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Chromatin Assembly at Kinetochores Is Uncoupled from DNA Replication

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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 [^{3}H]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 NH_2-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

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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 [^{3}H]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, 20 mM EDTA. Equivalent samples were taken for each time point for analysis of total [^{3}H]thymidine uptake. Immunoprecipitation was performed by adding 0.1% NP-40 and mAb 12CA5 IgG (25 μg) or a human scleroderma serum (hACA-M; 1 μl) followed by incubation overnight at 4°C on a rocker. Immune complexes were recovered in a 2-h incubation by addition of 50 μl of a 50% slurry of protein A sepharose (Sigma-Aldrich). Recovered immune complexes were washed six times with buffer A plus 300 mM NaCl, 0.1% NP-40 before scintillation counting. For flow cytometry, cells were fixed in 70% ethanol, then stained in 40 μg/ml propidium iodide with 100 μg/ml RNase A in PBS.

Immunocytochemistry and Microscopy

Immunofluorescence was performed essentially as described previously (Sullivan et al., 1994), with specific antibodies cited in the figure legends. 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′-diamido-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)[1] was performed per 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 antiserum 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®.

[1]*Abbreviation used in this paper:* BrdU; bromodeoxyuridine.

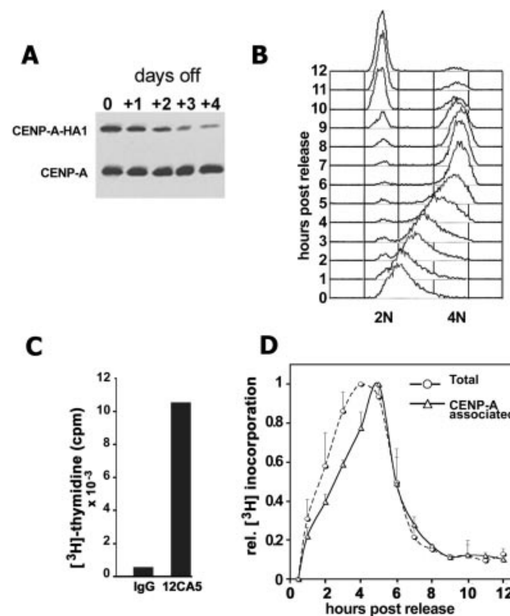

Figure 1. Timing of kinetochore-associated DNA replication. (A) CENP-A is a long lived protein. CENP-A–HA1 synthesis was induced in HeLa Tta-CENP-A–HA1 cells for 2 d (lane 0) and then repressed for 4 d. Cells were harvested at daily intervals (+1 through +4) and equal numbers of cells were analyzed by SDS-PAGE and Western blot using human autoimmune serum hACA-M, which recognizes both endogenous CENP-A (CENP-A) and epitope-tagged CENP-A (CENP-A–HA1). CENP-A–HA1 is stable, remaining detectable after four cell generations, and the ratio of CENP-A–HA1 to endogenous CENP-A decreases with each generation. (B) Synchrony of HeLa Tta-CENP-A–HA1 cells. Synchronized cells containing CENP-A–HA1 were released into S phase and sampled at hourly intervals. DNA content histograms reveal synchronous progression through S phase, with mitosis taking place ~9–11 h after release. (C) Specificity of chromatin immunoprecipitation by mAb 12CA5. Asynchronously growing cultures were pulsed with [^{3}H]thymidine for 16 h. Mononucleosomal chromatin was immunoprecipitated with mAb 12CA5 or a control mouse IgG2b. Labeled DNA was specifically recovered with mAb 12CA5. (D) Kinetochore DNA replication timing. Synchronized cells were pulse-labeled with [^{3}H]thymidine for 30 min and then mononucleosomal chromatin was prepared for immunoprecipitation. Incorporation of [^{3}H]thymidine into total DNA was assayed by liquid scintillation counting of 10^5 isolated nuclei. Data are plotted as relative values normalized to peak ^{3}H labeling. Total DNA (○) is replicated over the course of ~8 h, peaking 4 h into S phase. CENP-A–associated DNA synthesis (△) lags behind total replication and peaks 5 h after release.

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 inducibly 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

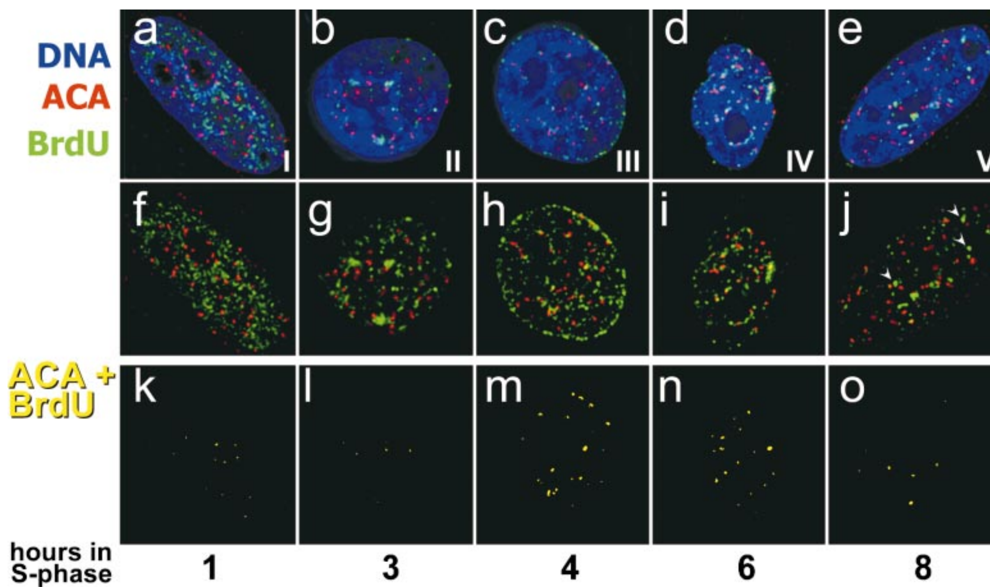

Figure 2. Spatial and temporal organization of centromere replication. HeLa Tta-CENP-A–HA1 cells were synchronized as described in the legend to Fig. 1. At hourly intervals, replicating DNA was pulse labeled with BrdU. Cells were then fixed and subjected to immunofluorescence analysis to localize DNA 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.

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 [^{3}H]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 (○, Fig. 1 D). Soluble mononucleosomal chromatin was prepared by micrococcal nuclease digestion, CENP-A–HA1 nucleosomes were immunoprecipitated with anti-HA1 mAb 12CA5, and associated DNA synthesis was determined by counting [^{3}H]thymidine (Fig. 1, C and D). CENP-A–HA1-associated DNA synthesis was delayed slightly relative to total DNA, reaching a peak 5 h after release and showing the same kinetics of completion as for total replication (△, Fig. 1 D). Identical results were obtained using human anticentromere antiserum (hACA-M), confirming that the 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.

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-

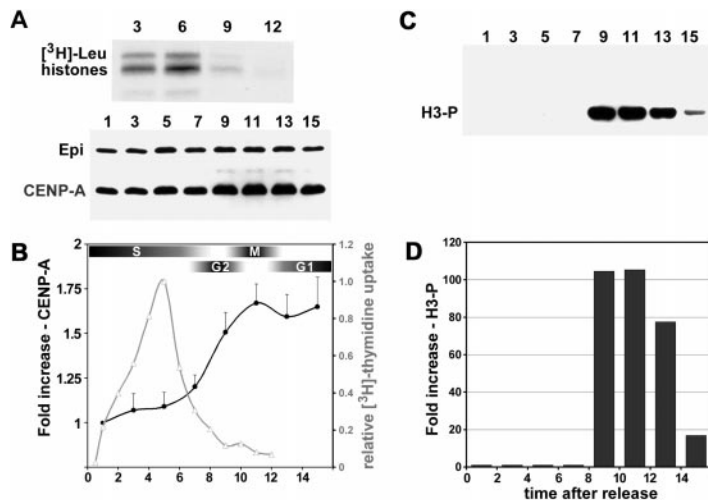

Figure 3. CENP-A accumulation occurs in G2. (A) CENP-A accumulation occurs in G2. HeLa Tta-CENP-A–HA1 cells were induced and synchronized as described above. The kinetics of histone synthesis was assayed by pulsing cells with [^{3}H]leucine for 3 h at different times after release, showing that synthesis peaks between 3 and 6 h into S phase (top, [^{3}H]-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 (Epi) 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 intensity 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 M cells. In C, significant H3 phosphorylation was detected by Western blot at 9 h after release. The fold change in phosphorylated histone H3 (bars) was estimated in one experiment, shown in D.

ble, this provides an internal standard for normalizing CENP-A protein abundance over the course of a cell cycle. For comparison, histone synthesis was assayed by SDS-PAGE fluorography after a 3-h pulse of [^{3}H]leucine (Fig. 3 A, top). Accumulation of CENP-A becomes detectable between 7 and 9 h after release into S phase, and is substantially complete by 11 h (Fig. 3, A and B). To more precisely stage CENP-A accumulation, Western blots were probed with an antibody to phosphorylated histone H3 (Fig. 3, C and D). Histone phosphorylation is a marker of late G2 and mitosis, beginning just before chromosome condensation and lasting until late anaphase (Hendzel et al., 1997). We find that significant histone H3 phosphorylation has occurred by the time CENP-A accumulation is detected at 9 h after release into S phase. These experiments demonstrate that CENP-A protein synthesis occurs in G2 phase. Thus, DNA replication at the prekinetochore is uncoupled from CENP-A synthesis.

The late synthesis of CENP-A relative to CENP-A–associated DNA contrasts with the tight coupling of bulk histone and DNA synthesis seen during S phase (Heintz et al., 1983). Normally, newly synthesized histone $(H3\text{-}H4)_2$ heterotetramers are deposited within minutes of DNA replication (Jackson, 1988). We examined the incorporation of CENP-A–HA1 within a single cell cycle using quantitative three-dimensional microscopy. Cells were synchronized at the G1/S boundary and released into S phase. CENP-A–HA1 expression was induced at the time of release and maintained throughout the experiment. Cells were fixed 10 h (late G2/M) and 22 h (late G1) after release, and CENP-A–HA1 signal was quantitated by three-dimensional microscopy (Fig. 4, A–E). Cells in G2/M, selected for positive reactivity with phosphohistone H3 antibody, exhibited low, relatively uniform levels of nucleoplasmic CENP-A–HA1 staining (Fig. 4, A and C). This is expected since most of the induction period spanned S phase, during which CENP-A is incorporated throughout chromatin (Shelby et al., 1997). Some focal incorporations of CENP-A–HA1 reaching a maximum of 2,000 on the intensity scale (Fig. 4 C, right) were detected with a low signal to noise ratio relative to nucleoplasm (Fig. 4 E, left) mostly at noncentromeric sites (Fig. 4 E, right). In contrast, CENP-A–HA1 in G1 cells showed an increase in signal intensity reaching a maximum of 12,000 (Fig. 4 D) at centromeric sites (Fig. 4 E, right), leading to a fourfold increase in signal to noise ratio, from 2.5 in G2 to 9.4 in G1 (Fig. 4 E, left). As a control, hACA-M signal to noise measured in the same cells did not exhibit any changes between G2 and G1 (Fig. 4 E, middle), demonstrating that CENP-A–HA1 was actively assembled into centromeres outside of S phase. We then asked whether CENP-A can be incorporated into centromeres in the absence of DNA replication. HeLa cells were transiently transfected with a plasmid expressing CENP-A–HA1 in medium containing 5 μg/ml aphidicolin, inhibiting DNA replication in >95% of cells. CENP-A–HA1-labeled centromeres could be detected in cells 12 h after transfection (Fig. 4 F). Together, these results demonstrate that CENP-A assembly can take place in the absence of DNA replication and that centromere-specific incorporation takes place outside S phase. Since endogenous CENP-A is synthesized in G2, we infer that CENP-A is assembled onto centromeres in the G2 phase of the cell cycle.

Our experiments demonstrate that the prekinetochore chromatin of human centromeres replicates through a distinctive pathway of uncoupled DNA replication and 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 promiscuously assembled throughout the chromosomes, and centromere-specific assembly cannot occur (Shelby et al., 1997). Regulation of DNA replication timing is thus un-

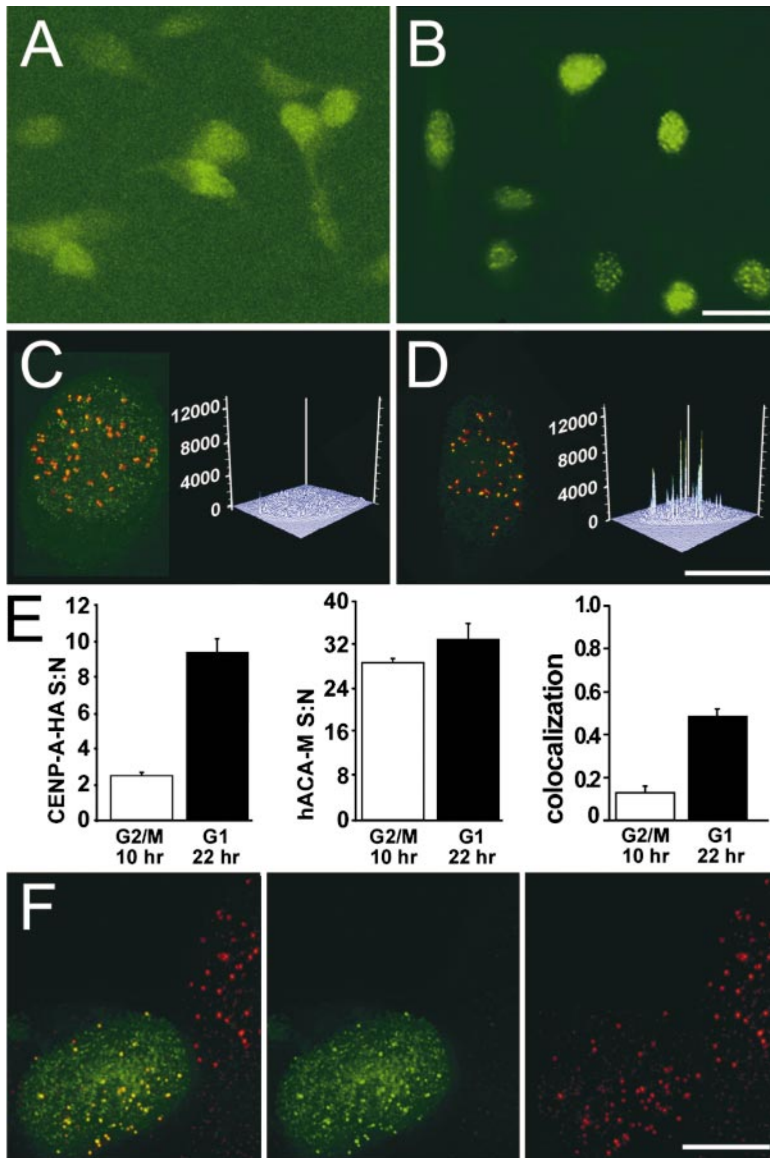

Figure 4. CENP-A assembles at centromeres without ongoing DNA replication. HeLa Tta-CENP-A–HA1 cells were synchronized without induction and then released into S phase while CENP-A–HA1 expression was induced. Cells were fixed after 10 h (A and C) or 22 h (B and D) and CENP-A–HA1 was detected with mAb 12CA5 (green) and endogenous centromere antigens with hACA-M (red in C and D) using three-dimensional microscopy. A and B show representative fields of cells fixed 10 h (G2 and A) or 22 h (G1 and B) after induction. Individual G2 (C) and G1 (D) cells are shown in projection for comparison. Note that for purposes of visualization the green signal intensity of G2 cells (A and C) has been increased by a factor of fivefold compared with G1 cells (B and C). To illustrate the relative signal intensities of CENP-A–HA1 in these cells, three-dimensional surface plots are shown that correspond to half of the sections projected for the cells shown in C and D. The z axis corresponds to signal intensity and the same scale is used for both. Signals reaching 12,000 on the intensity scale are observed 22 h after induction (right, D), whereas a maximum of 2,000 is found 10 h after induction (right, C). (E) Quantitative analysis of CENP-A–HA1 distribution. Five G1 and five G2 cells were analyzed for CENP-A–HA1 distribution within the nucleus. All the G2 cells analyzed were positive for phosphorylated histone H3. Incorporation was estimated using the ratio of focal fluorescence intensity (signal, S) versus nuclear background intensity (noise, N). The signal to noise ratio for CENP-A–HA1 is shown at left, with G2 in white and G1 in black bars. Note the significant increase of CENP-A–HA1 signal to noise ratio observed 22 h after induction relative to the 10-h induction. The signal to noise ratio for hACA-M staining (center) is unchanged during the cell cycle. Colocalization of newly synthesized CENP-A with centromeres was determined as the fraction of centromeric hACA-M signal contained within CENP-A–HA1 staining foci (right). Note that CENP-A–HA1 staining foci solely colocalize with centromeric sites 22 h after induction. (F) CENP-A–HA1 incorporates into centromeres in the absence of DNA replication. HeLa cells were transfected with pcDL-CA-HA1 (Shelby et al., 1997) in medium containing 5 μg/ml aphidicolin. Cells were fixed after 12 h. CENP-A–HA1 (green, center) and centromeres (red, right) were detected as above. The left panel shows the merged image where colocalization of CENP-A–HA1 with centromeres appears in yellow in the transfected cell, indicating that CENP-A assembly at centromeres can occur without ongoing DNA replication. Bars: (B) 25 μm; (D) 5 μm; (F) 2 μm.

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.

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