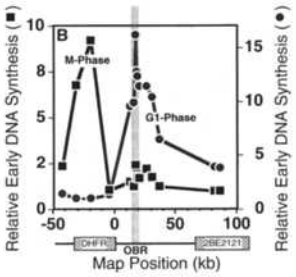


Figure 6. Replication initiates at ori-δ within nuclei assembled around CHOC 400 metaphase chromosomes. (*A*) CHOC 400 cells synchronized in either late G1 phase or metaphase were permeabilized with digitonin and introduced into activated *Xenopus* egg extract containing aphidicolin. After 2 h, nuclei were washed free of aphidicolin, and the ELFH assay was performed as described in Fig. 2. Replication intermediates were also labeled in nuclei from exponentially growing CHOC 400 cells (*Exponential*) and hybridized in parallel. *Corrected cpm* and Relative Early DNA Synthesis (*Rel. Early DNA Syn.*) were calculated as in Fig. 2 *A*. (*B*) The *Relative Early DNA Synthesis* values (defined in *A*) for either G1-phase (●) or M-phase (■) early replication intermediates were plotted vs the map positions of each probe as described in Fig. 2. Similar results were obtained in eight independent experiments.

in most (not all) cases. Since probe A is the DHFR cDNA, it was difficult to evaluate the position of peak early DNA synthesis. Since probe I overlaps exon 3 and probe J is near exon 6, the strong hybridization to probe A must have resulted from replication forks that were arrested either (*a*) at a single circumscribed site very close to exons 1 and 2 and/or exons 4 and 5, or (*b*) throughout a broad region between probe H and probe J, similar to the broad distribution of early replication intermediates at the ori-β locus. To distinguish between these possibilities, we purified additional DNA fragments from the DHFR structural gene to include in the ELFH assay and identified two that were free of repetitive sequences (Fig. 2 *C*). Probe T encompasses the DHFR promoter and exon 1. Probe N is located between exons 3 and 4. In addition, we acquired a segment of DNA located between exons 4 and 5 (a gift from L. Chasin) where a pair of scaffold attachment regions (SARs) have previously been identified (22; probe S in Fig. 2 *C*). To explore any potential role of such attachment regions in the selection of replication origins, a segment of DNA encompassing the MAR from within the ori-β locus (a gift from J. Hamlin) was also obtained (13; probe Q in Fig. 2 *C*).

CHOC 400 cells synchronized in metaphase were permeabilized with digitonin and introduced into *Xenopus* egg extract. The sites of initiation of replication were then mapped by the ELFH assay, including the new probes. The results (Fig. 7 *A*), presented as the mean of three independent experiments, revealed that the hybridization of early replication intermediates is distributed throughout the 5′ region of the DHFR gene, between probes H and S, with peak hybridization to probe N. Although probe A was not assigned a map position in these experiments (to avoid placing it adjacent to a particular probe), it was included in the analysis, and the results with this probe also supported a broad distribution of replication forks, reasoned as follows. Probes T, I, and S are very close or overlapping exons 1 and 2, exon 3, and exons 4 and 5, respectively. Thus, if replication forks were arrested within a circumscribed region that overlaps only one or two exons of the DHFR gene, then a probe located near those particular exons would be expected to hybridize to a significantly greater fraction of early replication intermediates than would probe A. On the other hand, if replication forks were arrested throughout a broad region, then probes distributed throughout that region would hybridize to early replication intermediates with an efficiency similar to that of probe A. Results showed that none of the exon probes hybridized more efficiently than probe A. Furthermore, probe N is located between exons 3 and 4, yet it hybridized to a greater fraction of early replication intermediates than probes located much closer to those

same exons. Hybridization to intron probe N was only slightly greater than that of A, giving a mean N/A ratio of 1.25. Thus, we conclude that aphidicolin-arrested early replication forks must be distributed throughout the region between probes H and S, encompassing exons 1–5 (which constitute 76% of probe A).

Results showed that the intragenic SAR did not constitute the most highly preferred initiation site within the ori-δ locus, since the values for probes T, I, and N were consistently higher than those for probe S. We also examined whether the MAR (probe Q) would constitute a highly preferred site relative to nearby probes under conditions that favored initiation in the ori-β locus. For these experiments, early replicating nascent DNA was labeled after intact G1-phase nuclei were introduced into activated egg extract supplemented with aphidicolin (Fig. 7 *A*) or after G1-phase cells were allowed to enter S phase in culture in the presence of aphidicolin (Fig. 7 *B*; *G1/S*) and hybridized to these same probes. As expected, hybridization to DHFR gene probes T, N, and S was weak and nearly the same as that to closely linked probes H, I, A (not shown), and J. Hybridization to probe Q was slightly higher than other probes distributed throughout the ori-β locus. However, it is difficult to distinguish whether this slightly greater value represents a true preference for initiation of replication at the MAR site, or normal variation inherent in the ELFH assay.

Thus, the pattern of initiation of replication at the ori-δ locus, where early replication forks were distributed over ~20 kb between probes H and S, resembles that at the ori-β locus, where early replication intermediates were distributed over ~30 kb between probes G and L. Although initiation within the ori-δ locus appears to be slightly more circumscribed than that at ori-β, it is possible that what we are referring to as the ori-β locus actually consists of two preferred loci, ori-β and ori-γ, that together lead to a broader distribution of early nascent DNA. As with ori-β and ori-γ, the ELFH assay cannot distinguish whether ori-δ consists of a highly preferred and localized initiation site, from which early replication forks traveled various distances in the presence of a leaky aphidicolin arrest, or a larger set of delocalized sites near which early replication forks were very effectively arrested by aphidicolin.

Discussion

Here we report that a novel and unanticipated pattern of initiation of DNA replication is revealed when DNA sequences from the CHO DHFR locus are presented to *Xenopus* egg cytosol in the form of condensed metaphase chromosomes. Whether CHO metaphase chromosomes served as the substrate for DNA replication, or whether CHO G1-phase nuclei were disassembled in a metaphase-arrested *Xenopus* egg extract and the resulting artificially condensed chromosomes served as the substrate, a unique nuclear structure was assembled by the activated egg extract that dictated preferential initiation of replication in the 5′ half of the DHFR gene, designated ori-δ. No investigator has previously reported replication initiation in this part of the DHFR locus in cultured CHO cells, and no investigator has previously reported site-specific initiation of replication in any nuclear structure assembled in *Xenopus* egg extracts, even with DNA templates from the DHFR locus (16). Thus, preferential initiation within ori-δ is unique to the interaction of activated *Xenopus* egg cytosol with condensed CHO chromosomes. Furthermore, since no preference for initiation at ori-β was detected within G1-phase nuclei cycled through a *Xenopus* egg mitosis unless nuclear envelope breakdown was prevented during the mitotic cycle, the components of the G1-phase nucleus that dictate initiation of replication at ori-β must be dismantled or masked by the events taking place during nuclear membrane breakdown.

The conditions that establish this unusual replication start site, while artificial, clearly demonstrate that origin choice in metazoan genomes can be influenced by complex features of higher order chromosome and nuclear structure. Previous studies have suggested that changes in nuclear structure occurring during G1 phase establish specific sites as origins of replication in mammalian cells (37). Since chromosome architecture is reestablished after each mitosis in all higher eukaryotic organisms, the results pre-

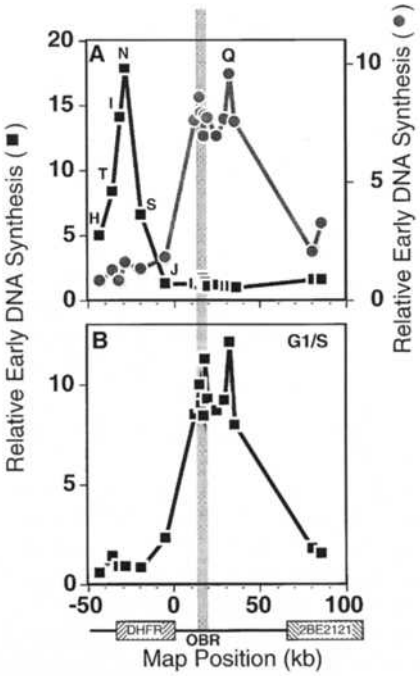


Figure 7. Aphidicolin arrests early replication intermediates throughout the region encompassing the first five exons of the DHFR gene, with most forks arrested between exons 3 and 4. (*A*) CHOC 400 cells synchronized in either G1 phase (●) or Metaphase (■) were permeabilized with digitonin and introduced into activated *Xenopus* egg extract containing aphidicolin. 2 h later, the sites of initiation of DNA replication were mapped by the ELFH assay, as described in Fig. 2, including the new probes T, N, S, and Q. Positions of these new probes are shown in Fig. 2 *C*. Shown are the mean values for three independent experiments. Probe names for new probes, and probes from within the ori-δ locus, are indicated next to their relative early DNA synthesis values. The value for probe A is not shown (see text). (*B*) CHOC 400 cells were synchronized in mitosis and allowed to proceed into S phase in the presence of 10 μg/ml aphidicolin to arrest cells at the G1/S border. Sites of initiation of replication in culture were then mapped by the ELFH assay as described (16), using the new probes.

sented here raise the possibility that it is a particular chromosomal architecture, established during G1 phase, that determines where replication will initiate in the upcoming S phase.

Our results strongly suggest that the topoisomerase II–mediated coiling of the metaphase scaffold axis is necessary for ori-δ recognition. Both modulating the ratio of chromatin to egg cytosol (Fig. 3) and inhibition of topoisomerase II activity (Fig. 4) prevented ori-δ recognition. Both of these manipulations have been shown to specifically inhibit coiling of the scaffold axis during chromosome formation (2, 29). Other modifications of nuclear structure—nuclear membrane breakdown, nuclear lamina solubilization, phosphorylation of histones H1 and H3, and the local condensation of 30-nm chromatin fibers—have been shown to be unaffected by these manipulations (29), and thus are not sufficient for ori-δ recognition. Moreover, ori-δ recognition did not require *Xenopus*-specific factors during nuclear membrane breakdown. Metaphase chromosomes condensed during a CHO mitosis also dictated initiation of replication at this novel site.

The fact that the same unique pattern of initiation was revealed with both naturally and artificially condensed metaphase chromosomes as a substrate demonstrates that the process of chromosome condensation in *Xenopus* egg extract is remarkably similar to that in cultured mammalian cells and must be highly conserved. Recognition of ori-δ clearly requires a specific chromosome structure that is assembled by either the condensation of G2-phase chromatin in cultured CHO cells or the condensation of G1-phase chromatin in *Xenopus* egg extract. This observation reinforces the utility of *Xenopus* egg extracts as a system with which to dissect the process of chromosome condensation in mammalian cells.

Modulation of Origin Choice by Alternative Nuclear Structures

We have now defined three patterns of initiation that can be elicited within the genomic DNA sequences comprising the CHO DHFR locus using different complex substrates for replication in *Xenopus* egg extract. We have previously demonstrated that *Xenopus* egg extract will initiate replication specifically at the ori-β locus, provided that the substrate DNA remains packaged within an intact, late G1-phase nucleus. With damaged nuclei or naked DNA as a substrate, replication initiated nonspecifically (16). We have also shown that *Xenopus* egg extracts initiate replication at random sites within nuclei isolated from CHOC 400 cells synchronized within the first 3 h after metaphase (pre-ODP). This allowed us to define a distinct transition during the cell cycle (ODP) at which CHO nuclei become committed to initiate at ori-β (37). Here we show that chromosome condensation during mitosis creates a third pattern of replication initiation. Furthermore, we have defined a series of events that can transform a nuclear structure committed to initiate replication at ori-β into a structure that leads to either of the other two patterns of initiation. Whenever post-ODP nuclei have retained an intact nuclear envelope, even after incubation in metaphase-arrested extracts, where membrane breakdown was inhibited by high concentrations of nuclei (Fig. 3; *250,000 nuclei/μl*), we have observed initiation at ori-β. If the nuclear envelope was perturbed or disassembled without mitotic chromosome condensation, the structural elements that commit nuclei to the ori-β locus were disrupted, leading to a complete loss of specificity. Finally, if mitotic chromosome condensation occurred, a third nuclear structure was assembled that favors initiation at the ori-δ locus. These results in *Xenopus* egg extracts suggest that site-specific initiation of DNA replication requires unique and dynamic elements of nuclear structure, whereas nonspecific initiation of replication occurs when these elements have not been assembled or if they have become damaged.

One of the most puzzling contradictions in the field of metazoan DNA replication is the occasional report of cloned DNA templates that have the ability to promote autonomous replication in mammalian cells, and the site-specific initiation of replication within those autonomous plasmids (3, 18, 35, 36). So far, these results have not been reproducible in the hands of other investigators (e.g., 15). Perhaps a unique set of circumstances or arrangement of DNA sequences can lead to the assembly of some cloned DNA templates into a structure that favors initiation at specific sites. Until we understand the nature of such structures, results using this approach to identify origins will be difficult to reproduce.

What Constitutes an Origin of Replication in Animal Chromosomes?

What is ori-δ, and what might it have to do with replication origins that are used in cultured animal cells? One clue might come from the observation that replication of metaphase chromosomes in *Xenopus* egg extract does not actually initiate until after the chromosomes have decondensed (see Figs. 1 and 5). This means that the recognition of ori-δ within a condensed chromosome architecture must be made in advance of replication initiation. It has been shown that, in activated *Xenopus* egg extract, replication enzymes aggregate on the surface of chromosomes immediately upon contact with the extract, before chromatin decondensation or nuclear membrane formation (1, 38). With the tightly coiled structure of metaphase chromosomes as a substrate, these prereplication complexes (pre-RCs) may only be able to access the DNA sequences that are exposed on the surface of the chromosome, establishing these sites as replication origins. *Xenopus* embryonic chromosomes, which are subject to the same mitotic cycles yet are replicated from randomly positioned origins (20), are organized into smaller chromosome loops and are much less compact than CHO chromosomes, which may allow the pre-RCs to access a greater number of sites. In fact, at the blastula stage of *Xenopus* development, as origins of replication become less frequent (and more specific) (21) and differentiation begins, there is an increase in chromosome loop size, and consequently a shortening and thickening of condensed mitotic chromosomes (27). The assembly of preinitiation complexes within such condensed chromosomes, at a time during development when other chromosomal functions such as transcription have become activated, might lead to a disastrous disruption of the normal pattern of replication fork polarity. The appearance of a G1 phase, which also occurs at the blastula stage of development, may allow remodeling of chromosomal architec-

ture before the assembly of pre-RCs to position the appropriate DNA sequences for their accessibility to pre-RCs. In support of this hypothesis, during early *Drosophila* development, chromosomes are not observed to occupy distinct domains within the nucleus until after the blastula stage, and it has been proposed that a lengthened G1 phase is necessary to allow specific chromosomal DNA sequences to find their characteristic positions within the nucleus (12). Perhaps the ODP represents the completion of chromosome repositioning. The model described here would predict that, in cultured cells, pre-RC association with DNA should be delayed until after the ODP. In fact, recent evidence suggests that pre-RC assembly in human cells does not take place until late G1 phase (28).

What might be the nature of such proposed higher order structures that assemble during G1 phase (and at the blastula stage of *Xenopus* development) to select replication origins? Decondensation of chromatin is not sufficient to select the ori-β locus, since decondensation is completed before the origin decision point (37). One event occurring in early G1 phase that has been suggested to affect the organization of chromosomes is the assembly of the nuclear lamina (19). Nuclear lamin proteins are required for the proper assembly of a nucleoskeleton, for the recruitment of replication enzymes to the sites within the nucleus where replication takes place, and for the initiation of DNA replication per se (19). It has been proposed that, as the nuclear lamina assembles, looped chromatin domains are reestablished by interaction between SARs or MARs and the nucleoskeleton. These sites would then act as replication centers to recruit essential replication enzymes (19). It is now of considerable interest to investigate the cell cycle–regulated alterations in the structure of chromosomes, the lamina, attachment of DNA to the nuclear matrix, and the activities of topoisomerase II that occur at the origin decision point.

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References

1. Adachi, Y., and U.K. Laemmli. 1994. Study of the cell cycle-dependent assembly of the DNA pre-replication centres in *Xenopus* egg extracts. *EMBO (Eur. Mol. Biol. Organ.) J.* 13:4153–4164.
2. Adachi, Y., M. Luke, and U. Laemmli. 1991. Chromosome assembly in vitro: topoisomerase II is required for condensation. *Cell.* 64:137–148.
3. Berberich, S., A. Trivedi, D.C. Daniel, E.M. Johnson, and M. Leffak. 1995. In vitro replication of plasmids containing human c-myc DNA. *J. Mol. Biol.* 245:92–109.
4. 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.
5. Blow, J.J., and A.M. Sleeman. 1990. Replication of purified DNA in *Xenopus* egg extract is dependent on nuclear assembly. *J. Cell Sci.* 95:383–391.
6. Buongiorno, N.M., G. Micheli, M.T. Carri, and M. Marilley. 1982. A relationship between replicon size and supercoiled loop domains in the eukaryotic genome. *Nature (Lond.).* 298:100–102.
7. Burhans, W., and J. Huberman. 1994. DNA replication origins in animal cells: a question of context? *Science (Wash. DC).* 263:639–640.
8. Burhans, W.C., L.T. Vassilev, M.S. Caddle, N.H. Heintz, and M.L. DePamphilis. 1990. Identification of an origin of bidirectional DNA replication in mammalian chromosomes. *Cell.* 62:955–965.
9. Caddle, M.S., and M.P. Calos. 1992. Analysis of the autonomous replication behavior in human cells of the dihydrofolate reductase putative chromosomal origin of replication. *Nucleic Acids Res.* 20:5971–5978.
10. Coverley, D., and R.A. Laskey. 1994. Regulation of eukaryotic DNA replication. *Annu. Rev. Biochem.* 63:745–776.
11. DePamphilis, M.L. 1993. Origins of DNA replication in metazoan chromosomes. *J. Biol. Chem.* 268:1–4.
12. Dernburg, A.F., K.W. Broman, J.C. Fung, W.F. Marshall, J. Philips, D.A. Agard, and J.W. Sedat. 1996. Perturbation of nuclear architecture by long-distance chromosome interactions. *Cell.* 85:745–759.
13. Dijkwel, P.A., and J.L. Hamlin. 1988. Matrix attachment regions are positioned near replication initiation sites, genes, and an interamplicon junction in the amplified dihydrofolate reductase domain of Chinese hamster ovary cells. *Mol. Cell. Biol.* 8:5398–5409.
14. Djikwel, P.A., and J.L. Hamlin. 1995. The Chinese hamster dihydrofolate reductase origin consists of multiple potential nascent-strand start sites. *Mol. Cell. Biol.* 15:3023–3031.
15. Gilbert, D., and S.N. Cohen. 1989. Autonomous replication in mouse cells: a correction. *Cell.* 56:143–144.
16. Gilbert, D.M., H. Miyazawa, and M.L. DePamphilis. 1995. Site-specific initiation of DNA replication in *Xenopus* egg extract requires nuclear structure. *Mol. Cell. Biol.* 15:2942–2954.
17. Hamlin, J.L., P.J. Mosca, and V.V. Levenson. 1994. Defining origins of replication in mammalian cells. *Biochim. Biophys. Acta.* 1198:85–111.
18. Holst, A., F. Müller, G. Zastrow, H. Zentgraf, S. Schwender, E. Dinkl, and F. Grummt. 1988. Murine genomic DNA sequences replicating autonomously in mouse L cells. *Cell.* 52:355–365.
19. Hutchison, C., J. Bridger, L. Cox, and I. Kill. 1994. Weaving a pattern from disparate threads: lamin function in nuclear assembly and DNA replication. *J. Cell Sci.* 107:3259–3269.
20. Hyrien, O., and M. Mechali. 1993. Chromosomal replication initiates and terminates at random sequences but at regular intervals in the ribosomal DNA of *Xenopus* early embryos. *EMBO (Eur. Mol. Biol. Organ.) J.* 12:4511–4520.
21. Hyrien, O., C. Maric, and M. Mechali. 1995. Transition in specification of embryonic metazoan DNA replication origins. *Science (Wash. DC).* 270:994–997.
22. Kas, E., and L. Chasin. 1987. Anchorage of the Chinese hamster dihydrofolate reductase gene to the nuclear scaffold occurs in an intragenic region. *J. Mol. Biol.* 198:677–692.
23. Kaufmann, W.K., J.C. Boyer, L.L. Estabrooks, and S.J. Wilson. 1991. Inhibition of replicon initiation in human cells following stabilization of topoisomerase-DNA cleavable complexes. *Mol. Cell. Biol.* 11:3711–3718.
24. Krysan, P.J., J.G. Smith, and M.P. Calos. 1993. Autonomous replication in human cells of multimers of specific human and bacterial DNA sequences. *Mol. Cell. Biol.* 13:2688–2696.
25. Little, R.D., T.H. Platt, and C.L. Schildkraut. 1993. Initiation and termination of DNA replication in human rRNA genes. *Mol. Cell. Biol.* 13:6600–6613.
26. Masukata, H., H. Satoh, C. Obuse, and T. Okazaki. 1993. Autonomous replication of human chromosomal DNA fragments in human cells. *Mol. Biol. Cell.* 4:1121–1132.
27. Micheli, G., A.R. Luzzatto, M.T. Carri, A. deCapoa, and F. Pelliccia. 1993. Chromosome length and DNA loop size during early embryonic development of *Xenopus laevis. Chromosoma (Berl.).* 102:478–483.
28. Murti, K.G., D. He, B.R. Brinkley, R. Scot, and S.-H. Lee. 1996. Dynamics of human replication protein A subunit distribution and partitioning in the cell cycle. *Exp. Cell Res.* 223:279–289.
29. Newport, J., and T. Spann. 1987. Disassembly of the nucleus in mitotic extracts: membrane vesicularization, lamin disassembly, and chromosome condensation are independent processes. *Cell.* 48:219–30.
30. Rattner, J., and C. Lin. 1985. Radial loops and helical coils coexist in metaphase chromosomes. *Cell.* 42:291–296.
31. Shinomiya, T., and S. Ina. 1991. Analysis of chromosomal replicons in early embryos of *Drosophila melanogaster* by two-dimensional gel electrophoresis. *Nucleic Acids Res.* 19:3935–3941.
32. Shinomiya, T., and S. Ina. 1994. Mapping an initiation region of DNA replication at a single-copy chromosomal locus in *Drosophila melanogaster* cells by two-dimensional gel methods and PCR-mediated nascent-strand analysis: multiple replication origins in a broad zone. *Mol. Cell. Biol.* 14:7394–7403.
33. Smythe, C., and J.W. Newport. 1991. Systems for the study of nuclear assembly, DNA replication and nuclear breakdown in *Xenopus laevis* egg extracts. *Methods Cell Biol.* 35:449–468.
34. Udvardy, A., and P. Schedl. 1991. Chromatin structure, not DNA sequence specificity, is the primary determinant of topoisomerase II sites of action in vivo. *Mol. Cell. Biol.* 11:4973–4984.
35. Virta, P.V., P.H. Gunaratne, and A.C. Chinault. 1993. Analysis of a replication initiation sequence from the adenosine deaminase region of the mouse genome. *Mol. Cell. Biol.* 13:5931–5942.
36. Wu, C., P. Friedlander, C. Lamoureux, H.M. Zannis, and G.B. Price. 1993. cDNA clones contain autonomous replication activity. *Biochim. Biophys. Acta.* 1174:241–257.
37. Wu, J.-R., and D. Gilbert. 1996. A distinct G1 step required to specify the Chinese hamster DHFR replication origin. *Science (Wash. DC).* 271:1270–1272.
38. Yan, H., and J. Newport. 1995. FFA-1, a protein that promotes the formation of replication centers within nuclei. *Science (Wash. DC).* 269:1883–1885.
39. Yoon, Y., A. Sanches, C. Brun, and J.A. Huberman. 1995. Mapping of replication initiation sites in human ribosomal DNA by nascent-strand abundance analysis. *Mol. Cell. Biol.* 15:2482–2489.