The replication timing program of the Chinese hamster β-globin locus is established coincident with its repositioning near peripheral heterochromatin in early G1 phase

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We have examined the dynamics of nuclear repositioning and the establishment of a replication timing program for the actively transcribed dihydrofolate reductase (DHFR) locus and the silent β-globin gene locus in Chinese hamster ovary cells. The DHFR locus was internally localized and replicated early, whereas the β-globin locus was localized adjacent to the nuclear periphery and replicated during the middle of S phase, coincident with replication of peripheral heterochromatin. Nuclei were prepared from cells synchronized at various times during early G1 phase and stimulated to enter S phase by introduction into *Xenopus* egg extracts, and the timing of DHFR and β-globin replication was evaluated in vitro. With nuclei isolated 1 h after mitosis, neither locus was preferentially replicated before the other. However, with nuclei isolated 2 or 3 h after mitosis, there was a strong preference for replication of DHFR before β-globin. Measurements of the distance of DHFR and β-globin to the nuclear periphery revealed that the repositioning of the β-globin locus adjacent to peripheral heterochromatin also took place between 1 and 2 h after mitosis. These results suggest that the CHO β-globin locus acquires the replication timing program of peripheral heterochromatin upon association with the peripheral subnuclear compartment during early G1 phase.

Introduction

Mammalian chromosomes are organized into domains that replicate in a precise temporal order and reside in specific compartments of the nucleus (Ferreira et al., 1997; Jackson and Pombo, 1998; Ma et al., 1998; Wei et al., 1998; Dimitrova and Gilbert, 1999a,b). Several observations suggest a relationship between the positions of chromosome domains within the nucleus, their replication timing, and their transcriptional potential. Transcriptionally active euchromatic domains are distributed throughout the interior of the nucleus, excluding the nucleolar regions, and replicate in the first half of S phase. Constitutive heterochromatin is located at the nuclear periphery, in the vicinity of the nucleolus, and at several interior domains of the nucleus and is replicated late in S phase. An intriguing question concerns the subnuclear localization of facultative heterochromatin, which contains developmentally regulated genes that often replicate late in most cell types and early in cell types in which they are expressed (Gilbert, 1986; Brown et al., 1987; Dhar et al., 1988, 1989; Hatton et al., 1988; Selig et al., 1992). Recent studies in human and mouse cells have shown that the silencing of certain genes is accompanied by their association with centromeric heterochromatin (Brown et al., 1999; Francastel et al., 1999; Schubeler et al., 2000). Similarly, position effect variegation in *Drosophila* is accompanied by association of the variegated locus with a heterochromatic environment (Csink and Henikoff, 1996; Dernburg et al., 1996). These data suggest that transcriptional repression can be accomplished by relocating genes to heterochromatic late-replicating compartments of the nucleus.

To investigate the relationship between replication timing and subnuclear position, we have exploited the ability of *Xenopus* egg extracts to initiate replication within mammalian nuclei isolated from cells staged at any time during G1 phase. With nuclei isolated 1 h after mitosis, heterochromatic and euchromatic domains are replicated in this in

vitro system in no particular order. However, with nuclei isolated 2 h after mitosis, the overall temporal order for replication of these domains is preserved in vitro (Dimitrova and Gilbert, 1999b). This time period (1–2 h after mitosis), designated as the timing decision point (TDP),* is coincident with the spatial repositioning of chromosomal domains within the nucleus, providing a provocative temporal coincidence between replication timing and subnuclear position. These previous experiments monitored the general positions of whole populations of chromosomal domains but did not examine individual genes or their relationship to transcription. Here, we compare the developmentally regulated β-globin locus, which is transcriptionally silent and late-replicating in CHO cells (Taljanidisz et al., 1989), to the active and early-replicating dihydrofolate reductase (DHFR) gene locus. The β-globin locus is an excellent candidate for a locus silenced by a developmentally regulated replication timing switch. At both the human and the mouse β-globin locus, over 200 kb of DNA is early-replicating and DNaseI sensitive in erythroid cells, but late-replicating and DNaseI resistant in nonerythroid fibroblasts (Dhar et al., 1988; Epner et al., 1988). In mouse–human hybrids, general deacetylation and transcriptional silencing of the human β-globin locus is accompanied by its localization adjacent to murine centromeric heterochromatin (Schubeler et al., 2000).

An important question is whether the β-globin locus acquires the replication timing program of the heterochromatin domain that it juxtaposes, and whether this juxtaposition is required to delay its replication timing program. In this report, we demonstrate that the CHO β-globin locus is localized close to the periphery of the nucleus and replicated in the middle of S phase, coincident with the replication of peripheral heterochromatin. By contrast, the DHFR locus is more internally located and is replicated within the first 30 min of S phase. We further demonstrate that the differential replication timing program of these two loci is established 1–2 h after mitosis and that, during this same period of time, the β-globin locus is localized to the periphery of the nucleus. These results are consistent with a model (Gilbert, 2001; Heun et al., 2001) in which replication timing of at least some loci is determined by association with a heterochromatic subnuclear compartment.

Results

CHO β-globin genes are replicated in the middle of S phase, coincident with the replication of peripheral heterochromatin

To examine the replication timing of the CHO β-globin locus, we used FISH to measure the number of copies of a given DNA segment per cell. This method can be performed with small numbers of cells (required for experiments in *Xenopus* egg extracts described below) and is the only method that allows one to simultaneously evaluate nuclear position and replication timing. Before replication of a chromosomal segment, single hybridization spots are detected within in-

terphase nuclei. After replication, doublets can be seen. Earlier replicating probes will display a higher percentage of doublets than later replicating probes. By hybridizing simultaneously with two differentially labeled probes and comparing the ratio of singlets to doublets for each probe, one can determine the order in which these segments replicate. One caveat with this technique is that it does not measure replication timing per se, but measures the time after replication at which the two daughter strands of DNA have separated in space to the extent that they can be scored as individual hybridization spots. The degree to which two segments can separate likely depends on the placement of cohesion molecules along the length of the sister chromatids during DNA replication (Nasmyth et al., 2000). A second limitation is that, conventional fluorescence microscopy will only discern postreplicative sequences as two separate spots if they are sufficiently separated in the x–y plane of space, relative to the observer. Confocal analysis can resolve two overlapping spots that are separated in the z axis. However, it is not practical to perform confocal sectioning analysis with the large numbers of individual cells required to ensure statistical significance of replication timing data. Hence, some doublets go undetected. Finally, doublet signals can occasionally be detected even in G1 phase cells (Bickmore and Carothers, 1995; Smith and Higgs, 1999), indicating that a proper baseline must be established. Labeling S phase cells with BrdU and restricting the analysis to S phase cells can alleviate some of this latter concern (Selig et al., 1992; Kitsberg et al., 1993a,b; Bickmore and Carothers, 1995). Despite these caveats, with the appropriate precautions, doublet analysis has proven to be a very effective means to measure replication timing (Boggs and Chinault, 1994, 1997; Subramanian and Chinault, 1997).

An example of FISH results with differentially labeled DHFR and β-globin probes is shown in Fig. 1 A. This example shows a nucleus with replicated doublets for both DHFR alleles and unreplicated singlets for both β-globin alleles. To verify that these probes could effectively discern a difference in replication timing between the early-replicating DHFR and late-replicating β-globin loci, the number of nuclei showing doublets for one or both of each allele was scored and the data were expressed in three ways (Fig. 1, B–D). First, the total percentage of doublet and singlet alleles revealed more than twice as many doublets for the DHFR locus (Fig. 1B). One advantage of the FISH method is that each cell can be examined individually. In principle, doublets for a late-replicating locus should only be observed within nuclei that already contain doublets for an early-replicating locus. When the percentage of nuclei showing more doublet alleles for one locus versus the other (1:0, 2:0, and 2:1) were scored (Fig. 1 C), a 10-fold preference for nuclei with a majority of doublet DHFR loci was observed. This preference for DHFR doublets increased to ∼100-fold when the analysis was restricted to the ∼30% of S phase nuclei that displayed doublets for both alleles of one locus and singlets for both alleles of the other locus (Fig. 1 D). Although we cannot rule out the possibility that the β-globin locus replicated before the DHFR locus in a few cells, the rare exceptions likely reflect the frequency with which DHFR doublets are obscured from vision. These results

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*Abbreviations used in this paper: DHFR, dihydrofolate reductase; TDP, timing decision point.
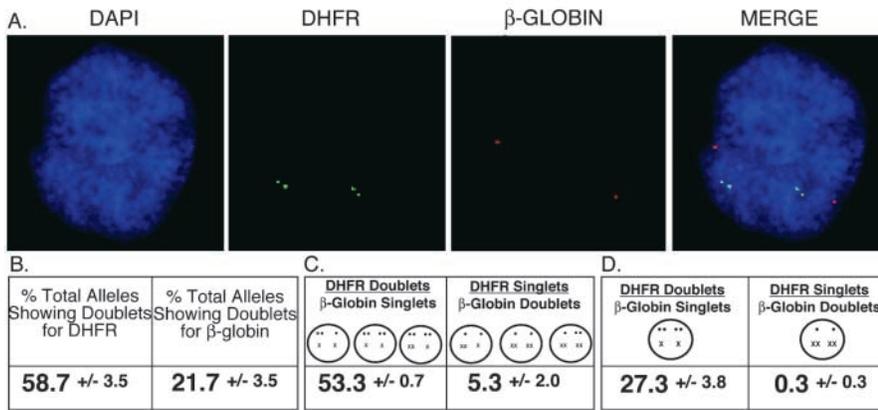
Figure 1. **Relative replication timing of DHFR and β-globin loci.** (A) Asynchronously growing CHO cells were pulse labeled with BrdU and hybridized in situ with digoxigenin-labeled DHFR cosmid cSc26 and biotin-labeled β-globin phage λJHC2. Sites of hybridization were visualized with FITC-conjugated antidigoxigenin antibodies (DHFR, green) and Texas red avidin (β-globin, red). BrdU label was detected with AMCA-labeled anti-BrdU antibodies (blue). In the image shown, BrdU foci are not visible because nuclei were additionally stained with DAPI (blue) to highlight the entire nucleus. (B–D) The number of DHFR and β-globin singlets and doublets per nucleus was evaluated. (B) The percentage of total alleles scored that displayed doublets for each locus was calculated. (C) The percentage of nuclei that displayed more doublets for one locus than the other was calculated. (D) The percentage of nuclei that showed doublets for both alleles of one locus and singlets for both alleles of the other locus was calculated. (C and D) Schematic representations of the arrangement of the DHFR (dot) and β-globin (x) alleles are included. The mean values ± SEM are shown for three independent experiments in which >100 BrdU-positive nuclei each were scored. All sets of data in this report include hybridizations in which both DHFR and β-globin probes were labeled with both biotin and digoxigenin and used in either combination, to control for any differences in labeling and detection of different nucleotide analogues. Only nuclei showing clear signals for both DHFR and β-globin loci were scored.

% Total Alleles Showing Doublets for DHFR	% Total Alleles Showing Doublets for β-globin
58.7 +/- 3.5	21.7 +/- 3.5

DHFR Doublets β-Globin Singlets	DHFR Singlets β-Globin Doublets
53.3 +/- 0.7	5.3 +/- 2.0

| DHFR Doublets β-Globin Singlets	DHFR Singlets β-Globin Doublets
27.3 +/- 3.8	0.3 +/- 0.3

agree with previous studies of replication timing in CHO cells (Taljanidisz et al., 1989) and verify the utility of FISH methodology as an assay for replication timing at these loci.

Previous studies of β-globin replication timing have measured the fraction of genomic DNA duplicated at the time of β-globin replication (Hatton et al., 1988; Taljanidisz et al., 1989; Cimbora et al., 2000), but did not distinguish whether inactive β-globin loci replicate during the euchromatic or heterochromatic spatial periods of replication. This can be addressed by examining the overall spatial pattern of DNA synthesis within nuclei at the time of β-globin replication. CHO AA8 cells were synchronized in mitosis by mechanical shake-off after a brief nocodozole block, and then accumulated at the G1/S border by plating them into medium containing aphidicolin. At 2-h intervals after release from aphidicolin, cells were pulse labeled with BrdU and stained with fluorescent antibodies to BrdU. Fig. 2 A shows examples of these spatial patterns during the progression of S phase in CHO AA8 cells. During the first 6 h of S phase, replication takes place within many foci distributed throughout the euchromatic regions of the nuclear interior. In keeping with previous characterization of these patterns in other CHO cell derivatives (O'Keefe et al., 1992; Dimitrova and Gilbert, 1999b), we designate these type I/II patterns. Several sets of type I/II foci are activated over the course of the first half of S phase, each completing DNA synthesis within ~60 min (Manders et al., 1996; Ma et al., 1998; Dimitrova and Gilbert, 1999b). In mid–S phase, replication takes place at the nuclear periphery and nucleolar regions (type III). In the last few hours of S phase, replication occurs first within several relatively large foci throughout the nucleus (type IV) and subsequently within a few very large internal or peripheral foci (type V). Fig. 2 B shows the percentage of cells displaying each pattern type at various time points after release from aphidicolin.

FISH analysis of asynchronously growing cells (Fig. 1 A) revealed that β-globin genes are located near the periphery of the nucleus, suggesting that they might replicate during type III DNA synthesis. To obtain more direct evidence for this notion, we first set out to determine whether β-globin doublets appear coincident with replication of the peripheral heterochromatin. One challenge to this analysis was that the conditions used to detect specific sequences by FISH also distort the patterns of BrdU labeling within the nucleus (Fig. 2 A). Hence, we began by correlating the appearance of doublets during S phase to the appearance of each pattern, determined independently. Aliquots of the same populations of synchronized cells used to monitor the patterns of DNA synthesis in Fig. 2 B were hybridized with β-globin and DHFR probes, and the percentage of alleles present as doublets for either the DHFR or β-globin loci was evaluated (Fig. 2 C). Before release from the aphidicolin block, ~10% of both DHFR and β-globin alleles scored as doublets, even though very little DNA is synthesized under these conditions. This provides a baseline of experimental error for subsequent analyses. Within 30 min after release into S phase, ~40% of the DHFR alleles were detected as doublets, whereas the β-globin locus did not show a significant number of nuclei with doublets until the middle of S phase. By 4.5 h after release from aphidicolin, 30.6 ± 9.5% of nuclei displayed type III patterns (Fig. 2 B), yet FISH analysis detected only 11 ± 3.5% (background levels) of alleles with doublets for β-globin (Fig. 2 C). By 6.5 h, 33 ± 2.6% of β-globin alleles were detected as doublets (Fig. 2 C) at a time when 49.2 ± 5% of nuclei still displayed middle-replicating patterns (Fig. 2 B). These data demonstrate that β-globin doublets appear after most cells have started to replicate peripheral heterochromatin, but before most cells have initiated late (type IV and V) DNA synthesis.

To more directly assess whether β-globin doublets appear during replication of the peripheral heterochromatin, the percentage of doublets for DHFR versus β-globin was scored as a function of the replication pattern during S phase in nuclei that were simultaneously stained for BrdU and subjected to FISH. To accomplish this, we had to be confident that the distorted replication patterns shown in Fig. 2
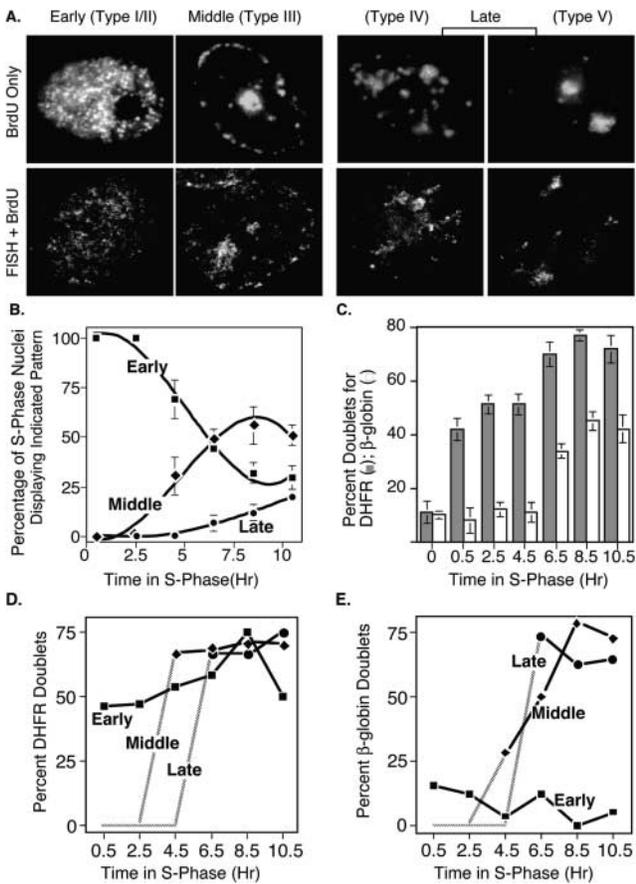
Figure 2. **The CHO β-globin locus is replicated during the stage of peripheral DNA synthesis.** (A) Examples of early (type I/II), middle (type III), and late (type and type V) replication patterns, revealed by pulse labeling CHO AA8 cells with BrdU and staining with fluorescent anti-BrdU antibodies. (Top) Shows cells fixed with ethanol and stained only for anti-BrdU, as described (Dimitrova and Gilbert, 1999b). (Bottom) Shows the pattern of anti-BrdU staining with cells that were first subjected to FISH. Conditions used for FISH distort the pattern of BrdU staining; however, it is still possible to distinguish the different pattern types. (B) CHO AA8 cells were synchronized in mitosis and released into medium containing aphidicolin to accumulate cells at the G1/S border. Cells were then released from the G1/S block, pulse labeled with BrdU at various times thereafter, and stained with anti-BrdU antibodies as in A (top). Shown are the percentage of BrdU-positive cells that exhibited early (■), middle (▲), or late (●) replication patterns at each time point. (C) Aliquots of the same cells from B were hybridized with β-globin and DHFR probes by FISH, and the percentage of doublet alleles for each locus was determined. Results in B and C show the means ± SEM (when >2) for three independent experiments in which >100 nuclei each were scored. (D and E) Cells synchronized as in B were subjected to FISH with either a DHFR (D) or β-globin (E) probe and subsequently stained with anti-BrdU antibodies as in A (bottom). The percentage of doublet alleles was scored as a function of the early (■), middle (▲), or late (●) BrdU patterns. The dashed lines for middle- and late-replication patterns indicate the time of appearance of these patterns during S phase (i.e., middle patterns first appeared between 2.5 and 4.5 h, and late patterns first appeared between 4.5 and 6.5 h).

A (FISH + BrdU) could be correctly categorized into early, middle, and late patterns. We defined early patterns as those displaying diffuse BrdU staining throughout the interior of the nucleus but noticeably absent BrdU staining at the nu-

clear periphery. Middle-replicating patterns were defined as nuclei with BrdU staining at the periphery and internal areas with significantly less BrdU staining. Late replication patterns displayed several large foci. Using these criteria, it was found that the percentage of nuclei displaying each pattern at various times during S phase was very similar to that shown in Fig. 2 B. Next, the percentage of either DHFR (Fig. 2 D) or β-globin (Fig. 2 E) alleles present as doublets within nuclei of each pattern was scored. As expected, DHFR doublets appeared in nuclei with early replication patterns within 30 min after the release from the aphidicolin block. Middle and late replication patterns were first observed at 4.5 and 6.5 h into S phase, respectively, and a high percentage of these nuclei harbored DHFR doublets at all time points after their appearance. By contrast, β-globin doublets were not observed above background levels in nuclei with early replication patterns at any time during S phase. Nuclei with middle-replicating patterns had an increasing percentage of β-globin doublet versus singlet alleles as cells proceeded through S phase, whereas nuclei with late-replicating patterns harbored a high percentage of doublet alleles from the onset of their appearance. Moreover, 73.3% of the β-globin doublets observed during mid–S phase were within 1 μm of the periphery, whereas only 22.2% of DHFR doublets were found within this zone (not shown). These data clearly demonstrate that β-globin doublets appear at the periphery during the middle replication pattern.

As discussed above, theoretically, the appearance of doublets is not necessarily coincident with replication. DHFR alleles were largely separated into distinct FISH signals within 30 min after the onset of S phase and so must have physically separated in less than 30 min. However, we can conclude that the β-globin loci are replicated during the middle S phase replication pattern only if we assume that the β-globin loci also separate shortly after replication. Moreover, with both the correlative method (Fig. 2 C) and the direct method (Fig. 2 D), there was an increase in the percentage of DHFR alleles displaying doublets as cells proceeded from early to middle S phase, coincident with the appearance of β-globin doublets. The simplest explanation for this increase is that chromosomal domains adjacent to the DHFR locus may replicate in mid–S phase, resulting in further separation of daughter DHFR strands and enhanced doublet resolution. However, it was formally possible that there is a general increase in the separation of daughter strands in mid–S phase, and that β-globin alleles are actually replicated earlier but take a longer time to separate than do DHFR alleles. To address this concern, we evaluated the colocalization of β-globin and DHFR doublets with BrdU label. Colocalization of FISH and BrdU signals is complicated by the highly variable signal-to-noise ratios obtained with FISH and the variable intensity of BrdU signal at different sites within the nucleus. However, computer-assisted colocalization analysis using a confocal microscope allowed us to identify alleles showing significant colocalization of the FISH signal with BrdU signal (Fig. 3). As the analysis of individual nuclei by this method was laborious, we concentrated on doublets within type III or IV nuclei. Within experimental error, yellow doublets represent alleles that have both replicated and separated within the BrdU pulse label-
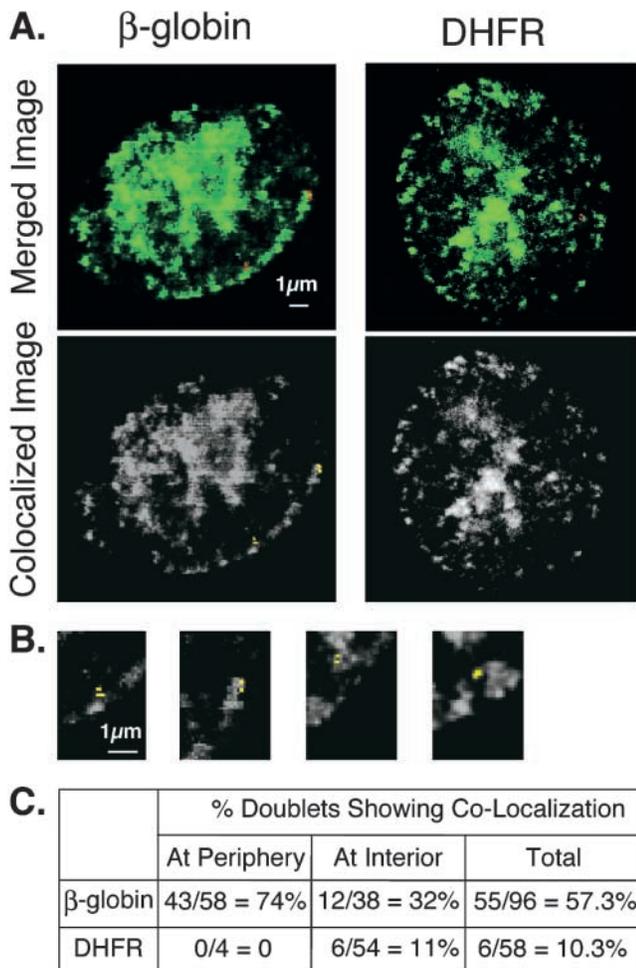
Figure 3. **Colocalization of β-globin versus DHFR doublets with sites of DNA synthesis during middle to late S phase.** CHO AA8 cells pulse labeled with BrdU were subjected to FISH with either a β-globin or a DHFR probe (red) and then stained with anti-BrdU antibodies (green). Analysis was concentrated on nuclei that displayed type III or IV BrdU patterns (described in legend to Fig. 2). (A) (Top) Show examples of merged confocal images. (Bottom) Show the same images after computer-assisted colocalization analysis. After background subtraction, those pixels still showing colocalization of red and green signals were colored in yellow, whereas pixels showing no colocalization were colored in white. (B) Sections of the nuclear periphery from exemplary nuclei showing the proximity of β-globin doublets to the peripheral heterochromatin. (C) The percentage of doublets within type III and IV nuclei that colocalized with BrdU signal was scored. Data were scored independently for doublets found either at the periphery (defined as <1 μm from the edge of DAPI staining) or the interior of the nucleus.

ing time. As shown in Fig. 3 C, β-globin doublets colocalized with BrdU at a much higher frequency than DHFR doublets. In many cases, the β-globin doublets appeared to be very close to peripheral heterochromatin (Fig. 3 B). Very few DHFR doublets were found within the peripheral zone (defined here as <1 μm from the perimeter of DAPI staining), and none of these colocalized with BrdU. These data strongly suggest that daughter β-globin loci are separated shortly after their replication, providing direct evidence for the replication of β-globin alleles in mid–S phase, coincident with the replication of peripheral heterochromatin.

Early G1 phase establishment of the β-globin late replication program
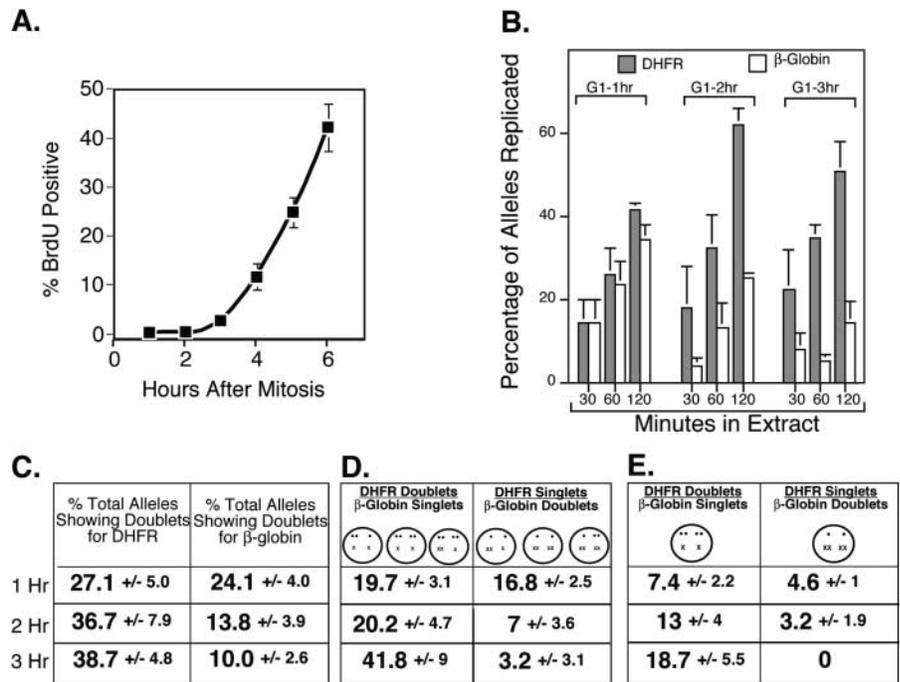
To evaluate when the replication program for the β-globin locus is established, we took advantage of the *Xenopus* cell-free system. We have previously characterized the optimal conditions for DNA replication that preserve physiological replication timing and origin specificity when G1 phase nuclei are introduced into *Xenopus* egg extracts (Dimitrova and Gilbert, 1998, 1999b). Replication initiates efficiently after a 10–20-min lag period within 100% of nuclei from cells isolated at any time during G1 phase. To ensure that nuclei introduced into the extract are derived from G1 phase cells that had not preinitiated DNA replication, it was first necessary to determine the length of G1 phase. Hence, CHO AA8 cells were synchronized in mitosis by selective detachment and released into G1 phase, and aliquots of these cells were pulse labeled with BrdU at hourly intervals thereafter. Results (Fig. 4 A) revealed that nearly all cells are in G1 phase for the first 3 h after mitosis, with the first 10–12% of the population entering S phase by 4 h after mitosis. To determine when the late replication program for the β-globin locus is established, we introduced nuclei from cells synchronized 1, 2, and 3 h after mitosis into a *Xenopus* egg extract and incubated for 30, 60, and 120 min in vitro. Results (Fig. 4 B) revealed that, with nuclei isolated from cells 1 h after mitosis, replication of the β-globin locus took place before replication of the DHFR locus as frequently as the reverse. However, with nuclei isolated 2 or 3 h after mitosis, the DHFR locus was clearly replicated before the β-globin locus. The results, quantified in Fig. 4, C–E, using the same three methods of data display shown in Fig. 1, B–D, demonstrate that nuclei experience a transition between 1 and 2 h after mitosis that delays the initiation of replication within the β-globin locus. This defines a timing decision point for the β-globin locus.

Early G1 phase repositioning of the β-globin locus

We have previously shown that the establishment of a global program for replication timing takes place coincident with the repositioning of chromosomal domains within nuclei. Since the β-globin genes are localized to the periphery of the CHO nucleus, and replicate during the type III stage of S phase, we monitored the return of labeled type III foci to the nuclear periphery after mitosis. Asynchronously growing CHO AA8 cells were pulse labeled with BrdU and chased for 3.5 h, a period of time sufficient to allow cells labeled in mid–S phase to approach mitosis. Cells were then synchronized in mitosis and released into G1 phase. At 1, 2, and 3 h thereafter, aliquots were fixed and stained with anti-BrdU antibodies, and the percentage of nuclei exhibiting a peripheral BrdU labeling pattern was scored. At 1 h after mitosis, <5% of nuclei could be identified as harboring a type III pattern. By 2 h after mitosis, 35% of nuclei from these same cell populations displayed clearly organized type III patterns (Fig. 5 A). These data are consistent with our previously reported results with an independent CHO cell line (Dimitrova and Gilbert, 1999b) and indicate that the repositioning of peripheral type III replication domains takes place between 1 and 2 h after mitosis, coincident with the TDP for β-globin.

A.

B.

Figure 4. Late replication of the β-globin gene locus in CHO cells is established 1–2 h after mitosis. (A) CHO AA8 cells were synchronized in mitosis and released into G1 phase. At the indicated times thereafter, cells were pulse labeled with BrdU, stained with anti-BrdU antibodies, and the percentage of BrdU positive nuclei was scored. (B) Nuclei isolated either at 1, 2, or 3 h after mitosis were introduced into *Xenopus* egg extracts. Aliquots of these nuclei were then subjected to FISH analysis at 30, 60, and 120 min thereafter. The percentage of total DHFR or β-globin alleles displaying doublets was calculated for >100 nuclei per time point. Shown are the means ± SEM for three independent experiments. (C–E) Results shown in B for 30, 60, and 120 min in vitro were averaged together and displayed by the same three methods described in the legend to Fig. 1, B–D.

C.

	% Total Alleles Showing Doublets for DHFR	% Total Alleles Showing Doublets for β-globin
1 Hr	27.1 +/- 5.0	24.1 +/- 4.0
2 Hr	36.7 +/- 7.9	13.8 +/- 3.9
3 Hr	38.7 +/- 4.8	10.0 +/- 2.6

D.

	DHFR Doublets β-Globin Singlets	DHFR Singlets β-Globin Doublets
1 Hr	19.7 +/- 3.1	16.8 +/- 2.5
2 Hr	20.2 +/- 4.7	7 +/- 3.6
3 Hr	41.8 +/- 9	3.2 +/- 3.1

E.

|	DHFR Doublets β-Globin Singlets	DHFR Singlets β-Globin Doublets
1 Hr	7.4 +/- 2.2	4.6 +/- 1
2 Hr	13 +/- 4	3.2 +/- 1.9
3 Hr	18.7 +/- 5.5	0

Next, we examined the appearance of type III DNA synthesis in *Xenopus* egg extract. We have previously shown that, in order to observe a transition from early to middle replication patterns in *Xenopus* egg extract, nuclei must be derived from cells that have established a spatiotemporal program (Dimitrova and Gilbert, 1999b). Nuclei from cells isolated at 1, 2, or 3 h after mitosis were introduced into *Xenopus* egg extract and pulse labeled with biotin-dUTP at 30, 60, and 120 min during the in vitro replication reaction. Nuclei were then stained with fluorescent streptavidin, and the percentage of nuclei showing either type I/II (early S) or type III (middle S) patterns were evaluated (type IV and V patterns are rarely observed since extracts lose DNA synthetic capacity within 3 h; Dimitrova and Gilbert, 1998, 1999b). As shown in Fig. 5 B, replication within 1 h nuclei continued with a type I/II pattern throughout the in vitro reaction, whereas nearly half of both 2- and 3-h nuclei transitioned to type III patterns within 60 min. These data provide further evidence that the type III peripheral replication pattern is established between 1 and 2 h after mitosis.

The ability to identify specific loci by FISH provided us with the opportunity to determine whether peripheral localization of the β-globin locus is established coincident with the establishment of its replication timing. At various time points after mitosis, the closest distance of each β-globin and DHFR allele to the periphery of the nucleus was measured and normalized to the nuclear radius. To control for the possibility of invaginations in the nuclear envelope, which are not readily identified with DNA stains, the nuclear periphery was highlighted with an antibody against the nuclear lamina (Fig. 5 C), as has been done in similar studies (Dernburg et al., 1996; Parreira et al., 1997). Results (Fig. 5 D) revealed that, at 1 h after mitosis, the β-globin locus was no closer to the periphery than the DHFR locus, and the distribution of these two genes within the nucleus was similar. However, at 2 and 3 h after mitosis, the median distance of

the β-globin locus to the periphery was significantly smaller than the DHFR locus. In fact, at 2 and 3 h after mitosis, the majority of nuclei had at least one β-globin allele with a distance/radius ratio of 0.2, whereas very few nuclei had DHFR alleles that close to the periphery. We conclude that the β-globin locus is positioned adjacent to the periphery of the CHO nucleus between 1 and 2 h after mitosis, coincident with the establishment of a replication timing program.

Discussion

A flurry of recent investigations has provided us with a preliminary glimpse of how the vast amount of information stored within the chromosomes of eukaryotic organisms is organized to carry out its myriad functions. One of the emerging concepts is the existence of subnuclear compartments where functionally related chromosomal domains cluster, creating environments that favor particular functional states of chromatin (Gasser, 2001; Gilbert, 2001). The organization of these compartments is not only spatial, as would be found in a typical filing cabinet, but is temporal. Compartments are replicated in a particular temporal sequence during the cell cycle that is generally related to their function. It is therefore likely that an understanding of the temporal regulation of DNA replication will provide new insights into the functional organization of chromosomes within the nucleus. We recently characterized a cell-free system in which to study replication timing in mammalian cells and demonstrated that the overall temporal program for replication of the genome is established early in G1 phase, coincident with the repositioning of sequences within the nucleus after mitosis (Dimitrova and Gilbert, 1999b). Here, we have focused on a particular well-studied mammalian genomic locus that has been demonstrated to show programmed changes in replication timing that accompany changes in transcriptional activity. We demonstrate that, in
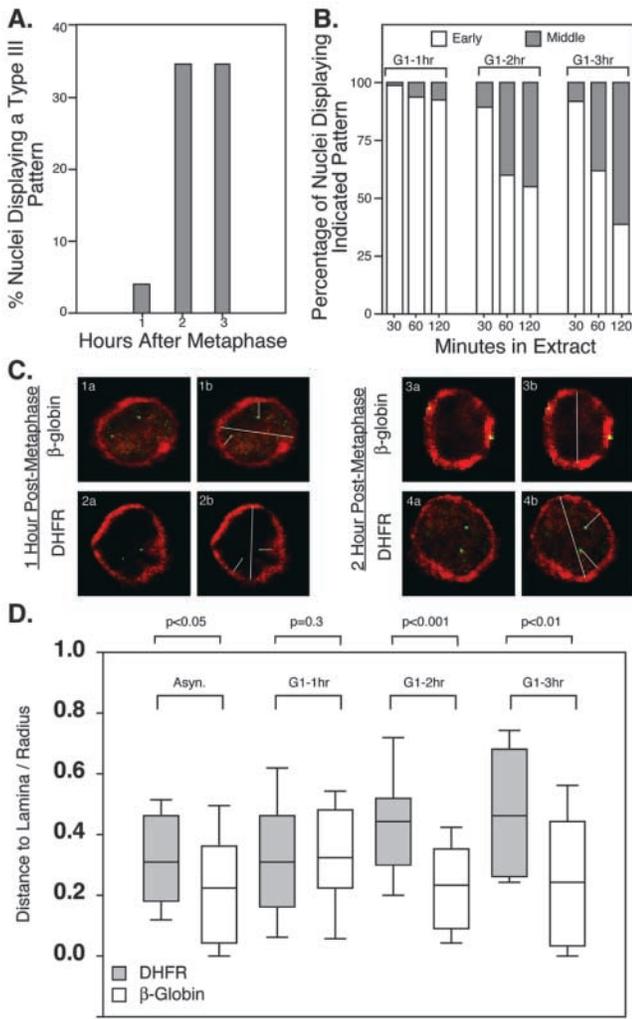
Figure 5.   **The repositioning of type III sequences and the peripheral localization of the β-globin locus are completed 1–2 h after mitosis.** (A) Asynchronous cultures of CHO AA8 cells were pulse labeled with BrdU for 30 min. Metaphase cells were harvested 3.5 h thereafter, creating populations of cells in which late-replicating sequences were tagged with BrdU (~1/3 of which were type III). At 1, 2, and 3 h after release into G1 phase, cells were fixed and stained with anti-BrdU antibody, and the percentage of nuclei displaying a type III spatial pattern, represented by peripheral and nucleolar BrdU staining, was calculated. (B) Nuclei were isolated from CHO AA8 cells synchronized at 1, 2, or 3 h after mitosis and introduced into *Xenopus* egg extracts. At the indicated time points, reactions were pulse labeled in vitro with biotin-11-dUTP, and nuclei were stained with Texas red streptavidin. The percentage of nuclei from each time point that displayed either early (many internal punctate foci) or middle (peripheral and nucleolar DNA synthesis) replication patterns was calculated. (C and D) Asynchronously growing cultures (Asyn.), as well as cells synchronized at 1, 2, or 3 h after metaphase, were subjected to FISH with DHFR or β-globin probes and then stained with an anti-lamin A/C antibody. Confocal images were collected and the distance from each allele to the nuclear lamina was measured and divided by the radius of the nucleus at its widest point. (C) Examples of images found for either DHFR or β-globin at either 1 or 2 h after mitosis. A copy of each image is shown to its immediate right, with white lines to denote the measurements taken for the diameter and the shortest distance from the FISH signal to the nuclear envelope. The lamina was used as an indicator of the nuclear periphery rather than DAPI (as in Fig. 1) due to the occasional invaginations of the nuclear envelope that are not always detectable with the use of DNA dyes. An example of such an

a cell line in which the β-globin locus is not expressed, the late replication timing program is established coincident with its repositioning near the peripheral heterochromatic compartment of the nucleus. Once positioned to the periphery, the β-globin locus appears to acquire the replication timing program of the peripheral heterochromatin. These results support the existence of a subnuclear compartment at the periphery of the mammalian nucleus that can influence the structure and function of chromatin.

We were surprised to find that the CHO β-globin locus associates with the periphery rather than centromeres. Several reports, including studies of the β-globin locus, have found transcriptional silencing in both mouse and human cells to be correlated with localization close to centromeric heterochromatin (Brown et al., 1999; Francastel et al., 1999; Schubeler et al., 2000). In addition, we found the β-globin locus adjacent to centromeres in mouse C127 fibroblasts (unpublished data). On the other hand, there is extensive evidence in *S. cerevisiae* that genes can be silenced by their localization to the periphery (Andrulis et al., 1998; Cockell and Gasser, 1999; Heun et al., 2001). Our results suggest that the nuclear periphery can also serve as a silencing compartment in mammalian cells. Since both mouse and human cells appear to have late-replicating hypoacetylated chromatin at the nuclear periphery (Croft et al., 1999; Taddei et al., 1999), it is likely that some silenced genes will be found localized to the periphery in these species as well.

The fact that the peripherally localized and transcriptionally silent β-globin locus is replicated in mid–S phase, distinctly before the latest replicating sequences, raises two important questions. First, are silenced genes ever replicated as late as constitutive heterochromatin, or is the middle of S phase reserved for replication of facultative heterochromatin? Second, do silenced genes generally take on the replication timing of the compartment with which they associate, as the β-globin genes appear to have done in CHO cells? Obviously, this report provides only a beginning, concentrating on a single locus that has been shown to switch its replication timing program during development. There is clearly a need for additional studies relating transcriptional silencing, association with heterochromatin, and replication timing in order to address these questions.

Our results provide additional evidence that nuclear position is established at a distinct point after nuclear formation (Csink and Henikoff, 1998; Dimitrova and Gilbert, 1999b; Bridger et al., 2000). However, they provide only a series of snapshots and cannot evaluate the mobility of these sequences during the cell cycle. In fact, the stability of these positions is still unresolved and may vary for different se-

invagination is shown (2, a and b). Two β-globin alleles at the periphery, appearing as yellow FISH foci, which would be scored as a distance of zero, are also shown (3a and 3b). (D) Box plot representing the shortest distance between DHFR or β-globin alleles and the nuclear lamina. Data for each cell population were collected from ≥37 nuclei. Horizontal bars represent the 10th, 25th, 50th (median), 75th, and 90th percentiles, and the p-values for each pair of samples are shown. This type of plot indicates that 50% of the population is found between the 25th and 75th percentiles, and is represented as a box. Gray boxes denote DHFR alleles, and white boxes denote β-globin alleles.

quences and under different metabolic conditions. Photobleaching studies of fluorescently labeled chromatin in living cells have detected very little movement during the cell cycle (Abney et al., 1997; Marshall et al., 1997; Zink et al., 1998; Manders et al., 1999). However, other studies, also in living cells, have revealed rather substantial movements of certain centromeres (Shelby et al., 1996) and transfected sequences (Li et al., 1998; Tumbar and Belmont, 2001). Furthermore, Bridger et al. (2000) reported that the peripheral localization of the largely late-replicating human chromosome 18 was lost upon entry of cells into quiescence. When stimulated to reenter the cell cycle, chromosome 18 remained late-replicating in the first S phase but did not return to the periphery until the second cell cycle. This experiment indicates that subnuclear localization is not necessary to maintain the replication timing program that was established at the TDP. This conclusion was recently supported by direct visualization of origin sequences in living yeast cells (Heun et al., 2001). These studies suggest that late-replicating sequences in budding yeast associate with the periphery during early G1 phase and acquire a chromatin modification that delays their replication time and persists even though the sequences may wander from the periphery later in the cell cycle. The lack of a perfect association of the β-globin locus with the periphery in the experiments reported herein is also consistent with some degree of dynamic movement of this locus within the nucleus. Hence, early G1 phase may represent a unique window of time during which association with heterochromatin can influence chromosome architecture and replication timing for the remainder of the cell cycle, regardless of the positional fate of those domains thereafter.

How might this occur? We have previously proposed a model in which proteins regulating chromosome architecture are removed from chromatin during mitosis and reassociate at the TDP (Dimitrova and Gilbert, 1999b; Gilbert, 2001). As sequences are repositioned after mitosis, the clustering of chromosomal segments that associate with similar proteins (e.g., constitutive heterochromatin and/or repetitive DNA segments) would seed a locally high concentration of these proteins, analogous to the concentration of Sir proteins at telomere clusters in *S. cerevisiae* (Cockell and Gasser, 1999; Gasser, 2001; Heun et al., 2001). Association with these nuclear compartments could influence the structure of facultative heterochromatin. Once established, this chromatin architecture may be stable for the remainder of the cell cycle, whether or not subnuclear position is maintained. Particular chromatin configurations could establish thresholds for the accessibility of replication origins to initiation factors, thereby influencing when during S phase replication origins can fire. The results presented here support this model by demonstrating that a specific developmentally regulated locus is positioned adjacent to peripheral heterochromatin early in G1 phase, coincident with a modification of this locus that delays the timing of its replication during S phase.

Materials and methods

Cell culture and synchronization
CHO AA8 cells were cultured in DME supplemented with 5% fetal calf serum and nonessential amino acids. Synchronization in mitosis was performed by selective detachment, as described (Wu et al., 1997). Synchro-nization at the G1/S boundary was achieved by releasing mitotic cells into medium containing 5 μg/ml aphidicolin (Calbiochem) for 14 h.

Probes and FISH
Cosmid cSc26, encompassing ∼40 kb of DNA downstream of the DHFR gene, was a gift from J. Hamlin (University of Virginia, Charlottesville, VA). To clone the Chinese hamster (*Cricetulus griseus*) β-globin locus, we hybridized a Chinese hamster genomic lambda FIX II library (catalogue no. 946910; Stratagene) with a partial cDNA probe corresponding to exons 1 and 2, and part of exon 3 from the human β-globin gene (gift of M. Aladjem, National Cancer Institute, Bethesda, MD) and isolated lambda phage λMCB. A 600-bp SacI cross-hybridizing fragment derived from this phage was cloned (pSac600) and sequenced (EMBL/GenBank/DDBJ under accession nos. AF314660 and AF314661), revealing 88% identity to the previously cloned (Lee et al., 1992) golden hamster β-major globin chain cDNA. In addition, oligonucleotide primers corresponding to the golden hamster embryonic z gene (Li et al., 1992) were used to amplify a fragment from Chinese hamster genomic DNA that was then cloned (pHEB) and used to identify four overlapping phage (λJHC2, λJHC4, λJHC6, and λJHC8). A 400-bp PvuII/SacI PCR fragment from λJHC2 was then sequenced (EMBL/GenBank/DDBJ under accession nos. AF314658 and AF314659), revealing 96% homology to the golden hamster β–like y-globin gene cDNA. Phage DNA was purified with a Wizard lambda DNA purification kit (Promega) for making FISH probes.

FISH analysis was performed as described (Bickmore and Carothers, 1995; Boggs and Chinault, 1997) with some modifications. Cultured cells were pulse labeled with BrdU 90 min, detached with trypsin, swollen in 0.075 M KCl at 37°C for 17 min, and fixed at 0°C for 10 min with fresh 3:1 methanol/acetic acid (fixative). Cells were centrifuged and resuspended in fixative at 0°C for 20 min, centrifuged, and resuspended again in fixative, and then dropped onto clean slides and air dried. Slides were stored at −20°C until use. For in vitro reactions, intact G1 phase nuclei were prepared by digitonin treatment and introduced into *Xenopus* egg extracts at 10,000 nuclei/μl extract, as described (Wu et al., 1997). Reactions were stopped by diluting nuclei 1:10 in cold nuclear isolation buffer (NIB; 10 mM NaCl, 3 mM MgCl₂, 10 mM Tris-HCl, 0.5% NP40). 100 μl of nuclei were overlaid on 300 μl 1:2 glycerol/extraction buffer (3× EB; 100 mM KCl, 5 mM MgCl₂, 20 mM Hepes, pH 7.5, 2 mM 2-ME), cytocentrifuged onto clean glass slides, and air dried. Fixative was dropped onto the slides, which were then air dried and stored as above.

Probes cSc26 and either λJHC2 or λMCB were labeled with either digoxigenin-dUTP or biotin-dUTP using a commercial nick translation kit (Roche). For each slide, labeled DHFR and β-globin probes (50–100 ng each) were coprecipitated with 5 μg sheared herring sperm DNA (Sigma) and 1 μg sonicated hamster genomic DNA. Probes were denatured 5 min at 84°C and preannealed at 37°C for 15 min before hybridization. For hybridization, slides were treated with RNase A (100 μg/ml) in 2× SSC at 37°C for 1 h, rinsed in 2× SSC, dehydrated in 70, 90, and 100% ethanol for 5 min each, and air dried. Slides were then incubated in 70% formamide/2×SSC at 84°C (in our hands, 72°C was not sufficient) for 3 min, and then transferred quickly to ice-cold 70, 90, and 100% ethanol for 2 min each and air-dried. Denatured probes were applied to each slide under a coverslip, sealed with rubber cement, and incubated at 37°C overnight. Slides were then washed 4 times for 3 min each in 50% formamide/2× SSC at 45°C, 2× SSC at 45°C, and then 0.1× SSC at 60°C. Probe detection and BrdU detection (with an AMCA-labeled secondary antibody) were performed exactly as described (Bickmore and Carothers, 1995), and only BrdU-positive nuclei were scored. For the characterization of replication patterns after FISH (Fig. 2), hybridization was performed for only a single probe (detected with Texas red), and BrdU-labeled DNA was detected by FITC-conjugated anti–mouse IgG (Jackson Laboratory) at a final concentration of 14 μg/ml. Nuclei were observed with a Nikon Labophot-2 equipped with a 100× Planapo lens (NA = 1.4), and images were collected with a 35-mm camera and P1600 slide film. Slides were scanned with a Nikon 2000 slide scanner and composed in Adobe® Photoshop™ software using only standard brightness and contrast adjustments.

To examine colocalization between FISH and BrdU, images were collected by confocal microscopy (MRC 1024ES; Bio-Rad Laboratories). The red and green images in the same focal plane were scanned sequentially to prevent bleed-through and were then merged. Background was subtracted, and colocalization analysis was performed with LaserSharp software (Bio-Rad Laboratories).

Labeling of nascent DNA in vivo and in *Xenopus* egg extracts
Xenopus egg extracts were prepared and handled as described (Wu et al., 1997; Dimitrova and Gilbert, 1998). The labeling of nascent DNA with 30

μg/ml BrdU in cultured cells, 50 μM biotin-11-dUTP in *Xenopus* egg extracts, and the detection of BrdU- and biotin-substituted DNA were performed as described (Dimitrova and Gilbert, 1999b).

Determination of gene position

Simultaneous detection of DHFR or β-globin and the nuclear lamina was performed as described (Itoh and Shimizu, 1998), except that probes were denatured and preannealed (as described above) before adding to slides. One digoxigenin-labeled probe was used at a time. After secondary antibody incubation, the nuclear lamina was detected using mouse monoclonal anti-lamin A&C IgM (Covance) and Texas red–conjugated goat anti–mouse IgM (Vector Laboratories). Images were collected by confocal microscopy (MRC 1024ES; Bio-Rad Laboratories) with 0.5 μm Z-series. The shortest distance from the brightest FISH pixel(s) to the center of the lamina signal, and the longest nuclear diameter within the medial plane were measured with LaserSharp-Processing software.

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