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Spatio-Temporal Organization of DNA Replication in Murine Embryonic Stem, Primary, and Immortalized Cells

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Abstract The extent to which chromosomal domains are reorganized within the nucleus during differentiation is central to our understanding of how cells become committed to specific developmental lineages. Spatio-temporal patterns of DNA replication are a reflection of this organization. Here, we demonstrate that the temporal order and relative duration of these replication patterns during S-phase are similar in murine pluripotent embryonic stem (ES) cells, primary adult myoblasts, and an immortalized fibroblast line. The observed patterns were independent of fixation and denaturation techniques. Importantly, the same patterns were detected when fluorescent nucleotides were introduced into living cells, demonstrating their physiological relevance. These data suggest that heritable gene silencing during commitment to specific cell lineages is not mediated by global changes in the sub-nuclear organization and replication timing of chromosome domains. J. Cell. Biochem. 95: 74–82, 2005. © 2005 Wiley-Liss, Inc.

Key words: DNA replication patterns; living cells; primary and immortalized cells; replication timing

Mammalian DNA replication is defined by a cellular program that dictates different parts of the genome to replicate at specific times during S-phase and in certain sub-nuclear locations. Proper control of replication timing is involved in the maintenance of normal cell cycle progression and genome stability, ensuring that the entire genome is efficiently duplicated exactly once per cell cycle [Loupart et al., 2000; Pflumm and Botchan, 2001]. The organization of this cellular program is thought to be linked to gene expression in metazoa, as transcriptionally active euchromatin tends to be replicated earlier in S-phase than transcriptionally silent heterochromatin [Gilbert, 2002; Goren and Cedar, 2003]. In support of this hypothesis, regional differences in chromatin structure between different cell types are linked to differences in their replication timing [Forrester et al., 1990; Bulger et al., 2003], and several proteins involved in chromatin maintenance colocalize with sites of active DNA synthesis [McNairn and Gilbert, 2003].

During each cell cycle, chromatin is reorganized within the nucleus in early G_1-phase, directing the re-localization of chromosomal domains to specific sub-nuclear compartments [Dimitrova and Gilbert, 1999; Li et al., 2001]. Nuclear DNA synthesis is defined by the spatial clustering of synchronously firing replication origins that can be visualized by labeling actively replicating DNA with nucleotide analogs [Jackson and Pombo, 1998; Ma et al., 1998; Dimitrova and Gilbert, 1999]. The reproducible and stable spatial arrangement of these replicon clusters throughout interphase is dependent upon the reorganization that occurs post-mitosis, and this takes place coincident with the establishment of a replication timing program [Dimitrova and Gilbert, 1999; Li et al., 2001]. This supports the existence of a causal relationship linking sub-nuclear position to replication timing. Given that euchromatin and heterochromatin occupy separate

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sub-nuclear compartments [Arney and Fisher, 2004], and the molecules that anchor chromatin within these compartments are being revealed [Hediger et al., 2002; Taddei et al., 2004], molecular mechanisms that link chromatin structure, sub-nuclear positioning, and replication timing may be identified in the near future.

Metazoan development is characterized by changes in the expression of genes that direct cells to their specific fates during the course of differentiation [Fisher, 2002]. At least some of these genes change their replication timing upon activation or silencing during differentiation [Hiratani et al., 2004]. In addition, changes in the expression and replication timing of some genes are coupled to changes in their sub-nuclear position [Zhou et al., 2002]. The fraction of the genome involved in such spatio-temporal reorganization is currently unclear. However, nuclear re-organization may involve more than just the subset of differentially expressed genes. In fact, it has been suggested that commitment to specific cell lineages may involve the global spatial reorganization of chromosomal domains and/or the progressive accumulation of heterochromatin to heritably silence genes whose expression is no longer required [Gilbert, 2001; Gasser, 2002; Marshall, 2003].

In this study, we examined whether there are significant differences in the global spatio-temporal regulation of DNA synthesis in cells isolated at different developmental stages. Using undifferentiated mouse embryonic stem (ES) cells, primary myoblasts, and an immortalized cell line, we show that the spatial patterns of DNA synthesis at specific times during S-phase are similar in all three cell types. These results suggest that there are no major differences in the large-scale spatio-temporal organization of DNA replication during development. Finally, we show that the patterns of replication foci observed in all of these cell lines using two different immunofluorescence staining techniques can also be visualized in living cells, and are therefore highly likely to reflect the physiological organization of replicons in living cells.

MATERIALS AND METHODS

Cell Culture and Synchronization

Mouse ES cells (HM1; gift of Prim Singh, Roslin Institute) were cultured in 0.1% gelatin-coated flasks in Glasgow minimum essential medium (Sigma, St. Louis, MO) supplemented with 5% fetal bovine serum (GIBCO, Carlsbad, CA), 5% newborn calf serum, 2 mM L-glutamine, 1× non-essential amino acids, 1 mM sodium pyruvate, 1× penicillin/streptomycin, 0.1 mM β-mercaptoethanol, and 10^4 U/ml leukemia inhibitory factor (LIF, Chemicon International, Temecula, CA). Adult mouse myoblasts (WG00; gift of Charles Thornton, University of Rochester) were cultured in flasks precoated with 0.1% gelatin in Dulbecco's minimum essential medium (GIBCO) containing 20% fetal bovine serum (GIBCO), 1× penicillin/streptomycin, and 10 ng/ml fetal growth factor (Promega, Madison, WI). Whenever either HM1 or WG00 were plated to glass coverslips or chambered coverglass, the coverslips or coverglasses were also coated with 0.1% gelatin. C127 were cultured in DMEM supplemented with 10% fetal bovine serum. Synchronization of the cells in mitosis by nocodazole treatment was performed as previously described [Wu et al., 1997; Dimitrova et al., 1999].

Nucleotide Labeling

In fixed cells. Exponentially growing cells plated on glass coverslips were labeled with 30 μg/ml 5′-bromo-2′-deoxyuridine (BrdU) (Sigma) and then fixed in either 70% ethanol indefinitely or in 4% paraformaldehyde for 10 min. For pulse-chase-pulse labeling, cells were labeled with 30 μg/ml 5′-chloro-2′-deoxyuridine (CldU) (Sigma) for 10 min, rinsed with warm PBS, chased in fresh medium containing 200 μM thymidine, rinsed with warm PBS, and then labeled with 30 μg/ml 5′-iodo-2′-deoxyuridine (IdU) (Sigma) for 10 min. Cells were then fixed in ethanol as described above.

In living cells. Cells were grown in Lab-Tek 8-well chambered coverglass (Nalge Nunc International, Rochester, NY) to 70%–80% confluency and were rinsed once with prewarmed KHB (10 mM HEPES, pH 7.4; 30 mM KCl) [Koberna et al., 1999]. KHB supplemented with 0.1 mM Cy3-dUTP (Amersham Pharmacia Biosciences, Piscataway, NJ) was added to the cells for 5 min at 37°C, the cells were washed once in complete medium and then incubated in fresh medium for at least 4 h before microscopy.

Immunostaining and Immunofluorescence Microscopy

Two methods for visualizing BrdU incorporation were used. Both of these are modifications

of previously described methods [Dimitrova and Gilbert, 1999; Kennedy et al., 2000]. Method 1: BrdU-labeled cells fixed in EtOH were treated with 1.5 N HCl for 30 min as a denaturant, and nucleotide-labeled DNA was detected with anti-BrdU mouse monoclonal antibodies (Becton Dickinson, Franklin Lakes, NJ). Antibody incubations were carried out in humidified chambers for 1 h at room temperature unless otherwise described. Method 2: paraformaldehyde-fixed cells were first permeabilized with 0.5% Triton X-100 for 10 min and then treated with 100 U/ml DNase I denaturant simultaneously with primary mouse anti-BrdU antibody for 1 h at 37°C. Differential staining of CldU- and IdU-labeled DNA was performed as described previously [Dimitrova et al., 1999] with some modifications. CldU-labeled DNA was detected with rat anti-BrdU primary antibody (Harlan-Sera Lab, Leicestershire, UK) and FITC-conjugated donkey anti-rat secondary IgG (Jackson ImmunoResearch Laboratories, West Grove, PA). IdU-labeled DNA was detected with primary mouse anti-BrdU antibody and Texas red-conjugated anti-mouse secondary IgG. Incubation in a high salt buffer (400 mM NaCl, 0.2% Tween-20, 0.2% NP-40) for 15 min between the IdU primary and secondary antibody incubations eliminated potential antibody cross-reactivity.

Microscopy of fixed samples was performed using a Nikon 100× 1.4NA oil immersion Nikon PlanApo objective on an upright Nikon Labophot-2 microscope. After counting spatial patterns displayed by labeled nuclei, images of representative patterns were obtained using a CCD camera (SPOT RT Slider, Diagnostic Instruments, Sterling Heights, MI) and AutoDeblur deconvolution software from AutoQuant Imaging, Inc. (Watervliet, NY). Microscopy of living cells grown on chambered coverglasses was performed using a Nikon Plan Fluor 100× 1.25NA objective on an inverted Nikon Eclipse T5100 microscope. Prior to imaging the cells, the microscope stage was heated to 37°C with warm air, using an Air Stream Stage Incubator (Nevtek, Burnsville, VA, #ASI 400). Alternatively, cells were grown on glass coverslips, labeled with Cy3-dUTP and briefly transferred to a glass slide to capture images on the Nikon Labophot-2 upright microscope, and de-convolution was performed.

Determination of the Temporal Organization of DNA Replication

The number of cells proceeding from one replication pattern to another pattern or to G_2, M, or G_1 phases in the CldU pulse-chase-IdU pulse procedure was scored for each chase period. Approximately 200 cells were counted for each chase period. The percentage of cells with a designated CldU-labeled pattern that displayed a subsequent replication pattern during the IdU pulse was then determined. We calculated the half-time of each replication pattern as the time it takes for 50% of cells in each pattern to move into subsequent pattern(s) or G_2-phase. The duration of each pattern was calculated as twice the half-life, and the total duration of S-phase was calculated as the sum of all patterns. In order to directly compare the temporal organization of replication between cell types with different S-phase lengths, we calculated the percent of the total S-phase time occupied by each pattern in each cell type.

RESULTS

Replication Patterns in ES Cells and Adult Myoblasts

Most studies of the spatial distribution of DNA replication sites have been performed with established (i.e., immortalized) cell lines, and the spatial patterns in ES cells have not been reported. When ES cells were briefly labeled with BrdU and immunofluorescently stained with anti-BrdU antibodies to detect sites of active DNA synthesis, we observed spatial replication patterns that were consistent with those previously described in other cell lines [Dimitrova and Gilbert, 1999; Dimitrova and Berezney, 2002] (Fig. 1A). To determine the order of appearance of these patterns, ES cells were synchronized in mitosis by selective detachment, and then pulse-labeled at hourly intervals thereafter (Fig. 1C). The characteristics of these patterns that are reproducibly observed are described in detail in Figure 1.

Since fixation techniques always introduce the possibility of artifacts, we sought to determine whether the same well-established replication patterns are seen when DNA synthesis is observed in living cells. ES cells were loaded with fluorescently-tagged nucleotides (Cy3-dUTP) during a brief hypotonic shock [Koberna et al., 1999] and then allowed to continue

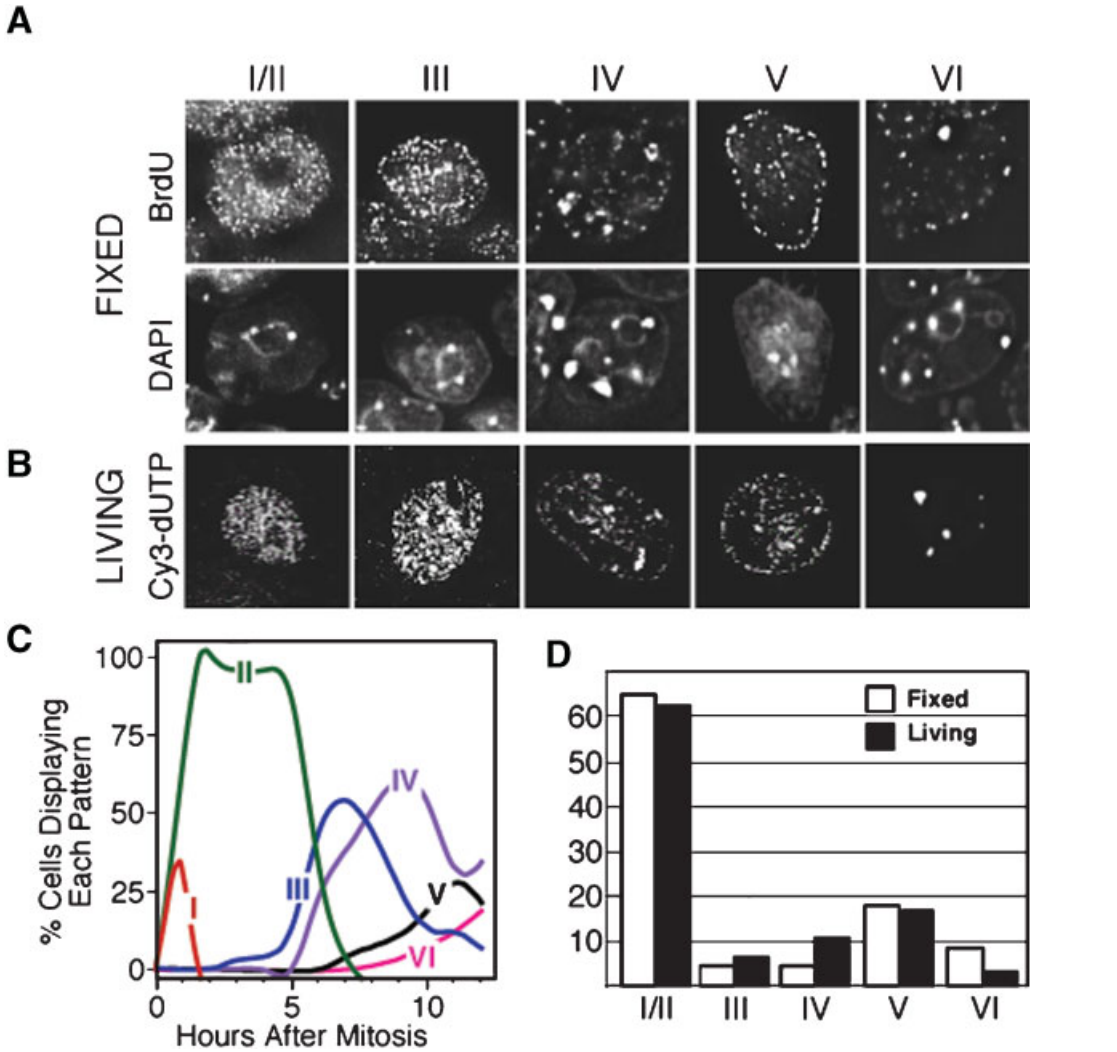


Fig. 1. Spatio-temporal replication patterns in murine embryonic stem (ES) cells. **A**: Asynchronously growing, undifferentiated mouse ES cells were labeled with 30 μg/ml 5′-bromo-2′-deoxyuridine (BrdU) for 30 min followed by fixation in 70% EtOH and HCl denaturation. Sites of BrdU incorporation were detected by anti-BrdU immunofluorescence staining. Shown are photographs of replication patterns representative of two independent experiments. These patterns were designated numerically (I–VI) based upon their order of appearance in synchronized cells, as shown in (**C**). **B**: Cells were subjected to a brief hypotonic shock in buffer containing 0.1 mM Cy3-dUTP for 5 min at 37°C, washed and placed in fresh medium. After a 4 h incubation, the cells were placed on a heated, inverted microscope stage. In three independent experiments, the replication patterns shown here were identified and categorized as in (A). C: To define the temporal order of the observed patterns, ES cells were synchronized in mitosis, released in fresh medium for hourly time points, labeled with BrdU, fixed, and stained as in (A). Based on data from counting 100 to 200 cells at each release time, the percentage of total cells displaying each of the patterns shown in A and B was scored. The order of their appearance is similar to that previously described in other cell types [Dimitrova and Gilbert, 1999]. Description of characteristics of each pattern: early S-phase is characterized by first a small (red, Type I; very brief and likely a precursor to Type II) and then a large (green, Type II) number of small replication foci distributed throughout the interior of the nucleus but excluding the nuclear periphery, peri-nucleolar and centromeric (dense DAPI staining) regions. As cells approach middle S-phase, foci at the nuclear periphery begin to replicate coincident with internal foci (blue, Type III). Thereafter, internal foci largely cease DNA synthesis while centromeric clusters (DAPI-dense) begin replicating (purple, Type IV). Toward the end of S-phase, replication takes place almost exclusively at the nuclear periphery and peri-nucleolar regions (black, Type V), and finally, the end of S-phase is characterized by the replication of large internal foci that are neither centromeric nor peri-nucleolar (pink, Type VI). **D**: The percentage of S-phase cells (of a total of ~200 each) displaying each replication pattern in either fixed or living preparations was scored for each replication pattern.

growing in fresh medium before imaging. As shown in Figure 1B, the same spatial replication patterns that are evident in fixed cells (Fig. 1A) were observed in living cells. Furthermore, the percentage of asynchronously growing cells in S-phase exhibiting each pattern is similar to that seen after fixation and immunostaining (Fig. 1D).

The spatial patterns of DNA synthesis in primary mammalian cells have recently become the subject of considerable debate. In the hands of some investigators [Kennedy et al., 2000; Barbie et al., 2004], replication in primary cells differs from that in immortalized cells in that a large fraction of early DNA synthesis takes place within 5–20 peri-nucleolar foci. Others have reported what appear to be directly contradictory results; primary and immortalized cells display the same spatial replication patterns regardless of fixation, denaturation or staining technique utilized [Dimitrova and Berezney, 2002]. To investigate this further, we applied several different replication labeling techniques to WG00, a primary adult myoblast cell line. Although we could occasionally see some primary cells displaying replication patterns resembling these sparse peri-nucleolar foci (data not shown), we were unable to consistently detect such patterns, using either ethanol fixation and acid denaturation (Fig. 2A), or paraformaldehyde fixation and denaturation via DNase I (Fig. 2B), the two methods used most commonly by many different groups. Importantly, the same characteristic replication patterns that appeared in fixed cells were evident in living adult mouse myoblasts, labeled with Cy3-dUTP by brief hypotonic shock (Fig. 2C). The percentages of S-phase nuclei displaying each pattern in either fixed or living preparations of the myoblasts were also similar (Fig. 2D). We conclude that the patterns observed using traditional fixation and denaturation methods accurately reflect the patterns of DNA synthesis that take place in living undifferentiated ES cells and primary myoblasts, supporting the physiological relevance of these spatio-temporal patterns.

Similar Duration of Patterns in ES and Differentiated Cells

The results shown in Figures 1 and 2 indicate that the spatial patterns and their temporal order are not obviously different between ES cells and primary myoblasts. However, it is possible that the amount of the genome organized within each spatial pattern could differ, which would be reflected as a difference in the total fraction of S-phase time devoted to each replication pattern. For example, if adult myoblasts have significantly more heterochromatin than ES cells, we would expect a higher fraction of the genome to be organized into heterochromatic, late S-phase replication foci, which would be revealed as an increased duration of late replication patterns.

To more accurately measure the duration of each of these patterns in these cell types, we employed a previously described method [Dimitrova and Gilbert, 1999] that measures these parameters dynamically in asynchronously growing cells. This pulse-chase-pulse method avoids the need for cumbersome synchronization methods that often produce different results in different cell types. Asynchronous cells were first pulsed with CldU, chased in thymidine-supplemented medium for various lengths of time, and then pulsed with IdU. After fixation, antibodies specific for each nucleotide analog allowed for differential visualization of the sites of DNA synthesis labeled during each pulse, with CldU stained with FITC and IdU stained with Texas red (Fig. 3A). Merging the fluorescent images confirmed the temporal order of these various patterns by establishing which patterns reproducibly follow each other. The duration of each replication pattern was estimated by determining the chase time necessary for any given cell to transition from one pattern to the next (Fig. 3B). C127 fibroblasts were used as a control to provide a comparison of the duration of each of these patterns in an immortalized cell line, as replication timing has already been extensively characterized in several established cell lines [Nakayasu and Berezney, 1989; Ma et al., 1998; Dimitrova and Gilbert, 1999], including C127 [Wu et al., 2005 (in press)]. To compare the fraction of S-phase occupied by each of these different patterns in the different cell types, which have different S-phase lengths, the duration of each pattern was expressed as a percentage of total S-phase time for each cell type (Fig. 3C). From these results, we conclude that, although there are small differences in the duration of some of the spatio-temporal patterns that could reflect differences at the level of individual chromosomal domains between these cell lines, there are no global quantitative or qualitative changes in the spatio-temporal order

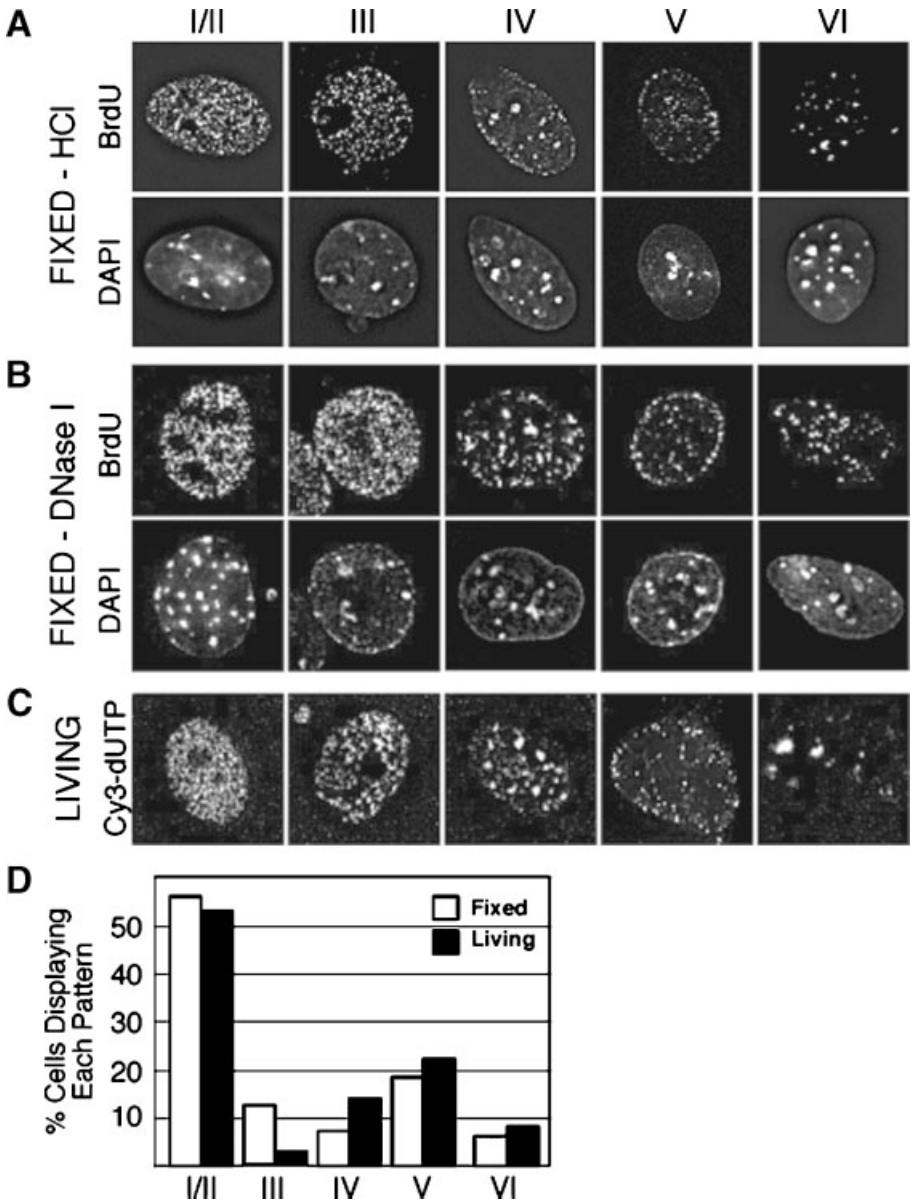
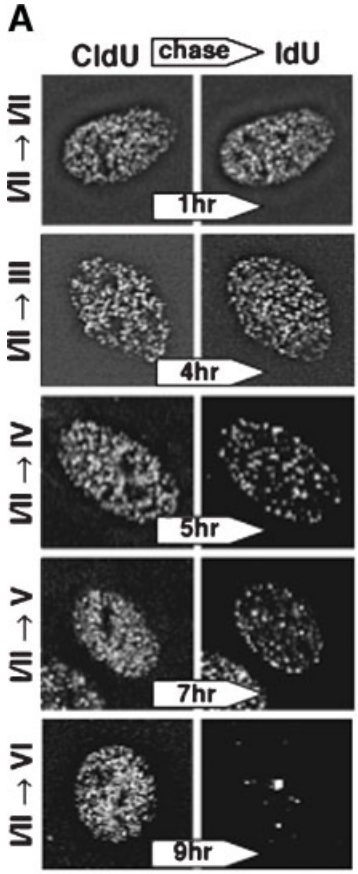


Fig. 2. Spatio-temporal replication patterns in murine myoblasts. **A**: Adult myoblasts were labeled with BrdU, fixed in EtOH, and immunostained as in Figure 1A, and the replication patterns shown here were identified in three independent experiments. **B**: Myoblasts labeled with BrdU for 30 min were fixed in 4% paraformaldehyde and permeabilized in 0.1% Triton X-100. DNase I treatment was performed simultaneously with anti-BrdU immunostaining, and the same typical replication patterns were seen as shown. **C**: Replication patterns in living cells were determined and imaged as in Figure 1C. **D**: The percentage of S-phase cells displaying each replication pattern was scored as in Figure 1D.

of the patterns of DNA synthesis between pluripotent ES cells, primary adult myoblasts, and immortalized fibroblasts.

DISCUSSION

Taken together, these results demonstrate that in mouse cells isolated at different developmental stages, the spatial distribution and temporal organization of DNA replication foci are similar. We provide the first characterization of these patterns in pluripotent cells, demonstrating that similar patterns of replication are found in cells with widely different potentials for gene expression. Furthermore, we demonstrate that spatio-temporal patterns of replication are similar between primary and immortalized cells independent of the method used to visualize these patterns, consistent with a previous report [Dimitrova and



B

ES

Chase time (hrs)	Percentage of cells proceeded from each pattern to a subsequent one during chase					
	I/II	III	IV	V	VI	G2/M/G1
1	1.9	28 5	9.1	2.5	23 3	13 5
3	39 1	100	90	83 7	100	51 7
6	94 8	100	100	91 3	100	90 9
9	97 9	100	100	100	100	91 4

myoblasts

Chase time (hrs)	Percentage of cells proceeded from each pattern to a subsequent one during chase					
	I/II	III	IV	V	VI	G2/M/G1
1	8.2	0	42.9	26.7	83.3	0
3	39.5	86.7	80	93.8	100	17.8
6	84.4	100	100	100	100	18.5
9	100	100	100	100	100	51.5

immortalized

Chase time (hrs)	Percentage of cells proceeded from each pattern to a subsequent one during chase					
	I/II	III	IV	V	VI	G2/M/G1
1	0	16 7	18 8	5.3	22 2	3.7
3	20 4	86 7	75	29 2	100	6.7
6	76	100	100	100	100	25 9
9	100	100	100	100	100	30 4

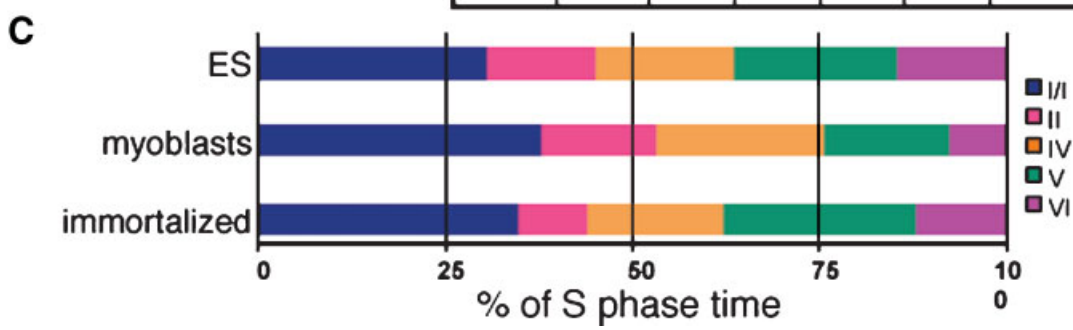


Fig. 3. Replication timing is similar in pluripotent ES and differentiated cells. ES cells, primary myoblasts, and C127 immortalized fibroblasts were pulse-labeled with 30 μg/ml 5′-chloro-2′-deoxyuridine (CldU) for 10 min, chased for several periods of time up to 9 h in thymidine-supplemented medium, then pulse-labeled with 30 μg/ml 5′-iodo-2′-deoxyuridine (IdU) for 10 min. Cells were fixed in 70% ethanol, denatured with 1.5 N HCl, and then stained with primary antibodies specific for each nucleotide analog. **A**: Exemplary pairs of images from nuclei engaged in Type I/II synthesis during the CldU label, that had been chased for the indicated times before IdU labeling. The CldU staining pattern is shown on the **left**, and the IdU staining pattern on the **right** for each nucleus. The spatial patterns detected by CldU and IdU staining are indicated to the left of each pair of images, and were identified as described in detail in Figure 1. **B**: The percentage of cells in (A) that had progressed from each individual pattern to subsequent patterns for each chase time was determined (from approximately 200 nuclei for each chase period). **C**: The amount of time it took for 50% of cells beginning in one pattern to proceed to subsequent patterns was normalized to the total length of S-phase as described in ''Materials and Methods.'' Shown is a schematic diagram of the percentage of S-phase in each of these cell lines that is devoted to each of the six spatio-temporal replication patterns.

Berezney, 2002]. Finally, we extend this previous report to show that the same patterns observed in fixed preparations can also be observed in living cells, confirming their physiological relevance.

Our results address spatio-temporal patterns of replication, but they do not rule out the possibility that committed or differentiated cells contain larger amounts of heterochromatin. They also do not rule out changes in sub-nuclear

organization at the level of individual genes. In fact, microarray data suggest that ES cell differentiation induces less than a 10% change in overall gene expression [Kelly and Rizzino, 2000; Loring et al., 2001; Sato et al., 2003]. Even if most of these genes were changing sub-nuclear position, it would escape the level of resolution afforded by our experiments. Here, we demonstrate that heritable silencing during commitment to specific cell lineages is not mediated by global changes in the sub-nuclear organization and replication timing of chromosome domains.

An interesting question raised by these results is how the fate of genes that switch their replication timing during differentiation is affected. We have recently provided the first evidence for changes in replication-timing in response to differentiation and our results predict that such changes may affect up to one-third of all genes [Hiratani et al., 2004]. Others have shown that genes with different replication timing programs can localize to different sub-nuclear regions [Zhou et al., 2002]. A question of interest to us now is whether the domains that contain these switching genes, which are clearly replicated in different spatio-temporal periods, are organized into different replication domain structures in different differentiation states, despite the globally similar spatio-temporal organization between different cell-types.

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