

Immunolocalization of CENP-A suggests a distinct nucleosome structure at the inner kinetochore plate of active centromeres

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The trilaminar kinetochore directs the segregation of chromosomes in mitosis and meiosis. Despite its importance, the molecular architecture of this structure remains poorly understood [1]. The best known component of the kinetochore plates is CENP-C, a protein that is required for kinetochore assembly [2], but whose molecular role in kinetochore structure and function is unknown. Here we have raised for the first time monospecific antisera to CENP-A [3], a 17 kD centromere-specific histone variant that is 62% identical to the carboxy-terminal domain of histone H3 [4,5] and that resembles the yeast centromeric component CSE4 [6]. We have found by simultaneous immunofluorescence with centromere antigens of known ultrastructural location that CENP-A is concentrated in the region of the inner kinetochore plate at active centromeres. Because CENP-A was previously shown to co-purify with nucleosomes [7], our data suggest a specific nucleosomal substructure for the kinetochore. In human cells, these kinetochore-specific nucleosomes are enriched in α -satellite DNA [8]. However, the association of CENP-A with neocentromeres lacking detectable α -satellite DNA, and the lack of CENP-A association with α -satellite-rich inactive centromeres of dicentric chromosomes together suggest that CENP-A association with kinetochores is unlikely to be determined solely by DNA sequence recognition. We speculate that CENP-A binding could be a consequence of epigenetic tagging of mammalian centromeres.

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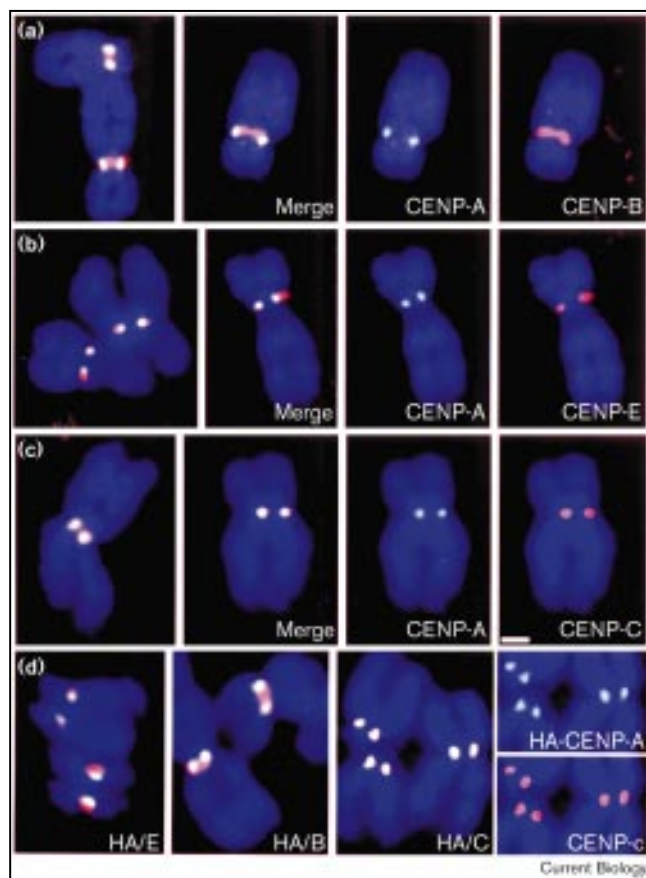
Results and discussion

Immunolocalization of CENP-A on human metaphase chromosomes yielded a characteristic double-dot staining at every centromere (Figure 1, Figure 2b, inset). Simultaneous co-localization experiments with antibodies to CENP-B, -C and -E then revealed that CENP-A is external to CENP-B (Figure 1a), internal to CENP-E (Figure 1b), and co-localizes with CENP-C (Figure 1c). Because the locations of CENP-B, -C and -E have all been determined by immunoelectron microscopy (throughout the centromeric heterochromatin [9], in the inner kinetochore plate [10] and in the kinetochore fibrous corona respectively [C.A.C., W.C.E., unpublished observations]) this enables us to triangulate the position of CENP-A with sufficient precision to localize the protein in or near the inner kinetochore plate. Thus, if as previously published experiments indicate, CENP-A defines a special subset of nucleosomes [7], those nucleosomes are localized within the kinetochore itself and are not distributed throughout the bulk of the centromeric heterochromatin. This model of kinetochore organization is consistent with our previous demonstration by immunoelectron microscopy that the inner kinetochore plate contains DNA [11].

Although the anti-CENP-A antibody fails to recognize endogenous CENP-A in immunoblots of isolated cell nuclei (Figure 2a, lane 5), it does recognize a CENP-A-HA fusion protein that is overexpressed in HeLa τ TA-CAHA1 cells under control of the tetracycline-regulated promoter (Figure 2a, lane 6) [12]. This CENP-A-HA fusion protein was also recognized by autoantiserum GS [3] and by the HA monoclonal antibody (Figure 2a, lanes 2,4). Although the anti-CENP-A antibody recognized a number of proteins in HeLa τ TA-CAHA1 nuclei, the overexpressed band of CENP-A-HA was recognized only in the induced cells. Furthermore, preincubation of anti-CENP-A with the CENP-A peptide abolished all specific staining of centromeres (Figure 2b) but had no effect on the simultaneous recognition of kinetochores by a CENP-C-specific antibody (Figure 2c).

Independent confirmation of the distribution of CENP-A within the kinetochore was obtained using the HeLa

Figure 1

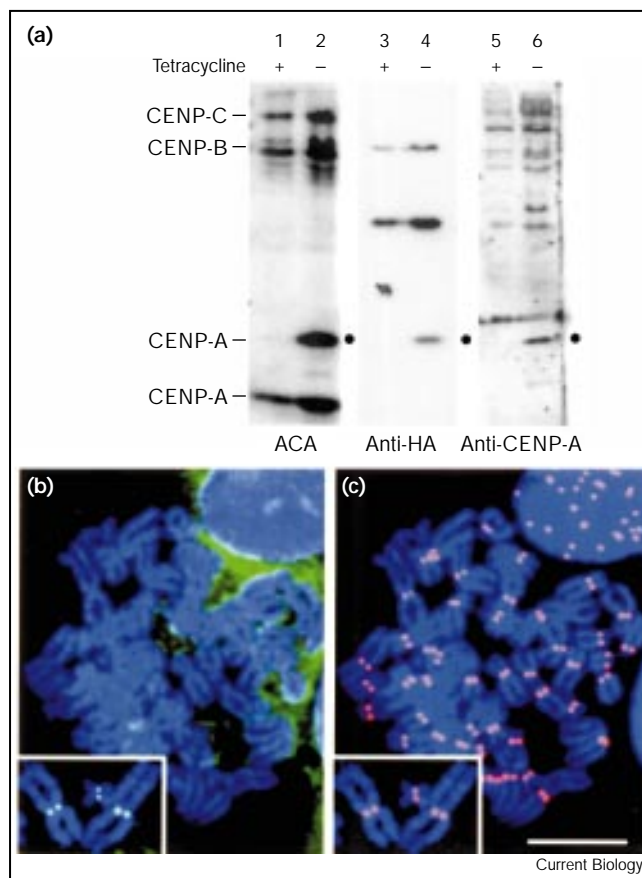


CENP-A is localized in or near the inner kinetochore plate. (a) CENP-A (green, but appearing aquamarine where it coincides with the blue staining of the chromosomes) is external to CENP-B (pink). (b) CENP-A (green) is internal to CENP-E (pink). (c) CENP-A (green) and CENP-C (pink) co-localize. (d) CENP-A-HA is distributed identically to endogenous CENP-A with respect to CENP-B, CENP-C, and CENP-E (pink). In all figures, chromosomes were counterstained with DAPI (blue). Scale bar = 2 μ m.

tTA-CAHA1 cells. When these cells were induced to express CENP-A-HA [12] and the fusion protein localized with the anti-HA monoclonal antibody, an identical distribution of CENP-A was observed: CENP-A-HA was external to CENP-B, internal to CENP-E, and co-localized with CENP-C (Figure 1d). The anti-HA monoclonal antibody failed to stain centromeres in uninduced HeLa tTA-CAHA1 cells.

We next exploited the use of variant human chromosomes to examine the relationship between CENP-A binding and the DNA sequence composition of centromeres. We first assayed for the presence of CENP-A on the centromeres of two independent stable dicentric chromosomes that have one active and one inactive centromere. Centromere inactivation is not understood, but it is likely to

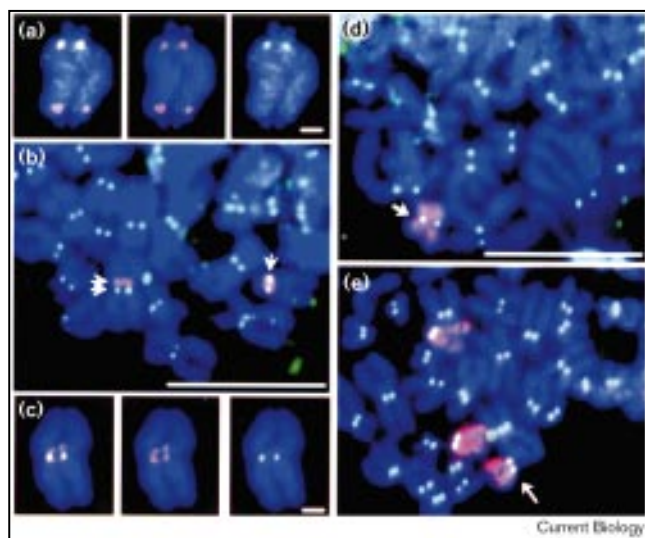
Figure 2



Characterization of the anti-CENP-A antibody. (a) Nuclei (5×10^5 per lane) were isolated from HeLa tTA-CAHA1 cells containing tetracycline-repressible HA-tagged CENP-A [12] either with (lanes 2,4,6) or without (lanes 1,3,5) the induction of CENP-A-HA expression for 72 h. Blots were probed with human autoantiserum GS containing anti-centromere antibodies (ACA) (lanes 1,2) [3], anti-HA monoclonal 12CA5 (lanes 3,4), or anti-CENP-A (lanes 5,6). CENP-A-HA was detected only in the induced cells by all three antibodies. Lanes 1,2, [125 I]protein A; lanes 3–6, enhanced chemiluminescence (ECL). (b) CENP-A double-dot staining (green) is abolished by preincubation of anti-CENP-A with 0.5 mg ml^{-1} CENP-A peptide (compare with inset – no CENP-A peptide). This preincubation with peptide also blocks the recognition of the CENP-A-HA polypeptide in immunoblots of nuclei from HeLa tTA-CAHA1 cells (data not shown). (c) Same field as in (b). Kinetochore staining with anti-CENP-C (pink) is unaffected by preincubation with the CENP-A peptide. Scale bar = 10 μ m.

involve loss of an epigenetic mark. The involvement of epigenetic marking in centromere structure and activity has previously been suggested by genetic analysis of fission yeast and *Drosophila* centromeres [13,14] and the fact that CENP-B binding to murine centromeres is regulated by DNA methylation [15]. The first dicentric examined here is a fusion of the distal q arms of chromosome 13 dic(13) [16]. CENP-A was detected only at the active centromere of this chromosome despite the presence of chromosome 13 α -satellite at both centromeres (Figure 3a).

Figure 3



Analysis of CENP-A (green) on variant human centromeres. (a) CENP-A is seen only on the active centromere of a dic(13) chromosome [16]. Chromosome 13-specific α -satellite DNA (pink) is seen at both centromeres. (b) CENP-A is seen only on the active centromere (cen 14 [21]) of the RT14q15q dicentric chromosome (double arrow); chromosome 15-specific α -satellite signal (pink) is adjacent to, but distinct from, the site of CENP-A binding. On the normal chromosome 15 (single arrow), anti-CENP-A and α -satellite DNA signals overlap. (c) CENP-B (pink) is detected at both centromeres of the RT14q15q dicentric chromosome: CENP-A is only seen at the active centromere. (d) CENP-A is found at the invYq neocentromere (white arrow). Distal Yq heterochromatic probe DYZ1 (pink) labels most of this chromosome. (e) CENP-A is detected at the neocentromere of the inv13q chromosome (white arrow), identified with a chromosome 13 painting probe (pink) that also hybridizes to the two normal chromosome 13. CENP-A localization near one end of inv13q is consistent with sites of sister chromatid cohesion seen in late metaphase chromosomes (data not shown). Scale bar = 2 μ m (a,c); 10 μ m (b,d,e).

CENP-C exhibits a similar distribution ([16] and data not shown), suggesting that CENP-A is located only at centromeres that nucleate formation of a functional kinetochore. Similar results were obtained using a cell line containing a dicentric Robertsonian translocation chromosome (RT14q15q, Figure 3b,c).

We next examined the distribution of CENP-A on two human chromosomes with neocentromeres — functional centromeres that derive from chromosomal regions that are distinct from the centromere and that lack detectable centromeric DNA sequences (reviewed in [17]).

The first of these neocentromeres is found in a metacentric chromosome generated by an inverted duplication of Yq (qter-q11.2::q11.2-qter). This chromosome (invYq) consists of Yq heterochromatin and distal Yq euchromatin but lacks detectable α -satellite DNA as judged both by *in situ* hybridization and by quantitative Southern blotting (G.G.,

C.T-S., unpublished observations). The second neocentromeric chromosome (inv13q), a duplication-inversion of 13q (qter-13q21.3::13q21.3-qter), also lacks detectable α - and β -satellite DNA as judged by *in situ* hybridization (D.W., unpublished observations). Both neocentromeres showed normal levels of double-dot staining with anti-CENP-A (Figure 3d,e). Although the presence of a minute amount of α -satellite on these chromosomes cannot yet be strictly ruled out, it is clear that CENP-A binding to centromeres does not reflect the quantity of α -satellite present.

It has been generally assumed that recognition of centromeric DNA sequences (e.g. α -satellite) has an important role in targeting CENP-A to centromeres. Indeed, recent immunoprecipitation studies confirm the association of CENP-A with α -satellite DNA [8]. The results shown here, however, reveal that CENP-A fails to bind to both the α -satellite DNA in the central domain of normal centromeres (Figure 1), and the large arrays of α -satellite DNA (and other putative kinetochore sequences) present at inactive centromeres of dicentric chromosomes (Figure 3a–c). Furthermore, CENP-A associates as intensely with neocentromeres that lack detectable α -satellite DNA as with centromeres that contain up to several million base pairs of it (Figure 3d,e). These findings support the suggestion [12] that CENP-A targeting is directed either by some aspect of DNA structure other than primary sequence, or by interaction with one or more protein components of the kinetochore.

In support of this model, CENP-A is concentrated in distinct pre-kinetochores in interphase nuclei, as are the other CENP antigens. Thus, the association of newly synthesized CENP-A with late S/G₂-replicating nucleosomes already containing CENP-A [12] could provide a mechanism for the sequence-independent perpetuation of centromere position. Bovine CENP-A has been reported to be quantitatively retained in sperm [4] despite the displacement of the great majority of histones during mammalian spermatogenesis. Together, these observations raise the possibility that CENP-A could provide an epigenetic mark for the kinetochore inner plate that persists through both DNA replication and the dramatic chromatin remodeling events that accompany spermatogenesis.

Materials and methods

Immunofluorescence

The 25 amino acid immunogen, SPSPTPTGPSRRGSLGASSHQHS (residues 17–42 of the human CENP-A sequence [5]), synthesized on a 4-branch multiple antigenic peptide backbone, was injected into guinea pigs (Hazleton HRP Inc.). The resulting antibody was used for immunofluorescence at a 1:500 dilution. Anti-CENP-B (RaACA-2 and mACA-1) [18], anti-CENP-C (serum 558) [10], anti-CENP-E (HX-1) [19] and anti-HA-tag (12CA5) were previously described. Immunofluorescence was performed using published protocols [18,20] with cytological spreads obtained using a Heraeus Megafuge cyto-system. In certain experiments, peptide competitor was preincubated with the diluted antibody for 1 h at 37°C prior to application to slides. Slides were examined using a Zeiss

Axioplan II with a Micromax cooled CCD camera (Princeton Instruments) using IPLab Spectrum (Signal Analytics) and further processed using Adobe Photoshop. All immunolocalizations were performed or verified using wedge error free filters (Zeiss), as well as by imaging fluorescent 2 μ m spherical beads (data not shown).

In situ hybridization

Following antibody labeling, slides were air dried 1 h to overnight and subjected to a standard *in situ* hybridization protocol [20] using human Cot-1 (Gibco) and salmon sperm DNA (1 mg per slide) as competitor. Probes were labeled by nick translation incorporating either biotin-16-dUTP or digoxigenin-11-dUTP (Boehringer Mannheim) and detected with fluorescein- or Texas red-conjugated streptavidin or rhodamine-conjugated sheep anti-digoxigenin (Vector), respectively. Digoxigenin-labeled α -satellite probes specific for chromosomes 13, 14 and 15 and chromosome 13 painting probe are available from Oncor. Slides were mounted in anti-fade (Vectashield) containing 5 mg ml⁻¹ DAPI under sealed cover slips.

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