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A new light on DNA replication from the inactive X chromosome

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Abstract

While large portions of the mammalian genome are known to replicate sequentially in a distinct, tissue-specific order, recent studies suggest that the inactive X chromosome is duplicated rapidly via random, synchronous DNA synthesis at numerous adjacent regions. The rapid duplication of the inactive X chromosome was observed in high-resolution studies visualizing DNA replication patterns in the nucleus, and by allele-specific DNA sequencing studies measuring the extent of DNA synthesis. These studies conclude that inactive X chromosomes complete replication earlier than previously thought and suggest that the strict order of DNA replication detected in the majority of genomic regions is not preserved in non-transcribed, “silent” chromatin. These observations alter current concepts about the regulation of DNA replication in non-transcribed portions of the genome in general and in the inactive X-chromosome in particular.

Keywords

cell cycle; chromatin; DNA replication; epigenetics; inactive X chromosome

Introduction

Chromosome duplication in somatic metazoan cells proceeds in a consistent, pre-determined and tissue-specific order. Because the order of replication is remarkably consistent, it is possible to create maps dividing the genome into concomitantly replicating regions (“replication timing domains”) that are distinct for each cell type and differentiation stage [1]. Replication timing domains form distinct patterns in three-dimensional nuclear space and share common characteristics, including comparable frequencies of expressed genes, similar distributions of repetitive elements and equivalent incidences of chromatin modifications [2–4]. Although it is not immediately evident that a strict order of replication should be important for cell survival and genomic stability, pathological chromosomal rearrangements in several rare genetic disorders [5, 6] and in cancer cells [7–11] are often accompanied by altered replication timing.

The consistency and tissue-specificity of the replication timing program suggest that the order of replication reflects a fundamental property of chromatin [12] that might be associated with gene expression patterns [13]. Indeed, the early- and late-replication portions

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of chromatin exhibit contrasting properties. Early-replicating chromatin contains a high proportion of active genes, mostly resides internally in three-dimensional nuclear space and commonly exhibits a decondensed (“open”) conformation. Conversely, late-replicating chromatin mostly contains transcriptionally inactive regions, primarily resides in the nuclear periphery and is often densely packed. Since enzymes that facilitate tight chromatin packaging (such as DNA methyltransferases and histone methylases) are often highly abundant in the later stages of S-phase, replication in a pre-defined order can facilitate correct packaging of DNA [1, 3, 14]. Consistent with this suggestion, transcriptional activation during differentiation often associates with advanced replication time, whereas replication delay often accompanies transcriptional silencing [3, 15–17]. In concordance, a strict replication program is not established in metazoan embryos prior to the activation of transcription; in such embryos, replication is completed much faster than in somatic cells and both the location and timing of DNA replication appear random and stochastic. Replication slows down when cells start transcription and undergo chromatin remodeling, establishing a distinct order of replication [18].

Imprinted genes, which exhibit allele-specific patterns of gene expression, provide a clear example of the association between replication timing and transcriptional activity. Differentially expressed alleles often replicate asynchronously, the expressed allele replicating earlier than the silent allele [19–22]. Notably, replication of the two X chromosomes is almost entirely asynchronous in somatic mammalian female cells, which achieve dosage compensation by random inactivation of transcription from one of the two X chromosomes ([23]; see [24–27] for review). The inactive X chromosome resides in a distinct chromatin compartment (Barr body) and exhibits heterochromatic marks including trimethylation of lysine 27 on histone H3 (H3K27me3), histone hypoacetylation, incorporation of macroH2A and DNA methylation. Nucleotide incorporation patterns in the two X chromosomes [28] and bi-phasic patterns of replication measured by whole genome sequencing [29] both implied that X chromosome replication occurs late during the S-phase of the cell cycle.

Within the replication domains, DNA replication starts from distinct sequences termed replication origins. Each replication-timing domain contains multiple potential replication origins, which often start replication alternatively so that most replication origins are only utilized in fewer than one-fifth of cell cycles [12, 30, 31]. The organization of replication domains and the choice of replication origins within those domains seem to be determined independently [32]. In female mammalian cells, both X chromosomes share at least some replication origins [33], suggesting that the inactivated and active X might follow a similar spatial replication program at different times during S-phase.

Recently, several interesting findings have prompted the community to change the way it thinks about replication timing in general and the relationship between replication timing and X chromosome inactivation in particular. First, high-resolution visualization of DNA replication patterns had concluded that inactive X-chromosome duplication occurs more rapidly than expected, and is completed earlier in the cell cycle than previously thought [34]. Second, recent studies using novel sequencing technology provide support for the observed rapid pace of DNA synthesis, and suggest that the strict order of replication detected in the

majority of genomic regions is not preserved in the non-transcribed, “silent”, chromatin of the inactivated X-chromosome [35].

The inactivated X chromosome exhibits unique replication patterns

Casas-Delucchi and colleagues [34] recently reported experiments measuring the replication times of the active and inactive X chromosomes in mouse and human cells, identifying the inactivated X chromosome by hybridization with the X-inactive specific transcript (Xist) RNA or by specific histone marks such as H3K27Me3. These studies confirmed that the inactive X chromosome started replicating later than its active counterpart. However, in contrast to the active X chromosome, which replicated similarly to the autosomes in a discontinuous manner throughout S-phase, the bulk chromatin of the inactivated X chromosome replicated in an apparently synchronous manner within a limited time frame of about 1–2 hours from mid S phase. A drug that inhibits histone hyperacetylation, TSA, disrupted the rapid replication pattern.

Koren and McCarroll [35] employed genetic sequencing technology that allowed identification of chromosome-specific single nucleotide polymorphisms (SNPs) for precise mapping of the replication timing patterns in active and inactive X chromosome separately. Sequencing DNA from both parents and an offspring in two families, and using lymphocyte populations in which the same X chromosome was inactive in >90% of the cells, the SNP calling technique facilitated the identification of DNA sequences derived from each of the two (active and inactive) X chromosomes. The assignment of replication data to genomic regions from each chromosome confirmed that X chromosome inactivation was accompanied by a replication delay. As expected, the active X chromosome exhibited distinct replication timing domains, which were duplicated according to a clear and consistent order. Unexpectedly, with the exception of one region, no defined replication order was observed in the inactive X chromosome. Consistent with the patterns revealed by the cytogenetic visualization experiments described above, DNA replication progressed markedly faster in the non-transcribed portion of the inactive X chromosome. This rapid and apparently temporally random replication pattern could be explained by synchronous initiation from many replication origins in the inactive X chromosome and was similar to the replication pattern observed during early embryogenesis in organisms including flies and frogs, which do not activate transcription during the first several cell divisions. Further mapping suggested that autosomal inactive non-transcribing chromatin in imprinted, inactive portions of the genome also replicates faster and in an apparently random manner. While these observations are in apparent contradiction with the earlier reports suggesting that inactivated X chromosomes exhibit consistent banding patterns [26, 28, 36], they are consistent with the observations by Casas-Delucchi et al., and the different scales might reconcile those differences in future studies.

What might be the mechanism of synchronous replication?

The apparently synchronous mode of DNA replication within the inactive X chromosome requires a deviation from the current model whereby replication starts sequentially from a series of replication origin sequences. To start replication during the S-phase of the cell

cycle, replication origins bind a pre-replication complex that gradually recruits elements of the replication machinery [13, 30, 31, 37, 38]. The pre-replication complex is anchored by the origin recognition complex (ORC), which binds chromatin throughout interphase. During the G1 phase of the cell cycle, ORC recruits components of the pre-replication complex, which contains Cdc6, Cdt1, and the minichromosome maintenance (MCM) complex [31, 39–44] and renders chromosomes competent (licensed) for duplication. Components of the pre-replication complex are recruited onto chromatin in inactive forms and their activation depends on the recruitment of other proteins that are themselves phosphorylated by Dbf4-dependent and cyclin-dependent kinases (DDKs and CDKs, respectively) during the S-phase of the cell cycle. Replication complexes disassemble from chromatin following replication and reassemble after mitosis. The assembly and disassembly of pre-replication complexes ensures that the genome is replicated precisely once during each cell cycle, but the mechanisms that determine which specific pre-replication complexes are activated each cell cycle remain unclear [12, 13, 31, 37, 45].

The gradual assembly of pre-replication complexes in an inactive form and the requirement for an activation step are consistent with a model suggesting at the local, individual origin level, initiation of DNA replication is subject to accessibility or availability of one or several limiting factors that could govern origin choice and control the timing of initiation. Indeed, recent studies in yeast suggest that recruitment of proteins that interact with the pre-replication complex (Sld3, Sld7, and Cdc45) may act as an initiation-limiting factor, since initiation events were frequently shown to depend on the levels of those proteins or the activation of the DDK that facilitates their recruitment [46, 47]. In agreement with this, the frog orthologs of Sld3 and Sld2 (Treslin and RecQ4, respectively), their activating kinase Drf1 (Dbf4 dependent kinase in humans) and their interacting protein Cut5 (Dbp11 in yeast, TopBP1 in human) were identified as critical determinants of replication initiation rates as well as transcription during early *Xenopus* development [48]. Human CDC45 was proposed as a limiting factor governing origin activation in mammals [49]. In addition, proteins that affect higher-order chromatin structure, such as Rif1, which regulates the organization of chromatin loops, can catalyze concomitant initiation at a distinct group of replication timing domains possibly by modulating interactions with the nuclear lamina [50–52]. Synchronous initiation of DNA replication at distinct chromatin domains can result from concurrent recruitment of a limiting factor, or a timely removal of an inhibitory modification that prevents activation of pre-replication complexes prior to a particular time during S-phase. The nature of the limiting factor(s) and the exact mechanism by which synchronous replication is mediated await further investigation.

On the inactive X chromosome, synchronous replication could imply that the distinct program of origin activation does not occur within heterochromatin, and that replication initiates in a disorganized manner from random sequences, or that all origins within an expanded replication timing domain should initiate replication concomitantly. Since silenced portions of inactive X chromosomes do not engage in transcriptional activity, it is tempting to assume that those regions replicate in a random manner without assignment of distinct replication origins, analogous to the situation in embryos before the establishment of a transcription program [18, 53]. However, at least for some regions within the inactive X chromosome, replication starts from the same origins identified in the active X [33], and

evidence emerging from whole-genome studies is consistent with that observation ([54], K. Utani and MIA, unpublished observations). If the same origins are used, one should postulate synchronous assembly of pre-replication complexes or synchronous activation of licensed, inactive pre-replication complexes in the inactive X chromosome (Fig. 1). For example, if the local concentration of DDK and CDK targets within the chromatin compartment occupied by the inactive X chromosome is sufficiently high (Figure 2), many replication origins could start replication concomitantly, facilitating the rapid synchronous replication [46, 47].

Replication time at the inactive X can be determined by combined genetic and epigenetic factors

A combination of genetic and epigenetic features can determine the order of replication and the organization of replication domains, which reflects high-order chromatin association maps [1–4, 12, 13]. At the DNA sequence level, the timing of DNA replication at particular origins can be determined by both proximal and distal DNA sequences, such as yeast late replication elements [55], chromatin insulators and locus control regions [56–58]. While silencers and insulators often delay replication, potential replication origins can advance replication time while preventing the formation of heterochromatin [59]. At the epigenetic level, chromatin modifiers seem to play an important role in determining replication sites and timing. Examples include the effect of the histone deacetylase, Sir2, on replication and transcriptional silencing in yeast [60], in which the extent of ORC-origin interactions at distinct groups of origins correlates with variations in nucleosome architecture [61]; combined effects of *Drosophila* activating chromatin marks dictating ORC binding sites [62] affecting global locations and timing of replication-initiation events [17]; the role of histone acetylation [63] and the Myb complex [64, 65] in recruiting the *Drosophila* ORCs to replication origins; and the effect of transcription and differentiation factors in determining replication sites and timing in mammals [66–68].

It is not yet clear whether chromatin modifications or DNA sequences that delay or advance the timing of replication interact with replication origins directly to start replication or exert their influence epigenetically by altering chromatin structure. For example, the histone acetylase, HBO1, directly interacts with the Cdt1 component of the pre-replication complex and acts as its co-activator [69–71]; and transcription factor HOXC13 interacts with the *TOP1* and *MCM4* replication origins [72] and recruits members of the ORC to the *lamin B2* origin [73]. Since the inactive X chromosome exhibits a condensed structure and a distinct combination of chromatin marks [24], those features could affect accessibility to replication factors. Notably, the lack of histone acetylation is a hallmark of the inactive X [36], and interference with histone acetylation patterns affects X chromosome replication patterns [34]. Identification of the distinct factors that determine the replication landscape of the inactive X chromosome and possibly contribute to synchronous replication awaits a detailed delineation of replication origins and proteins that interact with those origins within the inactive X-chromosome chromatin compartment.

Xist ncRNA coats the inactive X chromosome and participates in the transcriptional silencing of gene expression [24, 27], notably forming a silent nuclear compartment devoid

of active chromatin marks that recruits silenced genes during the process of X inactivation [25]. Although Xist-mediated silencing is accompanied by delayed replication, the Xist RNA does not act simply to delay replication because murine cells in which Xist was deleted exhibit very late X chromosome replication [74]. The direct or indirect role of Xist ncRNA in determining the replication timing of the inactive X chromosome is consistent with a role of ncRNA in imprinting at other loci and with the observation that autosomal ncRNAs (for example, Asynchronous replication and Autosomal RNA on chromosome 6 – ASAR6) might play a role in a replication delay of entire autosomes in cancer cells [7, 75, 76]. These observations open the intriguing possibility that the order of replication of entire chromosomes is determined genetically by loci that encode long, intergenic non-coding RNAs [77]. The mechanism by which a non-coding RNA might interact with replication origins to induce synchronous replication at a specific time, or initiate replication from regions that are not licensed for replication, remains to be studied.

Rapid replication at the inactive X might implicate genomic stability

Since the inactive X chromosome can apparently replicate more rapidly than the rest of the genome, why does the rest of the genome replicate more slowly and what could be the purpose of the strict order of replication observed in the rest of the genome? As discussed above, slower replication that obeys a strict order may ensure coordination between replication and transcription and expression-specific chromatin packaging [63, 66, 78, 79] -- processes that might not be exhibited in the inactive X. However, replication timing clearly plays a role in insuring genomic stability, since altered replication timing domains are associated with genomic rearrangements in cancer [7–11], and the stress induced by rapid replication might lead to shattering of incompletely replicated whole chromosome (chromotripsis) [80]. Rapid replication can further affect the ability of cells to recover from replication stress, because such recovery is often accomplished by initiating replication from “dormant” replication origins adjacent to slow or stalled replication forks [12, 13, 81, 82]. Synchronous, frequent or promiscuous initiation at the inactive X chromosome might prevent efficient recovery from replication stress by depleting the reservoir of inactive origins that could be activated upon replication stress. In agreement with this, the inactive X chromosome exhibits a high mutation rate in cancer cells [83]. Higher rates of single nucleotide substitutions are also detected in late-replicating chromatin than in earlier replicating chromatin [84]. These observations are consistent with slower DNA repair in heterochromatin, presumably due to lower accessibility of repair factors.

Conclusions and outlook

The discoveries described above suggest that inactive X-chromosome replication is accomplished synchronously rather than through gradual activation of replication initiation events, and is not delayed until the very end of the S-phase. These new insights impact our understanding of how the division of the genome into chromatin compartments coordinates replication and transcription and preserves genomic stability. Obvious short term goals for future analyses include resolving apparent contradictions between the observations described above and previous cytological [26, 28, 36] and sequencing-based [29] findings, which suggest that the inactive X chromosome exhibits an intrinsic replication order and

replicates very late in S-phase. A detailed mechanistic understanding of replication within the inactive X will require combining origin sequencing and SNP calling to delineate inactive-X-specific replication origins, a task that can be achieved with current sequencing resolution ([54]; E. Bouhassira and MIA, unpublished). Once inactive X – specific origins are identified, DNA-protein interactions could be elucidated, delineating how those origins interact with components of pre-replication complexes to identify limiting factors.

Because replication seems more rapid and less confined to a pre-determined order in both inactive X chromosomes and non-transcribing early embryos, the general notion that replication from consistent origins in a strict order serves primarily to coordinate replication with transcription seems to hold. However, inactive X chromosomes differ from embryonic chromosomes in several important aspects and a simple association between late replication and absence of transcription clearly needs refinement. It would be important to figure out whether the apparently distinct and mutually exclusive forms of replication observed in active and inactive X chromosomes represent two distinct states or two points on a continuum of replication dynamics, ranging from orderly to truly stochastic replication. Further analyses are clearly required to tease out epigenetic determinants that govern those replication modes and determine their effects on genomic stability.

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Abbreviations

CDK	cyclin-dependent kinase
DDK	Dbf4-dependent kinase
ORC	origin recognition complex
SNP	single nucleotide polymorphism
Xist	X-inactive specific transcript

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Figure 1.

Regulating replication by limiting replication factor(s). A single molecule of a limiting factor (magenta) activates unwinding at replication origins sequentially in the early-replicating regions of the active X chromosome. The functional concentration of limiting factors at the late-replicating regions of the inactive X chromosome is higher, hence unwinding at replication origins could occur synchronously. Light blue, DNA; pink, unwound DNA at replication origins; green, nucleosomes. (Note that for simplicity, only one strand is drawn before and after replication.)

Figure 2.

Distribution of limiting replication factors in nuclear space according to the model presented in Figure 1. Replication of the early-replicating components of the active X chromosome is facilitated by (an) essential limiting factor(s) localized in a diffuse pattern in the nucleus. The late-replicating components of the inactive X chromosome are packed in a relatively homogenous condensed chromatin structure. After the completion of the early-replication stage, this compact structure traps the limiting replication factor(s) and causes a high local concentration of those factors in the inactive X, facilitating replication within a short time frame.