Published in final edited form as:

Biochim Biophys Acta. 2012 July ; 1819(7): 794–801. doi:10.1016/j.bbagr.2012.01.017.

The chromatin backdrop of DNA replication: lessons from genetics and genome-scale analyses

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Introduction

A complete copy of the entire cellular genome must be replicated during each cell cycle, and the replication process must insure that all genetic and epigenetic information is accurately transferred to the daughter cells. While the biochemical principles underlying DNA replication have been extensively studied, it is yet unclear how replication proceeds along with chromatin condensation and remodeling while ensuring the fidelity of the replicated genome. In most somatic cells, DNA replication starts from consistent multiple initiation sites on each chromosome and advances in a precise temporal and tissue-specific order. It is postulated that this temporal and spatial consistency reflects a tight orchestration of replication initiation events that is necessary to coordinate replication with other chromatin transactions such as transcription.

Clues for the coordination of DNA replication and transcription can be derived from biochemical mapping of replication initiation sites on a whole-genome scale. The timing of DNA replication during the S-phase exhibits a clear association with transcription and chromatin condensation, as earlier replicating regions tend to consist of transcribed genes [1] exhibiting high chromatin accessibility [2]. The biochemical details of the replication process in eukaryotes and the regulatory processes that insure that mitotic cells replicate their entire genome once per cell cycle have been summarized in excellent reviews [3–12]. Here we discuss recent insights obtained from genome-scale analyses of replication initiation sites and transcription in mammalian cells, evaluating the possible relationships between transcription and replication initiation events and the effects of chromatin modifications on replication initiation. We also discuss DNA sequences, such as insulators and replicators, which modulate replication and transcription of target genes, and use genome-wide maps of replication initiation sites to evaluate possible commonalities between replicators and chromatin insulators.

Dynamics of replication initiation: first lessons from genome-scale analyses of DNA replication

The initiation of DNA replication requires DNA sequences, termed replicators, that contain information required to start replication at particular chromosomal locations [13]. In eukaryotes, replication initiation events are detected throughout the genome and replicators can be isolated based on their ability to facilitate replication when moved from their original

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location to ectopic sites. However, replication initiation sites are more numerous than potential replicators and not all replicators are used during each cell cycle [14–16]. Replication also requires initiators, protein complexes that bind replicators to recruit members of the replication machinery. Initiators, but not replicators, are highly conserved in eukaryotes. Initiators form pre-replication complexes during the early stages of the G1 phase of the cell cycle [6] [8]. These complexes are recruited gradually onto chromatin in an inactive form, are activated by cyclin dependent kinases during the S-phase of the cell cycle and are disassembled from chromatin following replication and reassemble after mitosis [17] [12] [4] (Figure 1). Assembly and disassembly of pre-replication complexes allow cells to reprogram replication origins and allows for flexible determination of the likelihood of initiation at each potential replicator each cell cycle [14, 15, 18]. Reprogramming of replication initiation events might facilitate accurate genome replication only once throughout each cell cycle, but the rules governing the decision of which particular replication origins will initiate replication each cell cycle are yet unclear [3, 4, 6, 14, 15, 19].

The DNA sequence is not the only determinant of origin usage. Even in yeast, where ORC consensus sequences are well defined, only 400 out of the 12,000 potential initiator (Origin Recognition Complex, ORC) binding sites are functional [20]. The ORC1 subunit of ORC contains a conserved N-terminal bromo-adjacent homology domain that plays a role in both replicator binding and transcription [21]. Yeast Sir2, a histone deacetylase, binds replicators that also act as transcriptional silencers [22] and the interaction between Sir2 and some replicators regulates replicator-specific initiation of DNA replication [23, 24]. In metazoa, ORC does not exhibit sequence specific DNA binding [25, 26] beyond a general preference for AT-rich sequences [27–31]. Nucleosome positions, which are determined in part by DNA sequence [32, 33], also play a role in replication initiation sites selection in yeast and in metazoa [34–36]. For example, in *S. cerevisiae*, nucleosomes are arranged primarily by ORC, and when the configuration of the nucleosomes are disrupted or altered, replication initiation is impaired [26]. A recent study has shown through genome-wide nucleosome profiling, that in *S. pombe*, nucleosome-depleted regions do not colocalize with DNA replication origins [37]. However, it should be noted that these studies focused on fission yeast replication, whereas our study focuses on metazoans. Chromatin modifiers including histone acetyl transferases and chromatin remodeling complexes also affect replication initiation sites. Examples include the heterochromatin protein HP1, which interacts with ORC [38, 39] and with fission yeast Dfp1/Dbf4-dependent kinase Cdc7 [40] and modulates replication timing of pericentric heterochromatin in yeast [40], *Drosophila* [41] and mammalian cells [42]. Another example is the HBO1 histone acetyltransferase, which is needed for the loading of MCM onto chromatin in human cells and *X. laevis* extracts. HBO1 also interacts with the pre-replication complex during normal growth [43–45] and with the tumor suppressor p53 to modulate replication initiation events following hyperosmotic stress [46].

Coordination of transcription and replication—Transcription factors are known to be involved in specification of initiation sites. For example, the initiation of viral replication often colocalizes with transcription regulatory elements and those elements are essential for replication [47]. In yeast, binding of ORC to specific chromatin sites was enhanced by a yeast transcription factor, ScAbf1. ORC was then able to facilitate DNA replication by changing the chromatin structure and positions of the nucleosomes [48]. Other examples include the *Drosophila* Myb complex [49] and the *Rb* protein [50] which are both essential for replication of the amplified chorion locus, which require histone acetylation to recruit the ORC complex [51]. In vertebrates, replication is affected by transcription factor binding, as observed at the human *c-myc* replicators [52] in *Xenopus* egg extracts [53]. This is consistent with the observation that origin usage changes dynamically during differentiation and development [54] [1, 6, 14, 15, 55]. However, it should be noted that replication patterns

can also change, genome-wide, in response to metabolic signals that do not directly involve transcription such as changes in nucleotide pool levels [56].

Genome-scale maps of replication initiation sites show that these sites are enriched with transcription factor binding motifs [57, 58] and CpG islands [59–61]. In human cells, microarray-based analyses of nascent DNA [58] [57] and replication intermediates [62] have identified several hundred replication initiation sites. Although the concordance between the identified replication initiation sites was not high, a common characteristic in all those studies was that replication initiation events were significantly enriched around transcription start sites (TSSs). For example, in the Karnani et al study [58] a third of initiation events occur at or near the RNA polymerase II binding sites and approximately 50% of the early origin activity was localized within 5 kb of transcription regulatory factor binding region clusters and were particularly enriched in DNase I-hypersensitive sites, H3K4-(di- and tri)-methylation and H3 acetylation modifications on histones. Replication preferentially starts in transcribed gene regions [34] [62, 63] and associates with genomic regions exhibiting DNase hypersensitivity [61]. These analyses are consistent with replication timing studies suggesting that accessible chromatin replicates before highly condensed chromatin [1, 2, 35, 55, 64]. Interestingly, although replication initiation events are enriched in regions straddling transcription start sites, recent higher resolution analyses have shown that the transcription start sites themselves seem depleted in replication initiation events. This bimodal initiation pattern in regions flanking the transcription start site was observed in *Drosophila*, murine [34] and human [61] cells.

One possible explanation for initiation in regions flanking transcription start sites is that transcription initiation complexes interfere with the formation of replication initiation complexes. In our study [61], we tested this hypothesis directly by utilizing actual transcription information for the cell lines in which replication was mapped, comparing transcription levels with the frequency of replication initiation events in defined cell lines grown under identical conditions. This comparison was performed by utilizing information from the NCI datasets characterizing in detail the properties of cancer cell lines (<http://discover.nci.nih.gov>) [65]. This comparison showed that the depletion of replication initiation events from transcription start sites was notably prominent only in genes that are actually transcribed in the cell lines in which replication initiation events were mapped [61]. Untranscribed regions exhibited fewer initiation events overall, and no depletion near the transcription start sites. Interestingly, this direct comparison between active transcription and initiation of DNA replication also revealed that although moderately transcribed genomic regions were enriched in replication initiation events, regions exhibiting high transcription rate exhibited very low frequency of initiation, further supporting the assumption that transcription interferes with the initiation of DNA replication. These observations are consistent with earlier studies suggesting that active transcription excludes replication initiation events [66] [63] [67] and with the hypothesis that one of the advantages of consistent locations of initiation events is coordination of replication and transcription [5]. Studies at the human β -globin locus show that when silenced genes are activated and actively transcribed, there is a shift in replication timing from late-replication to early-replication [68, 69]. A similar activation of early replication was seen in T-cell receptor loci [70] as well as immunoglobulin IgH [71].

Chromatin modifications that affect replication—A recent genome-scale study of replication sites in mouse and *Drosophila* showed that in both species most CpG islands (CGI) initiate replication [72]. This is consistent with previous observations suggesting that replication often starts at CpG islands [60]. Methylation, which is nearly absent in *Drosophila*, was not crucial for defining the activated origin. Genome scale analyses in human cells evaluated replication initiation events in cells in relation with methylation levels

in CpG regions. These studies revealed that the highest enrichment in initiation events was exhibited by methylated CpG regions and genes undergoing moderate transcription [61]. These findings seem contradictory because of the known association between CpG methylation and silencing of gene expression. However, it should be noted that recent studies suggest that CpG methylation is not limited to promoters that are negatively regulated and gene-body methylation is associated with active genes [73]; Recent analyses have also demonstrated that methylation in gene bodies is associated with early replication [74].

Notably, although genome-scale studies revealed that methylation of CpG sequences strongly affects the location of replication initiation sites, the same studies have demonstrated that histone modifications and transcription factor binding exhibit only a moderate correlation with replication initiation events [61]. These data are consistent with earlier studies suggesting that histone modifications and chromatin accessibility partially correlate with the locations of replication initiation events [57, 58]. Although acetylation of histones H3 and H4 modulate replication timing [75–77] and seem to be required for replication specific initiation of DNA replication in yeast [45], whole genome mapping studies have not found a single modification that correlates with genome-wide high frequency of initiation in several human cells. This does not rule out, however, that there are classes of replicators that are differentially affected by chromatin modifications, masking genome-wide analyses. For example, a recent study shows that HBO1 acetylates histone H4 and this interaction plays a role in replication [78]. It should be noted, however, that HBO1 also co-activates Cdt1 [79], which is essential for replication licensing, so it remains unclear what the precise role the acetylation of histone H4 plays in replication.

Although we have not yet found histone modifications that directly mark replication initiation sites, the emerging correlations with gene expression and the distinct effect of CpG methylation and DNase hypersensitivity on the frequency of replication initiation events clearly indicates that replication initiation events reflect chromatin modifications. The absence of a single distinct histone modification in replication sites might highlight the potential combinatorial nature of the interactions that determine replication initiation events. Alternatively, chromatin might contain several classes of replicators, each potentially activated at a different stage of the cell cycle or under different conditions to coordinate replication and gene expression. The emerging correlations between chromatin accessibility and replication timing [2, 75] and the involvement of DNA sequences that modify chromatin in genetic determination of DNA replication initiation events (as described below) strengthen this hypothesis.

Distal regulation of DNA replication and the role of replicators as chromatin modifiers

Genetic studies suggest that distal DNA elements, which do not start replication but facilitate chromatin remodeling, interact with replicators and are required for initiation of replication at a number of loci. Conversely, replicator sequences themselves can affect chromatin structure. For example, replicators prevent transcriptional silencing [80] by facilitating an interaction between a locus control region and a chromatin remodeling complex [81].

Role of distal interactions—DNA sequences that participate in distal interactions with replicators play a role in chromatin modifications and transcriptional regulation. In *Drosophila*, an Amplification Control Element is essential for replication initiation at the amplified chorion locus [82] and is also required for the recruitment of transcription factors that play a role in regulating replication, such as Myb [49] and Rb [50]. The replicator at the human c-myc locus binds a protein, DUE-B, which recognizes unwound DNA and interacts

with members of the pre-replication complex [83] and initiation is affected by histone acetylation. Replication initiation at the Chinese hamster *DHFR* locus requires a transcriptional promoter 17 kb 5' to the 55 kb initiation zone [84], and initiation at the human beta-globin locus requires an upstream 40 kb region known as the locus control region (LCR) [85]. Similarly, initiation of DNA replication from the mouse *IL-4/IL-13* locus also requires a distal transcriptional regulatory element (CNS-1: conserved noncoding sequence 1), which includes a DNase hypersensitive site [86].

Several observations suggest that the effect of distal transcriptional regulatory sequences on replication initiation is mediated through chromatin modifications and not by direct sequence specific interactions. For example, a heterologous promoter can substitute for the native Chinese hamster *DHFR* promoter, which is required for initiation of DNA replication from the *DHFR* intergenic region [66]. Similarly, replicators in the beta-globin locus, which require LCR sequences to initiate replication at their native location, can replicate at ectopic sites without the LCR, presumably interacting with distal elements at the new locations [87]. In addition, the lamin B replicator can interact with the human beta-globin LCR to facilitate early initiation when inserted at ectopic sites ([80] and see below). These observations are consistent with the hypothesis that the role of distal sequences is to promote or stabilize an environment that is permissive for the initiation of replication. According to this hypothesis, once a permissive environment is established, replication can start from any available replicator located within the permissive region.

The above observations raised the possibility that replicators are similar to DNA sequences, known as insulators, which play crucial roles in gene expression and anti-silencing/silencing of genes. Chromatin insulators are naturally occurring DNA elements that help form functional boundaries between adjacent chromatin domains [88–90]. One type of insulators known as “enhancer blocking” insulators function by blocking promoters from their distal sequences. The blocking or “insulating” of the target sequences, such as enhancers, can prevent inappropriate expression of a gene [89]. Insulators can also prevent chromatin modifications, such that DNA methylation and spread of heterochromatin. In some cases, insulators halt the spread of chromatin that leads to silencing of target genes and the insulator is therefore acting as an anti-silencer [88, 90–92].

Human Replicators that exhibit anti-silencing activity—Human replicators have been shown to prevent gene silencing while also preventing replication delay and histone deacetylation [80]. The locus control region (LCR) of the human beta-globin locus controls the expression of human beta-globin genes and has been shown to delay replication when moved to ectopic chromosomal sites [63, 93, 94]. Two replicators known as Rep-P and Rep-I [94], found on the human beta-like promoter of the beta-globin locus, can prevent transcriptional gene silencing. In that study, a vector that included LCR, a promoter and enhanced green fluorescent protein (EGFP) inserted into a late-replicating site in MEL cells delayed replication and expression was silenced. However, when Rep-P, Rep-I or a replicator from the human lamin B2 locus was inserted downstream of the LCR on the same plasmid, early replication occurred and EGFP was transcribed. Histone acetylation status also correlated with transcriptional status.

A recent study has identified a replicator-binding protein complex was essential for a physical interaction between LCR and an essential domain within Rep-P, and this interaction was required for the anti-silencing effect of the LCR/Rep-P interaction. The complex was identified as the LCR-associated remodeling complex (LARC), shown previously to bind LCR interacts with essential parts of Rep-P, and this interaction was needed to prevent transcriptional gene silencing [81]. Another protein complex (DARRT) binding at the HS4 site of the LCR contains DNA repair proteins and also affects replication and transcription

[95]. Interestingly, the DARRT complex binds at a second essential sequence within Rep-P (Figure 2).

Several chromatin insulators in mammalian cells have been identified based on their ability to facilitate stable transcription and prevent gene silencing. It is intriguing to ask whether such insulators also exhibit replicator activity, testing the hypothesis that replicators might act as chromatin organizers to provide an accessible environment that facilitates both transcription and replication. Genome-scale mapping of replication initiation sites afforded some clues to assess whether such insulators also act as genomic replicators. Some examples are listed below.

The APOA1/C3/A4/A5 gene cluster, located on human chromosome 11q23.3, contains sequence elements that bind CTCF and/or the RAD21 component of cohesin [96]. Enhanced CTCF/RAD1 binding insulator sites were found specifically from 116,120kb-116,260kb. Although CTCF binding is not associated with overall genomic enrichment of replication initiation sites [61], the APOA1 region exhibits an enhanced level of replication initiation events in several cancer cell lines (K562, HCT116, MCF7), embryonic stem cells and basic erythrocytes (Fig 3A).

Human gamma-satellite DNA consists of large stretches of DNA sequences that are non-coding and repeating [97]. Human gamma-satellite DNA can be found in most chromosomes in the pericentromeric regions. In a recent study, DNA-induced epigenetic silencing of transgene expression in RL5 cells was overcome when human pericentromeric DNA was added to the vector. The gamma-satellite DNA region from chromosome 8 showed the strongest anti-silencing effect. Tests for promoter or enhancer activity was performed for the gamma-satellite DNA region and the results were negative. This showed that the anti-silencing effect of the DNA region was not due to the transgene transcription's up or down-regulation. The authors concluded from this study that the gamma-satellite DNA regions might be capable of halting heterochromatin from spreading from vector DNA [98]. The region cloned in the insulator assay, human chromosome 8: 47,138,647-47,140,586 (FIGURE 3B), shows strong initiation activity in several human cancer cell lines [61].

The ubiquitously acting chromatin opening element (UCOE) [99, 100] is derived from a methylation-free CpG island encompassing two divergently transcribed promoters, *HNRPA2B1* and *CBX3*. A second UCOE from the similar TBP/PSMB1 locus exhibits the same properties. Gene therapy vectors that include the *HNRPA2B1-CBX3* UCOE do not show transgene silencing and exhibit stable transgene expression even when integrated within centromeric heterochromatin [99]. Interestingly, both UCOEs regions exhibit a high frequency of replication initiation events (FIGURE 3C, D).

The colocalization between certain replicators and insulators, as described above, underlies the potential role of replicator sequences as chromatin organizers that coordinate the timing and location of replication initiation events with transcription and chromatin modifications. Elucidating proteins that bind replicators and the signaling networks that interact with those proteins might help understand how replication and transcription occur concomitantly on chromatin without endangering genomic stability. Pinpointing the DNA-protein interactions that stabilize gene expression concomitant with activating replicators might also have a practical use in improving gene therapy vectors. Understanding how replicator binding proteins affect replication initiation events might help elucidate the molecular interactions underlying insulator activity and perhaps this will help identify required interventions for gene therapy. In addition, maps identifying replication initiation sites at particular genomic sites in distinct differentiation states can help identify potential insulators based on replication initiation frequency in specific cells.

Insulators have important uses in addressing two critical problems encountered in gene therapy. Integrated DNA sequences are subject to chromosomal position effects – variations due to the genomic sequences and/or overall chromatin structures flanking the sites of vector integration [101, 102]. The tendency for transgene silencing presumably reflects, at least in part, the ease of spread of heterochromatin in higher eukaryotes [88, 89], resulting in the silencing of transgenes that integrate within or near heterochromatin, or in genomic loci that become heterochromatinized during differentiation. Conversely, vector sequences can influence the expression of cellular genes flanking the sites of integration. As many gene therapy vectors integrate preferentially within or close to active transcriptional domains [103, 104], enhancer elements associated with transcription regulatory elements might have a potential for deleterious mutagenesis by affecting the expression of adjacent genes. Insertional mutagenesis can often cause disruption or dysregulation of cellular gene expression, and in the most extreme cases can lead to clonal expansion and even oncogenic transformation [90]. It is therefore important to develop safer vectors that are capable of establishing and maintaining a transcriptionally competent chromatin domain, which give rise to reproducible and stable transgene expression without potential mutagenesis. It is also of interest to evaluate insulator activity, which can be used to reduce retroviral vector-mediated genotoxicity [90], associated with a high replication initiation frequency. Recent genome-wide maps of replication initiation events along with transcription frequency allow investigators to evaluate whether anti-silencer sequences used in other studies exhibit initiation of DNA replication.

In summary, the available data from genome-scale analyses of DNA replication represent the first step towards understanding the relationships between replication, transcription and chromatin modifications. As we continue to mine the replication initiation dataset and perform correlative studies between replication initiation, replication timing and chromatin modifications, we hope to understand how cells determine where and when DNA replication occurs in cancer and normal cells and how the time and location of replication initiation events affect genomic stability. Integrating maps of replication initiation sites with information about transcription and chromatin modifications might help predict which regions will be useful as insulators for gene therapy purposes and analyses of replicator binding proteins might provide insights into the mechanisms underlying chromatin insulator activity. Functional genetic assays for replicators and insulators combined with data from genome-wide analyses might help decipher the rules governing chromatin spreading and replication origin activation that facilitate stable and accurate transmission of genetic and epigenetic information each cell division.

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Highlights

Genome-scale maps and genetic analyzes probe how replication coordinates with transcription

Replication starts preferentially at accessible chromatin and at methylated CpG regions

Replication starts at regions that straddle but do not overlap transcription start site

Replication starts preferentially at moderate, but not highly transcribed, chromatin regions

DNA sequences that start replication might colocalize with chromatin insulators

Figure 1.

Assembly of pre-replication complexes on replicators [16, 105, 106].[107]. Based, with permission, on [14]. Replicators contain genetic information essential for initiation of DNA replication. Shortly after mitosis, replicators bind a six-subunit complex called the origin recognition complex (ORC) by recruiting the largest subunit of ORC, ORC1, to chromatin. ORC serves as a platform for the assembly of pre-replication complexes that include CDC6 and CDT1, which facilitate the loading of a helicase complex consisting of MCM (minichromosome maintenance) proteins 2–7 (licensing). The resulting complex is termed the pre-replication complex (PreRC). The PreRC is activated to create the pre-initiation complex (PreIC) by recruitment of additional factors including CDC45, SLD2–3 (Treslin), TOPBP1, the GINS complex (SLD1 and PSF1–3) and MCM10. Assembly and activation of the pre-initiation complex by cyclin dependent kinase mediated phosphorylation of SLD3 in yeast and mammalian Treslin leads to initiation of DNA replication.

Figure 2.

The Rep-P replicator as a template for DNA-protein complexes that facilitate interactions with the distal locus control region [81, 95, 108]. A. Organization of LARC and DARRT binding sites on the Rep-P replicator in the context of the map of the beta globin locus. B. DARRT complexes bind both LCR and Rep-P; LARC mediates interactions between LCR and Rep-P. Please note that the number of complexes binding to LCR and Rep-P was not determined experimentally and the duplication in the DARRT complex signifies that it is unclear whether the same complex binds both to LCR and Rep-P. See text for details.

Figure 3.

Replication initiation profiles at human chromatin insulators. Replication initiation profiles obtained through massively parallel sequencing of nascent DNA strands ([61] and Fu et al., unpublished) are visualized on the integrated genome viewer (<http://www.broadinstitute.org/igv/>). Nascent strands are defined as short, RNA primed DNA sequences that are above the size range of Okazaki fragments isolated from asynchronous cell cultures by size fractionation of chromatin followed by exposure to lambda exonuclease [61]. Nascent strands are sequenced and mapped to the human genome (hg19) as described. The frequency of replication initiation events is calculated for each chromosomal location as the ratio of sequence reads in the nascent strand preparation to the sequence reads aligned to the same location (100 bp bins) in genomic DNA. The panels show replication initiation data for four genomic loci. For each locus, a chromosome map is shown at the *top*. The analyzed region is shown *underneath* the ideogram, with map coordinates indicated. The experimental tracks (MCF7, K562 and HCT116 nascent strand [NS]/genome ratios) show the distribution of sequence reads (aligned with the indicated region) obtained from massively parallel sequencing of nascent strands from MCF7 breast cancer cells, K562 erythroleukemia cells or HCT116 colon sarcoma cells. All data are shown as histograms depicting the ratio of sequence reads obtained from a nascent strand preparation and reads obtained from a corresponding control genomic DNA preparation. For each track, the *y*-axis indicates the nascent strand/genomic DNA ratio. Reads were calculated as reads per kilobase per million mapped reads (RPKM). RefSeq genes are aligned under the nascent strand distribution. A. The APOA1/C3/A4/A5 gene cluster. B. The gamma satellite region from human chromosome 8. C. The CBX3 locus. D. The TBP/PSMB locus.