Early-replicating heterochromatin

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Euchromatin, which has an open structure and is frequently transcribed, tends to replicate in early S phase. Heterochromatin, which is more condensed and rarely transcribed, usually replicates in late S phase. Here, we report significant deviation from this correlation in the fission yeast, Schizosaccharomyces pombe. We found that heterochromatic centromeres and silent mating-type cassettes replicate in early S phase. Only heterochromatic telomeres replicate in late S phase. Research in other laboratories has shown that occasionally other organisms also replicate some of their heterochromatin in early S phase. Thus, late replication is not an obligatory feature of heterochromatin.

Results and Discussion

Previous results: telomeres

Chromatin near fission yeast telomeres is heterochromatic, because bringing normally active genes into the proximity of telomeres epigenetically inactivates the genes, and this inactivation is partially dependent on standard heterochromatin proteins including Swi6 (Nimmo et al. 1994, Allshire et al. 1995; Ekwall et al. 1995, 1996). Epigenetic inactivation of euchromatic genes introduced into telomere-proximal positions is called Telomere Position Effect (TPE) and is conserved from budding yeast (Gottschling et al. 1990; Tham and Zakian 2002) to humans [Baur et al. 2001].

Using several independent synchronization procedures, we have demonstrated previously that the terminal HindIII restriction fragments of fission yeast chromosomes I and II replicate in very late S phase [Kim and Huberman 2001], consistent with the paradigm of late heterochromatin replication. However, we were surprised when the same studies revealed that the outer portions of fission yeast centromeres replicate in early S phase.

Previous results: outer centromeres

Fission yeast centromeres consist of variable numbers of outer repeat [otr] sequences arranged, in inverted orientation, around an inner portion consisting of chromosome-specific innermost repeats [imr] inverted around a central sequence [cnt] that is fully or partially unique for each chromosome (Chikashige et al. 1989; Takahashi et al. 1992; Smith et al. 1995).

The otr region consists primarily of dg and dh repeats. These repeats are heterochromatic, because an indicator gene transplaced into otr becomes silenced [Allshire et al. 1995], and otr is associated with the heterochromatin protein, Swi6 (Partridge et al. 2000). Our previous results led to the surprising conclusion that the HindIII fragments from the dg repeats [also called K repeats] within the otr portions of the centromeres contain active chromosomal replication origins and replicate in very early S phase [Kim and Huberman 2001].

Inner centromeres

In fission yeast, the chromatin within the inner portions of centromeres is heterochromatic in the sense that euchromatic genes introduced into inner centromeres become epigenetically silenced [Allshire et al. 1994, 1995]. However, in fission yeast as in all eukaryotic organisms, histone H3 within the inner centromeres is replaced by a histone H3 paralog, frequently called CENP-A [for review, see Smith 2002]. Because the N-terminal portion of CENP-A differs substantially from that of histone H3, CENP-A is not subject to K9 methylation, and it does not bind H1/Swi6-like proteins. Instead, inner centromeres are associated with the Mis6 protein [Partridge et al. 2000, Takahashi et al. 2000; Knioła et al. 2001].

The surprising early replication of the outer portions of the centromeres raised the following question: Do the inner portions, which have a different heterochromatin
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The remaining well-characterized heterochromatic region in fission yeast is the region containing the silent mating-type cassettes. There are two mating types in fission yeast, plus and minus. Wild-type fission yeast cells have three sets of genes (cassettes) encoding mating-type information, Plus or Minus. The three cassettes—mat1, mat2, and mat3—are located within 30 kb of each other on chromosome II. This wild-type configuration, h\(^{\text{wt}}\), is diagrammed in Figure 2 (top panel). The region between mat1 and mat2 is called the “L region,” and that between mat2 and mat3 is called the “K region.” The genes [Plus or Minus] in mat1 are expressed (mat1-P or mat1-M). Those in mat2 (usually Plus; mat2-P) and mat3 (usually Minus, mat3-M) are silenced by heterochromatinization that is partially dependent on Swi6. The silenced state is indicated in the diagram by a gray (rather than black) plus sign or minus sign. The silenced, heterochromatic region is located between two inverted repeats that form boundaries between heterochromatin and euchromatin (IR-L and IR-R; Noma et al. 2001; Singh and Klar 2002; Thon et al. 2002). Halfway between these inverted repeats is a stretch with strong sequence similarity to a portion of the otr of CEN2. This “CEN homology” domain contributes significantly to formation of regional heterochromatin (Hall et al. 2002).

To measure replication timing in the mating-type region, we used fission yeast strains bearing the cdc10-M17 mutation, which facilitates cell cycle synchronization [Kim and Huberman 2001]. These are haploid strains with alterations of their mating-type regions that inhibit mating-type switching. The alterations are called h\(^{\text{+}}\) and h\(^{\text{−}}\), and they are diagrammed in Figure 2 (bottom panel). h\(^{\text{+}}\) strains arise from mat1-M h\(^{\text{wt}}\) cells by recombination between homologous sequences at the borders of mat2-P and mat3-M, leading to deletion of the K region (Beach and Klar 1984). In h\(^{\text{−}}\) strains, the retained silent cassette contains Minus information. In Figure 2 [middle panel, h\(^{\text{−}}\)], the silent cassette is designated mat2:3-M, to indicate that the sequences to its left are identical to those to the left of mat2 in h\(^{\text{wt}}\), and the sequences to its right are identical to those to the right of mat3 in h\(^{\text{wt}}\). Similarly, h\(^{\text{−}}\) strains arise from mat1-P h\(^{\text{wt}}\) [Beach and Klar 1984], but in this case, an extra K region is inserted by recombination into mat1. The leftmost cassette (mat1:2-P) in the h\(^{\text{−}}\) configuration remains transcriptionally active and expresses Plus information. The second cassette (mat3:1) is inactive for transcription, but it retains the cis-acting sequences necessary for mating-type switching, and it is capable of switching. The switching has no phenotypic consequences, as the second cassette is silenced.

The results of our analyses of mating-type region replication timing with cells synchronized by cdc10 block and release [Kim and Huberman 2001] are shown in Figure 3, and the locations of the tested fragments are shown as horizontal boxes in Figure 2 (middle and bot-
tom panels). The probes used to detect the restriction fragments in Southern blotting are indicated by thick black lines immediately below these boxes. It is evident in Figure 3 that all tested fragments replicated in early S phase. The maximum abundance of RIs occurred at 40–60 min after temperature downshift. In contrast, the maximum abundance of RIs from telomeres occurred at 90–100 min after downshift in similar experiments (Kim and Huberman 2001).

Two features of the results in Figure 3 merit comment. First, the probe used to detect restriction fragment 3b (EcoRI, 4.0 kb) also detects restriction fragment 3a (EcoRI, 3.9 kb; Figs. 2, bottom panel, 3B, top panel). These two fragments are too close in size to be resolved. Therefore, we cannot determine whether the bubble arc detected by this probe arises from fragment 3a, fragment 3b, or both. It is interesting that this probe also detects a spot near the apex of the Y arc (Fig. 3B, top panel). This spot is probably due to replication fork pausing in one or both of the detected restriction fragments.

Another result that deserves comment is the behavior of the two h^+N EcoRI fragments (5a and 5b) detected by the probe used in the bottom two-dimensional gel panel of Figure 3B. Generation of fragment 5a requires an EcoRI site that is located within Minus—but not within Plus—sequences. It can be calculated from the information in Figure 2 (bottom panel) that the overall signal strength from fragment 5a should be three times greater than from fragment 5b. This ratio is consistent with our observations.

We have used two additional synchronization procedures to confirm the early replication timing of some of the restriction fragments from the mating-type region. Use of HU block and release showed that restriction fragment 2 (Fig. 2) accumulated RIs in the presence of HU, and the abundance of RIs decreased after HU was removed (Fig. 4A). Thus, this restriction fragment is also replicated in early S phase (Kim and Huberman 2001).

We found previously that sufficient synchronization of passage through S phase could be obtained by centrifugal
elutriation to allow distinction between early- and late-replicating restriction fragments [Kim and Huberman 2001]. In this procedure, the smallest cells in an unsynchronized population growing in rich medium are separated from the other cells by centrifugation and permitted to continue growing in rich medium. The small cells, which are initially in early G2 phase, continue through G2 and mitosis and then through G1 and S phase of the following cell cycle. Although the quality of the S-phase synchrony is not as good as with the other procedures described here, and only low numbers of cells are obtained, this procedure has the advantage that no cell cycle blocks are required. Consequently, this procedure should be free of potential artifacts that might be produced by temporarily blocking the cell cycle.

For the experiment in Figure 4B, two samples were collected from the synchronized culture for analysis of RIs. One sample, called “Early” in Figure 4B, contained mostly cells in early S phase with very few cells in late S phase. The other sample, called “Early and Late,” contained cells in both early and late S phase [Kim and Huberman 2001]. The proportions of cells in early and late S phase in these two samples can be estimated from the quantitative fluorescence microscopy data in Figure 1 of Kim and Huberman [2001]. The RIs detected by the 6a+6b and 3a+3b probes appear stronger in the “Early” sample, but the spot detected by the 3a+3b probe that is probably due to replication fork pausing appears stronger in the “Early and Late” sample, consistent with the relative apparent strengths of the Y arc and probable pause spot at different time points in the top panel of Figure 3B. Thus, both of the mating-type probes used in this experiment detect restriction fragments that are replicated predominantly in early S phase. For comparison, Figure 4B also shows results obtained with a probe specific for cnt2 (roughly equal signal strengths in the “Early” and “Early and Late” samples, consistent with cnt2 replication in mid-early S phase) and with a telomere probe (much stronger Y arc signals in the “Early and Late” sample, consistent with late telomere replication).

Occasional early heterochromatin replication in other organisms

Heterochromatin sometimes replicates early in other organisms, but examples in other organisms have been sufficiently rare that they have not called into question the paradigm of late heterochromatin replication. These examples include [1] early initiation of replication within bovine satellite I, which comprises 5% of bovine genomic DNA and is located in the centromeric heterochromatin of most chromosomes [Matsumoto and Gerbi 1982], [2] early initiation of replication of the pericentric heterochromatin and centromeres of mouse chromosomes [Hollo´ et al. 1996]; [3] early replication of some human telomeres [Wright et al. 1999], despite the fact that human telomeres exhibit TPE [Baur et al. 2001]; [4] occasional early replication of autosomal genes inactivated by spreading of heterochromatin from a translocated human X chromosome [Sharp et al. 2001]; [5] early replication of inner centromeres in Drosophila [Ahmad and Henikoff 2001, 2002; Sullivan and Karpen 2001]; and [6] early replication of Drosophila β-heterochromatin (the form of heterochromatin containing occasional expressed genes and located immediately distal to pericentric α-heterochromatin, Schübler et al. 2002).

No essential connection between heterochromatin and late replication

These examples from other organisms suggest that formation and maintenance of heterochromatin do not necessarily require late replication, but the surprising early replication of most heterochromatin in fission yeast forces us to recognize that the current paradigm of late heterochromatin replication requires revision. We do not make this statement lightly, because the association between heterochromatin and late replication is a strong one in most eukaryotic organisms. Yet the early-replicating heterochromatin in fission yeast appears to resemble heterochromatin in other organisms in all essential respects except replication timing. The best-studied fission yeast heterochromatin [in the outer centromeres and in the mating-type region] is physically associated with the Hp1-like Swi6 protein and with hypoacetylated histones. Its histone H3 is undermethylated on Lys 4 and overmethylated on K9. Normally active genes introduced into this heterochromatin become epigenetically inactivated [for review, see Grewal and Elgin 2002]. The RNA interference machinery [RNAi] contributes to establishment and [in the case of centromeres] maintenance of this heterochromatin [Hall et al. 2002, Volpe et al. 2002]. During interphase, the centromeres are condensed and associated with the spindle pole body at the nuclear periphery [Funabiki et al. 1993; Ekwall et al. 1995; Kniola et al. 2001]. The mating-type region is also condensed and frequently associated with centromeres and/or telomeres at the nuclear periphery [Ekwall et al. 1995]. Nevertheless, despite possessing all of these properties of classic heterochromatin, the fission yeast centromeres and mating-type region replicate in very early S phase. Thus, if there is a link between late-replication
timing and heterochromatin, that link must involve a nonessential feature that is commonly, but not universally, associated with heterochromatin. In many cell types, heterochromatin is located predominantly at the nuclear periphery, where it replicates in late S phase. For these reasons, the hypothesis that late replication is determined by peripheral nuclear positioning is currently popular (for review, see Gilbert 2002). As indicated above, however, peripheral location is not a guarantee of late replication timing in fission yeast. It is particularly interesting that in some fission yeast cells early-replicating mating-type regions appear to colocalize with late-replicating telomeres in regions that contain abundant Swi6 protein [Ekwall et al. 1995]. This observation argues that cis-acting sequences (which may be involved in specifying type of heterochromatin) are likely to be more important than nuclear position as primary determinants of replication timing in fission yeast. Results from other laboratories suggest that the same may be true in budding yeast [Zappulla et al. 2002] and in mammals [Simon et al. 2001].

Materials and methods

Strains

Easily synchronized cdc10-M17 ade6–704 leu1–32 ura4–D18 strains (kindly sent to us by Tony Carr [Sussex University, Falmer, Brighton, UK]) were used for all experiments. The mating-type configuration was h− unless indicated otherwise in the text and figure legends.

Cell cycle synchronization and flow cytometry

Synchronization by centrifugal elutriation, cdc10 block and release, and HU block and release was carried out as described previously (Kim and Huberman 2001). Evaluations of cell cycle position by flow cytometry or by quantitative fluorescence microscopy were as described [Kim and Huberman 2001].

Two-dimensional agarose gel analyses

Our procedures for purification of DNA from synchronized cell populations and for detection of RIs by two-dimensional gel electrophoresis have been described previously [Kim and Huberman 2001].

Hybridization probes

To detect telomeres, we used a previously described [Nimmo et al. 1994; Kim and Huberman 2001] 750-bp TAP probe. Cont1 and Cont2 were detected using a 3.4-kb EcoRI–dIII fragment from cont1, whereas cont2 was detected using a 2.15-kb EcoRI–dIII fragment. The ctn1 probes were kindly sent to us by Mary Baum [University of California at Santa Barbara] and Louise Clarke [University of California at Santa Barbara]. Probes for the mating-type region were obtained by appropriate restriction digestion of plasmids kindly sent to us by Amar Klar (Frederick Cancer Research and Development Center, Frederick, MD). In h− cells, the 2.5-kb EcoRI and 6.0-kb HindIII fragments were detected by a 700-bp EcoRV–HindIII probe. In h+ cells, the 3.9- and 4.0-kb EcoRI fragments were detected by an 890-bp XbaI–HindIII probe. The 3.7-kb EcoRI fragment was detected by a 2.1-kb HindIII–EcoRI probe. The 5.7- and 8.1-kb EcoRI fragments, and the 4.2- and 8.4 kb HindIII fragments were detected with a 1.4-kb HindIII–NsiI probe.

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