Chromatin Assembly at Kinetochores Is Uncoupled from DNA Replication

Richard D. Shelby, Karine Monier, and Kevin F. Sullivan

Department of Cell Biology, The Scripps Research Institute, La Jolla, California 92037

Abstract. The specification of metazoan centromeres does not depend strictly on centromeric DNA sequences, but also requires epigenetic factors. The mechanistic basis for establishing a centromeric “state” on the DNA remains unclear. In this work, we have directly examined replication timing of the prekinetochore domain of human chromosomes. Kinetochores were labeled by expression of epitope-tagged CENP-A, which stably marks prekinetochore domains in human cells. By immunoprecipitating CENP-A mononucleosomes from synchronized cells pulsed with [3H]thymidine we demonstrate that CENP-A–associated DNA is replicated in mid-to-late S phase. Cytological analysis of DNA replication further demonstrated that centromeres replicate asynchronously in parallel with numerous other genomic regions. In contrast, quantitative Western blot analysis demonstrates that CENP-A protein synthesis occurs later, in G2. Quantitative fluorescence microscopy and transient transfection in the presence of aphidicolin, an inhibitor of DNA replication, show that CENP-A can assemble into centromeres in the absence of DNA replication. Thus, unlike most genomic chromatin, histone synthesis and assembly are uncoupled from DNA replication at the kinetochore. Uncoupling DNA replication from CENP-A synthesis suggests that regulated chromatin assembly or remodeling could play a role in epigenetic centromere propagation.

Key words: kinetochore • centromere • chromatin • DNA replication • CENP-A

Introduction

Specification of centromeres on metazoan chromosomes appears to involve both DNA sequence determinants and epigenetic factors such as chromatin structure and replication (Harrington et al., 1997; Karpen and Allshire, 1997; Ikeno et al., 1998; Murphy and Karpen, 1998). Although human centromeric alpha-satellite DNA is able to promote centromere formation in certain instances (Harrington et al., 1997; Ikeno et al., 1998), alphoid DNA is neither necessary nor sufficient for centromere formation (Barry et al., 1999). An alternative view is that centromere identity is specified by an epigenetic mark on the chromosome that is independent of its underlying DNA (Karpen and Allshire, 1997). Candidates for such a mark include DNA methylation (Mitchell et al., 1996), chromatin structure (Ekwall et al., 1997; Vafa and Sullivan, 1997; Willard, 1998; Williams et al., 1998), and compartmentalized replication timing for centromeric DNA (Csink and Henikoff, 1998).

CENP-A is a specialized histone H3-like protein localized in the inner kinetochore plate of mammalian mitotic chromosomes (Palmer et al., 1991; Sullivan et al., 1994; Warburton et al., 1997). It is present throughout the cell cycle and therefore constitutively marks a “prekinetochore” domain of the centromere destined to become the mitotic kinetochore (Brenner et al., 1981; Sullivan et al., 1994). The CENP-A motif, comprising a histone H3-like histone fold domain coupled to a unique NH2-terminal domain, appears to be a widely conserved feature of centromeres (Sullivan et al., 1994; Stoler et al., 1995; Buchwitz et al., 1999; Henikoff et al., 2000; Takahashi et al., 2000). For bulk chromatin, most new histone synthesis is tightly coupled with DNA replication during S phase (Wu and Bonner, 1981). However, if CENP-A expression is experimentally limited to S phase using a replication-dependent histone H3 vector, centromere-specific assembly is abolished (Shelby et al., 1997). Endogenous CENP-A mRNA accumulation is maximal in the G2 phase of the cell cycle, suggesting that the timing of CENP-A expression plays an important role in centromere targeting (Shelby et al., 1997). Thus, if CENP-A expression is coupled to kinetochore DNA replication, then kinetochore DNA replication must occur quite late in the cell cycle. Such a mechanism has been proposed as a means of maintaining the unique identity of centromeres (Csink and Henikoff, 1998). Alternatively, the synthesis of CENP-A could be uncoupled from kinetochore DNA replication in S phase. If this were so, it would point to a distinctive mechanism.
for postreplicative chromatin assembly at the kinetochore. To distinguish between these possibilities, we directly measured the replication timing of prekinetochore DNA and CENP-A synthesis during the cell cycle.

Materials and Methods

DNA Replication Analysis

Kinetochore labeling was performed by inducing CENP-A–HA1 expression in HeLa Tta-CENP-A–HA1 cells for 2 d (Shelby et al., 1997). CENP-A–HA1 expression was repressed and cells were synchronized by a double thymidine block (2 mM thymidine in complete DME for 15 h each, separated by a 9-h interval). Cells were released into S phase by removal of thymidine and sampled at hourly intervals. DNA replication was assayed with a 30-min pulse of medium containing 5 μCi/ml [3H]thymidine. Cells were washed with PBS, and nuclei were harvested directly from dishes in nuclear isolation buffer (20 mM KCl, 3.75 mM Tris-Cl, pH 8.0, 0.05 mM spermine, 0.125 mM spermidine, 0.5 mM EDTA, and 0.5 mM DTT) with 0.1% digitonin. Washed nuclei were digested with micrococcal nuclease at 250 U/ml for 1 h at room temperature in buffer A (15 mM Tris-Cl, pH 7.4, 60 mM KCl, 15 mM NaCl, 0.2 mM EDTA, 1 mM DTT, 15 mM spermine, 0.5 mM spermidine, and 0.22 M sucrose). Reactions were stopped and nuclei lysed by addition of an equal volume of buffer A plus 600 mM NaCl, 0.1% NP-40 before scintillation counting. For flow cytometry, cells were fixed in 70% ethanol, then stained in

Immunocytochemistry and Microscopy

Immunofluorescence was performed essentially as described previously (Sullivan et al., 1994), with specific antibodies cited in the figure legends. Immunocytochemistry and Microscopy was performed with a widefield optical sectioning microscope (Deltavision; Applied Precision) and images were processed using constrained iterative deconvolution. Fluorescence signal intensities were quantitated using SoftWorx® analysis software (Applied Precision). Total signal intensities were determined in each cell by summing signal intensity for each probe within the whole nuclear volume as defined by DAPI (4',6'-diamidino-2-phenylindole) staining; signal intensity in discrete stained foci was determined using an intensity thresholding step and a three-dimensional polygon building algorithm. The signal to noise ratio was calculated as the ratio of signal intensity in discrete foci versus background signal intensity (total signal intensity minus the summed intensity in discrete foci). Colocalization of newly synthesized CENP-A with centromeres was assayed by determining the amount of CREST antibody signal contained within CENP-A–HA1 stained foci and is expressed as a fraction of the total signal intensity. Detection of DNA replication with bromodeoxyuridine (BrdU) was performed by the manufacturer’s instructions (Roche Molecular Biochemicals). Prints were prepared by assembling digital images with Adobe PhotoShop®.

Protein Analysis

Electrophoresis and Western blot techniques were performed as described previously using antibodies specified in the text (Shelby et al., 1997). For quantitation, the dynamic range of X-omat AR film (Eastman Kodak Co.) was determined empirically and Western blots were processed under conditions of linear response. Integrated intensities were quantitated using Image Pro Plus® (Media Cybernetics) from images digitized at 300 dpi using a flatbed scanner. For CENP-A, the integrated intensity in each lane was normalized against CENP-A–HA1. Anti-phospho H3 antibody was a gift from David Allis (University of Virginia, Charlottesville, VA). The range of phosphorylated histone H3 abundance exceeded the dynamic range of the film and was estimated by correcting for the time required to obtain similar band intensities from mitotic versus S phase time points. Images were assembled from scanned films using Adobe Photoshop®.

Results and Discussion

The prekinetochore chromatin domain of HeLa centromeres was labeled with an epitope-tagged derivative of CENP-A, using a stably transfected cell line that inductively expresses HA-1 epitope tagged CENP-A (Shelby et al., 1997). CENP-A–HA1 faithfully localizes to the inner kinetochore region (Warburton et al., 1997) and can be immunoprecipitated as mononucleosomes in association with alpha-satellite DNA (Shelby et al., 1997; Vafa and Sullivan, 1997). Like core histones, CENP-A is quite stable. This...
was shown in an epitope pulse–chase experiment in which CENP-A–HA1 expression was induced for 2 d and then repressed for several cell generations and assayed by Western blot (Fig. 1 A). On a per cell basis, CENP-A–HA1 decreases by ≈50% per generation and is readily detectable 4 d after repression (Fig. 1 A), indicating that the protein half-life is significantly greater than the cell cycle time. Immunofluorescence demonstrated that CENP-A–HA1 is retained at prekinetochores for multiple generations with individual cells exhibiting uniform labeling of centromeres. This suggests that parental CENP-A is equally partitioned to daughter centromeres with each round of replication as has been demonstrated for histone H3/H4 heterotetramers on bulk chromatin (Jackson, 1988). Therefore, CENP-A–HA1 is a suitable biochemical marker for kinetochore-associated DNA throughout the cell cycle.

For kinetochore DNA replication analysis, CENP-A–HA1 expression was induced for 2 d and then repressed as cells were synchronized at the G1/S boundary by double thymidine block. After release, cells proceeded through S phase, G2/M, and into the subsequent cell cycle as assayed by flow cytometry (Fig. 1 B). Replicating DNA was pulse labeled with [3H]thymidine at hourly intervals over a 12-h time course, sufficient for >90% of cells to complete mitosis and enter the subsequent G1 phase of the cell cycle. Total DNA replication begins shortly after release into S phase, peaks after 4 h, and is completed by 7–8 h (O’Keefe et al., 1992; Haaf and Ward, 1994). Second, there is no time when centromeres are the only loci being replicated. In all cells exhibiting BrdU uptake, noncentromeric replication foci were always present in cells that had replicating centromeres. Thus, centromeres do not comprise a uniquely late replicating component of human chromosomes.

Previous experiments demonstrated that CENP-A mRNA accumulation begins late in S phase and peaks in G2 (Shelby et al., 1997). Here, we examined the timing of CENP-A protein accumulation in the cell cycle by Western blot analysis (Fig. 3, A and B). Kinetochores were first labeled by expression of CENP-A–HA1 and then repressed as described for Fig. 1. Since CENP-A–HA1 is sta-

Figure 2. Spatial and temporal organization of centromere replication. HeLa Tta-CENP-A–HA1 cells were synchronized as described in the legend to Fig. 1. At hourly intervals, replicating DNA was pulse labeled with BrdU. Cells were then fixed and subjected to immunofluorescence analysis to localize DNA replication (BrdU, green) and centromeres (hACA, red). Cells representative of the five classes of S phase described by O’Keefe et al. (1992) are shown with time after release indicated at the bottom. The BrdU image was used to mask the centromere image in Photoshop®, and the resulting image (k–o) reveals centromere-associated DNA replication. Consistent with metabolic labeling, centromere replication appears to peak in the latter half of S phase. Centromeres were never the only loci undergoing replication, nor did we observe cells in which all centromeres were undergoing replication.

behavior documented for CENP-A–HA1 is reflective of endogenous CENP-A. Previous analysis of bulk alpha-satellite DNA showed that it is replicated in mid-to-late S phase (Ten Hagen et al., 1990; O’Keefe et al., 1992). Thus, the DNA of the prekinetochore domain replicates during the canonical S phase with timing similar to that of the total alpha-satellite DNA fraction.

The kinetic and spatial organization of centromere DNA replication was also examined in a cytological assay. Synchronized cells were pulsed with BrdU at hourly intervals after release into S phase. DNA replication sites and centromeres were then localized by immunofluorescence microscopy (Fig. 2). The characteristic spatial evolution of DNA replication (O’Keefe et al., 1992) was evident and examples of each class of replication pattern are shown in Fig. 2. The BrdU image was used to mask the centromere image and the resulting image, which shows replicating centromeres, is shown in Fig. 2, k–o. Centromere replication was highest between 4 and 6 h after release, consistent with the metabolic labeling experiments shown in Fig. 1. This experiment demonstrates two additional features of centromere replication. First, centromeres replicate asynchronously, consistent with previous reports of alpha-satellite DNA replication (O’Keefe et al., 1992; Haaf and Ward, 1994). Second, there is no time when centromeres are the only loci being replicated. In all cells exhibiting BrdU uptake, noncentromeric replication foci were always present in cells that had replicating centromeres. Thus, centromeres do not comprise a uniquely late replicating component of human chromosomes.

Spatial and temporal organization of centromere replication appears to peak in the latter half of S phase. Centromeres were never the only loci undergoing replication, nor did we observe cells in which all centromeres were undergoing replication.
tensity of CENP-A–HA1 in the experiment. The fold change in CENP-A (y axis, left) is plotted relative to its value at the time of release (solid black line). Values shown are the average of three independent experiments (with one standard error shown). For comparison, CENP-A–associated DNA synthesis (y axis, right), determined in Fig. 1, is plotted (solid gray line). (C and D) Histone H3 phosphorylation in synchronized cells. Parallel samples were probed for the presence of phosphorylated histone H3, a marker for late G2 and mitosis, beginning just before chromatin assembly. Unlike bulk chromatin, in which new histone synthesis is tightly coupled to DNA replication (Wu and Bonner, 1981), CENP-A is available for assembly only after DNA replication has occurred. Indeed, if CENP-A expression is restricted to S phase, it is progressively assembled throughout the chromosomes, and centromere-specific assembly cannot occur (Shelby et al., 1997). Regulation of DNA replication timing is thus un-

Copyright © 2000 The Rockefeller University Press. All rights reserved.

Figure 3. CENP-A accumulation occurs in G2. (A) CENP-A accumulation occurs in G2. HeLa Tta-CENP-A–HA1 cells were induced and synchronized as described above. The kinetics of histone synthesis was assayed by pulsing cells with [3H]leucine for 3 h at different times after release, showing that synthesis peaks between 3 and 6 h into S phase (top, [3H]-Leu histones). At intervals, whole cell extracts were prepared by lysis in sample buffer and subjected to SDS-PAGE and Western blotting with hACA-M, allowing detection of both CENP-A–HA1 (Eps) and endogenous CENP-A (CENP-A) (bottom). Numbers refer to the time, in hours, of sample collection. Although levels of CENP-A–HA1 remained constant, endogenous CENP-A increased in abundance beginning between 7 and 9 h after release. (B) Quantitative analysis of CENP-A accumulation. Western blots were scanned and the integrated signal intensity was determined for CENP-A–HA1 and endogenous CENP-A. CENP-A–HA1 was used to correct for lane loading differences using the ratio of CENP-A–HA1 intensity in that lane to the maximal inten-

Copyright © 2000 The Rockefeller University Press. All rights reserved.

Figure 3. CENP-A accumulation occurs in G2. (A) CENP-A accumulation occurs in G2. HeLa Tta-CENP-A–HA1 cells were induced and synchronized as described above. The kinetics of histone synthesis was assayed by pulsing cells with [3H]leucine for 3 h at different times after release, showing that synthesis peaks between 3 and 6 h into S phase (top, [3H]-Leu histones). At intervals, whole cell extracts were prepared by lysis in sample buffer and subjected to SDS-PAGE and Western blotting with hACA-M, allowing detection of both CENP-A–HA1 (Eps) and endogenous CENP-A (CENP-A) (bottom). Numbers refer to the time, in hours, of sample collection. Although levels of CENP-A–HA1 remained constant, endogenous CENP-A increased in abundance beginning between 7 and 9 h after release. (B) Quantitative analysis of CENP-A accumulation. Western blots were scanned and the integrated signal intensity was determined for CENP-A–HA1 and endogenous CENP-A. CENP-A–HA1 was used to correct for lane loading differences using the ratio of CENP-A–HA1 intensity in that lane to the maximal intensi-
likely to play a direct role in centromere maintenance. This would appear to rule out, at least for human centromeres, the “last to replicate” model of centromere maintenance in which centric DNA replication occurs uniquely late in order to couple with distinctive chromatin proteins expressed late in the cell cycle (Csink and Henikoff, 1998). Rather, our results point toward regulated chromatin assembly as a distinctive mechanism in centromere maintenance. Mechanisms that mediate nucleosome assembly without coupled DNA replication must exist, as replacement histones are efficiently incorporated in nonreplicating nuclei in numerous species (Pina and Suau, 1987; Thatcher et al., 1994). Indeed, *Tetrahymena thermophila* exhibits a specific requirement for constitutive histone H3 expression (Yu and Gorovsky, 1997). Although DNA synthesis-independent chromatin assembly has not been well characterized, general chromatin assembly is thought to occur through the action of one or more chromatin assembly factors that aid in deposition of histones on newly synthesized DNA (Verreault et al., 1996; Ito et al., 1997). A candidate for a CENP-A–specific assembly factor has been identified as the Mis6 gene in *Schizosaccharomyces pombe* (Takahashi et al., 2000). In the case of human CENP-A, the presence of parental CENP-A nucleosomes inherited by replicated sister kinetochores could serve as a mark to direct a chromatin assembly or remodeling factor to the kinetochore after DNA replication in S phase. Such a complex would serve as an epigenetic replicator, propagating protein complexes on the chromosome via protein–protein recognition events, without reference to the underlying DNA sequence.

This paper is dedicated to the memory of Douglas Palmer.
This work was supported by a grant (GM39068) to K.F. Sullivan from the National Institute of General Medical Science, National Institutes of Health. K. Monier had a fellowship from the French Association pour la Recherche contre le Cancer.

Submitted: 1 August 2000
Revised: 9 October 2000
Accepted: 11 October 2000

References


