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Replication timing and transcriptional control: beyond cause and effect. Part II

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SUMMARY

Replication timing is frequently discussed superficially in terms of its relationship to transcriptional activity via chromatin structure. However, so little is known about what regulates where and when replication initiates that it has been impossible to identify mechanistic and causal relationships. Moreover, much of our knowledge base has been anecdotal, derived from analyses of a few genes in unrelated cell lines. Recent studies have revisited longstanding hypotheses using genome-wide approaches. In particular, the foundation of this field was recently shored up with incontrovertible evidence that cellular differentiation is accompanied by coordinated changes in replication timing and transcription. These changes accompany subnuclear repositioning, and take place at the level of megabase-sized domains that transcend localized changes in chromatin structure or transcription. Inferring from these results, we propose that there exists a key transition during the middle of S-phase and that changes in replication timing traversing this period are associated with subnuclear repositioning and changes in the activity of certain classes of promoters.

INTRODUCTION

In every multi-cellular system examined, early replication and transcription are strongly correlated. This longstanding correlation has recently been confirmed statistically in *Drosophila*, human and mouse cells [1–8]. Moreover, an extensive developmental regulation of replication timing was rigorously demonstrated, associated with changes in transcription [8]. However, progress in understanding the underlying mechanisms remains sluggish. The relationship is clearly indirect, since 10–20% of late-replicating genes are expressed and some genes change transcription without changes in replication timing and vice versa [8]. The simplest explanation is that replication timing is related to features of chromatin and nuclear architecture rather than transcription per se.

Two genres of models, in which either chromatin dictates replication time or vice versa, were illustrated in a prior review (Figure 1 of [9]) and need no revision. Advances in the intervening years have underscored the complexity and variety of mechanisms by which chromatin can influence replication timing, with most effects surprisingly modest and some paradoxical. Ironically, some of our deepest mechanistic insights have come from budding and fission yeasts, yet there is no evidence for an association between replication timing and transcription in these unicellular organisms [10]. On the other hand, evidence that different types of chromatin are assembled at different times during S-phase remains indirect. A third genre of

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models suggests that replication timing is intimately linked to the 3-dimensional (3D) organization of the genome [11–14]. Recent findings have strengthened this association [15–17], but the relationship of 3D chromosome organization to chromatin states and transcription remains as elusive as replication timing [18].

Here, we summarize recent progress toward understanding this complex liaison between copying and reading genetic information. We begin with some basic facts about replication control that necessitate framing any discussion of the significance of replication to gene expression in terms of large chromosomal domains. Next, developmental changes in replication timing are discussed, which involve large chromosome segments and accompany spatial repositioning and transcriptional changes for certain classes of promoters. We will then discuss recent experiments that address mechanisms by which chromatin influences replication time and vice versa. Finally, we will discuss how these multiple mechanisms might be related to 3D genome organization.

Replication timing is regulated at the level of large chromosomal domains

In order to discuss how replication timing might influence transcription, it is important to appreciate that replication is regulated at the level of large chromosomal domains. In mammals, rates of fork elongation are on average 1–2 kb/minute and replication is bidirectional [19]. Hence, a single replicon (chromosomal DNA replicated from a single origin) will duplicate 100–200 kb within one hour of a 10 hour S-phase. Moreover, replication frequently proceeds via the nearly synchronous firing of several adjacent origins, resulting in the rapid coordinate duplication of multi-replicon, megabase-sized segments of chromosomes [19]. These segments replicate reproducibly at characteristic times during S-phase, punctuated by origin-less regions through which forks move unidirectionally until they encounter a fork from a neighboring replication domain [4,6–8,19–22]. Hence, any influence of replication timing over chromatin structure must extend over several hundred kilobases.

Mechanisms by which chromatin influences replication time are frequently discussed in terms of the effects of altered histone modifications on the accessibility of specific replication origins to the initiation machinery. However, such simplified models are difficult to reconcile with the concept of origin efficiency [23,24]. Any particular origin is utilized in only a fraction of cell cycles with each cell using a different collection of origins [21,24,25]. Hence, the inactivation of any particular origin may be of little consequence to replication time and frequently will increase the efficiency with which an adjacent origin is used [24]. On the other hand, a localized chromatin change that results in the earlier firing of an origin could potentially cause a replicon-sized shift in replication timing, but if confined to a single origin it would likely fire in only a fraction of cells. In fact, several observations suggest that replication timing is independent of where replication initiates. The human β -globin locus frequently replicates from one of two closely spaced origins while the mouse locus uses many widely dispersed origins yet replication timing is conserved [24]. Moreover, the replication time of chromosomal domains is re-established in each cell cycle prior to and independent of origin site specification [12,26]. Together, these results suggest that any working model for the influence of chromatin over replication timing must account for stable replication timing despite variable origin selection between individual cells.

Replication timing is regulated during development

If replication timing is related to transcription, then it should be subject to developmental control. The last several years witnessed first a challenge to and then a confirmation of the generality of replication timing changes during development. Until a few years ago, evidence other than during mammalian X-chromosome inactivation was restricted to a small number of genes whose replication time had been compared primarily between established, non-isogenic,

transformed cell lines [9]. Moreover, many genes replicated at similar times in different cell types [9]. More recently, comprehensive surveys found that replication timing correlates quite strongly with static sequence features of mammalian chromosomes such as isochore GC content and gene density [3,4,27], raising legitimate questions regarding the extent to which replication timing changes during development [16,27]. In fact, a microarray-based comparison of human chromosome 22 between fibroblast and lymphoblastoid cells revealed that only 1% of this chromosome differed in replication time [4]. It then became painfully obvious that there was not a single documented case of an autosomal replication timing change in response to differentiation cues analogous to what was seen during X-chromosome inactivation.

Dynamic changes in replication timing were first confirmed for several individual gene loci during mouse embryonic stem cell (ESC) differentiation to neural precursor cells (NPCs) [28,29]. Approximately one fourth of genes queried in these studies showed measurable changes in replication time and these genes all resided within AT-rich isochores, providing a potential explanation for why the GC-rich human chromosome 22 showed so few differences [4]. However, these studies were not sufficiently comprehensive to infer the frequency of such changes. The question was finally put to rest with a genome-wide study of replication timing during mouse ESC differentiation to NPCs in three independent cell lines using two differentiation schemes [8]. Replication timing changes were highly reproducible and affected up to 20% of the genome with changes at the level of large domains averaging 600 kb. As expected, replication-timing changes were coordinated with changes in transcription and furthermore accompanied subnuclear repositioning [8], supporting an earlier observation for the *Mash1* locus [30]. Most recently, extensive replication timing differences were reported between *Drosophila* Kc (embryonic origin) and C18 (from wing imaginal discs) cell lines [31].

Developmental changes: unique isochores facing opposing forces?

The mouse genome-wide study showed that domains that changed replication timing were AT-rich and above a certain threshold of LINE-1 transposon density [8], consistent with an earlier study [28]. Unexpectedly, it further revealed that these domains showed inverse correlation between GC content and gene density, which generally correlate with each other (Figure 1a). In general, isochore AT content is strongly associated with LINE-1 density, proximity to the nuclear periphery and late replication ([8] and references therein), which may involve chromatin association with the nuclear lamina [32–34]. In contrast, high gene density is associated with transcription, proximity toward the nuclear interior (where RNA polymerase II transcription factories are enriched [35,36]) and early replication. Therefore, depending on their transcriptional activity, chromosomal domains with inverse correlation between GC content and gene density (e.g. AT-rich/gene-rich isochores) may experience two opposing physical forces that influence their radial positioning and replication timing: an as-yet-undefined isochore sequence-based force (toward the nuclear periphery and late replication) and a transcriptional activity-based force (toward the interior and early replication). Moreover, mouse ESCs have substantially more smaller replication domains and do not show the strong relationship between replication timing and isochore GC content as compared to differentiated cells. This unusual replication domain organization is re-established when adult fibroblasts are induced to the pluripotent state (induced pluripotent stem [iPS] cells; [37]), suggesting that it is characteristic of the pluripotent state [8]. After ESC differentiation, early replication timing correlates better with GC content and radial subnuclear positioning, while maintaining its correlation to transcription and gene density. This suggests that an isochore sequence-based force becomes increasingly influential in shaping the functional and spatial organization of the genome upon differentiation.

Distinct classes of genes differ in their relationship to replication timing

While correlative, recent microarray studies have allowed us to sharpen hypotheses regarding the relationship between replication timing and transcription. It is now clear that the statistical relationship is similar across cell types and species and is confined to certain classes of genes. In both *Drosophila* and mouse, most genes replicate in the first third of S-phase and have an equally high probability of being expressed independent of their replication time within this period; a strong relationship between earlier replication timing and transcription is restricted to the ~25% of genes that replicate later during S-phase [1,8] (Figure 1b). In mammalian cells, early-replicating genes are enriched for high CpG-density promoters, while late-replicating genes are enriched for low CpG-density promoters [8] that maintain their repressed state even upon loss of DNA methylation or treatment with TSA [38]. Analyses of mESC differentiation revealed that high and low CpG-density promoters, which generally possess strong and weak promoter activity, respectively, showed distinct behaviors upon switching to a late-replicating environment, only CpG-poor promoters showing higher tendency toward transcriptional down-regulation [8]. Thus, the occasional strong promoter that finds itself located in a replication timing “switching” domain may “come along for the ride” but be unaffected by the replication timing change. This is consistent with reports in several systems that strong promoters can overcome heterochromatin silencing ([8] and references therein) and the observation that tethering a chromosomal region to the nuclear periphery represses some genes but not others [39–41]. Overall, these results reinforce the notion that a strong association exists between replication time and transcription for specific classes of genes.

Alterations in chromatin structure induce modest changes in replication timing

Several studies suggest that chromatin modifications directly regulate replication timing, but the effects of any particular modification are relatively minor. Chemical inhibition of histone deacetylases (HDAC) can partially advance replication timing of several mammalian genes [42] as well as the Epstein Barr Virus mini-chromosome [43], while over-expression of a chromatin remodeling complex NoRC delays replication of rRNA genes [44]. In budding yeast, silent chromatin proteins SIR3 [45] and the HDAC Rpd3 [46,47] delay the firing of specific origins, while tethering histone acetyltransferase (HAT) partially advances origin firing [46]. Recently, HAT/HDAC-tethering to the human β -globin origin in mouse cells caused similar partial changes (~20% of S-phase) [48]. Furthermore, modest changes in replication timing were detected for pericentric heterochromatin in Dnmt1, G9a, Eed, and Dicer mutant mouse ESCs, but rather surprisingly, 20 gene loci analyzed were unaffected [49]. In addition, a Suv39h1/h2 lysine methyltransferase (KMTase) mutation showed cell-type specific effects, slightly advancing replication timing of pericentric heterochromatin in embryonic fibroblasts, while slightly delaying it in ESCs [49,50]. Indeed, the role of specific histone modifications in regulating replication timing is still difficult to fathom, since even origins that fire synchronously within a replication domain have different histone modifications [51].

An elegant study in fission yeast provides our first glimpse into the mechanisms by which heterochromatin regulates replication timing in different contexts [52]. Heterochromatic pericentromeres and the *mat* locus are early-replicating [53] in a Swi6-dependent manner [Swi6: an HP1 (heterochromatin protein 1) ortholog] [52]. In a Swi6 mutant, late replication of the pericentromere but not the *mat* locus is dependent on the KMTase Clr4 [a Su(var)3–9 ortholog], whereas neither Swi6 nor Clr4 contributed to late replication of subtelomeric heterochromatin [52]. Importantly, Swi6 stimulated loading of the replication factor Sld3 onto origins at the pericentromere and the *mat* locus in a manner dependent on Dfp1 (Dbf4)-dependent kinase (DDK), an essential kinase required for the initiation of replication. Despite their varied regulation, targeting of Dfp1 advanced replication timing of all three loci.

Together, these results support the model (Figure 1 of [9]) that chromatin can control the accessibility of initiation factors to pre-replication complexes, but also underscore the complexity of the underlying mechanisms. To date, no epigenetic mark has been shown to correlate with replication timing significantly better than transcription itself [8,31]. Notably, however, the effects of most chromatin manipulations are modest and context-dependent, with a few exceptions [43,44,54,55].

Does replication timing affect chromatin structure?

Since chromatin is assembled at the replication fork, an appealing scenario is that replication timing dictates chromatin states that in turn regulate replication timing in the subsequent cell cycle, providing a means of epigenetic inheritance during somatic development. Moreover, since replication is regulated at the level of replicons, a change in replication timing could rapidly transmit a change in chromatin state to many genes simultaneously. Clearly this attractive model merits a definitive test, but it is currently impossible to manipulate replication timing without affecting other properties of a chromatin domain. The most compelling, albeit indirect, evidence that different chromatin structures are assembled at different times during S-phase is that reporter plasmids injected into early or late S-phase mammalian nuclei assembled into hyper- or hypo-acetylated chromatin, respectively [56]. This result prompted speculation that the replication fork scaffold protein PCNA might recruit different chromatin modifiers at different times during S-phase to assemble different types of chromatin [57]. Unfortunately, although dozens of proteins localize to replication forks, only HDAC2 [58] and MBD2-MBD3 [59] have been found to localize specifically to sites of late-replicating chromatin and both of these studies examined overexpressed epitope-tagged proteins. Hence, the existence of temporally regulated chromatin assembly mechanisms remains an attractive but unsubstantiated hypothesis.

Large replication timing changes accompany radial subnuclear repositioning

In every case examined, dynamic developmental changes in replication timing accompany subnuclear repositioning [8,30]. Although a genome-wide survey of such relationship is currently impractical, spatial patterns of DNA replication in the nucleus change dramatically as cells move through S-phase, demonstrating a global coupling of subnuclear repositioning with replication-timing changes [19,57] (Figure 1b). Moreover, replication timing is re-established during early G1-phase at the timing decision point (TDP), coincident with the repositioning of chromosomal domains in the nucleus after mitosis [12,13]. Consistently, chromatin mobility is relatively high during the first 1–2 hours of G1-phase, after which it is locally constrained through the remainder of interphase [60,61]. Moreover, inducible targeting of loci to the nuclear lamina requires passage through mitosis and takes place during late telophase to early G1-phase [39,40]. Together, these results predict that, during differentiation, subnuclear repositioning takes place at the TDP within the G1-phase preceding changes in replication timing. Testing this prediction will require an evaluation of the sequence of events during intermediate steps of differentiation. Notably, G1-phase length is highly variable between cell types, which may influence the extent to which nuclei are reorganized before replication initiates. In particular, the extremely short G1-phase in ESCs could result in replication starting before isochore sequence-based chromosome reorganization mechanisms (discussed earlier) are completed, explaining the larger number of smaller replication domains that do not replicate according to their isochore sequence properties.

The middle of S-phase represents a period of dramatic change, including: (1) a dramatic change in the spatial distribution of replication sites (Figure 1b, pattern II to III) [19,57]; (2) a transition from R to G band replication [17,62]; (3) a temporary reduction in replication fork movement [62]; (4) a sharp decline in the density of genes being replicated [5]; and (5) the onset of a

relationship between replication timing and transcription (Figure 1b) [8]. Thus, changes in replication timing that straddle mid S-phase are likely to accompany movements between subnuclear compartments (Figure 1b) or even formation of compartments that are more difficult to reverse once established (e.g. formation of a Barr body during X-chromosome inactivation [63]). In contrast, even large changes in replication timing that are restricted to the first half of S-phase may be less consequential (Figure 1b). Consistently, several early-replicating loci such as Oct-4 become slightly later replicating upon ESC differentiation but do not straddle mid S-phase [29,64] nor accompany radial subnuclear repositioning [8,65]. In addition, asynchronously replicated homologs of imprinted or monoallelically-expressed genes can show relatively small replication timing differences during mid S-phase but may exhibit significant radial position differences [42,66,67].

Conclusions and future directions

Our understanding of replication timing remains a fragmented set of half-truths that are currently impossible to integrate into absolutes. In fact, among the many experimental manipulations performed over the years, it is arguably only G1 nuclei before the TDP that display a globally disturbed replication-timing program [12,13]. In contrast, the majority of chromatin manipulations have resulted in relatively minor effects. Moreover, modifications of chromatin proteins generally persist through mitosis or are reinstated onto chromatin during mitotic exit, prior to the TDP [50,68–70]. This suggests that various chromatin structures analyzed are not sufficient to dictate the global timing program but their effects on replication timing may represent a secondary, fine-tuning role. Our current view is that replication-timing changes through the middle of S-phase are qualitatively distinct and more likely to involve subnuclear compartment changes (Figure 1b). Testing this hypothesis will require deciphering the principles of how nuclear genome reorganization occurs in early G1-phase, particularly the influence of isochore sequences and transcription. Since the molecular nature of these principles is still a mystery, comparative genome-wide analyses will continue to provide important insights to sharpen hypotheses. Studies of the effects of chromatin structures on replication timing regulation will continue to be important, particularly those that can tease out relationships to replication initiation mechanisms [52]. Also important are experiments to determine the extent to which replication time can influence chromatin assembly, for which at present evidence is scant. Finally, there are clearly non-transcriptional roles for replication timing such as the maintenance of genome stability [43,71,72]. At present, it appears that we are each collecting separate views of complex mechanisms linking genome structure and function, waiting for our various half-truths to intersect and reveal a more complete picture.

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Figure 1. Relationship between isochore properties and replication timing regulation, subnuclear position, and transcription

(a) Isochores with unusual sequence properties are subject to replication timing regulation. The mammalian genome is partitioned into isochores with different GC content and gene density. Isochores that are high in both [(GC%, gene density)=(High, High)] are replicated early in S-phase, while those that are low in both [(GC%, gene density)=(Low, Low)] are replicated late in S-phase. Exceptional isochores in which the two properties are inversely related [(GC%, gene density)=(Mid, Low) or (Low, Mid)] are frequently subject to replication timing regulation during mouse ESC differentiation (speculatively labeled "Facultative Heterochromatin") [8]. During neural differentiation of mouse ESCs, the former [(GC%, gene

density)=(Mid, Low)] tend to change from late to early replication, while the latter [(GC%, gene density)=(Low, Mid)] show an opposite directionality.

(b) Changes in replication timing that traverse the middle of S-phase accompany changes in subnuclear position and transcriptional potential. Replication early in S-phase (patterns I and II) takes place within the interior euchromatic compartment, while replication later in S-phase (patterns III, IV, and V) takes place at the nuclear periphery (pattern III), the nucleolar periphery (pattern III), and at internal blocks of heterochromatin (patterns IV and V) [19,57]. Note the dramatic transition from “euchromatic” to “heterochromatic” replication pattern during mid S-phase (from pattern II to III). In addition, a strong relationship between replication timing and transcription is observed for genes that replicate during mid to late stages of S-phase (“Strong Correlation”) [8]. In contrast, genes that are replicated in the first third of S-phase have equally high probability of being expressed (“No correlation”) and thus even large changes within this period may not accompany changes in subnuclear position or transcriptional competence. The figure illustrates how the durations of different spatial replication patterns (dotted lines) may relate to the probability of transcription [8,12]. These relationships imply that genome-wide replication timing analyses provide a means to infer changes in subnuclear position and transcriptional potential [8]. Photos are deconvolved images from Chinese hamster cells pulse-labeled with BrdU at different times during S-phase (J.L. and D.M.G., unpublished).