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REVIEW ARTICLE

Temporal regulation of DNA replication in mammalian cells

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Abstract

Eukaryotic cells follow a temporal program to duplicate their genomes. Chromosomes are divided into domains with a specific DNA replication timing (RT), not dictated by DNA sequence alone, which is conserved from one cell cycle to the next. Timing of replication correlates with gene density, transcriptional activity, chromatin structure and nuclear position, making it an intriguing epigenetic mark. The differentiation from embryonic stem cells to specialized cell types is accompanied by global changes in the RT program. This review covers our current understanding of the mechanisms that determine RT in mammalian cells, its possible biological significance and how unscheduled alterations of the RT program may predispose to human disease.

Keywords: *Chromosome domain; epigenetics; histone acetylation; nuclear position; replication timing*

The DNA replication timing (RT) program: general considerations

In a mammalian cell division cycle lasting approximately 24 h, up to 10 h are allocated for DNA replication. It is not obvious why this process should take so much time. The duplication of large genomes starts from multiple origins of replication, spaced every 100 Kb on average (Huberman and Riggs, 1968). Considering that new DNA is synthesized bidirectionally at a rate of 1–3 kb/min, the entire genome could be duplicated in less than one hour. Of course, this scenario would require all origins to fire *simultaneously* at the beginning of the S phase, and this is never observed. Instead, all eukaryotic cells in which DNA replication has been investigated follow temporal programs according to which certain chromosomal domains are replicated early in S phase and others are replicated late. The mechanisms and significance of the replication timing (RT) programs of different organisms have been under intense scrutiny over the years (reviewed by Lucas and Feng, 2003; Donaldson and Schildkraut, 2006; Gondor and Ohlsson, 2009; Hiratani and Gilbert, 2009; Hiratani *et al.*, 2009). For simplicity, this review is focused on the RT program in mammalian cells.

Chromosome "replication domains" were originally detected in the 1970s by pulse-labeling cells with nucleotide analog 5-bromo-2′-deoxyuridine (BrdU) at different times during S phase and visualizing its incorporation into discrete areas of condensed mitotic chromosomes (Latt, 1973; Stubblefield, 1975). It was noted that the cytogenetic bands generated in these chromosomes by classic quinacrine (Q) or Giemsa (G) staining coincided with late-replicating domains, whereas reverse (R) bands tended to be early-replicating (Dutrillaux *et al.*, 1976).

The dynamics of DNA replication can also be monitored by the detection of BrdU incorporation onto newly synthesized DNA during S phase. Replication foci appear as intranuclear punctuated structures (Nakamura *et al.*, 1986), which form different patterns as the S phase progresses. Early in S phase, hundreds of small foci are distributed all over the nuclei; in mid S, foci are preferentially assembled around the nucleoli and nuclear periphery; late S is characterized by clusters of foci that correspond to heterochromatic regions (O'Keefe *et al.*,

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1992; Dimitrova and Gilbert, 1999; Figure 1). Most foci remain active for 45–60 min before a different set of foci is activated (Jackson and Pombo, 1998). Foci are stable structures that can be detected throughout several cell cycles (Sparvoli *et al.*, 1994; Jackson and Pombo, 1998). Each one of them likely represents a replication domain with an average size of 1 Mb and contains a cluster of 5–10 origins that fire approximately at the same time (Nakamura *et al.*, 1986; Ma *et al.*, 1998). This implies two layers of control, a *global* mechanism that determines the RT of entire chromosomal domains and a *local* regulation of origin activity within each domain. In this review I discuss the global regulation of RT; other reviews have covered extensively the local control of origin activity (Mendez and Stillman, 2003; Sivaprasad *et al.*, 2006; Sclafani and Holzen, 2007).

Taking advantage of a cell-free DNA system that initiates DNA replication in mammalian cell nuclei incubated in Xenopus egg extracts, Dimitrova and Gilbert (1999) demonstrated that the RT for each chromosome domain is established early in the G1 phase, several hours before the onset of DNA replication. CHO nuclei from synchronized cultures that had proceeded for more than 2 h into G1 replicated in Xenopus following a standard RT program. In contrast, CHO nuclei isolated only 1 h into G1 failed to follow the RT and instead displayed early- and late-replication patterns simultaneously. These results indicated that a cellular event that occurs early in G1 establishes the RT, and the authors noted that this is precisely the time of nuclear repositioning of chromosomes after mitosis. The CHO/Xenopus heterologous system is not exempt of peculiarities (e.g. DNA replication is much faster than in CHO cells in culture) but the conclusions of this study have been highly influential in the field.

A strong correlation between active transcription and early RT has been inferred from many studies. Highly transcribed genes with housekeeping functions are replicated early in all cell types (Goldman *et al.*, 1984). Genes whose expression is tissue-specific display an earlier RT in the tissues where they are expressed than in tissues where they are inactive (Hatton *et al.*, 1988). A classic example is the human beta-globin locus, which is replicated early in erythroid cells and late in other tissues (reviewed by Aladjem, 2007). In the case of imprinted genes in which only one of the two parental alleles is active, the expressed allele replicates early and the repressed allele replicates late (Simon *et al.*, 1999). Other genes with monoallelic expression such as olfactory-receptor genes (Chess *et al.*, 1994) or genes undergoing V(D)J recombination in the immune system also display asynchronous replication. In the latter case, the repertoire of B- and T-cell receptors is achieved by gene rearrangements that almost always occur on the early-replicating allele (Goren and Cedar, 2003). Another striking example of asynchronous RT occurs during the X chromosome inactivation process. Upon accumulation of Xist RNA in the chromosome to be inactivated, the RT of the entire chromosome shifts from early to late (Keohane *et al.*, 1996). These correlations, however, do not imply that actual transcription is required for early replication. For instance, transcription of the beta-globin locus can be repressed without affecting its RT (Cimbora *et al.*, 2000; Reik *et al.*, 1998). The correlation could also be explained if the RT and transcriptional competence of a DNA element were regulated by specific chromatin contexts or nuclear position, as will be discussed later.

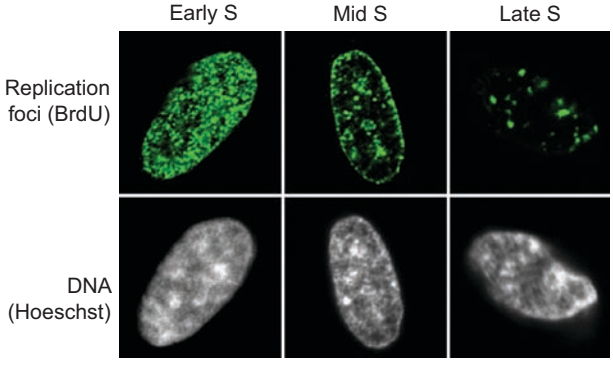

Figure 1. Patterns of replication foci in early, mid and late S phase. Replication foci were visualized by the immunodetection of BrdU after a 30 min pulse.

Traditionally, the RT of specific genomic loci has been determined using molecular methods relying either on PCR or fluorescence *in situ* hybridization (FISH). In the first type of approach, DNA from cells isolated at different times during S phase is subjected to PCR amplification with primers designed to the locus of interest. In a common version of this method, cells are labeled with BrdU to facilitate their sorting by flow cytometry during S phase. Because BrdU is photodegradable, BrdU-containing DNA is eliminated by UV irradiation and nuclease treatment, leaving unreplicated DNA as the only valid template for PCR amplification. Therefore, a comparison of PCR efficiencies at different time points allows to estimate the RT of any given sequence (Sinnett *et al.*, 1993). The second type of approach uses FISH to distinguish cells with a single hybridization dot per allele, before DNA replication, or two dots per allele after replication. In an asynchronous population, the ratio between the number of cells with two dots and those with one dot is directly proportional to the RT of the analyzed locus (Selig *et al.*, 1992).

Since the development of DNA microarray technology, the analysis of RT can be undertaken at the genomic level, as summarized in the next section. As with any other method, there are advantages and limitations to the genomic analysis of RT. The first obvious advantage

is the large amount of RT information generated by each experiment, which depends on the density of DNA probes in the microarray. Given the size of replicons, a resolution of at least 20–50 kb is desirable, but there is no real advantage in using extremely probe-dense arrays. For instance, the analyses of the mouse genome RT using an array with a probe every 6 kb or a tiling microarray with overlapping probes provided essentially the same information (Hiratani *et al.*, 2008; see below). A second advantage of these types of analyses is that they allow the visualization of "replication domains" along large chromosome territories. And more importantly, they facilitate the direct comparison of RT data with a range of genetic or epigenetic features, providing statistical significance to the correlations that may be established. There are also limitations, starting with the fact that a certain investment in DNA hybridization equipment and data analysis software is required. As with all experiments that involve microarray hybridizations, every effort must be made to establish reproducibility. Also, the RT of genomic regions rich in repeated sequences, such as telomeres and centromeres, is hard to determine as they are frequently under-represented in the arrays. Depending on the particular experimental set-up, interallelic variations of RT may also be difficult to analyze (see below).

High-density RT maps of the human and mouse genomes

In the last five years, the RT of the entire human and mouse genomes has been mapped using different methods. A straightforward approach relies on comparative genomic hybridization: DNA isolated from cells in G1 and S phase is differentially labeled and hybridized to DNA arrays containing an appropriate representation of the genome. For each sequence in the array, the ratio between S:G1 DNA is proportional to the average copy number in the asynchronous S phase, and therefore to its timing of replication. This method does not require the incorporation of nucleotide analogs and can be performed without aggressive synchronization procedures that affect the dynamics of DNA replication (Woodfine *et al.*, 2004).

Using this methodology, an RT map of the human genome has been determined with 1 Mb resolution in a lymphoblastoid cell line with normal karyotype (Woodfine *et al.*, 2004). Chromosomes 6 and 22 have been mapped at higher resolution using arrays with increased probe density, including overlapping tile path clones (White *et al.*, 2004; Woodfine *et al.*, 2004, 2005). As anticipated, chromosomes display many interspersed early- and late-replicating domains, 1 to several Mb in size, separated by relatively sharp boundaries. Each chromosome can be assigned a mean RT value, with chromosome 22 being the earliest and chromosome 4 the latest amongst the autosomes; both sexual chromosomes are late-replicating. At the chromosome level, earlier mean RT values correlated directly with higher gene density, GC content and Alu repeat density (Woodfine *et al.*, 2004). Not surprisingly, the high-resolution RT maps confirmed the excellent correlation with chromosome G+C isochore bands (Costantini and Bernardi, 2008; Woodfine *et al.*, 2004; Figure 2). In a second level of analysis, the RT value assigned to each probe in the microarray was compared to the DNA sequence features, reinforcing the correlation between GC content and early RT. It should be noted that this approach is very reliable for the identification of early and late-replicating domains, but not for mid-S replicating sequences, because an experimental result suggestive of mid-replication could also be caused by "pan-S" replication or by asynchronous allele replication of that specific sequence.

This limitation can be overcome with a technical variation in which cells are pulse-labeled with BrdU in early-, mid- and late-S phase. The BrdU-labeled nascent DNA strands are then purified and hybridized to DNA arrays. This approach has been used to determine the RT of HeLa cells using microarrays covering the 1% of the human genome studied by the ENCODE pilot

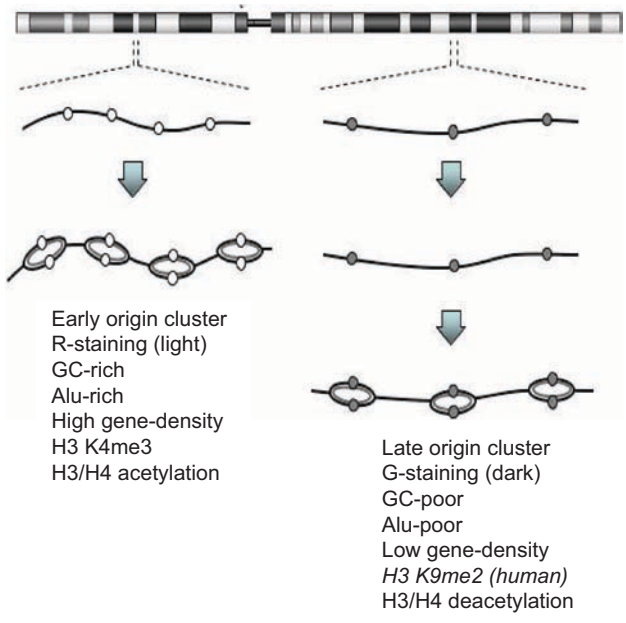

Figure 2. Genomic features that correlate with RT. Schematic of a human chromosome displaying Giemsa-staining banding. Reverse bands (R) correspond to early-replicating domains, and dark bands (G) correspond to late-replicating domains. Each domain consists of one or several clusters of origins arranged to fire simultaneously. Early origins are represented as white circles, late origins as gray circles

project (The ENCODE Project Consortium, 2007). In this study, 80% of the analyzed regions replicated with a specific RT (with approximately 1/3 being ascribed to early-, 1/3 to mid- and 1/3 to late-replicating patterns), while the remaining 20% of the genome displayed "pan-S" replication, mainly caused by interallelic variation (Karnani *et al.*, 2007). Because the DNA sequences covered by ENCODE are scattered throughout the genome, this study was not optimal to define large chromosomal replication domains, but instead it offered the possibility to directly compare RT with the large volume of data available about the same DNA regions. Notably, early RT regions were enriched in activating histone modifications whereas late RT regions were enriched in repressing ones (The ENCODE Project Consortium, 2007; Karnani *et al.*, 2007).

The RT of the mouse genome has also been mapped at approximately 22 kb resolution in a murine leukemia cell line (Farkash-Amar *et al.*, 2008) and at 6 kb resolution in pluripotent embryonic stem cells before and after differentiation to neural precursor cells (Hiratani *et al.*, 2008). Both mapping efforts confirmed the chromosomal organization in distinct domains with specific RT, and one of them suggested that up to 9% of the genome displays pan-S replication due to allelic variation, approximately half of the value reported in the human ENCODE study (Farkash-Amar *et al.*, 2008).

One of the two mouse genome RT studies (Farkash-Amar *et al.*, 2008) revealed that genes with "housekeeping" functions (metabolism, transport, transcription, cell cycle) are preferentially located in early-replicating domains, whereas tissue-specific genes not expressed in the lymphocytes used in the study, fall in the mid- and late-RT categories. Interestingly, genes involved in stress response and apoptosis, which are not expressed during normal proliferation but need to be accessible for rapid transcription in special circumstances, also fall within early-replicating domains. Therefore a correlation may exist between early RT and the *potential* for gene expression (chromatin structure) rather than actual expression. An example can be found in the major histocompatibility complex (MHC) class II region, an AT-rich isochore located near the centromere of human chromosome 6. These features are suggestive of late RT, but the region is very dense in inducible genes and it actually displays early- to mid-RT (Takousis *et al.*, 2007).

The other report (Hiratani *et al.*, 2008) mainly focused on the issue of RT changes occurring during cell differentiation, a notion that had been suggested for specific groups of genes (Hiratani *et al.*, 2004; Perry *et al.*, 2004). First, it was shown that the same chromosome replication domains, ranging in size from 0.2 to 2 Mb, are conserved in three independent embryonic stem cell lines. Then, global RT changes were observed during differentiation from stem to neural precursor cells. Embryonic stem cells have more replication domains, smaller in size, than neural precursors. During differentiation, some of the smaller domains are fused into larger ones. This phenomenon, termed "replication domain consolidation" affects a significant (20%) fraction of the genome. The domains that switch their RT have an unusual sequence composition, lacking a direct correlation between high GC content and gene density. Domains with inverse correlations between GC content and gene density are probably exposed to two opposing forces pulling towards early- and late-RT, and transcriptional activity may incline the balance towards one or the other (Hiratani *et al.*, 2009). During differentiation, GC-poor/gene-rich domains experience early-to-late transitions and GC-rich/gene-poor regions experience late-to-early transitions. After consolidation, the resultant larger replication domains show a high degree of alignment with chromosome isochore regions. Strikingly, these changes are reversible: the RT program of induced pluripotent stem (iPS) cells, reprogrammed from tail fibroblasts, was similar to that of embryonic stem cells (Hiratani *et al.*, 2008).

The human and mouse genome-wide analyses of RT have added more complexity to the connection between replication and transcription. Genes that changed their RT during differentiation from late to early were frequently up-regulated, whereas those in early-to-late switching domains remained unchanged or were down-regulated. However, the authors also report exceptions to this rule and their statistic analysis suggests that a direct correlation between the RT of a gene and its probability of expression is restricted to the second half of S-phase (Hiratani *et al.*, 2008). Regardless of the putative influence of gene expression in the RT of large chromosomal domains, origin activity is likely facilitated at the local level by transcription factors or transcriptional events (Aladjem *et al.*, 2006).

In the next section, the interconnection between RT and gene expression is discussed in the context of chromatin structure, and a model that integrates both programs in a compartmentalized cell nucleus is proposed.

RT, chromatin structure and transcription: nuclear position sets the mark

DNA sequence is not sufficient to establish the timing of replication, as indicated by the differential RT of imprinted alleles or the active/inactive X-chromosomes. DNA methylation also appears to be dispensable, because the RT of several individual loci did not change in DNA methyltransferase (DNMT)-deficient ES cells (Gribnau *et al.*, 2003; Jorgensen *et al.*, 2007). Instead, early- and late-replication could be determined by

different epigenetic signatures in the chromatin. Open, transcriptionally active euchromatin domains would replicate earlier than closed heterochromatin domains, probably because they compete for limiting DNA replication factors. In support of this idea, early RT domains in the human and mouse genome are enriched in histone marks characteristic of active chromatin (Karnani *et al.*, 2007; Hiratani *et al.*, 2008). The presence of repressive chromatin marks in late RT domains has been reported in the human ENCODE RT map (Karnani *et al.*, 2007) but not the mouse map (Hiratani *et al.*, 2008). The reason for this discrepancy is not clear, and it will be interesting to learn whether the observed correlation between repressive marks and late RT is still significative when the analysis is extended from the 1% ENCODE representation to the totality of the human genome.

The chromatin modification that correlates more strongly with early RT is histone acetylation. When Aladjem and colleagues inserted the human beta-globin locus into a murine chromosome, its expression and RT depended on the insert orientation. The transcription-competent orientation was packed in chromatin with acetylated histones and replicated early, whereas the transcription-inactive orientation was packed in non-acetylated histones and replicated late (Lin *et al.*, 2003). The latter effect can be reversed by the additional introduction of a functional replicator element that promotes initiation of DNA replication, accompanied by histone acetylation (Fu *et al.*, 2006). More recently, Cedar and colleagues used a related system to show that the recruitment of histone acetyl transferases (HATs) to the human beta-globin locus in a non-erythroid cell line changed its RT from late to early. Conversely, tethering a histone deacetylase (HDAC) to the locus in an erythroid cell line was sufficient to change its RT from early to late (Goren *et al.*, 2008). The correlation between histone acetylation and RT is reinforced by the fact that an HDAC inhibitor is sufficient to remove the replication imprinting signals in two loci on human chromosome 11p (Bickmore and Carothers, 1995). It is interesting to note that a HAT protein called HBO1 was originally identified via its interaction with the human origin recognition complex (Burke *et al.*, 2001; Iizuka and Stillman, 1999). HBO1 regulates origin licensing (Iizuka *et al.*, 2006), cooperating with CDT1 in the loading of the MCM helicase onto replication origins (Miotto and Struhl, 2008).

Besides histone acetylation, the influence of other DNA or histone modifications on RT is less certain and may be restricted to specific chromosome structures. In several murine ES cells lacking DNMT, histone methyltransferases and chromatin modifiers like NuRD or Dicer, changes in RT were observed in repetitive satellite DNA but not in many individual gene loci (Jorgensen *et al.*, 2007). Therefore, the RT of chromosome domains that are rich in satellite DNA, such as the pericentric regions, may be controlled by histone acetylation and additional parameters.

If chromatin structure influences the time of replication, is the RT program a passive element or does it serve any function? One exciting possibility is that after the RT program is established in G1, it could *in turn* dictate chromatin structure and transcriptional competence during S phase, creating an autoregulatory loop that would add stability to the system. This scenario would require that different types of chromatin were assembled onto early- and late- replicating DNA. Experimental evidence to support this notion comes from microinjection experiments conducted at different moments during S phase: a reporter gene microinjected into early S nuclei was assembled into nucleosomes with acetylated histones and expressed with higher efficiency than the same DNA microinjected in late-S nuclei, which was preferentially packed into deacetylated histone nucleosomes (Zhang *et al.*, 2002). This result, which strongly suggests that the timing of replication has the capacity to influence chromatin structure, has been supported by the recent demonstration that DNA sequences packaged in nucleosomes characteristic of late replication (i.e. containing deacetylated histones) can be reassembled with acetylated histones after shifting their RT to early S phase, and vice versa (Lande-Diner *et al.*, 2009).

An emerging concept is that RT and chromatin structure are both the consequence of a higher-order chromosome organization that is intimately related to nuclear position. In interphase, each chromosome occupies a distinct territory of the nucleus, anchored by the binding of centromeric and telomeric domains to the nuclear envelope or the nucleoli. The rest of the chromosome domains maintain some ability to move within the nucleoplasm, with the restrictions imposed by the anchor sites. Lamins extend from the nuclear envelope towards the interior, probably helping to structure dynamic subnuclear compartments that may contain high concentrations of the specific proteins required for replication, transcription or repair (reviewed by Gilbert and Gasser, 2006). These nuclear domains may be defined in part by the interactions between DNA and the nuclear lamina (Guelen *et al.*, 2008). Restricting DNA replication to specific nuclear compartments with defined chromatin-packing characteristics may be fundamental to maintain the transcriptional program. After all, the molecular mechanism of DNA replication involves chromatin disassembly and reassembly, with the potential to erase and/or reset histone modifications that are necessary to maintain gene expression programs. Establishing a layman parallelism, it could be argued that because we remove our clothes daily before going to sleep, every day provides an opportunity

to change our clothing style. This is technically true, but the next morning we are most likely to wear the type of clothes immediately available to us – those in our wardrobe. So, despite a daily opportunity to redefine styles, we perpetuate our dress code routine. Nuclear compartments would act as molecular wardrobes, exerting a strict influence in the type of chromatin packed onto newly synthesized DNA, therefore maintaining its epigenetic features like transcriptional competence and RT (Figure 3).

This model fits with the fact that the RT program is established in early G1, as this is the time of nuclear repositioning of chromosomes (Dimitrova and Gilbert, 1999), and may also account for the different patterns of BrdU incorporation during S phase (Figure 1). Additional support comes from an assortment of studies. The Igf2-H19 locus imprinting center directs the movement of the two alleles to distinct nuclear compartments where they acquire different RT (Gribnau *et al.*, 2003). In a recent systematic FISH analysis of over 60 very-early- and very-late-replicating foci, the former localized to nuclear interior sites whereas the latter had a preference for the nuclear periphery (Grasser *et al.*, 2008). Interestingly, changes in RT occurring during differentiation are also accompanied by shifts in nuclear position (Hiratani *et al.*, 2008).

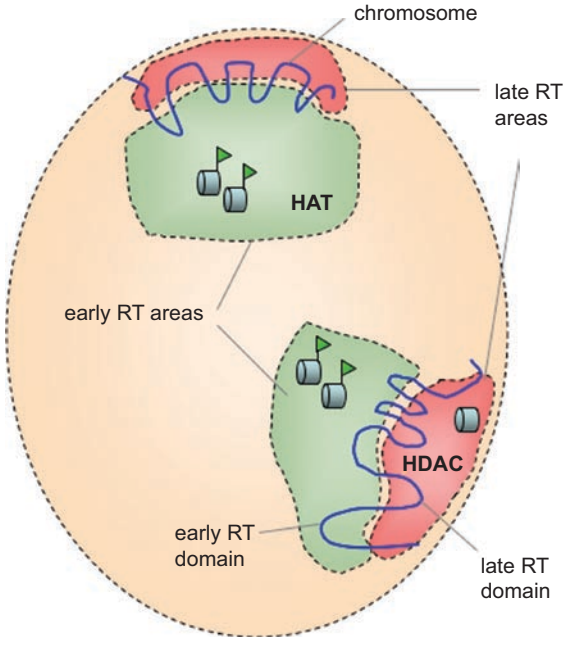

Figure 3. Nuclear position, chromatin structure and RT: a model. In this oversimplified, speculative model, certain compartments of the nucleus (represented as green/light gray areas) would be preferential zones for early replication and the assembly of "open" chromatin marked by acetylated histones (flags), while other compartments (red/dark gray areas) would favor late replication and the assembly of "repressed" chromatin marked by histone deacetylation and possibly other modifications. HAT suggests enrichment in histone acetyl transferases, and HDAC suggests enrichment in histone deacetylates in the indicated areas. The nature of the boundaries between compartments is unknown. Chromosomes (blue lines), known to occupy defined sites within the nucleus, would have domains located in both types of regions.

There is an additional, probably unrelated advantage of having a temporal program: it imposes certain limits to the number of replication forks that may be active at a given time. An abnormally low density of active origins and forks may cause aneuploidy in human cells (Ekholm-Reed *et al.*, 2004). On the other hand, the simultaneous activation of too many origins leads to a high frequency of stalled/collapsed forks that could activate the DNA damage response checkpoint and prevent cell proliferation (Syljuasen *et al.*, 2005). Perhaps the RT program contributes to maintain genomic stability by ensuring that in DNA replication, "slow and steady wins the race".

Bad timing? The RT program in health and disease

Even in healthy cells, there are inherent risks associated with the RT program. Late-replicating regions and the transition points between early- and late-replicating domains are likely more prone to suffer replicative stress. The molecular composition of the boundaries between replication domains needs to be explored in more detail, but they could contain fork-pausing or barrier elements to prevent forks from the early domains to progress into the late domains. Such fork barriers are hot spots for DNA breaks and chromosome rearrangements (reviewed by Rothstein *et al.*, 2000). Interestingly, several cancer and other disease-related genes are located in RT-switching regions and may therefore be more prone to inactivation (Watanabe *et al.*, 2002). Amplicons in chromosome 11q have also been mapped at RT-switching regions (Watanabe 04). Late-replicating regions are rich in single-nucleotide polymorphisms (Watanabe *et al.*, 2002) and display an increased mutation rate (Stamatoyannopoulos *et al.*, 2009). These intriguing properties of late-replication domains could be due to the inherent difficulties of replicating heterochromatic DNA, which could result in an accumulation of ssDNA that is more susceptible to damage. This mechanism would mainly affect genes that are sequestered in repressed nuclear compartments (Stamatoyannopoulos *et al.*, 2009). It should also be noted that the majority of common fragile sites and rare fragile sites are late-replicating (reviewed by Durkin and Glover, 2007).

Unscheduled alterations in the RT program may delay the process of chromosome condensation,

increasing chromosome instability. This sequence of events has been shown in a number of tumor cell lines and primary tumor samples (Smith *et al.*, 2001). Changes in RT have also been reported at the loci responsible for relatively rare conditions such as the Tourette, Di George and velocardiofacial syndromes (State *et al.*, 2003; D'Antoni *et al.*, 2004) and in a number of blood disorders (Amiel *et al.*, 2002). Allelic synchronization is frequently lost in cells derived from patients with various forms of leukemia, correlating with higher frequency of aneuploidy (Korenstein-Ilan *et al.*, 2002). In non-Hodgkin lymphoma, asynchronous allelic replication is indicative of higher risk of relapse (Amiel *et al.*, 2001). As the list of RT alterations associated to disease continues to grow, it will become very important to ascertain the specific contribution of RT changes to these phenotypes.

In summary, the control of RT is an active field of research that extends from basic cell biology to biomedicine. Naturally, more experiments will be needed to confirm the intriguing correlations between RT, chromatin structure, and nuclear position. The connection between RT and differentiation is a particularly interesting avenue of research. Because murine stem cells have specific chromatin and RT signatures (Azuara *et al.*, 2006; Hiratani *et al.*, 2008), a hot topic right now will be the elucidation of the RT maps of human stem and/or iPS cells, to gain a better understanding of the pluripotency status that is central to the future cellular therapy.

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