Centromeres Are Specialized Replication Domains in Heterochromatin

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Abstract. The properties that define centromeres in complex eukaryotes are poorly understood because the underlying DNA is normally repetitive and indistinguishable from surrounding noncentromeric sequences. However, centromeric chromatin contains variant H3-like histones that may specify centromeric regions. Nucleosomes are normally assembled during DNA replication; therefore, we examined replication and chromatin assembly at centromeres in *Drosophila* cells. DNA in pericentric heterochromatin replicates late in S phase, and so centromeres are also thought to replicate late. In contrast to expectation, we show that centromeres replicate as isolated domains early in S phase. These domains do not appear to assemble conventional H3-containing nucleosomes, and deposition of the Cid centromeric H3-like variant proceeds by a replication-independent pathway. We suggest that late-replicating pericentric heterochromatin helps to maintain embedded centromeres by blocking conventional nucleosome assembly early in S phase, thereby allowing the deposition of centromeric histones.

Key words: centromere • heterochromatin • DNA replication • histone • *Drosophila*

Introduction

Centromeres are the attachment points of chromosomes to the mitotic and meiotic spindles. Centromere activity in the budding yeast *Saccharomyces* is conferred by a specific DNA sequence (Clarke, 1990). However, the centromeres of chromosomes in complex eukaryotes are not defined by any distinct sequence: they are usually composed of highly repetitive satellite sequences that are indistinguishable from surrounding noncentromeric satellite blocks (Csink and Henikoff, 1998; Murphy and Karpen, 1998; Willard, 1998). In *Drosophila*, a centromere was mapped to a 420-kb region that carries full centromeric function, and which is composed of repetitive sequences that are found at noncentromeric regions of chromosomes (Murphy and Karpen, 1995; Sun et al., 1997). Minimal centromeres in mammalian cells are also hundreds of kilobases in size, and entirely composed of repetitive alphoid sequences (Lo et al., 1999; Yang et al., 2000). Because centromeres cannot be delineated from their flanking sequences by any sequence criteria, and analyzing highly repetitive DNA is difficult, the size and location of centromeres in most organisms remains unknown. For example, molecular mapping of functional centromeres in *Arabidopsis* could only map the centromeres to within the extensive repetitive regions of chromosomes that includes megabases of canonical 180-bp repeats, clusters of transposons, and even functional genes (Copenhaver et al., 1999).

Highly repetitive regions of chromosomes adopt a heterochromatic chromatin structure, with distinctive properties and chromatin components (Elgin, 1996). Centromeres have a different set of chromatin components, of which centromeric nucleosomes containing special histone H3-like proteins are most conspicuous. Nucleosomes are normally assembled during replication, and it has been suggested that the centromeric histone CENP-A is incorporated at the centromere at the time of its replication (Shelby et al., 1997). Heterochromatin replicates late (Lima-de-Faria and Jaworska, 1968), and so centromeres are also thought to replicate late. Very late replication of centromeres has been proposed to play a role in centromere function (Dupraw, 1968; Csink and Henikoff, 1998). In contrast to expectation, we show that centromeres replicate as isolated domains early in S phase. At this time, they are surrounded by heterochromatin that has not yet replicated. Therefore, a fundamental feature of DNA, replication timing, distinguishes centromeres from surrounding heterochromatin. We further show that nucleosome assembly using histone H3 is inhibited as centromeres replicate. We suggest that pericentromeric heterochromatin sequesters centromeres away from histone H3 and thereby participates in centromere maintenance.

Materials and Methods

Cell Culture and Immunostaining

All experiments were conducted using a *Drosophila* tetraploid *Kc* cell line. Culturing, transfection methods, and constructs were previously described (Henikoff et al., 2000), except that the colcemid treatment in cytological preparations was omitted. To label replicating DNA with nucleotide triphosphate analogues, we administered a 15-min hypotonic

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treatment using KHB buffer containing 0.1 mM digoxigenin (dig)[1]-dUTP (Boehringer; Koberna et al., 1999), and then returning cells to insect media for 15 min. For dual-pulse labeling, cells were treated with 0.1 mM dig-dUTP/KHB, resuspended in insect cell culture media for the chase interval, and then treated with 0.1 mM biotin (Bio)-dUTP/KHB (Boehringer).

Labeling of replicating DNA with 5-bromodeoxyuridine was performed by incubating cells in culture medium supplemented with 10 μg/ml BrdU and 10 μg/ml deoxycytidine for 1 h. Immunological detection of Cid was performed on immobilized cells, which were then fixed with methanol/acetic acid/H_2O (11:11:1). Digestion with exonuclease III (30 U in 100 μl buffer) was used to expose the BrdU epitope. BrdU was detected using a FITC-conjugated monoclonal anti–BrdU antibody (Boehringer), and dig-dUTP was detected using FITC- or Texas red–conjugated anti–dig antibodies (Jackson ImmunoResearch Laboratories). In dual-pulse experiments, the two nucleotide analogues were detected using FITC-conjugated anti–dig antibodies and Texas red–conjugated streptavidin (Pierce Chemical Co.).

Transfection efficiencies with histone-green fluorescent protein (GFP) constructs were typically 35–70%. To assay the localization of histone-GFP fusion proteins when replication was blocked, we split one transfected culture into a control and an experimental sample. Aphidicolin was added to the experimental culture at a final concentration of 0.1 mg/ml 5 min before heat shock induction. Cells were allowed to recover after induction for 2 h at 25°C.

HP1 or Cid proteins were detected using anti–HP1 rabbit antibodies (Smothers and Henikoff, 2001) or by using anti–Cid rabbit antibodies (Henikoff et al., 2000) followed by anti–rabbit IgG Cy5-conjugated goat antibodies (Jackson ImmunoResearch Laboratories). All images were collected as previously described (Henikoff et al., 2000).

Image Quantitation

Images were analyzed using DeltaVision software (Applied Precision). Cells were categorized according to their overall pattern of replication (euchromatic, scattered, or heterochromatic). Centromeres and nuclei were identified by thresholding anti–Cid signals and DAPI fluorescence, respectively.

To measure the amount of nucleotide incorporated in centromeres, we examined all optical sections that included an anti–Cid signal. We selected nuclei in which euchromatin was labeled, summed the intensity of the nucleotide analogue signal in an area defined by Cid signals, and divided this by the summed nucleotide signal intensity within the euchromatin of the same section. Both sums were corrected for background. The summed signal intensity indicates the amount of replication in a cell during the labeling period, and the average ratio from randomly selected early S phase cells ($n = 22$) is an estimate of the fraction of replication at centromeric foci relative to euchromatin. These integrated intensities allow an estimate of the amount of DNA replicated early in S phase at a centromeric replication focus. The amount of nucleotide analogue incorporated at centromeric replication foci above background relative to that incorporated in euchromatin throughout the early S phase period was 0.005 (SEM = 0.002). Since euchromatin contains ~120,000 kb of DNA (Adams et al., 2000), the average centromeric replication focus contains ~500 kb of DNA.

The *Hsp70* promoter has very low-level constitutive activity, so in all cases we determined the mean GFP signal from uninduced control samples and removed from the analysis any nuclei in which the GFP signal was within two standard deviations of this mean. We counted the number of nuclei with appropriate GFP signals (Cid-GFP localized to centromeres or H3GFP labeling replicating DNA), and scaled these numbers to the transfection efficiency (estimated as the number of nuclei showing localization after induction and with no aphidicolin treatment). The amount of histone-GFP fusion proteins incorporated at centromeres when produced from *cid* promoter constructs was estimated by comparing the integrated intensities from GFP in euchromatin to that in centromeres ($n = 23$ for *cid-H2BGFP*, $n = 10$ for *cid-H3GFP*).

Results

Replication of Euchromatin and Heterochromatin in Kc Cells

Immortal cell lines are traditionally used to examine replication timing in higher eukaryotes, because cell types are uniform and they can be easily manipulated. We used *Drosophila Kc* cells, which have been used in previous studies of replication timing, and have been documented as having a stable karyotype (Echalier, 1997). In *Kc* nuclei, there is a clear separation between the early replication of euchromatin and the late replication of heterochromatin during the ~10-h-long S phase (Barigozzi et al., 1966; Dolfini et al., 1970). Furthermore, the heterochromatic regions of chromosomes coalesce into a large irregular chromocenter that occupies ~25% of the nuclear volume and can be visualized by detection of the HP-1 protein (van Steensel and Henikoff, 2000; Fig. 1, A and B). Centromeres are embedded within the chromocenter (van Steensel and Henikoff, 2000). A chromocenter is not unique to the *Kc* nucleus, as we have observed consistent chromocenter formation in other dividing cells as well, including S2 and S3 cell lines (Ahmad, K., unpublished results). This chromocentric organization of the nucleus and the regularity of the cell cycle allowed us to precisely follow replication timing of centromeres in asynchronous *Kc* cell populations.

Pulse-labeling of *Kc* cells with the nucleotide analogue dig-dUTP confirms earlier studies of mitotic replication banding (Barigozzi et al., 1966; Dolfini et al., 1970) that heterochromatin and euchromatin replicate in distinct portions of S phase (Fig. 1, A and B). A typical experiment showed that 51% of nuclei were in S phase at the time of the pulse: 23% showed euchromatic replication patterns, 25% showed heterochromatic replication patterns, and 3% showed discrete foci scattered throughout both regions. This last type indicates that there is only minor overlap of the euchromatic and heterochromatic replication periods during the pulse. Euchromatin replicates in the first portion of S phase (early S), and heterochromatin in the last (late S) (Barigozzi et al., 1966; Dolfini et al., 1970). This spatial and temporal separation of replication in *Kc* cells facilitates a cytological analysis of centromere replication.

Centromeres Replicate in Early S Phase

We have previously described the *centromere identifier* (*cid*) gene in *Drosophila*, which encodes a centromeric histone H3-like protein (Henikoff et al., 2000). This protein is analogous to CENP-A in mammals (Palmer et al., 1991), Cse4p in *Saccharomyces* (Stoler et al., 1995), HCP-3 in nematodes (Buchwitz et al., 1999), and SpCenpA in *Schizosaccharomyces* (Takahashi et al., 2000). All of these proteins are thought to be assembled into specialized nucleosomes, although direct evidence has only been obtained for CENP-A (Palmer et al., 1987; Yoda et al., 2000). Because Cid appears to be a constitutive centromeric component, antibodies to Cid can be used to locate centromeres in interphase nuclei, and typically detect four to six centromeric spots (5.6 ± 2.1) in both diploid and tetraploid *Kc* cells (Henikoff et al., 2000). Chromosomes in *Drosophila* somatic cells are paired; therefore, each centromeric spot probably represents a cluster of centromeres from homologous chromosomes, regardless of their total number. These spots divide into individual centromeres only in late-G2 phase of the cell cycle (data not shown).

We used anti–Cid antibodies on nuclei that had been pulse labeled with nucleotide analogues to determine when centromeric DNA replicates. The Cid epitope and nucleotide analogues colocalize when euchromatin is also

[1]*Abbreviations used in this paper:* Bio, biotin; dig, digoxigenin; GFP, green fluorescent protein.

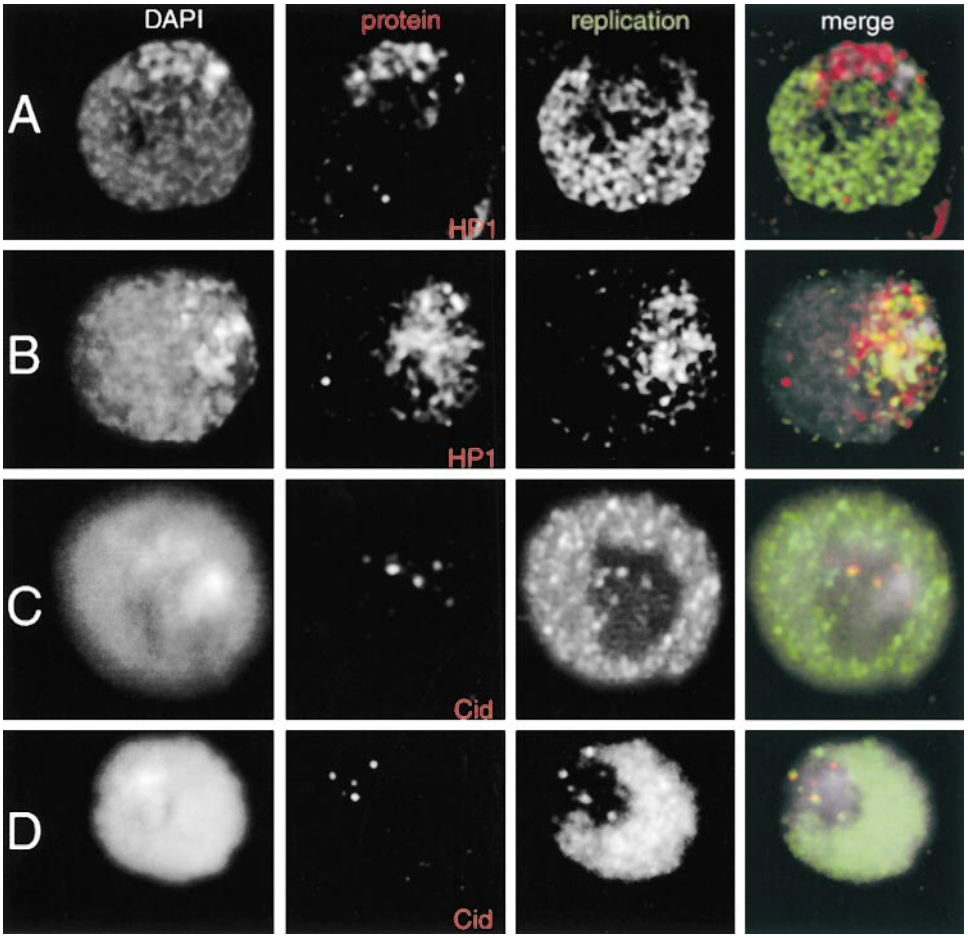


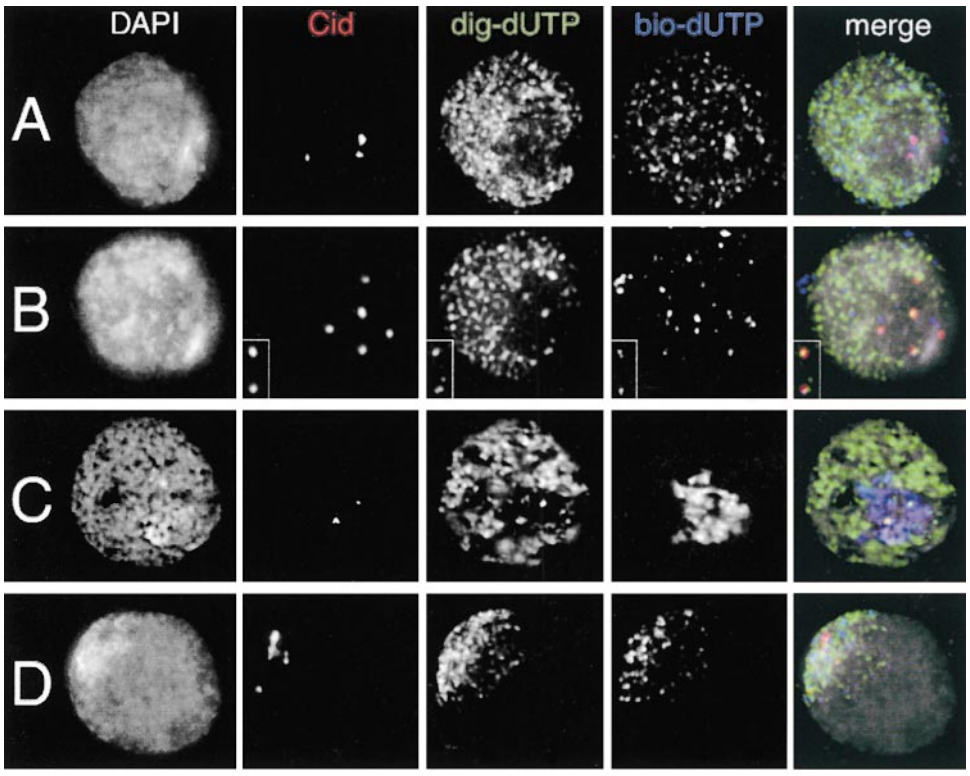
Figure 1. Euchromatin and heterochromatin replicate at distinct times in *Kc* nuclei. Newly replicated DNA was visualized by incorporation of dig-dUTP (A–C) or BrdU (D), and nuclei were counterstained with DAPI. (A and B) Detection with anti–HP1 antibodies stains the chromocenter; the remainder of each nucleus is euchromatin. (A) Euchromatic replication; (B) heterochromatic replication; (C and D) detection of centromeres by anti–Cid antibody. In the merged images, protein localization is in red and sites of replicating DNA are in green. Nuclei show on average 5.6 ($\pm$2.1, n = 254) centromeric spots throughout most of the cell cycle. Incorporation of the DNA label reveals that centromeres replicate with euchromatin. Single optical sections are shown (z = 0.2 μm).

replicating, revealing that centromeres replicate in early S phase (Fig. 1 C). Centromeres appear to be the only sites in the chromocenter to replicate at this time. Of 42 nuclei in which euchromatin was labeled, 20 also showed labeling at centromeres, indicating that centromeres are replicating through about one half of early S phase. However, within that period, centromeres must replicate asynchronously, since we observed incorporation at only a subset of centromeric spots in any one nucleus. Asynchronous replication of centromeric satellite has been observed in human cells (O'Keefe et al., 1992).

The nucleotide triphosphate pulse labeling procedure that we used allows for detection of incorporation without requiring DNA denaturation, thus providing excellent cytology. The brief hypotonic shock required to deliver nucleotide triphosphate analogues was found to have little or no ill effects on a wide variety of cell processes (Koberna et al., 1999). To rule out the possibility that the hypotonic treatment used to deliver nucleotide triphosphate analogues had affected DNA replication, we performed the incorporation using BrdU, which penetrates cells without hypotonic treatment. Identical results were obtained (Fig. 1 D). We conclude that replication at centromeres initiates substantially before that of the rest of the DNA in the heterochromatic compartment of the nucleus.

Prolonged Replication of Centromeres

We determined the relative timing of replication in different regions in a nucleus using two sequential pulses of nucleotide analogues. A pulse of dig-dUTP nucleotide analogue was administered, and this was followed by a chase in regular media and a second pulse of Bio-dUTP. Nuclei that had been labeled with a chase interval of 1, 2, or 3 h generally showed clear distinction between sites of dig- and Bio-dUTP incorporation, consistent with the completion of euchromatic replication in individual foci within these time intervals (Fig. 2, A and B; Manders et al., 1992). We categorized cells according to their overall pattern of nucleotide incorporation from these two pulses (Fig. 2 E: E/E, euchromatin/euchromatin; E/H, euchromatin/heterochromatin; or H/H, heterochromatin/heterochromatin), and then assessed incorporation at centromeres in each category. Some cells with an E/E pattern showed no incorporation of dig-dUTP at centromeres, but did show centromeric incorporation of the second nucleotide Bio-dUTP (Fig. 2, A and E). Incorporation of dig-dUTP but not Bio-dUTP at the centromeres of E/E pattern nuclei was never observed (n = 125). These results demonstrate that replication begins at euchromatic sites before centromeric origins fire. We also observed nuclei with an E/E labeling pattern in which both nucleotide analogues were incorporated into the same centromeric site, suggesting that this site has been replicating for the entire 1-h interval between the two pulses (Fig. 2 E, right-most column). The frequency of centromeres labeled with both nucleotide analogues suggests that replication continues for 2–3 h during the early S-phase period. Experiments in which the two nucleotide analogues were delivered 2 and 3 h apart



E

Nuclear pattern (dig/bio)	Labelling of centromeres dig: – bio: –	– +	+ –	+ +
E/E	14	12	0	19
E/H	5	1	27	5
H/H	38	2	2	0

Figure 2. Prolonged replication of centromeres. Cells were pulse labeled with dig-dUTP followed by a second pulse 3 h later with Bio-dUTP. Anti–Cid antibodies mark the positions of the centromeres. DAPI staining is white. In the merged images, Cid localization is in red, dig-dUTP in green, and Bio-dUTP in blue. Coincidence of dig-dUTP and Cid is yellow, and Bio-dUTP and Cid is magenta in the merged images. (A) An early S phase nucleus in which replication began in euchromatin before beginning in centromeres (incorporation of the second analogue only). (B) Colocalization of both nucleotide analogues at centromeres, indicating that some centromeres replicate for the entire period between the two pulses. (Insets) Two centromeres from this nucleus that display incorporation of both nucleotide analogues. (C) Labeling of euchromatin and centromeres with first pulse, and the chromocenter with the second pulse. (D) Labeling of distinct subsets of heterochromatin in the chromocenter by the two pulses, but not of centromeres. All images are single optical sections. (E) Number of centromeres labeled by nucleotide analogues. A dig-dUTP nucleotide pulse was delivered to cells and followed by a second pulse of Bio-dUTP 1 h later. Nuclei that had incorporated both nucleotide analogues were categorized by the overall replication patterns shown by each pulse (E/E, euchromatic/euchromatic; E/H, euchromatic/heterochromatic; H/H, heterochromatic/heterochromatic labeling). At least 10 nuclei of each combined pattern were examined. The "heterochromatin/euchromatin" pattern was never observed. Centromeres were scored for the incorporation of each nucleotide analogue (− or +), and the numbers of centromeres showing incorporation of either or both analogues are shown.

confirmed this calculation, because occasional nuclei were seen in which both dig- and Bio-dUTP were incorporated at a single centromeric spot (Fig. 2 B). This is an unusually prolonged period of replication since most sites complete replication in 40–60 min (Fig. 2; Manders et al., 1992; Ma et al., 1998). Based on the integrated intensity of nucleotide incorporation at centromeric replication foci (see Materials and Methods), we estimate that ~500 kb of DNA at each centromere within a centromeric replication focus is replicated in the early S-phase period.

The detection of early replication at centromeres is aided by their clear spatial separation from simultaneously replicating euchromatin. In nuclei with heterochromatic labeling patterns, many replication foci are near centromeres (Fig. 2, C and D), and the high density of foci might obscure centromere replication. To examine whether any replication occurs at centromeres in the late S period, we quantitated the amount of incorporated nucleotide analogue in centromeric spots in the early and late periods. These measurements show that ~90% of all detectable nucleotide incorporation at centromeres occurs in the early period (316 U mean intensity/pixel per centromere in the early period versus 34 U in the late period). Thus, the upper limit on the amount of replication at centromeres that occurs in the late S period is ~10%, but even this may be due to nearby replication foci that could not be distinguished from centromeres. We conclude that centromeric replication is largely complete before the rest of the heterochromatic chromocenter replicates.

Histone H3 Is Excluded from Centromeres

Models for centromere specification by unique DNA sequences are ruled out by the absence of centromere-specific sequences in natural centromeres (Karpen and Allshire, 1997), and the establishment of neocentromeres

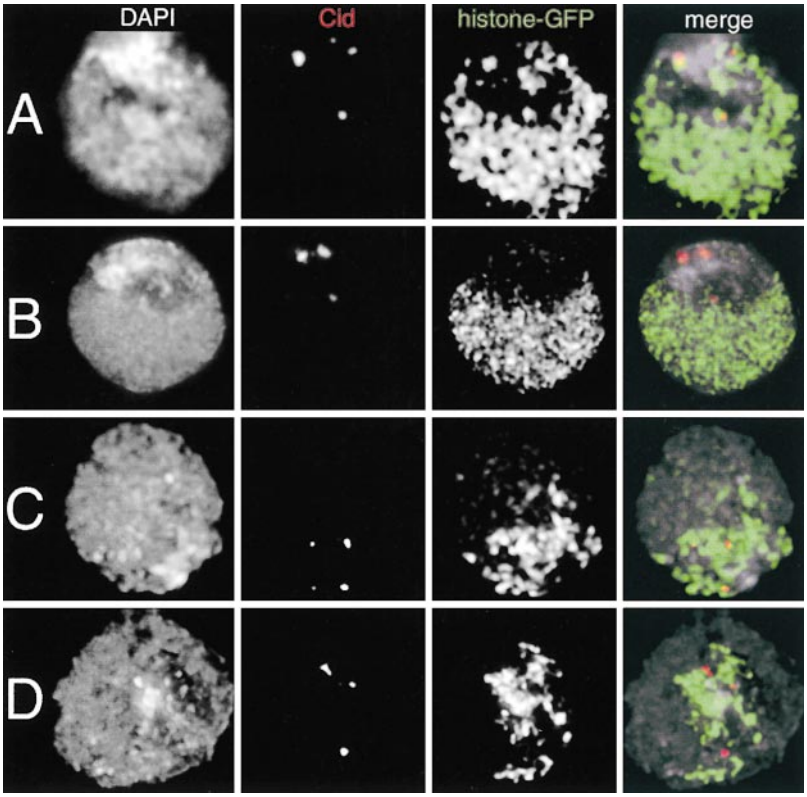


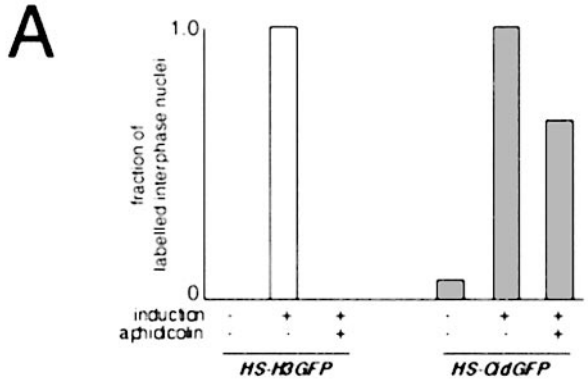
Figure 3. Localization of histone-GFP fusion proteins. (A and B) Expression of histones H2B-GFP and H3-GFP from the *cid* promoter, which is active early in S phase. DAPI staining is white. In the merged images, Cid localization is in red and histone-GFP in green. H2B-GFP localizes to euchromatin and to centromeres (A), but H3-GFP protein localizes only to euchromatin (B). For each histone-GFP fusion, we normalized the fluorescence intensity of GFP at centromeres to that in euchromatin in individual nuclei. The ratio of incorporation of H2B-GFP at centromeres relative to euchromatin was 0.016 (SEM = 0.01, $n = 23$), while for H3-GFP it was 0.0014 (SEM = 0.0004, $n = 10$). These ratios are significantly different when compared by a Mann-Whitney test ($P = 0.0002$). (C and D) Expression of H2B-GFP and H3-GFP, respectively, after induction of a heat-shock promoter. Both histone-GFP fusion proteins give similar labeling of heterochromatin in late S-phase cells. Single optical sections are shown.

at previously noncentromeric regions (Warburton et al., 2000). An attractive alternative to DNA sequence–based specification of centromeres is that centromeric nucleosomes define the centromere. In all eukaryotes, centromeres are likely to be contained in specialized nucleosomes that include an H3-like protein (Tyler-Smith and Floridia, 2000). We have previously shown that Cid-GFP fusion protein produced from the *cid* promoter localizes to centromeres, and that this promoter is active early in S phase (Henikoff et al., 2000). Thus, the normal localization of Cid takes place as centromeric DNA is replicating. However, the deposition of specialized nucleosomes at replicating centromeres must be difficult because histone H3 is in vast excess throughout S phase (Osley, 1991).

We wondered whether the deposition of Cid in centromeric replication domains might be facilitated by preventing the deposition of histone H3. Conventional nucleosomes are normally assembled during replication; therefore, we examined the deposition of conventional histones fused to GFP when centromeres replicate. Previous work has shown that histone-GFP fusion proteins can localize to chromatin (Kanda et al., 1998; Henikoff et al., 2000). We had previously characterized the deposition of H2B-GFP and H3-GFP fusion proteins by examining mitotic chromosomes. When expressed from the *cid* promoter, which is active early in S phase, these fusion proteins localize to the euchromatic arms of chromosomes (Henikoff et al., 2000). These experiments did not address whether small quantities of the fusion proteins were also incorporated at centromeres. Therefore, we examined the deposition of histone-GFP fusion proteins after production from transfected *cid*-promoter constructs in interphase nuclei to determine whether these histones were incorporated in centromeric replication domains. While H2B-GFP was readily deposited at centromeres, H3-GFP was not ($P = 0.0002$; Fig. 3, A and B). Both histone-GFP proteins give similar intense labeling in euchromatin, implying that the lack of H3-GFP at centromeres is not due to a general reduced deposition of H3-GFP. Labeling in heterochromatin by H3-GFP and H2B-GFP produced from heat-shock promoter constructs was also indistinguishable in intensity and pattern (Fig. 3, C and D). That the centromere may be deficient of histone H3 has been previously suggested, based on the absence of phosphorylated H3 antibody labeling (van Hooser et al., 1999), and our results with H3-GFP fusion protein support this idea. We conclude that the deposition of H3-containing nucleosomes is inhibited at the time that the *cid* promoter is active in early S phase.

Cid-containing Nucleosomes Are Assembled by a Replication-independent Pathway

The centromeric histone CENP-A can form nucleosomal particles in vitro (Yoda et al., 2000), and cofractionates with other histones in vivo (Palmer et al., 1987; Shelby et al., 1997). The similarity of *Drosophila* Cid to histone H3 suggests that Cid is also incorporated into nucleosomes. Since the deposition of histone H3 is inhibited as centromeres replicate, Cid-containing nucleosomes might be formed using an alternate pathway. To investigate the nature of a Cid deposition pathway, we tested the dependence of histone-GFP protein localization on DNA replication. A pulse of histone-GFP fusion proteins can be produced in cells by transfecting with a heat-shock promoter construct, and then inducing the promoter. Newly produced H2B-GFP and H3-GFP proteins localize in subnuclear patterns similar to patterns produced by pulses of



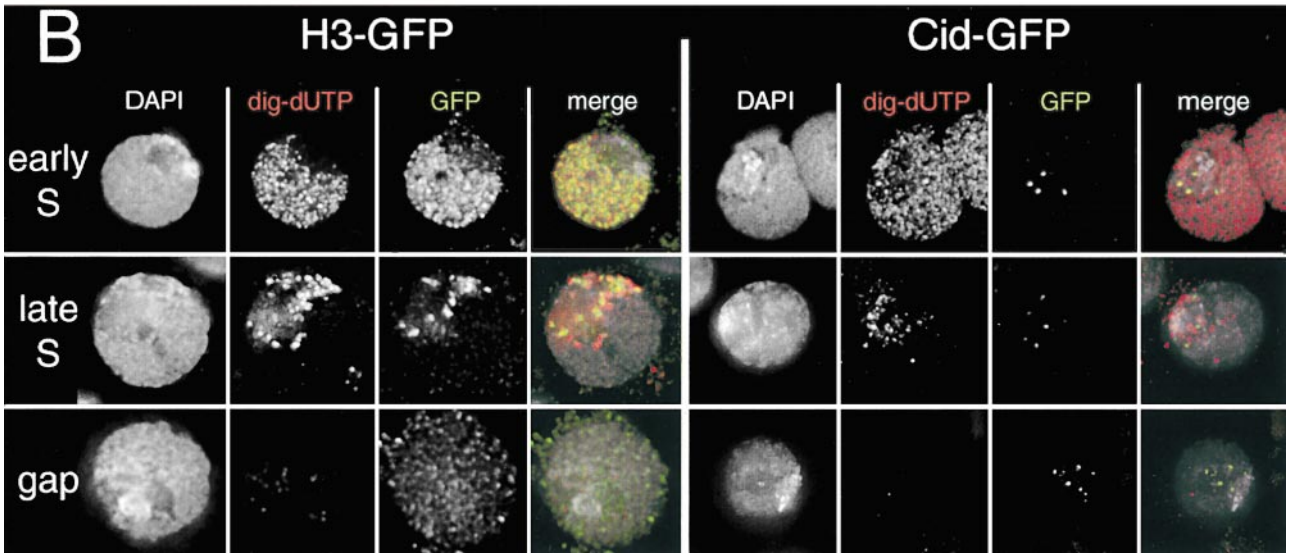


Figure 4. Cid-GFP is deposited at centromeres by a replication-independent pathway. (A) Cells transfected with *HS-H3GFP* (open bars) or *HS-CidGFP* (grey bars) were treated with aphidicolin to block DNA replication and then induced by heat-shock (no replication of DNA was seen in cells treated with aphidicolin, and then pulsed with dig-dUTP, data not shown). The number of cells showing GFP localization in centromeres (of Cid-GFP) or in any part of the nucleus (H3-GFP) was compared with the number observed in induced, untreated cells. Localization of H3-GFP is replication dependent, but that of Cid-GFP is not. For each sample, 100–200 nuclei were examined. (B) Cells transfected with heat-shock promoter-histone-GFP constructs were induced, and then loaded with dig-dUTP to mark replicating DNA. In the merged images, sites of replicating DNA are in red, and protein localization is in green. Nuclei from top to bottom are in early S phase, late S phase, and gap phase. H3GFP fusion protein localizes to replicating regions in S phase cells and does not localize in gap phase cells. Cid-GFP fusion protein localizes to centromeres in all cell stages. The intensities of GFP signals in each image are shown on the same absolute scale. Single optical sections are shown.

nucleotide analogues (Fig. 3; Henikoff et al., 2000), and fail to localize in cells that are blocked by the replication-inhibiting drug aphidicolin (Fig. 4 A). Thus, the deposition of these fusion proteins is coupled to the replication of the underlying DNA, just as is the formation of conventional nucleosomes (Adams and Kamakaka, 1999; Krude, 1999).

To determine whether the localization of Cid to centromeres is also coupled to replication, we transfected a heat shock promoter-Cid-GFP fusion construct (*HS-CidGFP*) into cells. Cells were then induced to express the fusion protein, or treated with aphidicolin to block DNA replication, and then induced. Centromeric localization of Cid-GFP occurred both in treated and untreated cells (Fig. 4 A), demonstrating that the deposition of Cid need not be coupled to DNA replication. The replication independence of Cid-GFP localization distinguishes it from that of the bulk of conventional histones.

We further examined the localization of Cid-GFP to centromeres when produced at different times in the cell cycle. The deposition of histones onto DNA is predominantly limited to cells that are in S phase of the cell cycle (Wu et al., 1986). We transfected cells with heat shock promoter-histone-GFP fusion constructs (*HS-H3GFP* or *HS-CidGFP*). We then induced the construct and pulsed these cells with nucleotide analogues to identify cells in S phase. A pulse of H3-GFP protein localized in early and late replication patterns similar to the nucleotide analogue in the same cells, and no detectable localization occurred in gap-phase cells (Fig. 4 B, left). However, we found that newly produced Cid-GFP protein localized to centromeres in

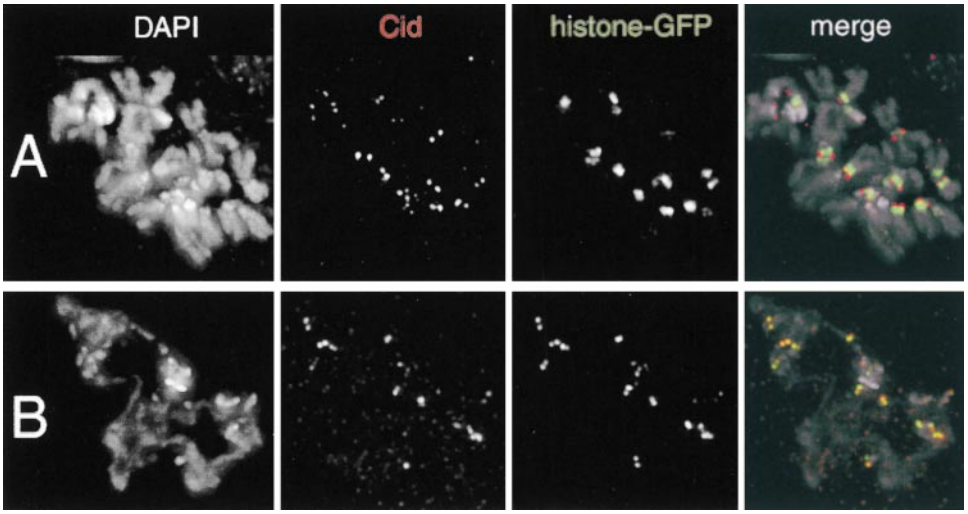


Figure 5. Deposition of Cid-GFP before mitosis. Cells transfected with *HS-H3GFP* or *HS-CidGFP* were induced and prepared for cytology after various chase times. We examined 50 metaphase figures from each time point and counted the number of labeled mitotic figures. In the merged images, Cid localization is in red and histone-GFP in green. Each image is a projection of multiple sections through the spread. (A) Mitotic chromosomes that show H3GFP labeling first appear 4 h after induction. H3GFP labels pericentric heterochromatin in these chromosomes, as expected for cells that were in late S phase at the time of induction. (B) Metaphase spreads with Cid-GFP at centromeres appear earlier, 2 h after induction, indicating that these cells were in G2 phase when induced.

both S-phase and gap-phase cells with similar efficiencies (Fig. 4 B, right). We confirmed that localization of Cid-GFP protein can occur in post-replicative cells, whereas that of H3-GFP is strictly replication dependent, by examining metaphase chromosomes at various times after induction of transfected constructs. In cells transfected with *HS-H3GFP*, occasional labeled metaphase figures first appear 4 h after induction and are labeled in heterochromatic segments (Fig. 5 A). The pattern of labeling and timing of these figures reflects the deposition of H3-GFP in late S-phase cells and the shortest time required to traverse the G2 phase of the cell cycle, and is consistent with previous observations in *Kc* cell populations (Barigozzi et al., 1966; Dolfini et al., 1970). However, Cid-GFP first appears at the centromeres in 48% of mitotic figures within 2 h of induction: these must be from cells that were induced during the G2 phase (Fig. 5 B). By 4 h s after induction, 100% of the mitotic figures from transfected cells show centromeric labeling. We conclude that the Cid deposition pathway is present and active throughout the cell cycle.

Discussion

Analysis of centromeres in complex eukaryotes has been hampered by the lack of sequence differences between the centromere and flanking heterochromatin, and the repetitive nature of these regions (Sun et al., 1997; Willard, 1998). These sequence commonalities have led to the attribution of heterochromatic features to the centromere, including late replication. Our analysis demonstrates that the replication of centromeres in *Drosophila* cells actually precedes that of pericentromeric heterochromatin. We estimate that, on average, ~500 kb of centromeric DNA is replicated in the early S phase period. This size is in agreement with a genetically defined fully functional centromere in *Drosophila* (Murphy and Karpen, 1995), suggesting that the early replication domain corresponds to the complete centromere. The early timing of its replication distinguishes the centromere from other repetitive sequences and rules out models for defining centromeres that have invoked their very late replication (Dupraw, 1968; Csink and Henikoff, 1998). Early replication appears to be a general feature of centromeres, as *Saccharomyces* centromeres are known to replicate early in S phase (McCarroll and Fangman, 1988).

Our observations on the controlled assembly of conventional (H3-containing) and specialized (Cid-containing) nucleosomes at replicating centromeres suggests that chromatin assembly is a critical step in centromere maintenance. The *cid* promoter drives expression early in S phase (Henikoff et al., 2000), and centromeres are replicating during this time. Therefore, Cid synthesis and centromere replication appear to be tightly coordinated. In *Schizosaccharomyces* yeast, the Cnp1 gene (encoding the centromeric SpCenpA histone) is also expressed early in S phase (Takahashi et al., 2000), and we expect that a similar coordination with centromeric replication will be found.

At the time that Cid is being deposited, H3 deposition is inhibited. It is striking that early replicating centromeres are typically surrounded by late-replicating heterochromatin, and we suggest that inhibiting histone H3 incorporation at centromeres when they replicate is one function of this juxtaposition. Inhibiting histone H3 incorporation at centromeres requires the uncoupling of conventional chromatin assembly and DNA replication. These two processes are thought to be linked by interactions between replication machinery and the CAF1 chromatin assembly factor (Krude, 1995; Verreault et al., 1996). Uncoupling may be accomplished if histone H3 or some component of its assembly machinery is excluded from the heterochromatic chromocenter early in S phase. Regions deficient in histone H3 would then be incorporated into Cid-containing nucleosomes by a replication-independent pathway.

The observation that centromeric histone H3-like proteins from worms and yeast preferentially localize to fly or human heterochromatin (Henikoff et al., 2000) suggests that heterochromatin sequesters centromeric H3-like pro-

teins in general. Sequestering Cid in the heterochromatic chromocenter would increase the local concentration of Cid around centromeres and thereby promote Cid deposition. Centromeres in many organisms are typically surrounded by heterochromatin, and genetic evidence suggests that heterochromatin is important for centromere function (Allshire et al., 1995). The centromeres of *Saccharomyces* chromosomes are the only known exception to this rule, but in this organism centromeric activity is conferred by a specific DNA sequence and associated DNA-binding proteins (Meluh and Koshland, 1997). The importance of heterochromatin for the function of complex centromeres is reinforced by the finding that a human neocentromere shows M31 staining (a marker for mammalian heterochromatin), whereas the parental chromosomal region does not (Saffery et al., 2000). Perhaps the exclusion of histone H3 during replication is one of the prerequisites for "centromerization" (Choo, 2000), thus necessitating that neocentromeres acquire heterochromatic proteins.

We expect that centromeres must be protected from conventional nucleosome assembly pathways in all dividing cells, but heterochromatin may not always perform this function. For example, distinct heterochromatin does not form in the rapidly dividing nuclei of *Drosophila* syncytial embryos (Hiraoka et al., 1993), and replication initiates throughout the chromosomes simultaneously (Kriegstein and Hogness, 1974). In these unusual nuclei, conventional nucleosome assembly might be prevented by excluding histone H3 from the apical edge of interphase nuclei, where centromeres lie (Foe et al., 1993). Similarly, varying local concentrations of proteins around nuclei have been proposed to explain progression of the syncytial cell cycle even though bulk cyclin levels are always high (Foe et al., 1993). In later cycles, it appears to be most efficient to produce Cid when centromeres replicate.

It has been of great interest to understand how the location of the centromere is stably maintained in successive cell divisions, because it does not appear that DNA sequence is responsible (Brown and Tyler-Smith, 1995; Karpen and Allshire, 1997). Nucleosome particles form the fundamental unit of chromatin, and so an attractive alternative to DNA sequence-based inheritance of centromere identity is that centromeric nucleosomes participate in centromere maintenance (Palmer et al., 1990). Replication initiation appears to depend on chromatin structure (Ofir et al., 1999; Stevenson and Gottschling, 1999) and we suggest that Cid-containing nucleosomes predispose DNA to replicate early. This early replication and the exclusion of histone H3 in heterochromatin would preclude conventional chromatin assembly, thus allowing the assembly of Cid-containing nucleosomes and ensuring early replication again in the next cycle. This process would maintain centromeres.

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