

# Spatial distribution and specification of mammalian replication origins during G1 phase

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We have examined the distribution of early replicating origins on stretched DNA fibers when nuclei from CHO cells synchronized at different times during G1 phase initiate DNA replication in *Xenopus* egg extracts. Origins were differentially labeled in vivo versus in vitro to allow a comparison of their relative positions and spacing. With nuclei isolated in the first hour of G1 phase, in vitro origins were distributed throughout a larger number of DNA fibers and did not coincide with in vivo

origins. With nuclei isolated 1 h later, a similar total number of in vitro origins were clustered within a smaller number of DNA fibers but still did not coincide with in vivo origins. However, with nuclei isolated later in G1 phase, the positions of many in vitro origins coincided with in vivo origin sites without further change in origin number or density. These results highlight two distinct G1 steps that establish a spatial and temporal program for replication.

## Introduction

DNA replication in mammalian cells proceeds via the synchronous firing of clusters of adjacent origins that together coordinate the replication of several hundred kilobases of DNA (for reviews see Berezney et al., 2000; Gilbert, 2002b). Each of these coordinately replicated chromosome domains replicates at a characteristic time during S phase of the cell cycle. Programmed changes in both the temporal order of domain replication and the choice of replication origin sites accompany key stages of development and are often coupled to changes in gene expression (Gilbert, 2002a,b; Schubeler et al., 2002). Hence, the spatial distribution of replication origins along the lengths of chromosomes is likely to be critical for both the timely completion of DNA replication and the organization of chromosomes into structural and functional domains. However, very little is known about how replication origin sites are determined or what regulates the order in which they initiate replication. Although the proteins that regulate the initiation of DNA replication and limit replication to exactly one round per cell cycle are highly conserved from yeast to man, the sequences that are used as initiation sites are highly divergent (Gilbert, 2001a). Initiation cannot occur at random positions within chromosomes because that would

risk placing origins too far apart to complete replication within the course of an S phase (Blow et al., 2001; Hyrien et al., 2003). In most systems, replication initiates at defined and reproducible replication origin sites. However, to date, no specific DNA sequence elements have been identified that predict the locations of origins in a multicellular eukaryote (Gilbert, 2001a). In fact, in the rapid early cleavage stages of *Xenopus* and *Drosophila* development replication initiates at random with respect to DNA sequence but is nonetheless organized to initiate at regular 5–15-kb intervals (Lucas et al., 2000; Blow et al., 2001).

To investigate the spatial and temporal organization of mammalian replication origins, we have exploited a cell-free replication system in which nuclei from mammalian cells staged at various times during G1 phase are introduced into extracts from *Xenopus* eggs. This system has allowed us to evaluate the preparedness of cells for executing a replication program. Using this in vitro system, we have defined several discrete steps during early G1 phase that establish a spatial and temporal program for replication. First is the assembly of prereplication complexes (pre-RCs)\* consisting of ORC, Cdc6, and Mcm proteins. Pre-RC assembly takes place during telophase as demonstrated by the physical association of these proteins with mammalian cell chromatin and the functional capacity of telophase chromatin to replicate in *Xenopus* egg

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\*Abbreviations used in this paper: CldU, 5'-chloro-2'-deoxyuridine; DHFR, dihydrofolate reductase; IdU, 5'-iodo-2'-deoxyuridine; ODP, origin decision point; pre-RC, prereplication complex; TDP, timing decision point.

extracts that have been rendered deficient for pre-RC assembly (Okuno et al., 2001; Dimitrova et al., 2002). Approximately 2 h after the assembly of pre-RCs, a replication timing program becomes established (timing decision point [TDP]), coincident with the relocalization of chromosome domains to defined compartments within the newly formed nucleus (Dimitrova and Gilbert, 1999; Li et al., 2001; Gilbert, 2001b, 2002b). However, establishing the order in which domains will replicate is not sufficient to dictate the sites of initiation within each domain (Dimitrova and Gilbert, 1999). Origin sites are selected at a distinct point (origin decision point [ODP]) after the TDP, likely through a mechanism that selects a subset of pre-RCs as the preferred origin sites (Wu and Gilbert, 1996; Okuno et al., 2001). All of these events are independent of serum mitogens and are upstream of the restriction point and phosphorylation of retinoblastoma tumor suppressor protein (Rb), which leads rapidly to the execution of the replication program established during G1 phase (Wu and Gilbert, 1997; Wu et al., 1998; Keezer and Gilbert, 2002).

These results suggest that there are two distinct points during G1 phase when the number of chromosomal sites available for initiation of replication at the onset of S phase becomes increasingly restricted. The TDP restricts early replication events to euchromatic chromosomal domains, whereas the ODP selects which specific sites within those domains will ultimately be used as replication origins. To date, the TDP and ODP have been measured using very different methods, each of which has its limitations. The TDP was identified by monitoring the replication timing of whole chromosomal domains using methods that could not directly examine the distribution of replication origins (Dimitrova and Gilbert, 1999; Li et al., 2001). The ODP was defined by studies of a single group of replication origins located within the dihydrofolate reductase (DHFR) locus in CHO cells (Wu and Gilbert, 1996; Okuno et al., 2001). Hence, it remained to be determined whether the ODP is a global event that specifies all replication origins, only a subset of origins, or whether different origins are specified at different times during G1 phase. Here, we have used DNA fiber methodology to compare the spatial distribution of replication origins in cultured cells to that seen when nuclei from cells staged at various times during G1 phase are introduced into *Xenopus* egg extracts. Our results suggest that potential origins are broadly distributed throughout the genome in newly formed nuclei. At the TDP (1–2 h after mitosis), origins used in vitro become more clustered into groups that fire synchronously but still do not coincide with sites used in vivo. However, at the ODP (2–5.5 h after mitosis) there is a significant increase in the frequency with which in vitro and in vivo origins coincide. We conclude that the TDP and the ODP are discrete G1 phase steps that reduce the number of replication origins that have the potential to fire at the onset of S phase.

## Results

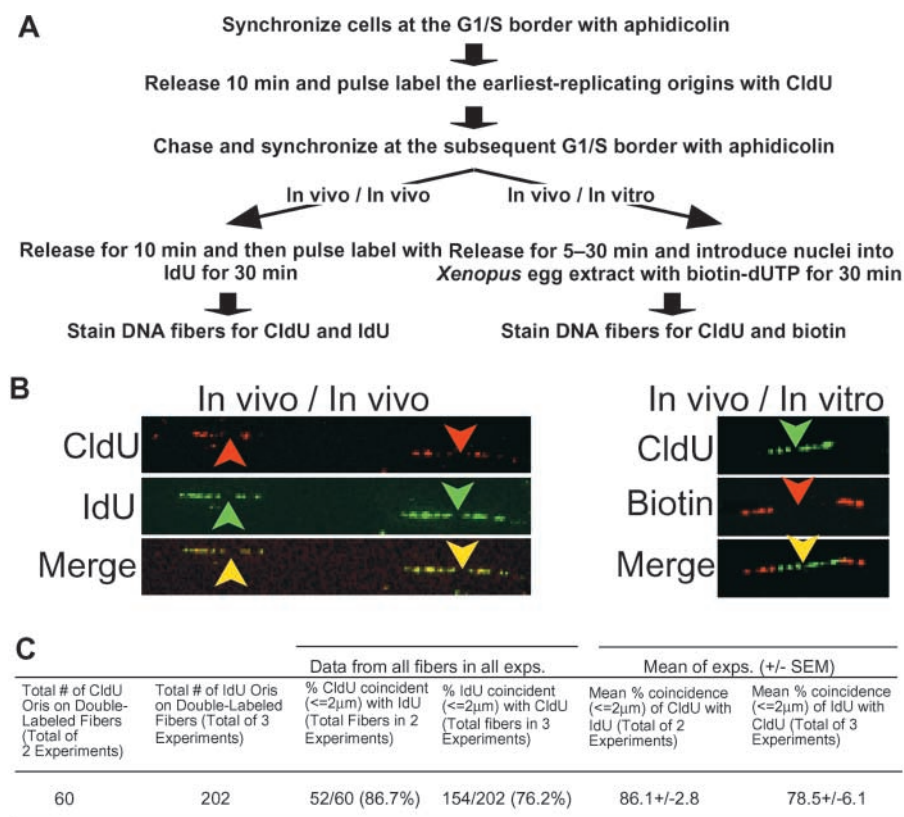
### Replication origins are specified at a discrete time during G1 phase

To examine origin specificity on individual DNA fibers, the population of earliest replicating origins were first labeled

with BrdU, and cells were then chased into the following cell cycle. When nuclei isolated from these cells at different times during G1 phase were introduced into *Xenopus* egg extracts, the specificity of in vitro initiation could be evaluated as the frequency with which the earliest initiation events in vitro coincide with BrdU-labeled origins. Critical to the interpretation of these experiments is the frequency with which the same origins are normally chosen in two consecutive cell cycles. To determine this frequency, cells in which early replicating origins were prelabeled with 5'-chloro-2'-deoxyuridine (CldU) were synchronized in mitosis and released in the presence of aphidicolin to accumulate cells at the G1/S border of the following cell cycle. These cells were then pulse labeled with 5'-iodo-2'-deoxyuridine (IdU) and chased for 6–13 h to allow complete replication of all DNA molecules. Stretched DNA fibers were prepared and the sites of CldU and IdU incorporation were revealed by staining the DNA fibers with differentially labeled fluorescent anti-IdU and anti-CldU antibodies (Fig. 1). Equal length tracks of labeled DNA separated by a small gap (the gap resulting from DNA replicated before metabolic labeling) were scored as origins of bidirectional replication (Fig. 1 B, left). Origins labeled with IdU and CldU were considered coincident if the centers of the corresponding gaps were within 2  $\mu\text{m}$  ( $\sim 5.2$  kb) of each other. The results (Fig. 1 C) revealed that among those DNA fibers that were labeled in both cell cycles, initiation sites coincided 70–90% of the time. These results are consistent with previous results using fiber methodology similar to that described here (Jackson and Pombo, 1998).

To confirm that the incubation of mammalian nuclei in *Xenopus* egg extracts does not alter the pattern of initiation sites established in vivo, cells prelabeled with CldU and arrested at the subsequent G1/S border with aphidicolin were released into S phase for various lengths of time and introduced into *Xenopus* egg extracts containing biotin-16-dUTP. In this case, pairs of biotin tracks could be seen emanating from the sites of CldU incorporation from the previous cell cycle 72% (31 out of 43 double labeled fibers) of the time (Fig. 1 B, right). These results demonstrate that replication forks assembled in vivo at the onset of the second cell cycle can be efficiently labeled with biotin in *Xenopus* egg extract and extend primarily from origins labeled in vivo in the previous cell cycle.

To determine whether the population of earliest replicating origins become specified at a particular time during G1 phase, cells with their earliest replication origins labeled with BrdU were synchronized in mitosis and collected either 1 (pre-TDP), 2 (pre-ODP/post-TDP), or 5.5 (post-ODP) h thereafter. Nuclei were prepared from these cells, and the earliest sequences to replicate in *Xenopus* egg extract were pulse labeled with biotin-16-dUTP. DNA fibers were stretched and double stained with fluorescent anti-BrdU antibodies and avidin. Unlike in Fig. 1, in this case biotin label was incorporated from the onset of DNA synthesis. Therefore, no gaps are expected within biotin-labeled tracks. Assuming bidirectional replication in vitro, the sites of initiation were defined as the center of each biotin track and were compared with the positions of the gaps at the center of BrdU tracks in double labeled fibers. Results (Fig. 2) revealed that, with nuclei prepared 1 or 2 h after mitosis, in



**Figure 1. Colocalization of origins used in two consecutive cell cycles.**

(A) Cells were synchronized at the G1/S border with aphidicolin, released for 10 min, and then pulse labeled with CldU for 10–60 min. Labeled cells were then synchronized in mitosis and blocked at the following G1/S. Aliquots were then released for 10 min and pulse labeled with IdU for 30 min and chased for 6–13 h. DNA fibers were prepared, and CldU and IdU were detected by indirect immunofluorescence (In vivo/In vivo). In parallel, aliquots were released for 5–30 min, and nuclei from these cells were introduced into *Xenopus* egg extracts containing biotin-16-dUTP. In vitro replication reactions were terminated at 30 min, and DNA fibers were stained for CldU and biotin (In vivo/In vitro). (B) Exemplary fibers for each of the methods described in A are shown. In these experiments, 1–5-μm gaps between similar length (1:1 to 1:2) stretches of label were considered to be origins. Based on the lengths of purified lambda DNA molecules, we estimate fibers to be ~2.6 kb/μm, which is in agreement with measurements made by others (Jackson and Pombo, 1998). Using this estimate, a 10-min in vivo pulse label highlights 10–20-kb stretches of DNA, in agreement

with the estimates of replication fork rates in mammalian cells (30 nt/s [Brown et al., 1987]). (C) Coincidence of origins labeled in two consecutive in vivo cell cycles (In vivo/In vivo). The distances between origins were measured as described in Materials and methods. Only double labeled (IdU and CldU) fibers were scored (note that most fibers were singly labeled). Origins were scored as coincident if the center of the gaps for CldU and IdU were within 2 μm (~5.2 kb) of each other. Shown are the percentage of CldU origins that were coincident with IdU origins (percentage of time that origins used in the first cell cycle were used in the second cell cycle) and the percentage of IdU origins that were coincident with CldU origins (percentage of time that origins used in the second cell cycle were used in the first cell cycle). Results of three independent experiments are shown (CldU coincident with IdU was scored in two of those three experiments) expressed as either the total data from all fibers in all three experiments or as the average coincidence for the three experiments and the variation between those experiments.

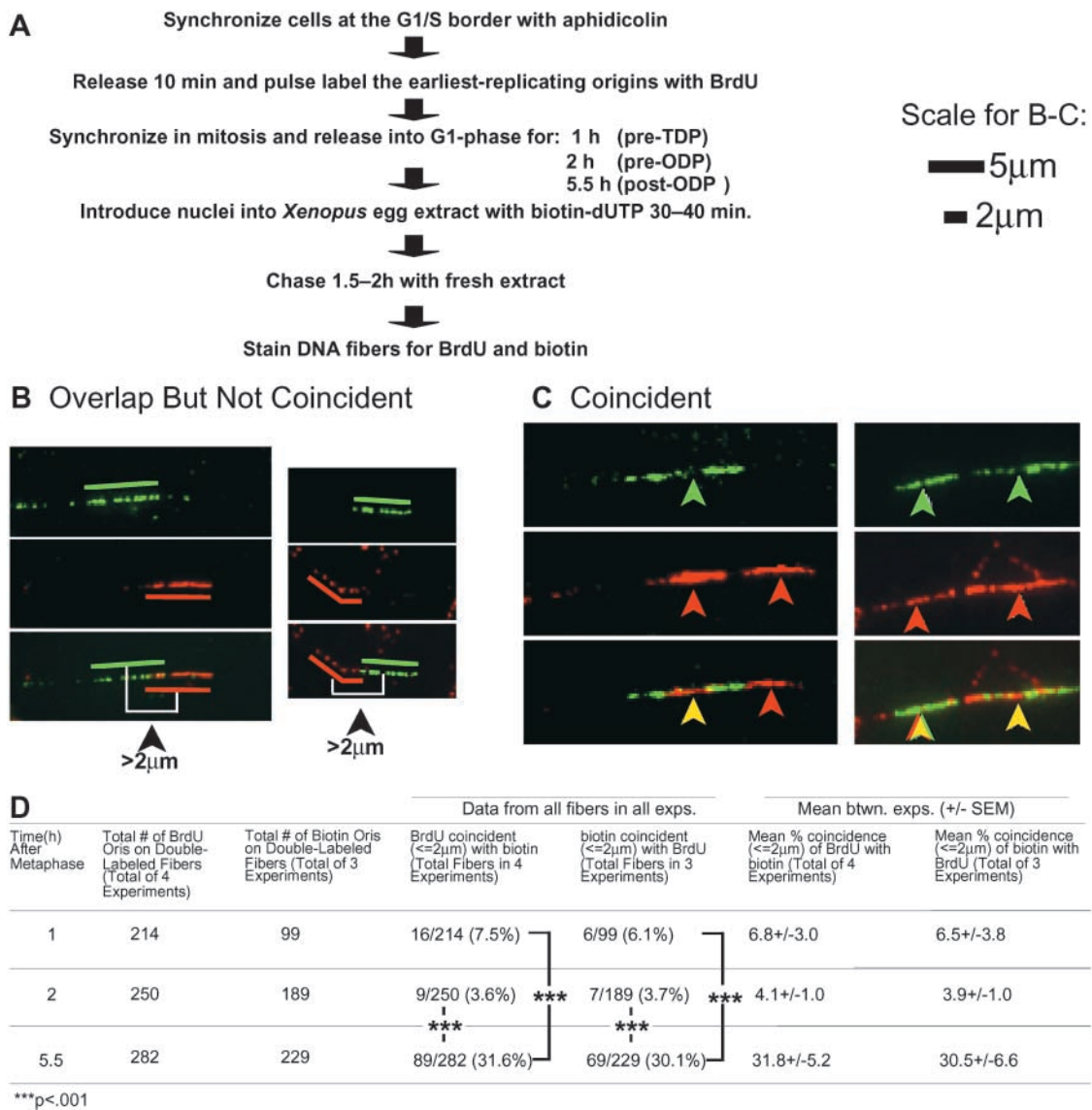
in vitro replication coincided with in vivo origins 4–7% of the time. However, when nuclei were prepared from cells at 5.5 h after mitosis, a significant rise in the frequency with which in vitro initiation events coincided with in vivo labeled origins (to >30%) was observed. These results provide the first evidence that origins other than those within the DHFR locus are specified at a distinct point during G1 phase.

We are not sure why the coincidence of biotin-labeled origins in vitro with the in vivo BrdU-labeled origins is lower than that for IdU-CldU origins labeled in two in vivo cell cycles (30–32% in vitro versus 76–87% in vivo). This could reflect a bonafide reduction of specificity in vitro relative to in vivo. Although the pattern of initiation at the DHFR origin locus is very similar in vitro versus in vivo (Gilbert et al., 1995), the resolution of these studies was not sufficient to rule out nearby but not coincident origins. Another possibility is that there is some bias against initiating in vitro replication within BrdU-substituted DNA. In fact, we note that if replication in vitro were to take place at random sites then there would be 16% chance for the center of a biotin track to fall within 2 μm of the center of a BrdU-labeled site (assuming an average interorigin distance of 25 μm; see Fig. 4), which is considerably higher than the coincidence observed for pre-ODP nuclei. A third possibility is that some origins

are specified even later during G1 phase. Unfortunately, this would be difficult to determine experimentally, since nuclei isolated more than 6 h after mitosis contain significant S phase contaminants (Wu and Gilbert, 1996). Regardless of the reasons why the coincidence is quantitatively less than expected, a substantial increase in coincidence is observed when cells pass through the ODP, to a level that is significantly higher than that expected from a random distribution ( $P < 0.05$ ; one-tailed test).

### The density of clustered origins is similar throughout G1 phase

One trivial explanation for the appearance of origin specification between 2 and 5.5 h after mitosis would be that *Xenopus* egg extract initiates replication at a higher localized density of sites within pre-ODP nuclei compared with post-ODP nuclei, which would reduce the percentage of coincidence but not the actual usage of in vivo origins. Previous experiments have demonstrated that the overall rate of DNA synthesis and replication fork extension are indistinguishable with pre- and post-ODP nuclei (Wu and Gilbert, 1996; Dimitrova and Gilbert, 1998; Okuno et al., 2001), suggesting that the same total number of origins are used within both pre- and post-ODP nuclei. However, these experi-

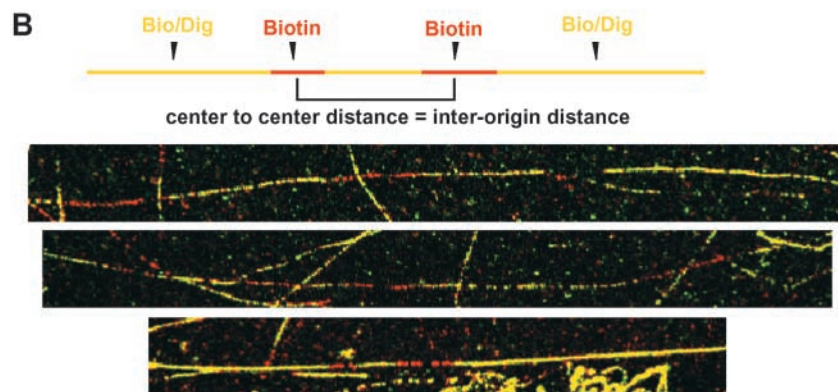
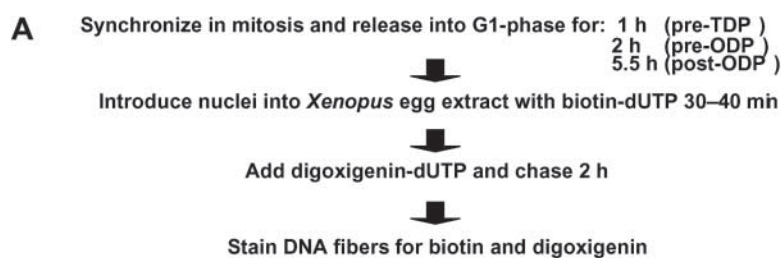


**Figure 2. Colocalization of origins selected for initiation in vitro with those selected in vivo.** (A) CHO 400 cells were synchronized at the G1/S border with aphidicolin and then released from the aphidicolin block for 10 min. Cells were then pulse labeled with BrdU for 20–30 min and synchronized in mitosis. At 1 (pre-TDP), 2 (post-TDP), or 5.5 (post-ODP) h after mitosis, cells were collected, and intact nuclei were introduced into *Xenopus* egg extract supplemented with biotin-dUTP. After 30 or 40 min, nuclei were transferred to fresh extract without biotin and further incubated for 1.5–2 h. DNA fibers were then stained for BrdU and biotin. Exemplary DNA fibers are shown that display either partial overlap between BrdU and biotin label that was not scored as coincident (B) or overlap that was scored as coincident (C; many fibers did not show any overlap). In C, the green arrowheads indicate BrdU-labeled origins, and red arrowheads indicate biotin-labeled origins. Yellow arrowheads indicate the overlap. Fibers do not always show yellow label. This may be due to the interference of antibody and avidin labeling on the same DNA sites or to recurrent binding of DNA fibers to the glass surface, which interferes locally with DNA denaturation and restricts the accessibility to epitopes (Pasero et al., 2002). (D) The percentage of BrdU origins that were coincident with biotin origins (percentage of time that origins used in vivo were used in vitro) and the percentage of biotin origins that were coincident with BrdU origins (percentage of time that origins used in vitro were used in vivo) was scored as in the legend to Fig. 1. Shown are the results of four independent experiments where data were collected independently by two different investigators in a double blind manner (coincidence of biotin with BrdU was only scored in three of those experiments). Data are presented as in the legend to Fig. 1 except that p-values are included to demonstrate the significance of the differences between results obtained with pre-ODP and post-ODP nuclei. Note that the increase in biotin origins on double labeled fibers is interpreted to reflect a clustering of origins at the TDP as shown in Fig. 5.

ments did not directly evaluate the localized density of replication origins. DNA fiber methodology described here provided an opportunity to measure interorigin distances directly under different experimental conditions.

In the experiments described above, independently labeled tracks were presumed to be on the same DNA fiber if they were oriented toward each other, well separated from

other labeled tracks, and within a distance of 50  $\mu$ m ( $\sim$ 130 kb). These experiments were designed to evaluate whether closely spaced in vitro and in vivo initiation sites were coincident. To measure origin spacing over larger distances, we performed sequential labeling with two different nucleotide analogs in the same round of replication, allowing the second label to paint the entire length of the DNA fibers (Fig. 3



**C**

Time (h) after mitosis	# of Biotin Labeled DNA Fibers		# of Biotin Labeled DNA Tracks		Tracks per Fiber (>=2)		Mean Center to Center Distance (kb)+/-SEM		Mean Length of Biotin Labeled DNA Tracks (kb)+/-SEM	
	30'	40'	30'	40'	30'	40'	30'	40'	30'	40'
1	26	43	56	102	2.2	2.4	59.8+/-7.5	76.4+/-8.3	19.8+/-1.6	23.4+/-1.6
2	26	27	73	64	2.8	2.4	40.3+/-3.4	57.0+/-4.7	15.1+/-0.8	21.0+/-1.8
5.5	26	26	69	61	2.6	2.4	57.2+/-7.3	75.9+/-7.0	16.6+/-2.1- ***	27.8+/-2.1

\*\*\*p<.001

**D**

Time (h) after mitosis	# of Biotin-Labeled DNA Fibers		# of Biotin Labeled DNA Tracks		Tracks per Fiber (>=2)		Mean Center to Center Distance (kb)+/-SEM		Mean Length of Biotin-Labeled DNA Tracks (kb)+/-SEM	
	+	-	+	-	+	-	+	-	+	-
1	50	13	120	29	2.6	2.3	76.2+/-7.3	66.0+/-8.3	22.3+/-1.3	27.3+/-2.3
2	34	27	76	60	2.1	2.3	55.6+/-4.2	54.3+/-5.2	21.0+/-1.6	23.4+/-1.8
5.5	32	22	75	53	2.3	2.4	73.3+/-6.0	71.5+/-6.5	27.3+/-1.8	21.6+/-1.8

\*\*\*p<.001

A). Nuclei isolated from cells at various times after mitosis were introduced into *Xenopus* egg extracts, and the earliest sequences to replicate were labeled briefly with biotinylated nucleotides. Digoxigenin-dUTP was then added to the in vitro replication reaction, and extracts were further incubated for 2 h in the presence of both biotin-dUTP and digoxigenin-dUTP. DNA fibers prepared from these samples were then stained for both biotin and digoxigenin. In this way, origins were labeled exclusively with biotin and appeared as red tracks, whereas the remainder of the DNA fibers were labeled with both biotin and digoxigenin and appeared as much longer yellow tracks (Fig. 3 B), facilitating our ability to trace individual fibers over large distances.

Results of these experiments (Fig. 3, C and D) revealed that the distances between multiple origins on the same DNA fiber were indistinguishable whether nuclei were isolated in the pre-TDP, pre-ODP, or post-ODP stages of G1 phase. Moreover, interorigin distances were much larger than previously observed with *Xenopus* sperm chromatin (Herrick et al., 2000; Blow et al., 2001) or naked DNA (Lu-

### Figure 3. Interorigin distances in vitro.

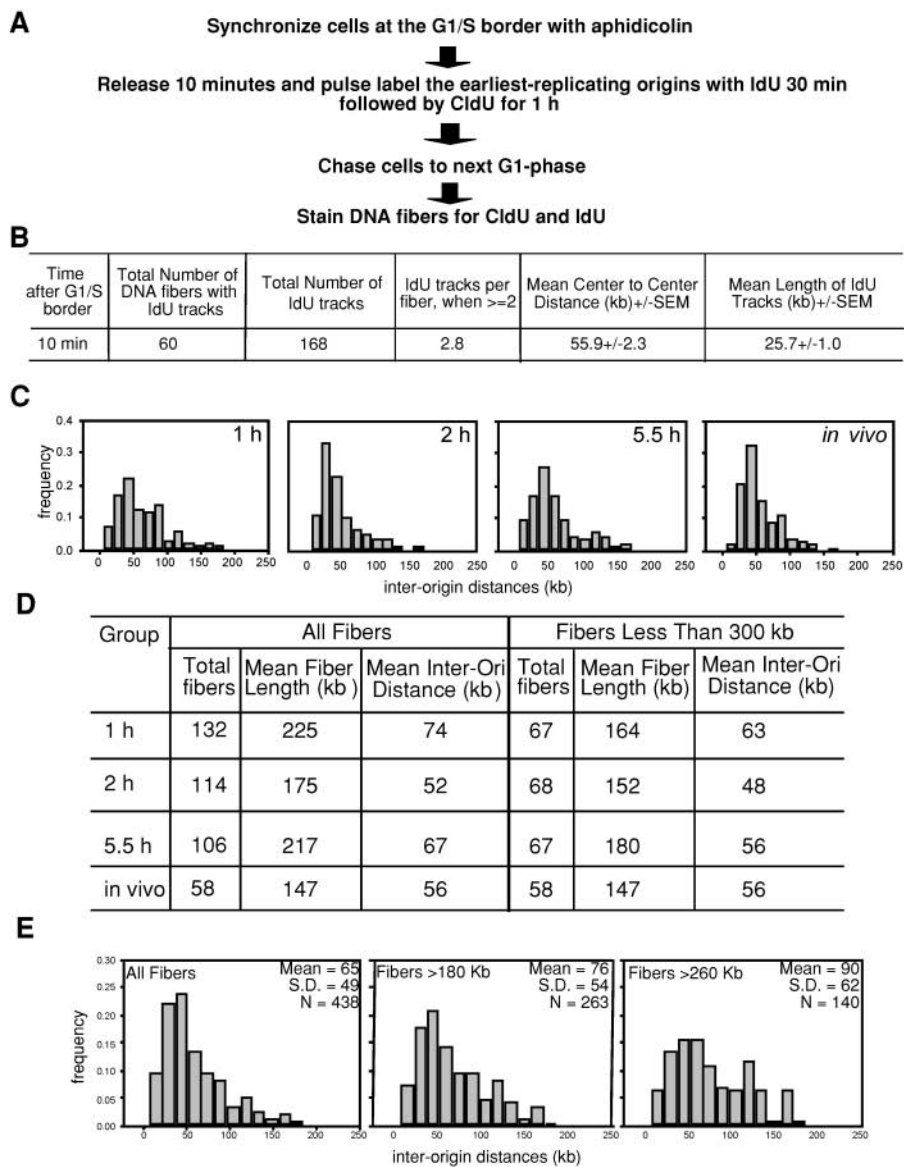
(A) Intact nuclei from cells synchronized at 1, 2, or 5.5 h after mitosis were introduced into *Xenopus* egg extract supplemented with biotin-dUTP for 30–40 min. Digoxigenin-dUTP was then added, and reactions were incubated for an additional 2 h. (B) Shown are three exemplary DNA fibers and a schematic diagram of how the interorigin distances were measured. (C) Images of DNA fibers were collected with a confocal microscope as indicated in Materials and methods, and both the lengths of the biotin labeled tracks, the number of tracks per fiber (on fibers containing more than one track), and the interorigin distances were scored. The differences in track lengths between 30 and 40 min are not generally statistically significant but are consistently longer for the 40-min labeling times, and the differences are consistent with prior measurements of the fork elongation rates with mammalian nuclei in *Xenopus* egg extracts (Dimitrova and Gilbert, 1998). (D) Same as C except nuclei were incubated in *Xenopus* egg extracts either in the presence (+) or absence (-) of a nondegradable derivative of geminin to inhibit pre-RC assembly in vitro. Biotin-dUTP labeling time was 40 min. For C and D, similar results were obtained in two independent experiments.

cas et al., 2000; Marheineke and Hyrien, 2001) as a substrate. To address the concern that replication forks emanating from closely spaced origins could have fused during the 30-min labeling period, which would bias the data toward larger interorigin distances, nuclei were labeled with biotin for either 30 or 40 min (Fig. 3 C). Although the length of biotin tracks increased by an amount consistent with our previous calculation of the average fork rate (4.5 nt/s or 2.7 kb in 10 min [Dimitrova and Gilbert, 1998]), interorigin distances were not affected, indicating that fusion of forks from closely spaced origins is not likely to be a major concern.

To confirm that the observed origin spacing is not influenced by the pre-RC assembly activities of the *Xenopus* egg cytosol, similar experiments were performed in the presence and absence of a nondegradable form of geminin, a potent inhibitor of pre-RC assembly in *Xenopus* egg extracts (Okuno et al., 2001; Tada et al., 2001; Dimitrova et al., 2002). As seen in Fig. 3 D, the presence of geminin had no effect on interorigin distances, confirming that the observed distribution of origins results from the initiation of replica-

**Figure 4. Interorigin distances in vivo.**

Cells were synchronized at the G1/S border with aphidicolin, and the earliest replicating origins were labeled with IdU as in the legends to Figs. 1–2. After a 30-min IdU labeling period, medium was changed to medium containing CldU, and cells were incubated an additional 1 h. To allow DNA fibers to complete replication, cells were allowed to proceed into the next G1 phase. DNA fibers were then stretched and stained for IdU and CldU as in the legend to Fig. 1. (B) Interorigin distances were measured as in the legend to Fig. 3. Shown are the average results from two independent experiments. (C) Histograms comparing the interorigin distances from all fibers analyzed in Figs. 3 and 4, grouping distances into bins of 15 kb starting from 5–20 kb. (D) Relationship of interorigin distances and total fiber lengths for each dataset. When all fibers longer than the longest fiber in the in vivo dataset (dataset with the shortest fibers) are removed from each dataset, interorigin distances come into even closer agreement than for the unadjusted dataset. (E) Data for all fibers was pooled and plotted in histograms that include all fibers, only fibers longer than 180 kb, or only fibers longer than 260 kb. Also shown are the means, SD, and total number of fibers (N) for each sampling.



tion at pre-RCs that were assembled in the mammalian nuclei before isolation.

**Interorigin distances are similar in vitro and in vivo**

To determine how closely these results match the distribution of origins when CHO cells initiate replication in vivo, cultured cells were labeled during the first 30 min of S phase with IdU (labeling  $\sim 50$  kb at 30 nts/s) and then chased for 1 h with CldU (labeling  $\sim 100$  kb/fork; Fig. 4 A). As can be seen from Fig. 4, B and C, interorigin distances in vivo did not differ significantly from those observed in *Xenopus* egg extracts and were similar to previous estimates in CHO cells (Laughlin and Taylor, 1979; Takebayashi et al., 2001). Furthermore, the histograms in Fig. 4 C reveal a much broader distribution of interorigin distances (from 10 to 185 kb, with an average of one origin every  $\sim 60$  kb) than has been observed previously with *Xenopus* sperm chromatin or naked DNA replicating in *Xenopus* egg extracts (evenly spaced every 5–15 kb [Herrick et al., 2000; Lucas et al., 2000; Blow et al., 2001]). The fact that the distribution of origins in vitro

closely matches that in vivo, is independent of the pre-RC assembly activities of the extract, and is significantly different from what is observed when *Xenopus* egg extracts initiate replication of other DNA templates, indicates that the distribution of origins in this system is largely determined by the nature of the chromatin template and/or the sites of pre-RC assembly within mammalian nuclei.

Considering the high concentration of replication factors in *Xenopus* egg extracts, this latter observation may come as somewhat of a surprise. In fact, in a typical in vitro replication reaction over half of the genome is synthesized in 2 h, more than twice the rate of in vivo genome replication (Dimitrova and Gilbert, 1998). Moreover, the fork elongation rate under these conditions is estimated to be 4.5 nt/s (Dimitrova and Gilbert, 1998), less than one-sixth the rate estimated in cultured mammalian cells ( $\sim 30$  nt/s [Brown et al., 1987]). Hence, more origins must be activated during replication in *Xenopus* egg extracts. Indeed, we routinely observed eight times as many biotin-labeled origins than BrdU-labeled origins in the double labeling experiments shown in Fig. 2, indi-

cating that there are more origins active at any one time in vitro versus in vivo even though the spacing between clusters of origins is the same. A simple explanation for these results is that the higher replication capacity of *Xenopus* egg extracts allows for the simultaneous initiation at groups of origins that would normally be activated sequentially over the course of a much longer period of time in vivo. This would also be consistent with previous results showing, when post-TDP nuclei are introduced into *Xenopus* egg extracts, spatial replication patterns proceed in the same temporal order but in a time frame that is compressed relative to that in vivo (Dimitrova and Gilbert, 1999; Li et al., 2001).

One concern with this type of analysis is that origins separated by distances greater than the mean length of observable DNA fibers would be under represented (Berezney et al., 2000). For example, it is clear from Fig. 4 D (left) that datasets with shorter DNA fibers had smaller interorigin distances. To determine the extent to which this affected our results, we performed two different tests. First, we removed all fibers that were longer than the longest fiber in the in vivo dataset (300 kb), which had the shortest mean fiber length (147 kb). In doing so, the mean interorigin distances between different samples became even more similar (Fig. 4 D, right), demonstrating that any differences between these samples can be largely accounted for by fiber length. Second, we pooled the data for all fibers, since the differences between these datasets were not significant, and replotted the data using all fibers, only fibers longer than 180 kb, or only fibers longer than 260 kb (Fig. 4 E). Restricting the dataset to increasingly longer fibers had a noticeable broadening affect on the interorigin distance histogram and significantly affected the variance. Hence, although the exact spacing of origins in these kinds of experiments can be significantly influenced by the underrepresentation of large interorigin distances, we conclude that when fiber sets of similar size ranges are compared the spacing between origins is similar in vitro and in vivo.

### Origins become clustered at the TDP

We have shown previously that *Xenopus* egg extracts initiate replication in pre-TDP nuclei at both early and late replicating sites, whereas in post-TDP nuclei replication initiates exclusively within the early replicating portions of the genome (Dimitrova and Gilbert, 1999; Li et al., 2001). This implies that the entire genome is available for initiation in pre-TDP nuclei, whereas a significant part of the genome is prevented from initiating early replication after the TDP. If this is correct and if the total number of origins that fire in vitro is the same with nuclei isolated at each time point, then one would predict origins to become clustered into a smaller number of DNA fibers at the TDP. Moreover, since BrdU-labeled fibers represent the earliest replicating segment of the genome, biotin-labeled origins should be found on BrdU-labeled fibers at a higher frequency after the TDP. In fact, the results shown in Fig. 2 D indicate an increase in the number of biotin-labeled origins on BrdU-labeled fibers after the TDP. Note that the results shown in Figs. 3 and 4, which reveal similar interorigin distances with pre- and post-TDP nuclei, are not in contradiction with these observations because they examine only those fibers that contain two or more origins

### A

Time (h) After Mitosis	Number of Biotin tracks per fiber	
	> 1	=1
1	75/235 (31.9%) ***	160/235 (68.1%)
2	116/202 (57.4%) ***	86/202 (42.6%) ***
5.5	110/196 (56.1%)	86/196 (43.9%)

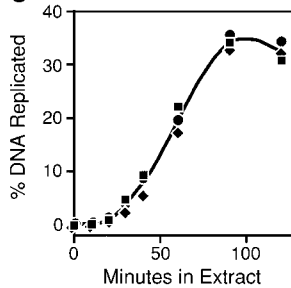
\*\*\*p<.001

### B

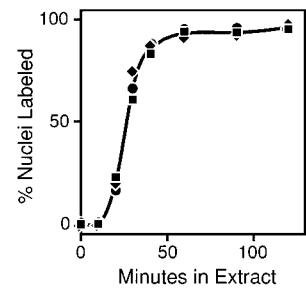
Time (h) After Mitosis	Ratio Biotin/BrdU Tracks	Ratio Fibers Labeled: BrdU Only / BrdU+Biotin
1	807/107 (7.52)	50/85 (59%) ***
2	955/128 (7.46)	19/56 (34%) ***
5.5	898/118 (7.61)	25/69 (36%)

\*\*\*p<.005

### C



### D



**Figure 5. A similar number of origins localize to fewer DNA fibers at the TDP.** (A) Using the fiber preparations collected in Fig. 3, the percentage of DNA fibers displaying only one versus more than one origin was determined. Results indicate a significant increase in the number of fibers containing two or more biotin tracks at the TDP, with no further increase at the ODP. (B) Using the fiber preparations collected in Fig. 2, the total number of biotin- and BrdU-labeled origins was scored for several fields of vision. For these same fields of vision, the number of fibers containing only BrdU tracks versus those containing both BrdU and biotin tracks was scored. Since preparations representing all three time points were derived from the same population of BrdU-labeled cells, these results indicate that a similar number of origins were used but that more origins were localized to early replicating DNA fibers after the TDP, with no further change at the ODP. (C) Aliquots of nuclei used for the experiments in Fig. 2, isolated from cells at 1 (squares), 2 (circles) or 5.5 (diamonds) h after mitosis, were introduced into *Xenopus* egg extracts supplemented with [ $\alpha$ - $^{32}$ P]dATP, and the percentage of input DNA replicated was determined at the indicated times by acid precipitation (e.g., 100% DNA synthesis would indicate replication of all genomic DNA). (D) In parallel, the same preparations of nuclei were incubated in extracts supplemented with biotin-dUTP, and the percentage of biotin-labeled nuclei was scored at the indicated time points. Given that the mean replication fork elongation rate is the same for each preparation (i.e., similar labeled track lengths; Fig. 3), the data in C and D provide independent support that the same number of replication origins are used with nuclei isolated at all three G1 phase time points.

and do not address the frequency with which individual origins are distributed amongst different DNA fibers.

To more directly examine whether post-TDP origins are clustered within a smaller number of DNA fibers, we determined the percentage of DNA fibers that harbored one ver-

sus more than one origin. These results (Fig. 5 A) revealed that with pre-TDP nuclei, the majority of fibers contain a single origin, whereas with post-TDP nuclei the majority of fibers contain two or more origins. To determine whether the same number of origins was used in each preparation, we examined the ratio of biotin-labeled origins to BrdU-labeled origins in each sample from Fig. 2. Since all three nuclear preparations were derived from the same population of BrdU-labeled cells, this ratio is directly proportional to the total number of origins used in vitro. Results (Fig. 5 B) revealed that this ratio was nearly identical for all three G1 phase time points. Furthermore, replication with all three nuclear preparations proceeded at the same overall rate (Fig. 5 C), with the same total number of nuclei participating in the in vitro replication (Fig. 5 D) and the same mean DNA track length or fork elongation rate (Fig. 3, C and D), providing independent confirmation that *Xenopus* egg cytosol initiates at a similar number of origins regardless of the time during G1 phase at which these nuclei are prepared. We conclude that a similar number of origins are clustered onto a smaller number of DNA fibers at the TDP and then redistributed from a more random to a more selected set of initiation sites at the ODP.

## Discussion

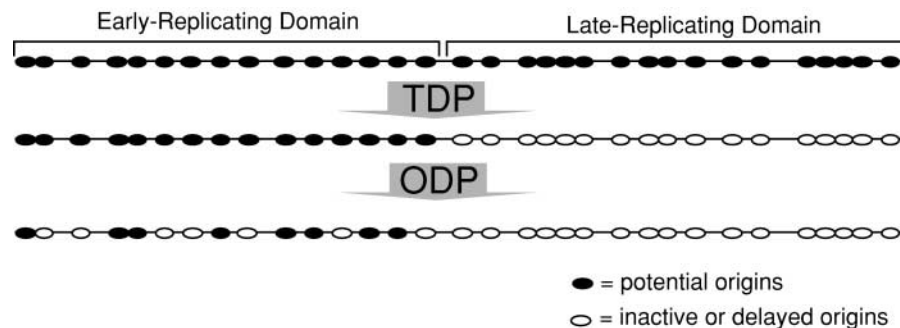
The results presented here demonstrate that the establishment of a replication timing program at the TDP and the specification of replication origins at the ODP are independent events, each of which occurs by a mechanism that restricts the pool of potential sites that can be used as replication origins at the onset of S phase (Fig. 6). Within the first hour after mitosis, many sites distributed broadly throughout the genome have the potential to serve as early replication origins. At the TDP, the pool of potential origins becomes restricted to only those within the early replicating subset of chromosomal domains, but there is not yet a preference for initiation at origins that are typically used at the onset of S phase. At the ODP, this pool becomes further restricted as specific sites from within the early replicating domains are designated for initiation at the onset of S phase. These results also provide the first evidence that the ODP is not unique to the DHFR origin locus but is a point at which many early replicating origins are specified, although further experimentation will be necessary to determine whether this

includes all origins. Extrapolation of the ODP beyond the DHFR origin underscores the importance of its role in the assembly of mammalian replication origins.

Previous results strongly suggest that the assembly of pre-RCs is not sufficient to establish either the replication timing program or the specification of replication origins, since the assembly of functional pre-RCs takes place during telophase (Okuno et al., 2001; Dimitrova et al., 2002). Hence, we favor a model in which pre-RCs form at many sites that initially share an equal potential of being selected as replication origins (Fig. 6). Subsequent events taking place at the TDP and ODP may potentiate some sites and/or inactivate others. This model predicts that there are significantly more pre-RCs assembled than there are origins, and several lines of evidence indicate that this is the case. In budding yeast, pre-RCs assemble on both active and inactive origins (Santocanale and Diffley, 1996), yet most origins are not used in every cell cycle (Lengronne et al., 2001; Poloumienko et al., 2001; Pasero et al., 2002). Replication origins in mammalian cells appear to be used even less efficiently than in yeast (Kalejta and Hamlin, 1996). Also, biochemical studies in *Xenopus* egg extracts demonstrate that there are ~10 times as many Mcm complexes assembled onto either *Xenopus* sperm chromatin or naked DNA than there are replication origins (Mahbubani et al., 1997; Edwards et al., 2002). In addition to the assembly of extraneous pre-RCs, additional plasticity in the choice of potential origin sites could exist if pre-RCs are repositioned during G1 phase. An important test of this model will be to directly determine the positions of pre-RCs at different times in G1 phase in relation to the positions of replication origins.

In any case, the available evidence suggests that the TDP and the ODP are replication regulatory steps that are distinct from each other and from other known G1 phase regulatory steps. In fact, site-specific origins in organisms such as *Xenopus* (Hyrien et al., 1995) and *Drosophila* (Sasaki et al., 1999) do not appear until after the midblastula transition, a time when transcription, cellular differentiation, and the appearance of a true G1 phase begins, demonstrating that the TDP and ODP are likely to be dispensable for regulating DNA replication itself. In mammalian cells, as well, the ODP can be abrogated without preventing entry into S phase (Wu et al., 1998), and both the temporal order of replication (Gilbert, 2002b) and origin specification (Zhou et al., 2002a,b) can change during development. It is tempting

**Figure 6. Model for the progressive restriction of initiation potential during G1 phase.** In early G1 phase, many sites distributed throughout the genome have an equal potential to be used as early replication origins. At the TDP, late replicating chromosomal domains become excluded from the pool of potential early replicating origins. At this time, origins within these early replicating domains still have an equal potential for initiation regardless of their position within the domain. At the ODP, a subset of these potential origins are chosen for initiation in the upcoming S phase.





to speculate that the TDP and ODP are important for aspects of DNA replication that transcend the basic need to duplicate the genome, such as coordinating replication with other chromosomal functions that vary during development (Gilbert, 2001a, 2002a,b).

## Materials and methods

### Cell culture and synchronization

CHO400 cells were grown in DME supplemented with 5% FBS. Cells were synchronized at the G1/S border by first arresting them in G1/Go with isoleucine-deficient medium (GIBCO BRL) and then releasing them in the presence of 5  $\mu\text{g}/\text{ml}$  aphidicolin (Calbiochem-Novabiochem) for 14 h as described (Wu and Gilbert, 1997). Cells were synchronized in mitosis by mitotic shake-off after a brief (4-h) incubation in 50 ng/ml nocodazole (Calbiochem-Novabiochem). Under these conditions, nearly 100% of cells complete nuclear membrane formation within 45 min (Wu et al., 1997; Okuno et al., 2001).

### DNA synthesis in *Xenopus* egg extracts

Intact nuclei were prepared by digitonin permeabilization (Wu et al., 1997; Dimitrova and Gilbert, 1998) and incubated in *Xenopus* egg extract prepared as described (Wu et al., 1997) at 10,000 nuclei/ $\mu\text{l}$ , which has been shown to be optimal for maintaining physiological patterns of replication (Dimitrova and Gilbert, 1998). Purified recombinant geminin<sup>DEL</sup> protein was used at a final concentration of 40 nM as described (Okuno et al., 2001). Measurement of total DNA synthesis by acid precipitation was done exactly as described (Wu et al., 1997). The percentage of nuclei labeled with biotin was determined as described (Wu et al., 1997) except that nuclei were labeled with 30  $\mu\text{M}$  biotin-16-dUTP instead of BrdUTP (methanol and denaturation steps omitted) and then stained for 30 min at RT with Texas red Streptavidin (Amersham Biosciences) diluted 1:200 in PBS/0.5% Tween 20. Coverslips were stained with 0.1  $\mu\text{g}/\text{ml}$  DAPI, washed twice in PBS, and mounted with Vectashield (Vector Laboratories).

### Labeling origins in two consecutive cell cycles

Cells arrested at the G1/S border were released for 10 min to allow recovery from the aphidicolin block (Gilbert et al., 1995) and then pulse labeled with 30  $\mu\text{g}/\text{ml}$  CldU (Sigma-Aldrich) for 10–60 min. Cells were then washed in medium containing 100  $\mu\text{M}$  thymidine (Sigma-Aldrich) and incubated in medium with 10  $\mu\text{M}$  thymidine for 8–9 h. Loosely attached cells were removed at this time, and thymidine-free medium with 50 ng/ml nocodazole was then added for 4 h before mitotic shake-off as described (Wu et al., 1997). To examine origins initiated in a second cell cycle in vivo, mitotic cells were plated in thymidine-free DME containing aphidicolin for 16–18 h to arrest them at the second G1/S border. Aliquots of these cells were released from aphidicolin for 10 min, pulse labeled with 30  $\mu\text{g}/\text{ml}$  IdU for 30 min, and then chased with thymidine as described above for 6–13 h. To examine the extension of in vivo-initiated replication forks by *Xenopus* egg extract, aliquots of these same cells were released from aphidicolin for 5–30 min, and intact nuclei were prepared by digitonin permeabilization (Wu et al., 1997; Dimitrova and Gilbert, 1998) and incubated for 30 min in *Xenopus* egg extract supplemented with 30  $\mu\text{M}$  biotin-16-dUTP (Roche). To compare sites of initiation in vivo to those in vitro, cells labeled at the G1/S border with BrdU, chased and subsequently synchronized in mitosis as described above, were plated into thymidine-free DME for 1, 2, or 5.5 h. Nuclei were prepared and incubated in *Xenopus* egg extracts containing biotin-16-dUTP for 30 or 40 min as described above. Under these conditions, replication initiates after a 10–20 min lag period (Gilbert et al., 1995). 30 min was determined empirically as the minimum labeling time to consistently observe clear tracks of biotin label at a reproducible density. Nuclei were then transferred to biotin-free extracts for 1.5–2 h to allow for completion of fiber replication.

### Double labeling origins in a single cell cycle

To double label origins in vivo, cells were synchronized at the G1/S border and labeled with IdU 30 min as described above except that instead of washing and chasing cells with thymidine, cells were chased with 30  $\mu\text{g}/\text{ml}$  CldU for 1 h. To double label origins in vitro, cells were synchronized in mitosis and released for 1, 2, or 5.5 h. Intact nuclei were incubated in *Xenopus* egg extract supplemented with biotin-16-dUTP for 30 or 40 min, and then 30  $\mu\text{M}$  digoxigenin-11-dUTP (Roche) was added and nuclei were further incubated in extract for 2 h.

### DNA fiber preparation

DNA fibers were stretched on glass microscope slides as described (Jackson and Pombo, 1998) with minor modifications. Nuclei containing labeled DNA were mixed with a fivefold excess of nuclei from unlabeled asynchronous cells to minimize confusion from too many labeled fibers (Jackson and Pombo, 1998). To avoid confusion due to the bundling of fibers (Berezney et al., 2000), slides were stained with 20  $\mu\text{M}$  YOYO-1 iodide (Molecular Probes), which allows direct visualization of DNA fibers, and areas with low density of DNA fibers could be marked with a diamond pencil. However, YOYO-1 staining is subsequently removed during later denaturation steps. With experience, informative areas of a slide could be identified without YOYO-1 staining based on the appearance of the dried DNA fibers on the slide. In either case, bundled fibers still must be carefully identified and avoided based upon the intensity of antibody staining, described below, always picking the area with a very low density of fibers, searching for straight fibers and avoiding areas with a lot of intersecting fibers. DNA fibers stretched with a combing machine were performed as described (Michalet et al., 1997) using silanized coverslips provided by A. Bensimon (Institut Pasteur, Paris, France) along with a combing machine purchased from A. Bensimon. Two of four experiments in Fig. 2 used the molecular combing method; all images of fibers shown in this report are images using the conventional stretching method. Although molecular combing produced DNA fibers that were straighter and more evenly separated than the stretching method, difficulties keeping sufficient numbers of denatured DNA fibers on coverslips that were not supplied by A. Bensimon precluded our ability to further exploit this method.

### Staining of stretched DNA fibers

Fibers substituted with halogenated deoxyuridine residues were denatured and washed as described (Jackson and Pombo, 1998). IdU was detected with mouse anti-BrdU (Becton Dickinson) and donkey anti-mouse IgG conjugated with Texas red or FITC (Jackson ImmunoResearch Laboratories). CldU was detected with rat anti-BrdU (Accurate) and donkey anti-rat IgG conjugated with FITC or Texas red (Jackson ImmunoResearch Laboratories). Simultaneous detection of biotin and digoxigenin were performed by six successive layers of antibody reactions as described by Herrick (2000) with the following modifications. The first layer consisted of Texas red avidin (Vector Laboratories) and sheep antidigoxigenin conjugated with fluorescein (Roche). The second layer was fluorescein rabbit anti-sheep IgG (Vector Laboratories). The third layer was biotinylated anti-avidin (Vector Laboratories) and mouse anti-rabbit IgG conjugated with FITC (Jackson ImmunoResearch Laboratories). The fourth layer was Texas red avidin, the fifth layer was biotinylated anti-avidin, and the sixth layer was Texas red avidin. For simultaneous CldU and biotin detection, the following five layers were used: (1) Texas red Avidin plus rat anti-BrdU; (2) goat anti-rat FITC (Jackson ImmunoResearch Laboratories) plus biotinylated anti-avidin; (3) Texas red Avidin; (4) biotinylated anti-avidin; and (5) Texas red Avidin. Simultaneous detection of BrdU and biotin was done similarly except that layer 1 used mouse anti-BrdU plus Texas red Avidin and layer 2 used goat anti-mouse IgG conjugated with FITC (Jackson ImmunoResearch Laboratories) plus biotinylated anti-avidin.

### Image collection and measurement

For measuring the coincidence of origins labeled in two different cell cycles, multiple images of labeled fibers were collected with a CCD camera, and measurements were made on the images with LaserPix software. Origins labeled in vivo after release from an aphidicolin block were defined as an obvious gap of 1–5  $\mu\text{m}$  between pairs of similar length (1:1 to 1:2) labeled DNA tracks, whereas origins labeled in vitro were assumed to be the center of biotin tracks. Segments <1  $\mu\text{m}$  and the fibers containing many unlabeled segments >2  $\mu\text{m}$  were excluded. Measurements of distances between origins and lengths of labeled tracks were performed using the LineProfile function of LaserSharp 2000 software after collecting images by confocal microscopy (MRC 1024ES; Bio-Rad Laboratories). These measurements were made in a double blind fashion by two investigators (F. Li and J. Chen) who independently labeled and exchanged slides without revealing the labeling system.

### Statistical analysis of data

Statistical significance was analyzed as described (Sokal and Rohlf, 1995). First, Model I analysis of variance (ANOVA) was applied. If significant differences were detected, we used the Tukey method to test which pairs of means are different from one another. The Kolmogorov-Smirnov test indicated that in general the data did not conform to the normal distribution, an assumption required for ANOVA. This was resolved after transformation of the data with a  $\log_{10}$  function. Histograms of center-to-center distances were

fit with the Poisson function. The parameters for the computed distributions were obtained from the sampled observations. Analysis and plotting were performed with software packages SigmaPlot and SigmaStat (SPSS Inc.).

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