Comment

Nuclear Position Leaves its Mark on Replication Timing

David M. Gilbert

Department of Biochemistry and Molecular Biology, State University of New York Upstate Medical University, Syracuse, New York 13210

The multiple replication origins of eukaryotic chromosomes are programmed to replicate at specific times throughout S phase of the cell cycle. The functional significance of this program is not understood, but, in general, transcriptionally active chromatin replicates early in S phase, whereas hypoacetylated, transcriptionally inactive chromatin replicates later. During metazoan development, multiple origins, encompassing megabase domains of chromosomes, exhibit replication timing switches that precede gene activation and accompany changes in chromatin structure (Selig et al., 1992). These early- and late-replicating chromosomal domains are localized to distinct spatial compartments of the metazoan nucleus where DNA synthesis can be observed to take place at defined times during S phase. Recently, it has been shown both in budding yeast (Raghuraman et al., 1997) and in mammalian cells (Dimitrova and Gilbert, 1999) that replication origins are marked in early G1 phase to establish a replication timing program. In mammalian cells, this time point coincides with the repositioning of chromosome domains after mitosis, suggesting that the temporal program for replication may be dictated by the spatial arrangement of sequences within the nucleus. However, it has been difficult to get a molecular handle on this level of nuclear organization. In this issue, Heun et al. (2001) tap the power of yeast biology to study the relationship between nuclear position and replication timing. Using both fluorescence in situ hybridization (FISH) and the targeting of GFP to specific chromosomal sites, they find that late-replicating origins tend to localize close to the periphery of the nucleus specifically during G1 phase while early-replicating origins are more randomly localized. Their results suggest that origins may be modified at the nuclear periphery during G1 phase to delay their initiation time. These findings open the door to the use of yeast genetics to dissect the complex relationship between replication timing and nuclear positioning and raise interesting parallels in the organization of nuclei between yeast and mammalian cells.

Address correspondence to David M. Gilbert, Department of Biochemistry and Molecular Biology, State University of New York Upstate Medical University, 750 East Adams Street, Syracuse, NY 13210. Tel.: (315) 464-8723. Fax: (315) 464-8750. E-mail: gilbertd@mail.upstate.edu

What Regulates Replication Timing?

While many laboratories have catalogued the replication timing of various genes and related this to their transcriptional activity and overall chromatin structure, very few studies have addressed the problem of what actually regulates replication timing. In mammalian cells, these studies are hindered by difficulties in identifying and manipulating specific DNA sequences that function as replication origins (Gilbert, 1998). However, in *Saccharomyces cerevisiae*, replication origins are well defined sequences that can be conveniently manipulated both on autonomously replicating sequence (ARS) plasmids and at their native sites in the chromosome. *S. cerevisiae* origins are also programmed to replicate at particular times during S phase (Diffley, 1998) and, similar to the situation in mammalian cells, large (>130 kb) domains can contain multiple origins that initiate coordinately (Friedman et al., 1996). In nearly all cases, the elements dictating replication time are clearly separable from the origins themselves. For example, core origin sequences cloned as autonomously replicating plasmids almost always initiate early, regardless of when they initiate in their native location. Adding flanking chromosomal sequences to these plasmids has allowed investigators to define elements that delay the initiation of replication. Prior studies set the stage for the Heun et al. (2001) paper and deserve detailed discussion.

The telomeres of *S. cerevisiae* provide a clear example of a chromosomal element that can delay origin firing. ARS501, located 31 kb from the right telomere of chromosome V, replicates very late in S phase. However, when 14.8 kb of subtelomeric DNA containing ARS501 as the only origin was cloned onto a circular plasmid, ARS501 initiated early (Ferguson and Fangman, 1992). Linearization of this plasmid and addition of telomeres restored late activation to ARS501, demonstrating that telomeres are sufficient to delay origin firing. Telomeres contain transcriptionally silent chromatin stabilized by Sir proteins that interact with the tails of hypoacetylated histones, H3 and H4. The abundance of Sir proteins in the cell determines how far the silent domain spreads from the telomere inward along the chromosome (Hecht et al., 1996). Recently, it was shown that the ability of the chromosome V telomere to delay initiation of very closely linked origins (Y′ ARS <1 kb, X-element ARS, <10 kb) requires a func-

tional Sir3 gene product (Stevenson and Gottschling, 1999). In fact, all yeast telomeres with the common subtelomeric Y′ ARS element replicated late in a *Sir3*$^{+}$ strain and early in a *sir3*$^{-}$ mutant. These results provide a convincing demonstration that late replication of origins <10 kb from telomeres is a direct consequence of telomeric chromatin structure. However, in this same study ARS501 (31 kb from the telomere) was not affected by the lack of Sir3p, indicating that ARS501 is delayed by an independent mechanism that, nonetheless, appears to require the telomere (Ferguson and Fangman, 1992; Raghuraman et al., 1997). A clue may come from a recent microarray analysis demonstrating that Sir mutations affect the expression of only a few genes immediately adjacent to telomeres, whereas depletion of histone H4 selectively affects the expression of a large number of genes up to 20 kb away from telomeres (Wyrick et al., 1999). This finding suggests a Sir-independent influence of telomeres on chromatin. It would be interesting to examine the replication time of ARS501 after H4 depletion, to determine whether the ARS501 initiation delay is, in fact, related to chromatin structure.

What about the many late-firing origins in *S. cerevisiae* that are not localized near a telomere? Surely, the telomere influence cannot extend more than a few tens of kilobases, as there are very early firing origins (e.g., ARS305) located within 40 kb of telomeres. Friedman et al. (1996) investigated a cluster of four late-firing origins distal (157–288 kb) from the chromosome XIV telomere. An early replicating origin (ARS1) fired late in S phase when inserted into this chromosomal region, suggesting the presence of dominant elements influencing replication timing. Two origins from this domain (ARS1412 and ARS1413) initiated replication early when the core origin sequences alone (each contained within <2 kb) were present on plasmids, but initiated late when these plasmids included various segments of flanking DNA. Several DNA segments scattered throughout this locus were able to delay initiation. Although different segments were interchangeable, no sequence homologies, repetitive elements, or patterns of sequence composition could be identified. Perhaps even more surprising, several actively transcribed genes exist within this locus, providing an apparent contradiction to the general rule that late replicating chromatin is hypoacetylated and transcriptionally silent. However, there are also exceptional cases of late-replicating active genes in metazoa, and there are even some genes that require heterochromatin to be expressed (Clegg et al., 1998). Clearly, further studies to examine the state of chromatin at the ARS1412 locus are warranted.

The Importance of Subnuclear Position

So what prompted Heun et al. (2001) to examine the subnuclear positions of early and late replicating origins? Again, telomeres provide a useful paradigm (Fig. 1 A). The 32 *S. cerevisiae* telomeres are clustered into 6–10 sites at the nuclear periphery. Telomere clusters provide a well developed model for a subnuclear compartment whose integrity is required to seed the assembly of silent chromatin (reviewed in Cockell and Gasser, 1999). Telomeres are tethered to the nuclear envelope through an interaction between the telomeric DNA-bound yKu heterodimer (Laroche et al., 1998) and proteins (Mlp1 and Mlp2) that extend from the nuclear pore via an interaction with the pore protein NUP145 (Galy et al., 2000). Both Ku and another telomere-bound protein (Rap1), recruit Sir proteins to the telomere clusters, creating microenvironments with high local concentrations of Sir proteins. Mutations that either disrupt the localization of telomeres (mutations in NUP145, yKu, or Mlp1/Mlp2 double mutants), or that cause Sir proteins to disperse throughout the nucleus (Rap1 mutations) also relieve telomeric silencing. Silencing under these conditions can be restored by overexpressing Sir proteins. Hence, Sir proteins are limiting in the overall nucleus but become concentrated at telomere clusters to a level sufficient to seed the assembly of silent chromatin (Fig. 1 A). In fact, a reporter gene flanked by a crippled silencer can be silenced, in a Sir-dependent fashion, by anchorage to the nuclear periphery (Andrulis et al., 1998), suggesting that the entire nuclear periphery constitutes a zone of high Sir concentration. How might this increase in local concentration of chromatin regulators influence replication timing? It has been proposed (Stevenson and Gottschling, 1999) that silent chromatin could restrict the access of replication proteins to origins (Fig. 1 A). These could be initiation factors that accumulate during S phase until their levels overcome the restriction imposed by silent chromatin. In support of this model, high concentrations of a transcriptional activator can overcome telomeric silencing of a reporter gene (Aparicio and Gottschling, 1994).

Since Sir proteins are required to delay the firing of Y′ telomeric origins, it is logical to presume that localization to telomeric clusters at the periphery should also be required for late initiation of these origins. However, do all late replicating origins localize to the nuclear periphery? This is the question that was addressed by Heun et al. (2001). Using high resolution FISH methods that preserve subnuclear structure, they examined the positions of six early- and six late-initiating nontelomeric origins relative to the positions of telomere clusters. The authors wisely chose to restrict their analysis to G1 phase cells, to avoid potential movements that might take place during S phase; movements which they later confirm can be quite dynamic. By dividing the nucleus into five concentric zones, they found that early origins were localized randomly throughout the nucleus, while late origins were preferentially localized to the most peripheral zone, albeit not as frequently as telomeres. Interestingly, clusters of early firing centromeres and 2μ plasmid DNA, were enriched in specific internal zones, hinting at the existence of additional levels of nuclear substructure. Next, they compared the localization of ARS1412 plasmids either with or without the flanking sequences shown by Friedman et al. (1996) to confer late replication. The late replicating ARS1412 plasmid containing flanking sequences was localized to the most peripheral zone at a frequency comparable to that of chromosomal ARS1412, while the early replicating core origin plasmid was found at random sites throughout the nucleus. One potential caveat to this result is that the late replicating plasmid was nearly three times larger than the early replicating plasmid (24 kb vs. 9.4 kb). However, others have shown that plasmid size per se does not influence replication timing (Friedman et al., 1996; Donaldson et al., 1998a) or the mobility of sequences in yeast nuclei (Marshall et al., 1997). Therefore, the simplest interpretation is that sequences flanking ARS1412, previously shown to delay replication timing, target ARS1412

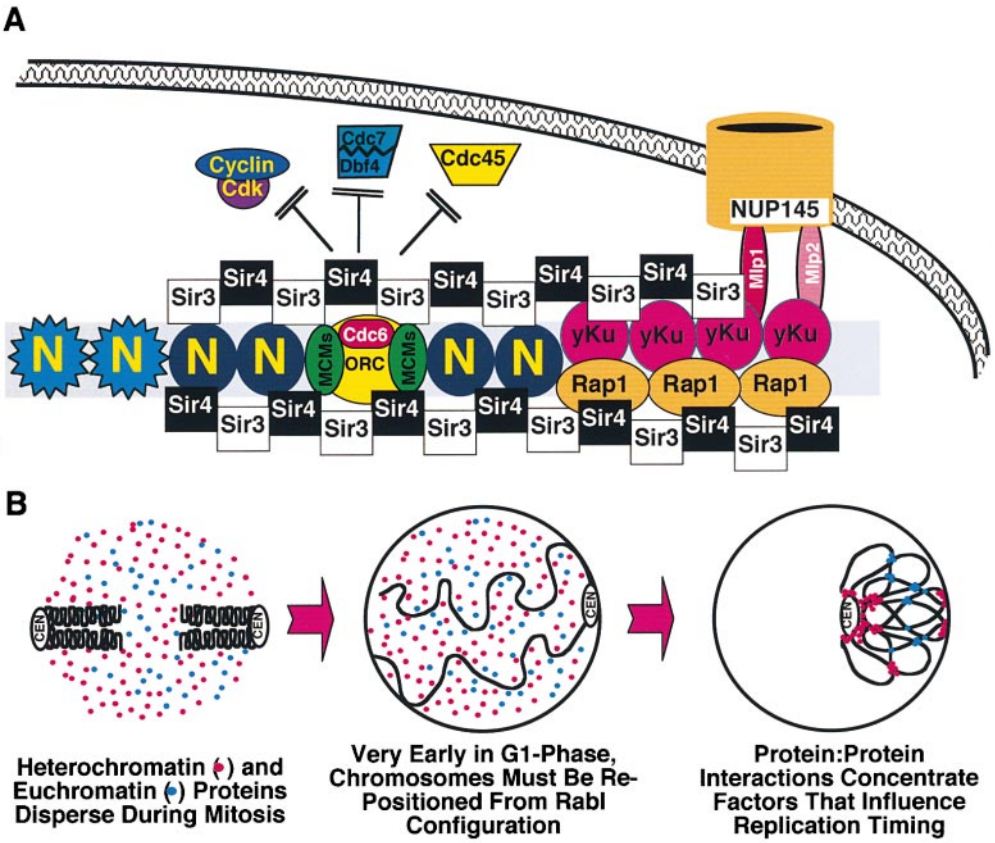

Figure 1. A, Telomere-dependent late replication. yKu binds both telomere DNA and proteins that extend from nuclear pores (Mlp1 and Mlp2; anchored to the pore through NUP145) to mediate the clustering of telomeres at the periphery (for simplicity, only one telomere is shown). yKu and the telomere binding protein Rap1 recruit Sir proteins. The increased local concentration of Sir proteins seeds the propagation of Sir complexes into the adjacent chromatin where they interact with and stabilize hypoacetylated nucleosomes (dark blue N, hypoacetylated; light blue N, acetylated), creating a silenced chromosomal domain. It has been proposed (Dimitrova and Gilbert, 1999; Stevenson and Gottschling, 1999) that such silenced domains set thresholds for the initiation of replication by restricting the access of initiation factors to origin-bound prereplication complexes, consisting of the origin recognition complex (ORC), Cdc6, and the Mcm complex. The nature of the limiting initiation factor(s) is unknown, but is likely to include the B-type cyclin-Cdk (Donaldson et al., 1998b) and Dbf4/Cdc7 (Donaldson et al., 1998a) protein kinases and/or Cdc45 (Aparicio et al., 1999). If the concentration of any one of these initiation factors is limiting at the onset of S phase, initiation would be restricted to those replication origins located within the most accessible domains. As the concentrations of initiation factors increase during S phase, the less accessible later initiating origins can then fire. B, Similar microenvironments may form at multiple sites in mammalian nuclei. See text for details.

to the nuclear periphery. The target is not a unique site on the periphery as the ARS1412 plasmids did not colocalize with chromosomal ARS1412 or with telomeric clusters. In fact, of four nontelomeric late-replicating chromosomal origins examined, none colocalized with telomeres. Hence, it is possible that nontelomeric late replicating origins associate with the periphery in a manner distinct from that which anchors telomere clusters. It will be interesting to find out whether localization of these origins is disrupted in Mlp1/Mlp2 or nuclear pore mutants.

Establishment but Not Maintenance?

One of the surprising findings of Heun et al. (2001) was the remarkable mobility of both early- and late-replicating sequences within the nucleus. A yeast strain developed earlier in which the late-replicating ARS501 was flanked by site-specific recombinase sites allows for the excision of a 30 kb circular fragment containing ARS501 upon inducible expression of the recombinase protein. Previous experiments (Raghuraman et al., 1997) had shown that excision during mitosis resulted in a circular ARS501 episome that replicated early in the ensuing S phase, while excision late during G1 phase resulted in a late-replicating ARS501 circle. Hence, a modification requiring the native chromosomal context takes place during early G1 phase to establish the late-replicating properties of ARS501. When Heun et al. (2001) followed the positional fate of these circles by in situ hybridization, excision either during metaphase or during G1 phase resulted in the movement of the circle away from its native location and the loss of association with the periphery. This result demonstrates that chromosomal elements flanking the excised 30 kb segment (most likely the telomere itself) are required to anchor ARS501 to the periphery. However, the result also shows that excision during late G1 phase results in a late-replicating circle that is not localized to the periphery, raising the interesting possibility that peripheral localization may be required only during the critical early G1 phase period. In other words, perhaps peripheral localization makes its mark on origins during early G1 phase but is not required for maintenance of that mark. Heun et al. (2001) went on to test this hypothesis through an impressive real time imaging experiment. By inserting lac operator sites adjacent to either the late-initiating ARS1413 on chromosome XIV or an early firing origin on chromosome IV, the positions of these origins could be monitored throughout the cell cycle in living cells expressing a GFP-tagged lac repressor. Results revealed that the early replicating origin was free to move throughout the nucleus in both G1 and S phase, while the late replicating ARS1413 oscillated in the vicinity of the nuclear periphery during G1 phase but, wandered further from the periphery during S phase

(Heun et al., 2001). Hence, early G1 phase may present a unique window of time during which nuclear position can program initiation time, regardless of the positional fate of the origin thereafter.

The mobility of these sequences was surprising in light of prior studies of chromatin dynamics. First, even the dampened G1 phase movements of the late-origin contrast with the relatively immobile telomeres observed in cells expressing GFP–Rap1 (Laroche et al., 2000), suggesting a fundamental difference in the way in which telomeric and nontelomeric late replicating origins associate with the nuclear periphery. Second, a similar live cell study of the GFP-tagged centromere-proximal LEU2 locus in yeast revealed only energy independent diffusion constrained to within a 0.3 μM radius (Marshall et al., 1997). Similarly, when mammalian nuclei labeled with an intercalating DNA stain were photobleached to produce 0.4 μM radius spots, these spots never recovered, indicating very little movement of chromatin (Abney et al., 1997). In contrast, Heun et al. (2001) observed two types of movements. One type was small and oscillatory (<0.3 μM), consistent with diffusion. However, the second type was significantly larger, extending >0.5 μM within a time scale of seconds. Clearly more studies of this type are needed to determine whether this second type of movement is the exception or the norm. It is worth mentioning, however, that other studies, also in living mammalian cells, have revealed rather substantial movements of certain centromeres (Shelby et al., 1996) and transfected sequences (Li et al., 1998) during the cell cycle.

A Model for Higher Eukaryotes?

The findings of Heun et al. (2001) suggest interesting parallels between yeast and higher eukaryotes, providing hope that yeast can provide a valuable model system for understanding nuclear organization in higher eukaryotes. In mammalian cells, the positions of chromosomal domains are also established early in G1 phase, within the same narrow window of time at which the replication timing program of these domains is established (Dimitrova and Gilbert, 1999). Furthermore, peripheral localization of the largely late-replicating human chromosome 18 is lost upon entry of cells into quiescence but, when cells are stimulated to enter S phase, the chromosome remains late-replicating without returning to the periphery until after mitosis (Bridger et al., 2000). Hence, as the Heun et al. (2001) results suggest for yeast, subnuclear localization in mammalian cells takes place early in G1 phase and may be necessary for establishment but not maintenance of a replication timing program.

Although telomeres neither replicate late (Ten-Hagen et al., 1990) nor localize to the periphery in mammalian cells, telomere clusters in *S. cerevisiae* provide a useful working model for silent compartments in metazoan nuclei, which may exist at many sites within the nucleus in addition to the periphery. In both *Drosophila melanogaster* and mammals silencing of certain genes is accompanied by their association with clusters of late-replicating heterochromatin and this association can vary dynamically during development (Csink and Henikoff, 1996; Dernburg et al., 1996; Brown et al., 1999; Francastel et al., 1999; Schubeler et al., 2000). Fig. 1 B schematically illustrates a model that we previously proposed for the establishment of replication timing in metazoa (Dimitrova and Gilbert, 1999). Proteins that modulate chromosome architecture, such as those of the chromodomain family, are removed from chromatin during mitosis and reassociate shortly thereafter. Chromodomain proteins are likely to be functional homologues of the yeast Sir proteins (Jones et al., 2000; Wang et al., 2000). Just as modulating the concentrations of Sir proteins in the cell can modulate the extent of silencing in yeast (Hecht et al., 1996), the spreading of PEV in *Drosophila* and mammals can be enhanced or suppressed by modulating the levels of chromodomain proteins (Festenstein et al., 1999). Both HP1 and PcG chromodomain proteins have been shown to dissociate during mitosis and reassociate shortly thereafter. Although *S. cerevisiae* nuclei do not break down during mitosis, partial release of Sir3 and Sir4 from telomere clusters during mitosis has been observed (Laroche et al., 2000). Both groups of proteins associate with a variety of other chromatin proteins and can either self-associate (e.g., HP1) or associate with other family members (e.g., Sir3 and Sir4). We speculate that the reassociation of these proteins during early G1 phase could bring chromosomal segments containing similar chromatin proteins into close proximity, increasing the local concentration of these proteins and creating microenvironments that seed the formation of particular chromatin architectures (Fig. 1 B). Once established, this chromatin architecture may be quite stable for the remainder of the cell cycle whether or not subnuclear position is maintained. As was proposed for telomere clusters (Stevenson and Gottschling, 1999), the architecture of chromatin, whether established by Sir-like or other chromatin-modifying proteins, could restrict the accessibility of replication origins to initiation factors (Fig. 1 A), setting thresholds that influence when replication can initiate during S phase. Of course, this model is grossly oversimplified and will inevitably have to be modified to account for the subtle differences in replication timing of origins that fire at specific intermediate times during S phase.

The underlying assumption linking the two models in Fig. 1 is that, ultimately, modifications of chromatin dictate when an origin will initiate replication during S phase. Can this explain the situation at the nontelomeric ARS1412 locus, where several actively transcribed genes are embedded within a late replicating locus? Often, the exceptions in nature provide the key to common threads linking similar mechanisms. The demonstration that both telomeric and nontelomeric origins localize to the periphery suggests that such a common thread will be found (Heun et al., 2001). Surely, further investigations into the gene products that regulate peripheral localization and replication timing at the ARS1412 locus will provide valuable clues. The growing parallels between the organization of nuclei in yeast and higher eukaryotes inspire confidence that the power of yeast biology can be applied to unravel the complex relationships between chromosome structure and function in all eukaryotes.

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