Molecular Cell, Vol. 4, 983–993, December, 1999, Copyright ©1999 by Cell Press

The Spatial Position and Replication Timing of Chromosomal Domains Are Both Established in Early G1 Phase

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Summary

Mammalian chromosomal domains replicate at defined, developmentally regulated times during S phase. The positions of these domains in Chinese hamster nuclei were established within 1 hr after nuclear envelope formation and maintained thereafter. When G1 phase nuclei were incubated in *Xenopus* egg extracts, domains were replicated in the proper temporal order with nuclei isolated after spatial repositioning, but not with nuclei isolated prior to repositioning. Mcm2 was bound both to early- and late-replicating chromatin domains prior to this transition whereas specification of the dihydrofolate reductase replication origin took place several hours thereafter. These results identify an early G1 phase point at which replication timing is determined and demonstrate a provocative temporal coincidence between the establishment of nuclear position and replication timing.

Introduction

Chromosomal domains replicate in a defined spatiotemporal program. Each domain occupies a specific nuclear position and replicates at a precise time during S phase (reviewed in Berezney et al., 1999; Dimitrova and Gilbert, 1999). In general, Giemsa-light (R) bands, containing transcriptionally active chromosomal domains, replicate earlier, and Giemsa-dark (G) bands, comprising transcriptionally silent heterochromatin, replicate later in S phase (Gilbert, 1986; Hatton et al., 1988; Drouin et al., 1990; Manuelidis, 1990). Changes in replication timing accompany key stages of metazoan development (Holmquist, 1987; Hatton et al., 1988; Selig et al., 1992) and are often coupled to changes in gene expression (Dhar et al., 1989). Taken together, these results suggest that structural, transcriptional, and replicational domains share topographical boundaries and represent basic units of chromosome organization.

It is not known whether components of replication origins dictate when domains replicate, or whether epigenetic mechanisms regulating domain structure and function dictate when origins will be activated. Translocations and deletions can influence the replication timing of large chromosomal domains (Calza et al., 1984; Forrester et al., 1990). At some loci, the developmental switch in replication timing is accompanied by a change in origin usage (Ermakova et al., 1999) whereas other loci initiate replication at the same origin whether early or late replicating (Kitsberg et al., 1993). Even in the yeast *Saccharomyces cerevisiae*, where origins are well defined (Gilbert, 1998), the determinants of replication timing have proven complex. In at least one case, the determinant of replication timing appears to lie within the origin sequences themselves (Bousset and Diffley, 1998). In other cases, it is clearly mediated by *cis*-acting elements separate from the origins. Two types of chromosomal context effects have been found to confer late initiation: proximity to a telomere (Ferguson and Fangman, 1992) and association with specific segments of chromosomal DNA (Friedman et al., 1996). In the absence of these sequences, many late origins will fire early in S phase. For one late-initiating origin located near a telomere, the signal for late replication is established between metaphase (nocodazole arrest) and START (α factor arrest) (Raghuraman et al., 1997). Finally, the ability of telomeres to delay replication of another late-initiating origin requires SIR3 (Stevenson and Gottschling, 1999), a silent chromatin component, suggesting that late replication of some origins is influenced by chromatin structure.

We have developed a cell-free system that efficiently initiates replication within mammalian nuclei isolated at different stages of G1 phase. When intact late G1 phase Chinese hamster ovary (CHO) nuclei are introduced into *Xenopus* egg extracts, replication initiates at the physiologically relevant origins within the CHO dihydrofolate reductase (*DHFR*) locus (Dimitrova and Gilbert, 1998). DHFR origin recognition requires nuclei isolated from cells that have progressed beyond a distinct point during G1 phase, the origin decision point (ODP) (Wu and Gilbert, 1996). The ODP takes place after the initial binding of the pre–replication complex (pre-RC) Mcm proteins to chromatin (Dimitrova et al., 1999) and prior to the restriction point (Wu and Gilbert, 1997). The existence of an ODP was subsequently confirmed in cultured cells without the use of *Xenopus* egg extract (Wu et al., 1998; D. S. D., unpublished data), demonstrating that novel G1 phase regulatory events can be identified using this heterologous cell-free system.

Here, we investigated whether replication timing in mammalian cells is also established during G1 phase. To address this question, we developed methodology to differentially tag early- and late-replicating domains of CHO cell chromosomes. This allowed us to monitor both their spatial distribution within the nucleus and their potential to be distinguished as early- or late-replicating domains in *Xenopus* egg extracts. Our results revealed that the temporal program for replication is established in early G1 phase, after the initial binding of Mcm2 proteins to chromatin but prior to the ODP. Intriguingly, establishment of a replication timing program took place within the same time interval as the spatial repositioning of chromosomal domains within the nucleus.

Results

Characterization of Spatiotemporal Replication Patterns in CHOC 400 Cells

DNA replication takes place at discrete sites that can be visualized by pulse-labeling cells with 5-bromo-2′-deoxyuridine (BrdU) and staining nuclei with fluorescent

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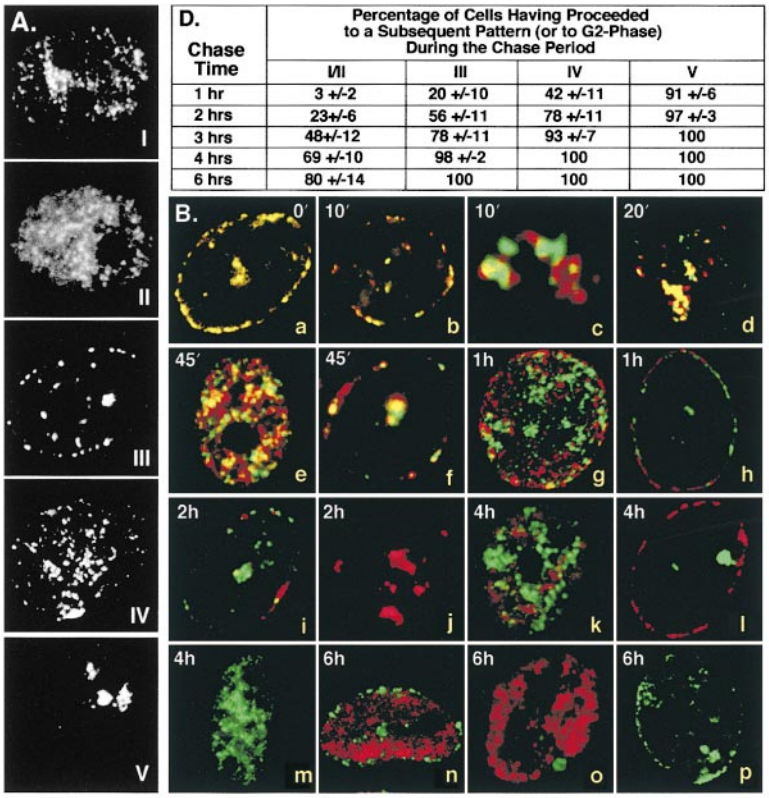


D.

Chase Time	Percentage of Cells Having Proceeded to a Subsequent Pattern (or to G2-Phase) During the Chase Period			
	I/II	III	IV	V
1 hr	3 +/-2	20 +/-10	42 +/-11	91 +/-6
2 hrs	23+/-6	56 +/-11	78 +/-11	97 +/-3
3 hrs	48+/-12	78 +/-11	93 +/-7	100
4 hrs	69 +/-10	98 +/-2	100	100
6 hrs	80 +/-14	100	100	100

C.

Chase Time	Percent of Labeled Nuclei Showing											
	100% Yellow Foci				Mixture of Red, Green and Yellow Foci				Complete Separation of Red and Green Foci			
	I/II	III	IV	V	I/II	III	IV	V	I/II	III	IV	V
0 min	100	100	100	100	0	0	0	0	0	0	0	0
10 min	96+/-2	85+/-10	54+/-10	49+/-13	4 +/-2	17+/-10	46+/-12	51+/-13	0	0	0	0
45 min	0	0	0	0	82+/-4	85+/-3	22+/-7	12 +/-5	18 +/-4	15 +/-3	78+/-7	88 +/-5
60 min	0	0	0	0	16 +/-5	16+/-6	9 +/-5	2 +/-2	84 +/-5	84 +/-6	91+/-5	98 +/-2

Figure 1. Spatiotemporal Patterns of DNA Replication in CHOC 400 Cells

(A) CHOC 400 cells were synchronized at the G1/S border and released into S phase. At various intervals thereafter, cells were pulse labeled for 5 min with BrdU and stained with anti-BrdU antibodies. Shown are examples of each labeling pattern (I–V).

(B) Ten asynchronous cultures of CHOC 400 cells were pulse labeled for 3 min with IdU. One of these cultures was labeled simultaneously with CldU (0′) whereas the rest were chased for the indicated times before they were pulse labeled with CldU. Sites of CldU (green) and IdU (red) incorporation were visualized using CldU- and IdU-specific antibodies. (c) is an expanded image of a single type V focus.

(C) The percentage of each type of nuclei (I/II–V) showing either complete colocalization (100% yellow foci), partial colocalization (mixture of red, green, and yellow foci), or little to no colocalization (complete separation of red, green, and yellow foci) was scored for chase periods less than 1 hr.

(D) For chase periods longer than 1 hr, the percentage of each type of IdU-labeled nuclei (I/II–V) that had proceeded to subsequent stages of S phase (or to G2 phase) during the chase was scored. In (C) and (D), the mean values are shown ±SEM for three independent experiments in which 100–200 nuclei each were scored.

(E) Schematic diagram approximating the duration of each replication pattern, derived from the independent approaches described in (A) (scoring the percentages of each pattern at various time intervals) and (D).

antibodies to BrdU (Berezney et al., 1999). We reasoned that a detailed characterization of these sites would provide the basis for an approach to rapidly evaluate the replication timing of whole groups of domains within individual nuclei replicating in our cell-free system. Figure 1A shows the spatiotemporal sequence of replication patterns in CHOC 400 cells. These patterns are designated types I-V, in accordance with previous nomenclature (O'Keefe et al., 1992). The type I pattern was observed only during the first few minutes of S phase, when the number of replication foci is relatively small. New foci emerge continuously, until there are several hundred foci throughout the internal, euchromatic regions of the nucleus, defining the type II pattern. Since type I is brief and is probably a direct precursor to the type II pattern, we refer to them collectively as type I/II. This pattern of labeling is observed throughout the first half of S phase (0–6 hr). At mid–S phase (6–9 hr), replication takes place almost exclusively at the nuclear periphery and nucleolar regions (type III). Late in S (9–12 hr), replication occurs first within several relatively large foci throughout the nucleus (type IV) and subsequently within a small number of large internal or peripheral foci (type V) that likely represent large stretches of tandemly repeated sequences, such as satellite DNA (Manuelidis, 1990; O'Keefe et al., 1992).

To confirm the distinct nature of these foci, we took advantage of the ability to distinguish DNA labeled with either 5-chloro-2′-deoxyuridine (CldU) or 5-iodo-2′-deoxyuridine (IdU) using specific antibodies (Aten et al., 1992). Exponentially growing CHOC 400 cells were pulse labeled with IdU, chased for different periods of time, pulse labeled with CldU, and then stained with red (for IdU) and green (for CldU) fluorochrome-conjugated antibodies (Figure 1B). When visualized through a dual red/green filter, foci that synthesized DNA throughout the pulse/chase/pulse period incorporated both IdU and CldU and appear yellow, IdU-labeled foci having completed replication during the chase label only in red, and foci that initiated replication during the chase label only in green. As expected, simultaneous IdU/CldU labeling revealed exclusively yellow foci (Figure 1Ba). After a 10 min chase, type I/II foci were still yellow, but separate red and green foci were evident in some type III (Figure

1Bb) and many type IV and V (Figure 1Bc) patterns, possibly reflecting a higher rate of replication fork movement during late S (Housman and Huberman, 1975). Increasing chase times led to an increase in the number of red- or green-labeled foci and a decrease in yellow foci (Figures 1Bd–1Bh and 1C). These results generally agree with previous reports concluding that early foci complete replication within approximately 60 min (Manders et al., 1996; Ma et al., 1998) but reveal considerable heterogeneity among individual foci and suggest that late foci may complete replication more rapidly.

Chase periods longer than 1 hr frequently revealed clearly demarcated foci of the same pattern type. Separate green and red type I/II patterns were observed after 1–6 hr chase periods (Figures 1Bg, 1Bk, and 1D). Separate type III patterns were observed in cell cultures chased for 1–3 hr (Figure 1Bh, 1Bi, and 1D). Separate type IV patterns were found after a 1 hr chase but rarely when chased for 2 hr, while type V patterns were largely completed within 1 hr (Figure 1D). The presence of foci labeled only with CldU within nuclei that still contain yellow foci (Figures 1Bb and 1Bd–1Bf) demonstrates that activation of new replication sites does not require the completion of all previous sites. Furthermore, the onset of a subsequent pattern could often be seen prior to the completion of the previous pattern. These results are consistent with the continuous activation of replication sites throughout S phase.

The length of the chase period sufficient for cells to switch from one type of replication pattern to subsequent patterns (Figure 1D) was consistent with the duration of each pattern (Figure 1E). Hence, nuclei were not observed to progress to the next pattern (type II to type III, Figure 1Bn) or more than one pattern ahead (type III to type V, Figure 1Bl; type II to type V, Figure 1Bo) until after the appropriate amount of time. Nuclei that were in G1 phase during the IdU label and in S phase during the CldU label stained only in green (Figures 1Bm and 1Bp), while nuclei that were in mid/late S phase during the IdU label and had moved out of S phase prior to the CldU label stained only in red (Figure 1Bj). These nuclei demonstrate the lack of cross-reactivity between the two antibodies. Together, these data provide a thorough description of the temporal sequence of replication focus activation in CHOC 400 cells (Figure 1E).

Replication in *Xenopus* Egg Extracts Respects the Proper Temporal Order

To determine whether replication in CHO nuclei by *Xenopus* egg cytosol proceeds according to the proper temporal program, exponentially growing CHOC 400 cells were pulse labeled with BrdU, and intact nuclei from these cells were then introduced into a *Xenopus* egg extract. At various times thereafter, the replication intermediates synthesized in vitro were briefly pulse labeled with biotin-dUTP, and nuclei were stained with both anti-BrdU antibodies (green) and Texas red–conjugated streptavidin (red; streptavidin specifically recognizes sites of biotin-dU incorporation). This protocol allowed us to distinguish cells in all phases of the cell cycle. Nuclei at various stages of S phase could be identified by their BrdU pattern. G1 phase nuclei could be identified by the appearance of biotin label after a 10–20 min lag period and the absence of BrdU label. G2 phase nuclei were not labeled with either nucleotide analog since intact G2 nuclei are not competent to replicate in *Xenopus* egg extract (Leno et al., 1992).

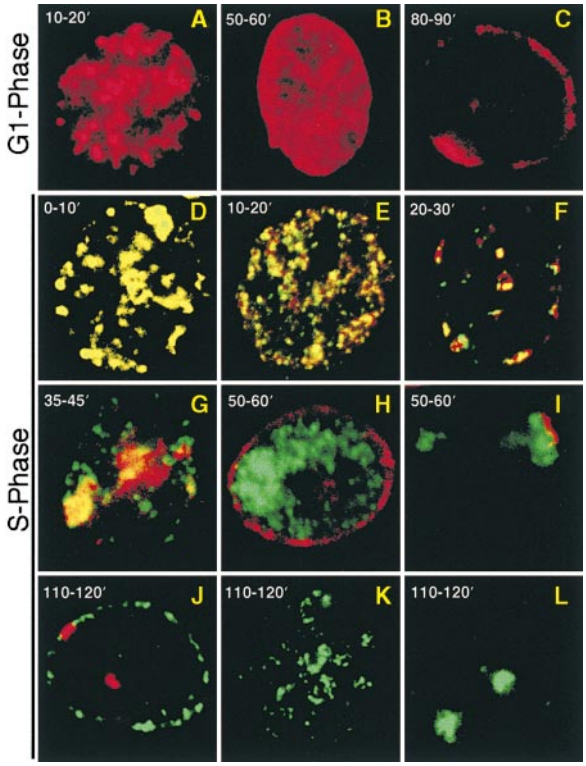


Figure 2. The Spatial Positioning and Temporal Order of Replication of Chromosomal Domains Are Maintained in *Xenopus* Egg Extracts

Exponentially growing CHOC 400 cells were pulse labeled for 20 min with BrdU before intact nuclei from these cells were prepared and introduced into a *Xenopus* egg extract. Aliquots of these nuclei were pulse labeled for 10 min with biotin-dUTP at the indicated time intervals in vitro. Nuclei were fixed and double stained for BrdU (green) and biotin (red).

Exemplary results from these experiments are shown in Figure 2. The earliest in vitro DNA synthesis in all G1 phase nuclei resembled type I/II patterns (Figure 2A). Nuclear staining became more diffuse and uniform later in the reaction (Figure 2B). Type III DNA synthesis (Figure 2C) began after 60 min, and by 2 hr, 50%–80% of G1 phase nuclei had progressed to the type III pattern. With S phase nuclei (BrdU-positive), in vitro biotin label colocalized with sites of in vivo DNA synthesis during the first 20 min (Figures 2D and 2E) and became increasingly separated from BrdU label thereafter (Figures 2F–2J). Nuclei labeled in a type I/II pattern in vivo progressed to type III in vitro (Figure 2H), type III progressed to type IV, and type IV progressed to type V (Figure 2G). Type IV and type V nuclei completed S phase in vitro (Figure 2I) and ceased to incorporate biotin label thereafter (Figures 2K and 2L). At late time points, some nuclei had progressed through more than one pattern (type III to type V, Figure 2J). No nuclei were observed to skip from early to late patterns without passing through the middle patterns, nor were any nuclei observed to repeat patterns already completed in vivo. As a control, aliquots of

these same nuclei were permeabilized under conditions that preserve replication efficiency (Dimitrova and Gilbert, 1998) prior to introduction into *Xenopus* egg extract. With these preparations, G2 phase nuclei were able to initiate replication, and S phase nuclei were observed to reinitiate earlier patterns already replicated in vivo (data not shown), consistent with the role of the nuclear membrane in preventing rereplication within one cell cycle (Leno et al., 1992). Importantly, replication patterns labeled in vivo, which reflect the relative positions of those labeled domains, remained intact throughout the duration of the in vitro reaction. Hence, with intact CHO nuclei as a substrate, *Xenopus* egg extract does not reposition chromosomal domains, and replication proceeds according to the proper temporal program.

Monitoring Early and Late Replication Domains throughout the Cell Cycle

To address whether the temporal order of replication is established at a specific point during G1 phase, we differentially tagged early- and late-replicating domains in vivo with CldU and IdU and prepared nuclei from these cells at various times during the following G1 phase. These nuclei were then introduced into *Xenopus* egg extracts and pulse labeled at various time points with biotin-dUTP. With this protocol, DNA synthesis taking place either early or late in vitro could be localized to either early- or late-replicating domains (Figure 3A). To verify that these manipulations do not disturb the replication program, several control experiments were performed. First, we verified that the chromosomal domains labeled at the onset of S phase in the first cell cycle are the earliest to replicate in the following S phase (Figure 3B). Cells synchronized at the G1/S border were pulse labeled with CldU (2–30 min pulses), collected in mitosis, and released in the presence of aphidicolin to accumulate cells at the G1/S border of the second cell cycle. These cells were then pulse labeled with IdU (2–30 min, respectively) and double stained for CldU (green) and IdU (red). The number of double-labeled type I foci increased with increasing pulse-labeling times, until foci began to resemble type II patterns, at which point there was nearly complete colocalization of the two labels. Shown is an example of a nucleus pulse labeled for 10 min in both cell cycles, exhibiting an intermediate level of colocalization typical for this pulse length (Figure 3B). We conclude that the same subset of early replication domains initiates within a period of approximately 30 min at the onset of each S phase, consistent with previous reports (Amaldi et al., 1973; Ma et al., 1998) and that this program is unperturbed by our protocol.

To verify that cells initiate at the appropriate origin sites after this complex synchronization protocol, cells were synchronized as in Figure 3A, and the sites of earliest DNA synthesis were mapped within the *DHFR* locus. Nascent replication forks within nuclei isolated at either the first or second G1/S boundaries were briefly labeled with [α-^{32}P]dATP. These short, pulse-labeled nascent strands were hybridized to probes derived from specific positions within the *DHFR* locus. The relative amounts of hybridization of these labeled strands were plotted versus the map position of each DNA segment, giving a distribution of the relative frequency at which replication initiated near each probe. Results (Figure 3C) were indistinguishable between one cell cycle and the next, demonstrating that our synchrony regime does not alter the pattern of initiation sites at the *DHFR* locus.

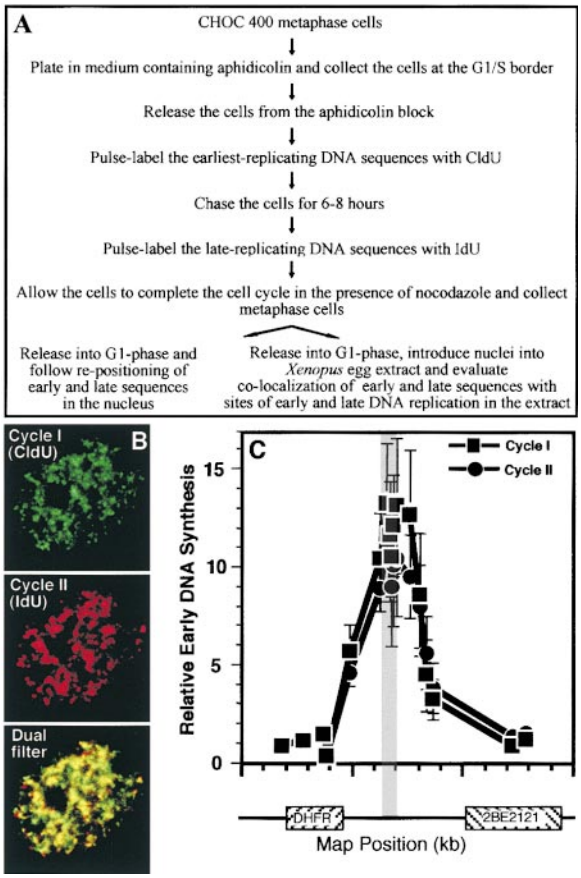


Figure 3. DNA Replication Initiates within the Same Replication Domains at the Beginning of Two Consecutive S Phases

(A) Pure populations of metaphase cells (95%–100%) were released for 12–14 hr into fresh medium containing aphidicolin to accumulate cells at the G1/S phase border. The earliest-replicating domains were labeled briefly with CldU; cells were then chased into late S phase and pulse labeled with IdU. Since cells do not remain completely synchronous throughout the chase period, individual cells incorporate IdU into either type III, type IV, or type V late-replicating patterns. These prelabeled cells were then resynchronized in the following mitosis and collected after different time intervals for use in the experiments described.

(B) Cells synchronized at the G1/S border as described above were pulse labeled for 10 min with CldU, collected in mitosis, and resynchronized at the following G1/S border by a second aphidicolin block. Cells were pulse labeled for 10 min with IdU, and the sites of CldU (green) and IdU (red) incorporation were visualized as in Figure 1B.

(C) Aliquots of cells synchronized at the G1/S border of either the first (squares) or the second (circles) cell cycle in the experiments described in (B) were used to analyze the distribution of early replication intermediates at the *DHFR* locus. Nascent DNA strands were labeled with [α-^{32}P]dATP, and the distribution of the radioactive replication intermediates was determined by hybridizing them to 17 unique DNA probes that encompass ~120 kb of the *DHFR* locus. The relative amounts of hybridization to each probe are plotted against the map position of each probe. The vertical shaded line highlights the positions of probes B–R, which encompass the region of peak DHFR initiation activity.

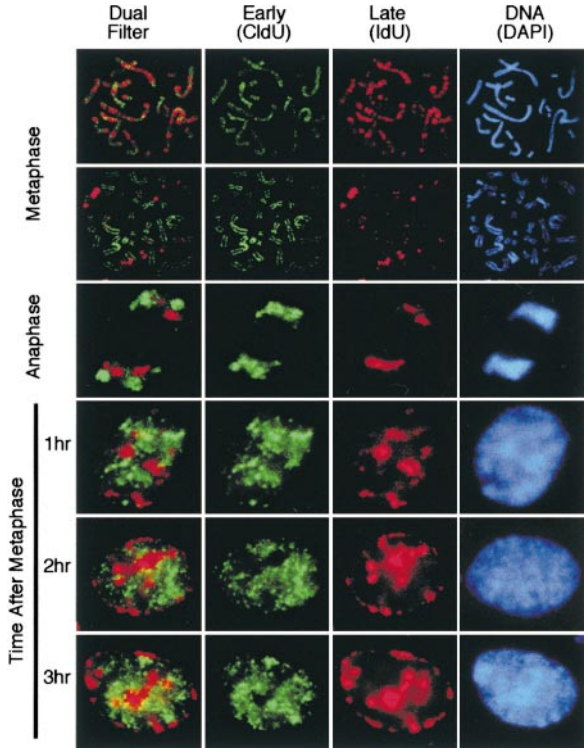


Figure 4. Nuclear Repositioning of Early- and Late-Replicating Chromosomal Domains Is Completed by 2 Hr Post Metaphase

Cells, prelabeled with CldU early in S phase and with IdU late in the same S phase as in Figure 3A, were synchronized in metaphase and released in the following G1 phase. Aliquots of cells were collected at various intervals thereafter and stained for CldU (green) and IdU (red) as in Figure 1B. DNA was stained with DAPI. Nearly 100% of nuclei from each time point gave results consistent with the images shown.

Replication Domains Form Stable Units that Are Repositioned in the Nucleus Early in G1 Phase

The spatial pattern of replication domains persists throughout multiple cell cycles (Sparvoli et al., 1994; Ferreira et al., 1997; Jackson and Pombo, 1998; Ma et al., 1998; Zink et al., 1998). To determine when replication domains are repositioned after mitosis, CHOC 400 cells were double labeled early and late in S phase with CldU and IdU as in Figure 3A, synchronized in metaphase, and released into G1 phase. Cells were then collected at various times after mitosis and stained with CldU- and IdU-specific antibodies (Figure 4). Metaphase chromosomes displayed a speckled/banded pattern of early- (green) and late-replicating (red) chromosomal domains, previously shown to correspond to R and G bands (Drouin et al., 1990; Sparvoli et al., 1994). Although type IV and type V domains could be easily identified at all cell cycle times, including metaphase (Figure 4, second row), it was difficult to determine when they reposition, since these domains persist as several large foci with no discriminating arrangement. However, type III foci provide a distinct marker for domain positioning, as they are almost exclusively perinuclear and perinucleolar. One hour after metaphase, type I/II and type III domains both exhibited a bundled appearance. At this time, only 3% of the total double-labeled nuclei displayed a typical type III pattern, although bundles of type III foci could be seen near the periphery in some nuclei. By 2 hr after metaphase, type I/II foci had lost their bundled appearance and had acquired the typical nucleolar exclusion pattern; type III patterns were now observed in 63% of double-labeled nuclei. The frequency of type III nuclei did not change at subsequent time points (3 hr, 66%; 4 hr, 56%; 6 hr, 66%; G1/S, 70%). We conclude that the general repositioning of replication domains takes place between 1 and 2 hr after metaphase.

The Temporal Order of Replication Is Established at the Time of Nuclear Repositioning

To determine whether replication timing is established at the ODP, CHOC 400 cells were double labeled as in Figure 3A, synchronized in mitosis, and released into G1 phase for either 2 hr (pre-ODP) or 6 hr (post-ODP). Intact nuclei were introduced into *Xenopus* egg extracts, pulse labeled with biotin-dUTP at various times, and then stained for biotin and either IdU or CldU. Results with both pre- and post-ODP nuclei were indistinguishable (pre-ODP shown in Figure 5A). The earliest sites of biotin label colocalized with early-replicating (CldU-labeled) domains and were excluded from late-replicating domains (IdU-labeled) (Figures 5A and 6E). Later in the in vitro reaction, the sites of DNA synthesis in most nuclei showed a striking colocalization with the type III IdU–labeled domains (Figures 5A and 6E), demonstrating that replication in these nuclei had redistributed from early- to late-replicating domains. Surprisingly, identical results were also obtained when aliquots of these nuclei were permeabilized (data not shown), which eliminates DHFR origin recognition within post-ODP nuclei (Figure 5B and Dimitrova and Gilbert [1998]). To verify that nuclei were in fact isolated from cells in the pre-ODP and post-ODP stages of G1 phase, we analyzed the in vitro sites of initiation of replication at the *DHFR* locus. As shown in Figure 5B, replication initiated at dispersed sites with intact pre-ODP nuclei or permeabilized post-ODP nuclei, and specifically within the *DHFR* origin locus with intact post-ODP nuclei. We conclude that the global replication timing program is established prior to, and is independent of, the specification of initiation sites within the *DHFR* locus.

To test whether replication timing might be a constitutive property of chromatin, metaphase chromosomes from prelabeled cells were introduced into *Xenopus* egg extracts, and the temporal order of replication was monitored as in Figure 5A. Sites of DNA synthesis in vitro were found to localize to both early and late replication domains at all times during the in vitro reaction (Figure 6E), indicating that the temporal order of replication was not maintained. One problem with the interpretation of these experiments is that in vitro replication of metaphase chromosomes requires assembly of a nuclear envelope mediated by *Xenopus* egg cytosol (Lawlis et al., 1996). Hence, it was possible that embryonic nuclear assembly factors had disrupted an existing temporal program. Alternatively, the replication timing program could be established between metaphase and 2 hr. To distinguish between these possibilities, we evaluated the temporal order of replication with nuclei prepared at 1 hr after metaphase, which have completed nuclear

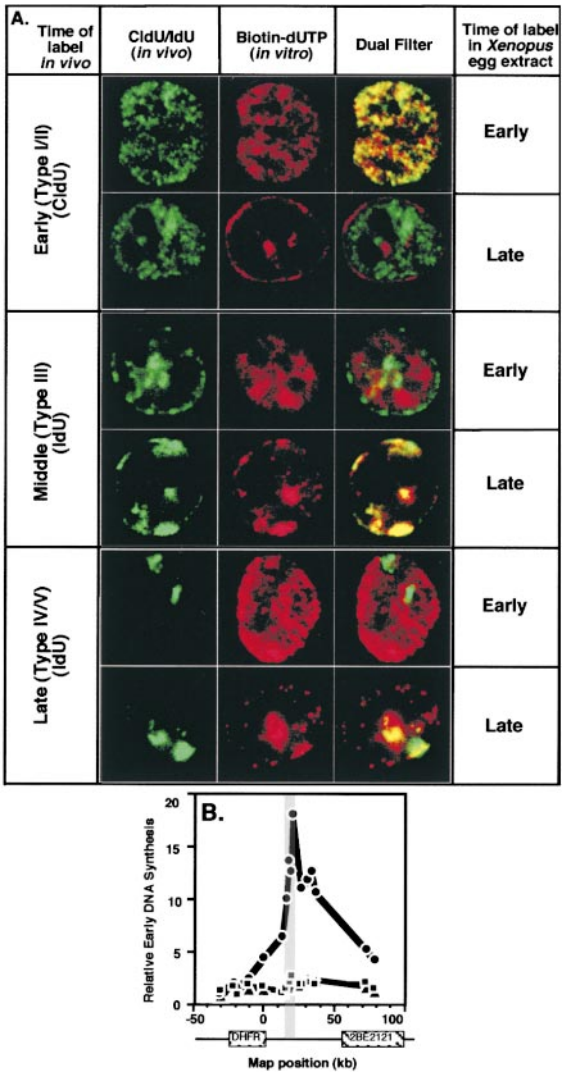
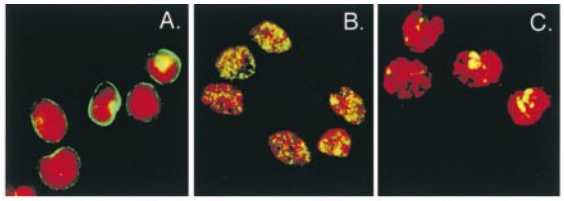


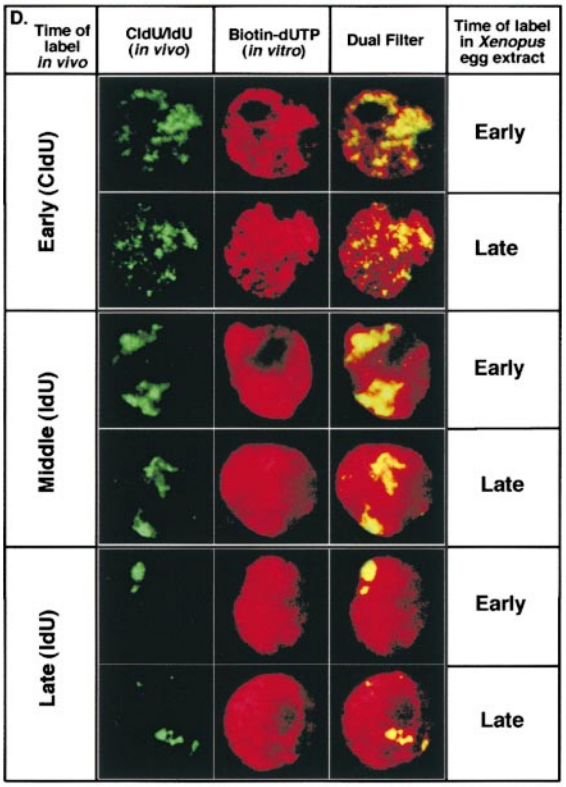
Figure 5. The Temporal Program for Replication in CHOC 400 Nuclei Is Established prior to the ODP

(A) Cells prelabeled early/late with CldU/IdU as in Figure 3A were synchronized in mitosis and released for 2 hr (pre-ODP) in the following G1 phase. Intact nuclei were introduced into *Xenopus* egg extract and pulse labeled with biotin-dUTP at various time intervals thereafter. Nuclei were then fixed and stained for biotin-dUTP (in vitro, red) and either CldU (early in vivo, green), or IdU (middle or late in vivo, green). Shown are examples of nuclei labeled either at 25–30 min (early) or at 115–120 min (late) in vitro. Similar results were obtained with intact nuclei prepared at 6 hr (post-ODP) after metaphase (Figure 6E) and with either 2 hr or 6 hr nuclei that had been permeabilized by exposure to higher concentrations of digitonin (not shown).

(B) Aliquots of intact 2 hr (square) or 6 hr (circle) nuclei from (A), as well as permeabilized 6 hr nuclei (triangle), were introduced into *Xenopus* egg extract, and the distribution of ^{32}P-labeled early replication intermediates at the *DHFR* locus was evaluated as described in the Experimental Procedures and plotted as in Figure 3C.

membrane formation (Wu and Gilbert, 1996; Dimitrova et al., 1999). Staining these nuclei with anti-lamin A/C and anti-lamin B antibodies revealed the presence of an assembled nuclear lamina in all nuclei (Figure 6A). Moreover, extraction with Triton X-100 and staining with anti-Mcm2 antibody revealed the tight association of





E.

Time After Metaphase That Nuclei Were Isolated	Time of *In Vitro* Biotin-dUTP Pulse	Percentage of Nuclei Displaying Colocalization (Yellow Foci)	
		With Early-Replicating Chromatin (CldU)	With Late-Replicating Chromatin (IdU)
0 hr and 1 hr	Early	78 +/- 6	73+/- 10
	Late	81 +/- 7	76 +/- 19
2 hr and 6 hr	Early	73 +/- 9	16 +/- 6
	Late	31 +/- 15	64 +/- 21

Figure 6. Replication Timing Is Established after Nuclear Envelope Assembly and the Binding of Mcm2 to Chromatin

(A–D) Cells prelabeled with CldU/IdU as in Figure 3A were synchronized in mitosis and released into G1 phase for 1 hr. (A) Cells were fixed with methanol and stained with anti–lamin B antibody (green). DNA (red) was counterstained with propidium iodide. (B and C) Cells were extracted with 0.5% Triton X-100, fixed with formaldehyde, and stained with an anti-Mcm2 antibody (red) and either (B) anti-CldU (green) or (C) anti-IdU (green) antibodies. (D) Intact nuclei were introduced into *Xenopus* egg extract, and the temporal order of replication was evaluated and displayed as in Figure 5A. Although domains are not repositioned, we have classified IdU label as middle or late based on the number and size of foci.

(E) The percentage of nuclei displaying significant colocalization of biotin label with either early- (CldU-tagged) or late- (IdU-tagged; types III–V) replicating chromatin at either early (first 60 min) or late (90–180 min) times during the reaction was scored. Results with metaphase chromatin (0 hr) and 1 hr nuclei were similar and were averaged together, as were the results with 2 and 6 hr nuclei. Shown are the mean values ± SEM for six independent experiments in which 100–200 nuclei were scored.

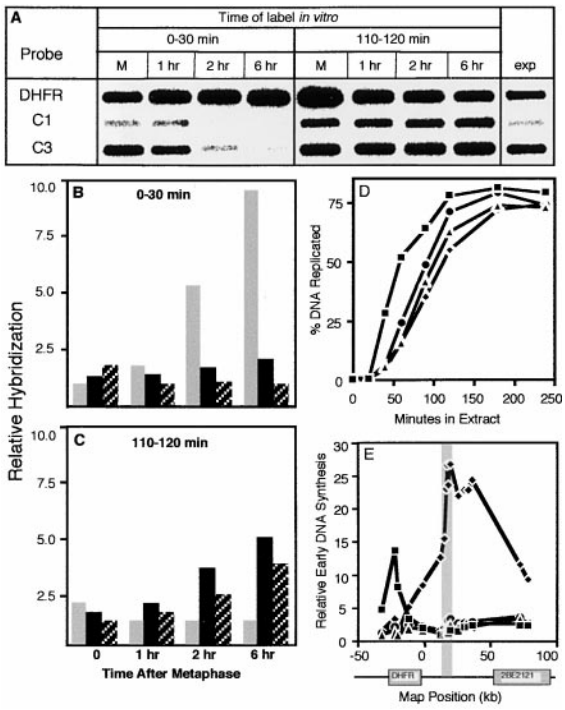


Figure 7. Hybridization of Nascent DNA Synthesized Early or Late In Vitro to Early- or Late-Replicating Hamster Sequences

(A) Metaphase chromatin (0 hr) and nuclei isolated at 1, 2, or 6 hr after metaphase were introduced into *Xenopus* egg extract. Replication intermediates were pulse labeled with [α-^{32}P]dATP either at the beginning (0–30 min) or at the second hour (110–120 min) of the in vitro reaction, and radiolabeled DNA was hybridized to early- (DHFR) and late-replicating (LINES C1 and C3) hamster DNA sequences.

(B and C) The relative cpm hybridized to the DHFR (gray bar), C1 (black bar), and C3 probes (hatched bar) with nascent DNA radiolabeled either early (B) or late (C) in vitro were detemined for each of the four types of G1 phase–staged nuclei.

(D) Aliquots of the same metaphase (closed quare), 1 hr (closed triangle), 2 hr (closed circle), or 6 hr (closed diamond) G1 nuclei were introduced into *Xenopus* egg extract supplemented with [α-^{32}P]dATP, and the percentage of input DNA replicated at the indicated times was determined.

(E) The same earliest ^{32}P-labeled replication intermediates from (A) (labeled 0–30 min without aphidicolin) were hybridized to the entire set of DHFR probes. Relative cpm from metaphase (closed square), 1 hr (closed triangle), 2 hr (closed circle), or 6 hr (closed diamond) G1 nuclei were plotted as in Figure 3C. For (A)–(E), similar results were obtained in two independent experiments.

Mcm2 proteins with both early- (CldU-labeled, Figure 6B) and late-replicating (IdU-labeled; Figure 6C) chromatin in all nuclei, consistent with previous results (Dimitrova et al., 1999). When these 1 hr nuclei were introduced into *Xenopus* egg extracts, replication initiated after the same lag period and proceeded at the same rate as 2 hr and 6 hr nuclei (exemplary results shown in Figure 7D). However, unlike 2 hr and 6 hr nuclei, early and late replication domains in 1 hr nuclei were replicated in no specific temporal sequence, similar to the results obtained with metaphase chromosomes as a substrate. At all times during the in vitro reaction, sites of DNA synthesis localized to both early and late replication domains and continued with a diffusely distributed pattern of foci throughout the duration of the in vitro reaction (Figures 6D and 6E).

To confirm these results using an independent method, replication intermediates synthesized in the extract were hybridized to specific hamster genomic sequences (Figure 7). The amplified *DHFR* genes initiate replication in early S phase and continue to replicate passively throughout much of S phase (Caddle and Heintz, 1990; Dijkwel and Hamlin, 1995). The long interspersed repeat sequences (LINES) C1 and C3 replicate during the second half of S phase (Holmquist and Caston, 1986). Metaphase chromosomes and nuclei prepared 1 hr, 2 hr, or 6 hr after metaphase were introduced into a *Xenopus* egg extract, and nascent DNA synthesized in vitro was pulse labeled with [α-^{32}P]dATP either at the onset or at the end of the second hour of the replication reaction. Radiolabeled DNA was isolated and hybridized to a probe from the *DHFR* locus and to segments of DNA containing the C1 and C3 repeats. The rate of replication (Figure 7D) and the specificity of initiation (Figure 7E) at the *DHFR* locus were evaluated for each nuclear preparation. Results revealed that both metaphase chromosomes and 1 hr nuclei showed no detectable preference for initiation within early-replicating sequences whereas both 2 hr and 6 hr nuclei initiated preferentially within early sequences (Figures 7A and 7B). Late in vitro, metaphase chromosomes and 1 hr nuclei still showed no significant preference for DNA synthesis within either early- or late-replicating sequences whereas both 2 hr and 6 hr nuclei were preferentially replicating DNA that hybridized to the late-replicating LINE sequences (Figures 7A and 7C). Hence, at 1 hr after metaphase—a time when the positions of sequences within the nucleus have not yet been reestablished—*Xenopus* egg cytosol cannot distinguish early- from late-replicating chromatin even when presented in the form of an intact nucleus. We conclude that the temporal order of replication of chromosomal domains is established between 1 and 2 hr after mitosis, coincident with the completion of chromosome domain positioning in the nucleus. We designate this novel cell cycle point as the replication timing decision point (TDP), to distinguish it from the previously described ODP.

Discussion

The observation that chromosome bands replicate at specific times during S phase was described more than two decades ago (Stubblefield, 1975). Since then, several investigators have catalogued the replication time of specific sequences, characterizing transitions between domains, and an overall correlation between transcriptional activity and replication timing. However, attempts to address the components of mammalian chromosomes that dictate replication timing have been largely unproductive (Gilbert, 1989). In this report, we show that our previously described cell-free replication system (Dimitrova and Gilbert, 1998) can recapitulate the proper replication timing of chromosomal domains. Using this system, we demonstrate that the establishment of temporally distinct replication domains is cell cycle dependent, taking place early in G1 phase. Interestingly, recent results in the yeast *S. cerevisiae* suggest that this early G1 phase establishment of replication timing may be evolutionarily conserved; the late replication program of

a telomeric origin was shown to be determined at some point between metaphase and START (Raghuraman et al., 1997). The ability to stage CHO cells at precise times in early G1 phase allowed us to demonstrate that replication timing is established after the binding of Mcm2 to chromatin and prior to specification of the *DHFR* origin locus, which is upstream of the restriction point. Replication timing was established during the same early G1 phase interval in which chromosomal domains were repositioned in the nucleus after mitosis.

A Cell-Free System to Study the Replication Timing of Mammalian Chromosomal Domains

Previous studies suggested that it might not be possible to study the spatiotemporal order of replication using *Xenopus* egg extracts. With *Xenopus* sperm nuclei as a substrate (Mills et al., 1989), discrete replication foci are evenly distributed throughout nuclei at all times during S phase. Experiments with *Drosophila* polytene (Sleeman et al., 1992), chicken erythrocyte (Leno and Laskey, 1991), or human fibroblast (Kill et al., 1991) nuclei observed exclusively type I/II patterns. These investigators concluded that *Xenopus* egg extracts remodel somatic cell nuclei, imposing upon them an embryonic replication pattern. We found no evidence of remodeling (Figures 2 and 5), even after prolonged incubation times. An important difference is that the prior studies employed nuclei prepared with detergents, which disrupts nuclear morphology and strongly inhibits DNA synthesis (Dimitrova and Gilbert, 1998). With nuclei prepared by controlled exposure of cells to digitonin, initiation of replication is rapid, synchronous (all nuclei initiate within 10–30 min), and efficient. In addition, previous reports employed much lower concentrations of nuclei, which can also disrupt replication patterns (Dimitrova and Gilbert, 1998). We conclude that the conditions for nuclear preparation and in vitro DNA replication described in this report preserve physiological properties of chromosomes, allowing replication to proceed according to the program specified in vivo.

Not all aspects of the in vivo replication pattern were recapitulated in *Xenopus* extracts. First, the total rate of genomic replication is accelerated in *Xenopus* egg extracts; nearly 80% of the genome is replicated within 2–3 hr (Figure 7D), and mid/late replication begins within 2 hr (Figures 2, 5, and 7). Since replication fork elongation rates in vitro are approximately one sixth of that observed in vivo (Dimitrova and Gilbert, 1998), the accelerated rate of genome duplication is likely due to increased origin activation. However, DHFR origin specificity is indistinguishable from the in vivo situation, suggesting that extracts do not activate origins that are not typically utilized in vivo. One possibility is that the entire set of type I/II foci (which take 6 hr to replicate in vivo) may be activated within 1 hr in vitro. Indeed, we observed a greater number of labeled foci with G1 phase nuclei in *Xenopus* egg extracts as compared to early S phase cells in culture (Figure 1 versus Figure 2B). A second difference is that G1 phase nuclei were rarely observed to progress to type IV or type V patterns de novo in the extract, and not more than 80% of the genome was replicated (Figure 7 and Dimitrova and Gilbert [1998]). Since type III S phase nuclei progressed to type IV and type V patterns in vitro (Figure 2), extracts are clearly capable of replicating these domains. Incomplete replication of G1 nuclei could result from exhaustion of extract components or the accumulation of a replication inhibitor(s) in the extract. Alternatively, it is possible that a mid–S phase transition may be important for replication of heterochromatin. In fact, remodeling of heterochromatin just prior to replication has been observed in cultured mammalian cells (Milner, 1969; Li et al., 1998). Further experiments will be required to distinguish these possibilities.

Relationship between Replication Timing and Nuclear Positioning

We demonstrate that nuclear envelope assembly, including a morphologically complete nuclear lamina and an intact nuclear membrane, is completed prior to the global repositioning of chromosomal domains. Consistent with these results, completed decondensation of chromonema fibers (Belmont and Bruce, 1994) and the peripheral localization of human chromosome 18 (J. Bridger and W. Bickmore, personal communication) require 2–3 hr after mitosis. Although we cannot determine whether each individual domain returns to precisely the same physical location within the nucleus, the overall distribution of these domains was preserved after mitosis, and the same domains initiated replication at the onset of two consecutive S phases.

Genetic evidence in *Drosophila* suggests that position effect variegation is correlated with a change in nuclear position of the variegated locus, mediated by the proximity to heterochromatin (Dernburg et al., 1996). In mammalian cells, silencing of certain genes during B and T cell development requires a protein (Ikaros) that appears to relocate these genes to centromeric heterochromatin (Brown et al., 1999). Our finding that the temporal order of replication is not established until sequences are repositioned within the nucleus provides a provocative coincidence between gene position, replication timing, and transcriptional function. Indeed, Ikaros-mediated gene silencing and repositioning is accompanied by a change in replication timing of those domains (K. E. Brown, M. Merkenschlager, and A. G. Fisher, personal communication). It is possible that replication timing is a consequence of nuclear position, which is itself a reflection of transcriptional potential. Accordingly, in *Xenopus* embryos there is no transcription and no evidence for a spatiotemporal order to replication, whereas in mouse embryos transcription begins at the end of the first cell cycle, and replication already follows a defined spatiotemporal pattern (Ferreira and Carmo-Fonseca, 1997). This model would imply that epigenetic regulation of chromosomal domains is dominant to the information contained within origin sequences themselves in controlling replication timing. Although we have analyzed only one replication origin, our finding that the overall replication timing program is maintained independently of conditions that alter origin specification (Figure 5) is consistent with this notion.

How could nuclear position dictate replication timing? Potentially, molecules recruited to the sites of specific chromosomal domains could establish microenvironments that modulate the ability of pre-RCs to compete

for limiting concentrations of initiation factors. For example, transcriptional silencing at *S. cerevisiae* telomeres requires a microenvironment containing high local concentrations of Sir3p that form foci at the nuclear periphery (Laroche et al., 1998), and nontelomeric loci can be silenced by anchorage to the nuclear periphery (Andrulis et al., 1998). Sir3p is also required for the late replication of at least one origin near the telomere (Stevenson and Gottschling, 1999), suggesting that the repressive chromatin structure created by this microenvironment delays replication. The in vitro system described here should allow us to test hypotheses about the molecules that establish replication timing differences.

Experimental Procedures

Cell Culture and Synchronization

CHOC 400 cells (CHO cell derivative containing ~1000 amplified copies of the *DHFR* gene) were maintained and synchronized as described (Gilbert et al., 1995; Dimitrova et al., 1999).

Labeling of Nascent DNA with Nucleotide Analogs

In Cultured Cells

Exponentially growing CHOC 400 cells were pulse labeled with 30 μg/ml BrdU (Sigma) for 2–30 min. For differential tagging of early- and late-replicating DNA domains, CHOC 400 cells synchronized at the G1/S border were released into S phase and labeled with CldU and IdU as described (Dimitrova et al., 1999).

In Xenopus *Egg Extracts*

Intact or permeabilized nuclei were prepared by digitonin treatment and introduced into interphase *Xenopus* egg extracts at a concentration of 10,000 nuclei/μl as described (Dimitrova and Gilbert, 1998). Aliquots were removed at different time points, biotin-11-dUTP (Sigma) was added to a final concentration of 50 μM, and reactions were further incubated for 5–10 min. Nuclei were fixed with 4% formaldehyde and transferred to polylysine-coated coverslips (Sigma) as described (Mills et al., 1989).

Rates and extent of genomic DNA replication were evaluated by measuring the amount of acid-precipitable [α-^{32}P]dATP as described (Dimitrova and Gilbert, 1998).

Immunofluorescent Microscopy

BrdU-substituted DNA was detected with mouse monoclonal anti-BrdU antibody (Becton Dickinson, #347580) followed by FITC-conjugated goat anti-mouse IgG (Jackson Labs, #115-095-146). The differential staining of DNA sites substituted with CldU or IdU was performed as described (Dimitrova et al., 1999). Biotin-11-dUTP incorporation was detected with Texas red–conjugated streptavidin (Amersham #RPN1233). Mcm2 proteins were detected as described (Dimitrova et al., 1999). The nuclear lamina was detected using monoclonal antibody LN43.2 (gift of C. Hutchison). DNA was stained with 0.1 μg/ml 4′,6-diamidino-2-phenylindole (DAPI) or 0.01 μg/ml propidium iodide (Sigma). All images shown were photographed directly from a Nikon Labophot-2 microscope with a 100× 1.4 NA oil immersion Nikon PlanApo objective through either single FITC and Texas red filters or a dual FITC + rhodamine fluorescence filter (#51004V2, F/R 712) as described (Dimitrova et al., 1999). Slides were scanned and figures assembled as described (Dimitrova et al., 1999). No computer merging was performed, and only standard brightness and contrast adjustments were made. All statistical data were collected using this microscope and filter sets. Confocal imaging (Dimitrova et al., 1999) of several selected images gave similar results.

Mapping Replication Origins by the Early Labeled Fragment Hybridization (ELFH) Assay

In Xenopus *Egg Extracts*

Specificity of initiation in the *DHFR* locus in pre- and post-ODP CHOC 400 nuclei was determined by the ELFH assay as described (Dimitrova and Gilbert, 1998). In brief, nuclei were introduced into a *Xenopus* egg extract supplemented with 100 μg/ml aphidicolin. Nuclei were then washed free of aphidicolin, and the earliest-replicating nascent DNA chains were labeled with [α-^{32}P]dATP. ^{32}P-labeled early replication intermediates were then hybridized to 17 unique probes distributed over a 120 kb region of the *DHFR* locus. Relative cpm were obtained by phosphorimaging analysis and adjusted for differences in probe size, deoxyadenine content, and hybridization efficiency by normalizing to the corresponding values for parallel hybridizations with labeled replication intermediates from exponentially growing cells.

In Cultured Cells

Radioactive labeling of the earliest synthesized nascent DNA strands was done as previously described (Gilbert et al., 1995), except that intact nuclei were prepared by digitonin permeabilization.

Replication Timing of Specific DNA Sequences in *Xenopus* Egg Extracts

5×10^5 digitonin-permeabilized metaphase or 1 hr, 2 hr, and 6 hr G1 phase cells were resuspended in *Xenopus* egg extract, and early or late replication intermediates were labeled with 100 μCi of [α-^{32}P]dATP during either the first 30 min or between 110 and 120 min of the in vitro reaction. Nuclei were washed, and labeled genomic DNA was purified as described (Gilbert et al., 1995). An equal number of cpm from each sample was hybridized to a panel of DNA plasmids (1 μg each) immobilized on nylon membranes. Probe R (Gilbert et al., 1995) from the *DHFR* locus was used as an early-replicating sequence, since it maps within the region of peak initiation activity. Late-replicating hamster DNA sequences (LINES C1 and C3) were a gift of G. Holmquist (Holmquist and Caston, 1986). Relative cpm were obtained by phosphorimaging analysis (Molecular Dynamics) and normalized to the corresponding values from parallel hybridizations with replication intermediates from exponentially growing CHOC 400 cells, labeled as described (Gilbert et al., 1995). This corrects for differences in probe size, deoxyadenine content, and hybridization efficiency. To express the relative preference for replication of early or late sequences (Figures 7B and 7C), results for each type of G1 phase–staged nuclei were normalized to the lowest value among the three probes.

Acknowledgments

We thank B. Knox for critical reading of the manuscript, K. Meiri and J. Schaefer for helpful discussions on the CldU/IdU staining procedure, I. Todorov for anti-Mcm2 antibody and C. J. Hutchison for anti-lamin antibody, and G. Holmquist for providing late-replicating DNA probes. This work was supported by March of Dimes grant #1-FY96-0610 and NIH grant #GM57233-01 to D. M. G.

Received March 8, 1999; revised September 17, 1999.

References

Amaldi, F., Buongiorno-Nardelli, M., Carnevali, F., Leoni, L., Mariotti, D., and Pomponi, M. (1973). Replicon origins in Chinese hamster cell DNA. II. Reproducibility. Exp. Cell Res. *80*, 79–87.

Andrulis, E.D., Neiman, A.M., Zappulla, D.C., and Sternglanz, R. (1998). Perinuclear localization of chromatin facilitates transcriptional silencing. Nature *394*, 592–595.

Aten, J.A., Bakker, P.J.M., Stap, J., Boschman, G.A., and Veenhof, C.H.N. (1992). DNA double labelling with IdUrd and CldUrd for spatial and temporal analysis of cell proliferation and DNA replication. Histochem. J. *24*, 251–259.

Belmont, A., and Bruce, K. (1994). Visualization of G1 chromosomes: a folded, twisted, supercoiled chromonema model of interphase chromatid structure. J. Cell Biol. *127*, 287–302.

Berezney, R., Dubey, D.D., and Huberman, J.A. (1999). Heterogeneity of eukaryotic replicons, replicon clusters, and replication foci. Chromosoma, in press.

Bousset, K., and Diffley, J.F. (1998). The Cdc7 protein kinase is required for origin firing during S phase. Genes Dev. *12*, 480–490.

Brown, K.E., Baxter, J., Graf, D., Merkenschlager, M., and Fisher,

A.G. (1999). Dynamic repositioning of genes in the nucleus of lymphocytes preparing for cell division. Mol. Cell. *3*, 207–217.

Caddle, M.S., and Heintz, N.H. (1990). The replication timing of the amplified dihydrofolate reductase genes in the Chinese hamster ovary cell line CHOC 400. Biochem. Biophys. Res. Commun. *170*, 134–139.

Calza, R.E., Eckardt, L.A., DelGiudice, T., and Schildkraut, C.L. (1984). Changes in gene position are accompanied by a change in time of replication. Cell *36*, 689–696.

Dernburg, A.F., Broman, K.W., Fung, J.C., Marshall, W.F., Philips, J., Agard, D.A., and Sedat, J.W. (1996). Perturbation of nuclear architecture by long-distance chromosome interactions. Cell *85*, 745–759.

Dhar, V., Skoultchi, A.I., and Schildkraut, C.L. (1989). Activation and repression of a β-globin gene in cell hybrids is accompanied by a shift in its temporal regulation. Mol. Cell. Biol. *9*, 3524–3532.

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

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

Dimitrova, D.S., and Gilbert, D.M. (1999). DNA replication and nuclear organization: prospects for a soluble in vitro system. Crit. Rev. Euk. Gene Exp. *9*, 353–361.

Dimitrova, D.S., Todorov, I.T., Melendy, T., and Gilbert, D.M. (1999). Mcm2, but not RPA, is a component of the mammalian early G1-phase pre-replication complex. J. Cell Biol. *146*, 709–722.

Drouin, R., Lemieux, N., and Richer, C.L. (1990). Analysis of DNA replication during S-phase by means of dynamic chromosome banding at high resolution. Chromosoma *99*, 273–280.

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

Ferguson, B.M., and Fangman, W.L. (1992). A position effect on the time of replication origin activation in yeast. Cell *68*, 333–339.

Ferreira, J., and Carmo-Fonseca, M. (1997). Genome replication in early mouse embryos follows a defined temporal and spatial order. J. Cell Sci. *110*, 889–897.

Ferreira, J., Paolella, G., Ramos, C., and Lamond, A.I. (1997). Spatial organization of large-scale chromatin domains in the nucleus: a magnified view of single chromosome territories. J. Cell Biol. *139*, 1597–1610.

Forrester, W.C., Epner, E., Driscoll, M.C., Enver, T., Brice, M., Papayannopoulou, T., and Groudine, M. (1990). A deletion of the human beta-globin locus activation region causes a major alteration in chromatin structure and replication across the entire beta-globin locus. Genes Dev. *4*, 1637–1649.

Friedman, K.L., Diller, J.D., Ferguson, B.M., Nyland, S.V., Brewer, B.J., and Fangman, W.L. (1996). Multiple determinants controlling activation of yeast replication origins late in S phase. Genes Dev. *10*, 1595–1607.

Gilbert, D.M. (1986). Temporal order of replication of *Xenopus laevis* 5S ribosomal RNA genes in somatic cells. Proc. Natl. Acad. Sci. USA *83*, 2924–2928.

Gilbert, D.M. (1989). Temporal order of DNA replication in eukaryotic cells: its relationship to gene expression. Ph.D. thesis, Stanford University, Stanford, California.

Gilbert, D.M. (1998). Replication origins in yeast vs. metazoa: separation of the haves and the have nots. Curr. Opin. Gen. Dev. *8*, 194–199.

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

Hatton, K.S., Dhar, V., Brown, E.H., Iqbal, M.A., Stuart, S., Didamo, V.T., and Schildkraut, C.L. (1988). Replication program of active and inactive multigene families in mammalian cells. Mol. Cell. Biol. *8*, 2149–2158.

Holmquist, G.P. (1987). Role of replication time in the control of tissue-specific gene expression. Am. J. Hum. Genet. *40*, 151–173.

Holmquist, G.P., and Caston, L.A. (1986). Replication time of interspersed repetitive DNA sequences in hamsters. Biochim. Biophys. Acta *868*, 164–177.

Housman, D., and Huberman, J.A. (1975). Changes in the rate of DNA replication fork movement during S phase in mammalian cells. J. Mol. Biol. *94*, 173–181.

Jackson, D.A., and Pombo, A. (1998). Replicon clusters are stable units of chromosome structure: evidence that nuclear organization contributes to the efficient activation and propagation of S phase in human cells. J. Cell Biol. *140*, 1285–1295.

Kill, I.R., Bridger, J.M., Campbell, K.H., Maldonado, C.G., and Hutchison, C.J. (1991). The timing of the formation and usage of replicase clusters in S-phase nuclei of human diploid fibroblasts. J. Cell Sci. *100*, 869–876.

Kitsberg, D., Selig, S., Keshet, J., and Cedar, H. (1993). Replication structure of the human β-globin gene domain. Nature *368*, 588–590.

Laroche, T., Martin, S.G., Gotta, M., Gorham, H.C., Pryde, F.E., Louis, E.J., and Gasser, S.M. (1998). Mutation of yeast Ku genes disrupts the subnuclear organization of telomeres. Curr. Biol. *8*, 653–656.

Lawlis, S.J., Keezer, S.M., Wu, J.-R., and Gilbert, D.M. (1996). Chromosome architecture can dictate site-specific initiation of DNA replication in *Xenopus* egg extracts. J. Cell Biol. *135*, 1–12.

Leno, G.H., and Laskey, R.A. (1991). The nuclear membrane determines the timing of DNA replication in *Xenopus* egg extracts. J. Cell Biol. *112*, 557–566.

Leno, G.H., Downes, C.S., and Laskey, R.A. (1992). The nuclear membrane prevents replication of human G2 nuclei but not G1 nuclei in *Xenopus* egg extract. Cell *69*, 151–158.

Li, G., Sudlow, G., and Belmont, A.S. (1998). Interphase cell cycle dynamics of a late-replicating, heterochromatic homogeneously staining region: precise choreography of condensation/decondensation and nuclear positioning. J. Cell Biol. *140*, 975–989.

Ma, H., Samarabandu, J., Devdhar, R.S., Acharya, R., Cheng, P., Meng, C., and Berezney, R. (1998). Spatial and temporal dynamics of DNA replication sites in mammalian cells. J. Cell Biol. *143*, 1415–1425.

Manders, E.M., Stap, J., Strackee, J., van Driel, R., and Aten, J.A. (1996). Dynamic behavior of DNA replication domains. Exp. Cell Res. *226*, 328–335.

Manuelidis, L. (1990). A view of interphase chromosomes. Science *250*, 1533–1540.

Mills, A.D., Blow, J.J., White, J.G., Amos, W.B., Wilcock, D., and Laskey, R.A. (1989). Replication occurs at discrete foci spaced throughout nuclei replicating in vitro. J. Cell Sci. *94*, 471–477.

Milner, G.R. (1969). Changes in chromatin structure during interphase in human normoblasts. Nature *221*, 71–72.

O'Keefe, R.T., Henderson, S.C., and Spector, D.L. (1992). Dynamic organization of DNA replication in mammalian cell nuclei—spatially and temporally defined replication of chromosome-specific alpha-satellite DNA sequences. J. Cell Biol. *116*, 1095–1110.

Raghuraman, M., Brewer, B., and Fangman, W. (1997). Cell cycle-dependent establishment of a late replication program. Science *276*, 806–809.

Selig, S., Okumura, K., Ward, D.C., and Cedar, H. (1992). Delineation of DNA replication time zones by fluorescence in situ hybridization. EMBO J. *11*, 1217–1225.

Sleeman, A.M., Leno, G.H., Mills, A.D., Fairman, M.P., and Laskey, R.A. (1992). Patterns of DNA replication in *Drosophila* polytene nuclei replicating in *Xenopus* egg and oocyte extracts. J. Cell Sci. *101*, 509–515.

Sparvoli, E., Levi, M., and Rossi, E. (1994). Replicon clusters may form structurally stable complexes of chromatin and chromosomes. J. Cell Sci. *107*, 3097–3103.

Stevenson, J.B., and Gottschling, D.E. (1999). Telomeric chromatin modulates replication timing near chromosome ends. Genes Dev. *13*, 146–151.

Stubblefield, E. (1975). Analysis of the replication pattern of Chinese hamster chromosomes using 5-bromodeoxyuridine suppression of 33258 Hoechst fluorescence. Chromosoma *53*, 209–221.

Wu, J.-R., and Gilbert, D.M. (1996). A distinct G1 step required to specify the Chinese hamster DHFR replication origin. Science *271*, 1270–1272.

Wu, J.-R., and Gilbert, D.M. (1997). The replication origin decision point is a mitogen independent, 2-aminopurine sensitve, G1-phase step that precedes restriction point control. Mol. Cell Biol. *17*, 4312–4321.

Wu, J.-R., Keezer, S., and Gilbert, D. (1998). Transformation abrogates an early G1-phase arrest point required for specification of the Chinese hamster DHFR replication origin. EMBO J. *17*, 1810–1818.

Zink, D., Cremer, T., Saffrich, R., Fischer, R., Trendelenburg, M.F., Ansorge, W., and Stelzer, E.H. (1998). Structure and dynamics of human interphase chromosome territories in vivo. Hum. Genet. *102*, 241–251.