

Centromere/Kinetochore Localization of Human Centromere Protein A (CENP-A) Exogenously Expressed as a Fusion to Green Fluorescent Protein

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ABSTRACT. Three human centromere proteins, CENP-A, CENP-B and CENP-C, are a set of autoantigens specifically recognized by anticentromere antibodies often produced by patients with scleroderma. Microscopic observation has indicated that CENP-A and CENP-C localize to the inner plate of metaphase kinetochore, while CENP-B localizes to the centromere heterochromatin beneath the kinetochore. The antigenic structure, called “prekinetochore”, is also present in interphase nuclei, but little is known about its molecular organization and the relative position of these antigens. Here, to visualize prekinetochore in living cells, we first obtained a stable human cell line, MDA-AF8-A2, in which human CENP-A is exogenously expressed as a fusion to a green fluorescent protein of *Aequorea victoria*. Simultaneous staining with anti-CENP-B and anti-CENP-C antibodies showed that the recombinant CENP-A colocalized with the endogenous CENP-C and constituted small discrete dots attaching to larger amorphous mass of CENP-B heterochromatin. When the cell growth was arrested in G1/S phase with hydroxyurea, CENP-B heterochromatin was sometimes highly extended, while the relative location between GFP-fused CENP-A and the endogenous CENP-C was not affected. These results indicated that the fluorescent CENP-A faithfully localizes to the centromere/kinetochore throughout the cell cycle. We then obtained several mammalian cell lines where the same GFP-fused human CENP-A construct was stably expressed and their centromere/kinetochore is fluorescent throughout the cell cycle. These cell lines will further be used for visualizing the prekinetochore locus in interphase nuclei as well as analyzing kinetochore dynamics in the living cells.

Key words: autoantigen/CENP-A/CENP-B/CENP-C/centromere/kinetochore

The kinetochore is morphologically defined as the microtubule attachment site at the primary constriction of metaphase chromosomes. It is assumed to be a highly organized chromatin structure specialized for capturing the spindle microtubules. Numerous investigations have focused on understanding the molecular components of the centromere/kinetochore of mitotic chromosomes. So far, mammalian kinetochore proteins have been divided into two categories, “constitutive” or “facultative”, depending on their localization during the cell cycle (see He *et al.*, 1998 for the classification). The former includes three major centromere autoantigens, CENP-A (CENtromeRe Protein A), CENP-B and CENP-C, originally identified by anticentromere autoanti-

bodies (Earnshaw and Rothfield, 1985; Muro *et al.*, 1990). They have been indicated to possess certain DNA binding activity and to localize at the centromeric region throughout the cell cycle (Sugimoto *et al.*, 1992b; Knehr *et al.*, 1996; Vafa and Sullivan, 1997). CENP-A is a histone H3-related 17-kDa protein that is conserved in mammals and yeasts (Sullivan *et al.*, 1994; Stoler *et al.*, 1995). CENP-B is an 80-kDa protein that binds a 15–17 bp motif which is present in human alphoid DNA and mouse minor satellites (Masumoto *et al.*, 1989; Sugimoto *et al.*, 1992a; Sugimoto *et al.*, 1998). CENP-C is a highly basic 140-kDa protein with DNA-binding and self-associating properties (Saitoh *et al.*, 1992; Sugimoto *et al.*, 1994; Sugimoto *et al.*, 1997). Immunostaining of metaphase chromosomes with monospecific antibodies against each of them indicated that CENP-A, CENP-C and recently identified CENP-G, were mapped to the inner plate of kinetochore, while CENP-B localized to the centromere heterochromatin beneath the kinetochore (Cooke *et al.*, 1990; Saitoh *et al.*, 1992; Warburton *et al.*, 1997; He *et al.*, 1998). In contrast, the “facultative” proteins include those involved in chromosome movement and

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Abbreviations: CENP, centromere protein; GFP, green fluorescent protein.

anaphase checkpoint such as CENP-E, CENP-F, ZW10, and hBUBR1 (Yen *et al.*, 1992; Liao *et al.*, 1995; Starr *et al.*, 1997; Chan *et al.*, 1998). They have been shown to transiently associate with kinetochore during mitosis (see Craig *et al.*, 1999 for a review).

Early microscopic observation with antacentromere autoimmune sera has indicated that the antigenic structure called "prekinetochore" exists even in interphase nuclei, duplicates in G2 phase and differentiates into the matured kinetochore during mitosis (Brenner *et al.*, 1981). However, little was known about the molecular organization and maturation process of mammalian interphase prekinetochore, because of the lack of appropriate molecular probes to specifically detect each antigenic peptide. In a previous study, we reported the immunological detection of two human centromere antigens, CENP-B and CENP-C, with monospecific antibodies against each of them (Sugimoto *et al.*, 1999). CENP-C remained as round discrete dots, whereas CENP-B often displayed the larger surrounding materials in interphase nuclei. Furthermore, CENP-C showed the limited localization on highly extended chromatin fiber of centromeric alphoid DNA, suggesting the significance of CENP-C in the kinetochore organization. Recently, CENP-A was also suggested to play an important role on kinetochore assembly, since, like CENP-C and CENP-E (Sullivan and Schwartz, 1995), it is present at the active centromere but not at the inactive one of mitotically stable dicentric chromosomes (Warburton *et al.*, 1997). Nevertheless, the relative position of these three antigens in human prekinetochores has not yet been compared precisely.

In this study, to visualize human prekinetochore in the living cells, we first constructed a stable human cell line in which human CENP-A antigen was expressed as a fusion protein to the green fluorescent protein of *A. victoria*. Centromere/kinetochore localization of the GFP-fused CENP-A was confirmed by simultaneously staining with monospecific anti-CENP-B and anti-CENP-C antibodies. We then expressed the same construct in other mammalian cell lines.

Materials and Methods

PCR cloning of human CENP-A cDNA

A forward primer (5'-TTCTCGAGCTCTGCGGCGTGTCATGG-3') and the reverse primer (5'-TTCTCGAGCCGAGTCCCTCCTCAAGG-3'), were used to amplify the entire coding region of human CENP-A cDNA, as described (Muro *et al.*, 2000). PCR products were first introduced into pGEM-T vector (Promega, Madison, WI, USA) and sequenced by ALF DNA sequencer (Pharmacia, Uppsala, Sweden). One of the clones, pGEM-CENP-A (#8), was digested with *SacI* and *XhoI* (underlined in the primer sequences) and the inserted 430 bp fragment was recloned into the *SacI-SalI* site of pEGFP-C1 (Clontech, Palo Alto, CA, USA), resulting in pEGFP-AF8.

Cell culture and electroporation

Human MDA435, mouse A9, bovine MDBK and porcine PK15 cells were grown in DMEM (Nissui Pharmaceutical, Tokyo, Japan) containing 10% FCS at 37°C in a 5% CO₂ atmosphere (Sugimoto *et al.*, 1992b). Plasmid DNA (16 µg) was mixed with 0.6 × 10⁷ cells in 0.5 ml of K-PBS (30 mM NaCl, 120 mM KCl, 8 mM Na₂HPO₄, 1.5 mM KH₂PO₄, 5 mM MgCl₂) and applied for electroporation in a cuvette with electrodes spaced at 0.4 cm intervals, at a setting of 220V and with a 960-µF capacitor using Gene Pulser and Capacitance Extender (Bio-Rad Laboratories, Hercules, CA, USA). After the pulse, electroporated cells were incubated with equal volume of serum-free MEM for 10 min at room temperature, and grown in an excess amount of fresh medium.

Immunofluorescence with antibodies to centromere proteins

Aliquot of the cells (1–3 × 10⁵) were plated on glass coverslips in 35 mm dishes, grown for 48 hr, fixed with 4% paraformaldehyde dissolved in PBS for 20 min and then placed in 0.1% Triton X-100 in PBS for 5 min. To obtain the cells arrested in G1/S phase, hydroxyurea (Sigma Chemical Co., St. Louis, MO, USA) was added into the medium to the final concentration of 2 mM and incubated for 12 hr, as described (He and Brinkley, 1996). Mitotically dividing cells were obtained from a randomly growing culture in a 90 mm dish by briefly tapping off and sedimented onto glass slides using Cytospin 2 (Shandon Southern Instruments, Sewickley, PA, USA). Monolayer cells and metaphase chromosome spreads were incubated with mouse anti-CENP-B serum (1:300; Sugimoto *et al.*, 1992b) and rabbit anti-CENP-C serum (1:1000; Sugimoto *et al.*, 1999), labeled with Cy3-conjugated goat anti-mouse and Cy5-conjugated goat anti-rabbit antibodies, respectively (Jackson ImmunoResearch Laboratories, West Grove, PA, USA), and observed under a fluorescence microscope (Eclipse E600, Nikon, Tokyo, Japan) equipped with a PlanApo 100x objective (NA 1.40, Nikon), a MicroMAX 1300Y cooled CCD camera (Princeton Instruments, Princeton, NJ, USA) and a BioPoint MAC3000 controller system for a filter wheel and a Z-axis motor (Ludl Electric Products). The light was filtered with a quad filter set (No. 84; Chroma Technology Corp., Battleboro, VT, USA). Images were acquired by MetaMorph software (Universal Imaging Corp., West Chester, PA, USA), by collecting a Z-series of optical sections (0.2–0.3 µm steps).

Results

Stable expression of GFP-fused human CENP-A in MDA435 cells

To visualize CENP-A in living cells, we expressed human CENP-A as a fusion protein to the green fluorescent protein of *Aequorea victoria* (Chalfie *et al.*, 1994), as we did for the visualization of heterochromatin protein I (HP1^{HSα}) (Yamada *et al.*, 1999). When GFP-fused CENP-A was transiently expressed in human MDA435 cells, we observed discrete fluorescent dots in interphase nuclei (data not shown). To precisely examine the behavior of GFP-fused CENP-A in the cell cycle, we obtained several stable transformants after addition of G418 to the medium. Figure 1

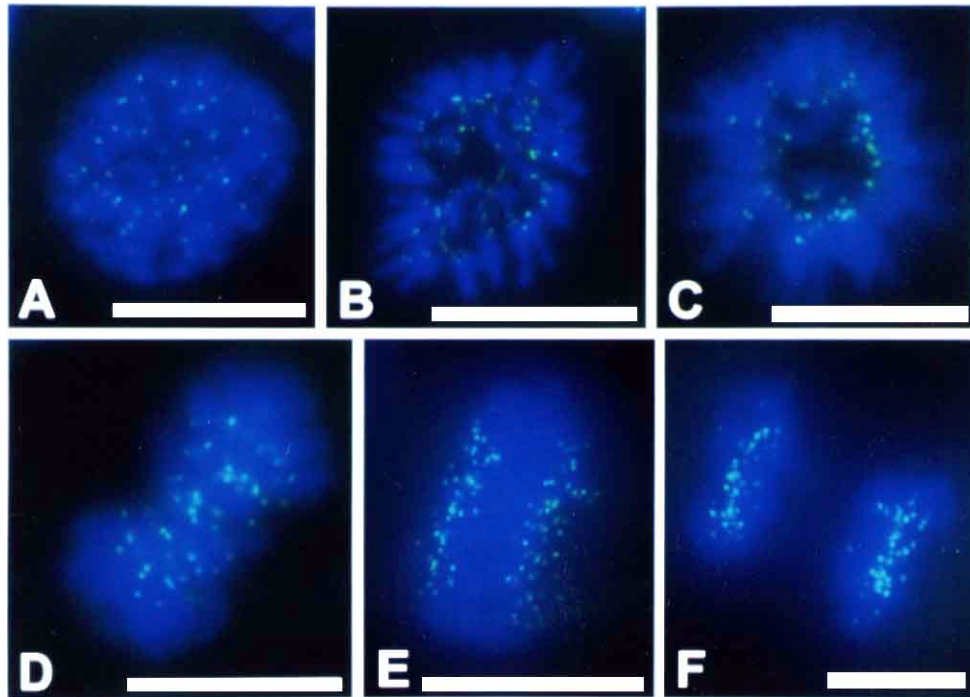


Fig. 1. Cellular localization of GFP-tagged human CENP-A. Human MDA435 cells were transfected with pEGFP-AF8. Mitotic cells were obtained from a random culture of MDA-AF8-A2, one of the G418-resistant stable transformants, fixed with 80% ethanol, counterstained with DAPI and observed under a fluorescent microscope. Interphase (A), prometaphase (B), prometaphase with a “rosette”-like structure (C), metaphase with twisted chromosomes (D), early anaphase (E) and late anaphase (F). Bar: 10 μ m.

shows its localization in a randomly growing culture of MDA-AF8-A2 cells, one of the G418-resistant transformed cell lines. We observed discrete fluorescent dots typical for the centromere staining in interphase and mitotic cells, consistent with a recent report on the endogenous CENP-A localization (Figueroa *et al.*, 1998). In addition, the fusion protein was detected in the primary constriction of every mitotic chromosome (Fig. 1B). The results indicated that the GFP-fused human CENP-A exogenously expressed in MDA435 cells localizes to the centromere, at least, of mitotic chromosomes. This forced us to use this cell line for the subsequent analyses below.

Relative position of GFP-fused CENP-A to centromere heterochromatin

To further compare the relative position of GFP-fused CENP-A to the centromere in interphase nuclei, the fluorescent MDA-AF8-A2 cells were stained with mouse anti-CENP-B antibodies that have been shown to detect human centromere heterochromatin throughout the cell cycle (Sugimoto *et al.*, 1992b). Figure 2 shows the representative staining patterns. GFP-fused CENP-A existed as small discrete dots again, while CENP-B displayed morphological changes from discrete dots to fused or extended dots de-

pending on the cell cycle. According to the previous report on the morphological changes of the prekinetochore structure (He and Brinkley, 1996), we tentatively arranged them in a cell-cycle dependent manner. As shown in Figures 2A–2C, for example, CENP-A (*green*) as well as most of CENP-B (*red*) showed discrete dots and colocalized with each other. The size of CENP-A dots was relatively uniform in this stage, probably in G1 phase. In some cell populations, we observed that certain CENP-A dots were slightly elongated along the extended CENP-B chromatin (see arrowhead in Fig. 2E). This kind of cell was likely to be in S phase. Figures 2G–2I show a typical G2 cell in which most of the CENP-A dots have duplicated, while CENP-B displayed a rather condensed amorphous mass. Interestingly, a pair of CENP-A dots seemed to be smaller than those observed in the earlier phase and surrounded by CENP-B heterochromatin.

When the mitotic cells were spread, CENP-A displayed a pair of dots situated at the periphery of the primary constriction of each chromosome (Fig. 3A). In contrast, the CENP-B heterochromatin often existed as a bar-like structure covering the whole region of the centromere, and the amount of CENP-B was apparently different from chromosome to chromosome. Figures 3B and 3E focus on several chromosomes that contain relatively small amounts of CENP-B

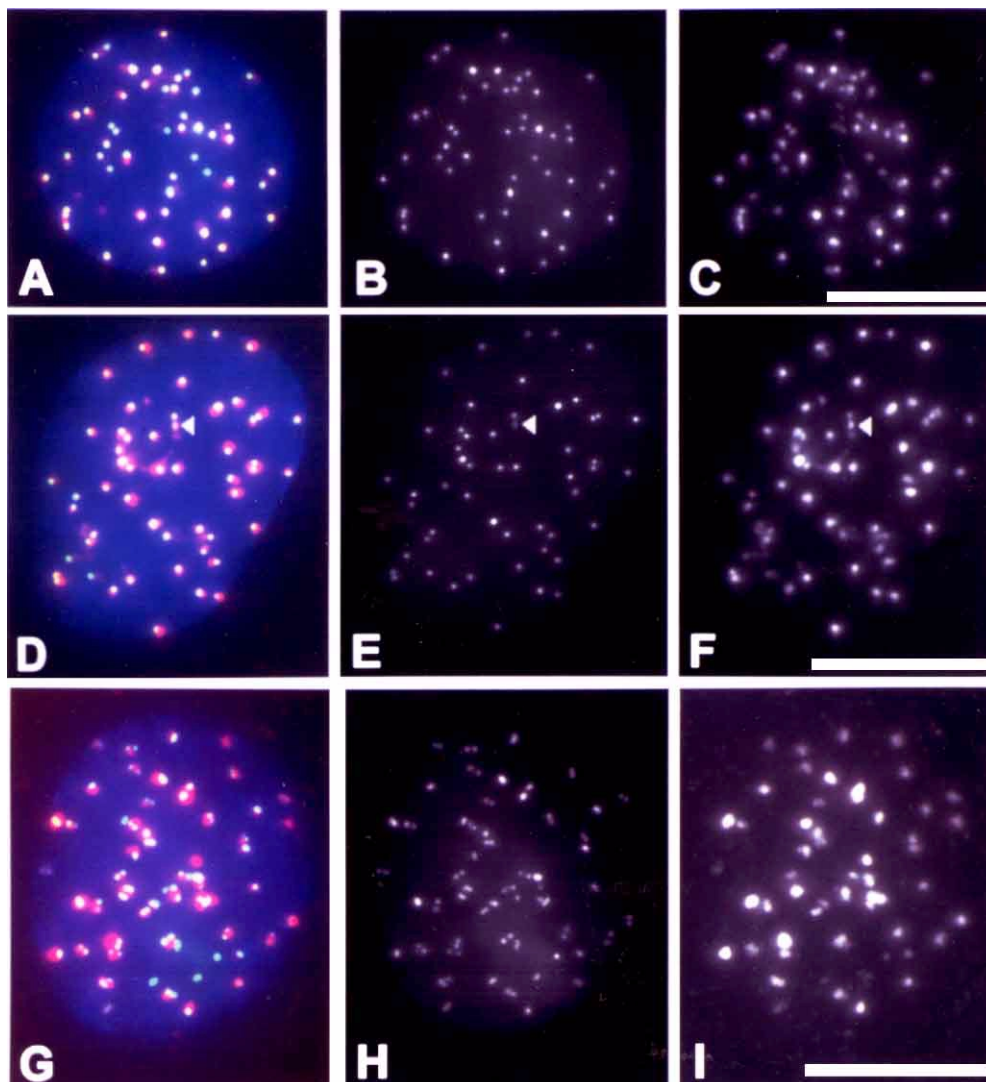


Fig. 2. Relative location of GFP-fused CENP-A to the endogenous CENP-B in interphase MDA435 cells. (A, D, G) MDA-AF8-A2 cells were fixed with 4% paraformaldehyde, incubated with mouse anti-CENP-B antibodies, stained with Cy3-conjugated anti-mouse antibodies (*red*) and then counterstained with DAPI (*blue*). Interphase cells were assigned to be in G1 (A–C), S (D–F), and G2 (G–I) according to a previous report (He and Brinkley, 1996). Separate images of GFP-fused CENP-A (B, E, H) and the endogenous CENP-B (C, F, I) are shown in black and white. Note an extended CENP-A dot (*arrowhead* in panel E) along the extended CENP-B heterochromatin (panel F). Bar: 10 μ m.

heterochromatin. The location of CENP-A was almost indistinguishable to that of CENP-B (compare Fig. 3C to Fig. 3D). Alternatively, a pair of CENP-A dots localized on a spiral fiber of CENP-B heterochromatin, as indicated by the arrowhead in Figures 3E–3G. Besides, as shown in Figures 3H–3J, CENP-A dots were clearly observed on all the chromosomes including Y chromosome where no CENP-B signal was detected. These results showed that the GFP-fused CENP-A localizes to the centromere/kinetochore of all human chromosomes throughout the cell cycle.

Simultaneous visualization of CENP-A, CENP-B and CENP-C in interphase nuclei

We previously reported the visualization of CENP-B and CENP-C in human interphase nuclei. The results indicated that CENP-C remained as round discrete dots, whereas CENP-B often displayed larger surrounding materials (Sugimoto *et al.*, 1999). To simultaneously visualize these three centromere antigens, the fluorescent MDA-AF8-A2 cells were incubated with rabbit anti-CENP-C and mouse anti-CENP-B antibodies. Both the CENP-A and CENP-C colocalized with each other and remained as discrete dots attached to the CENP-B heterochromatin (data not shown).

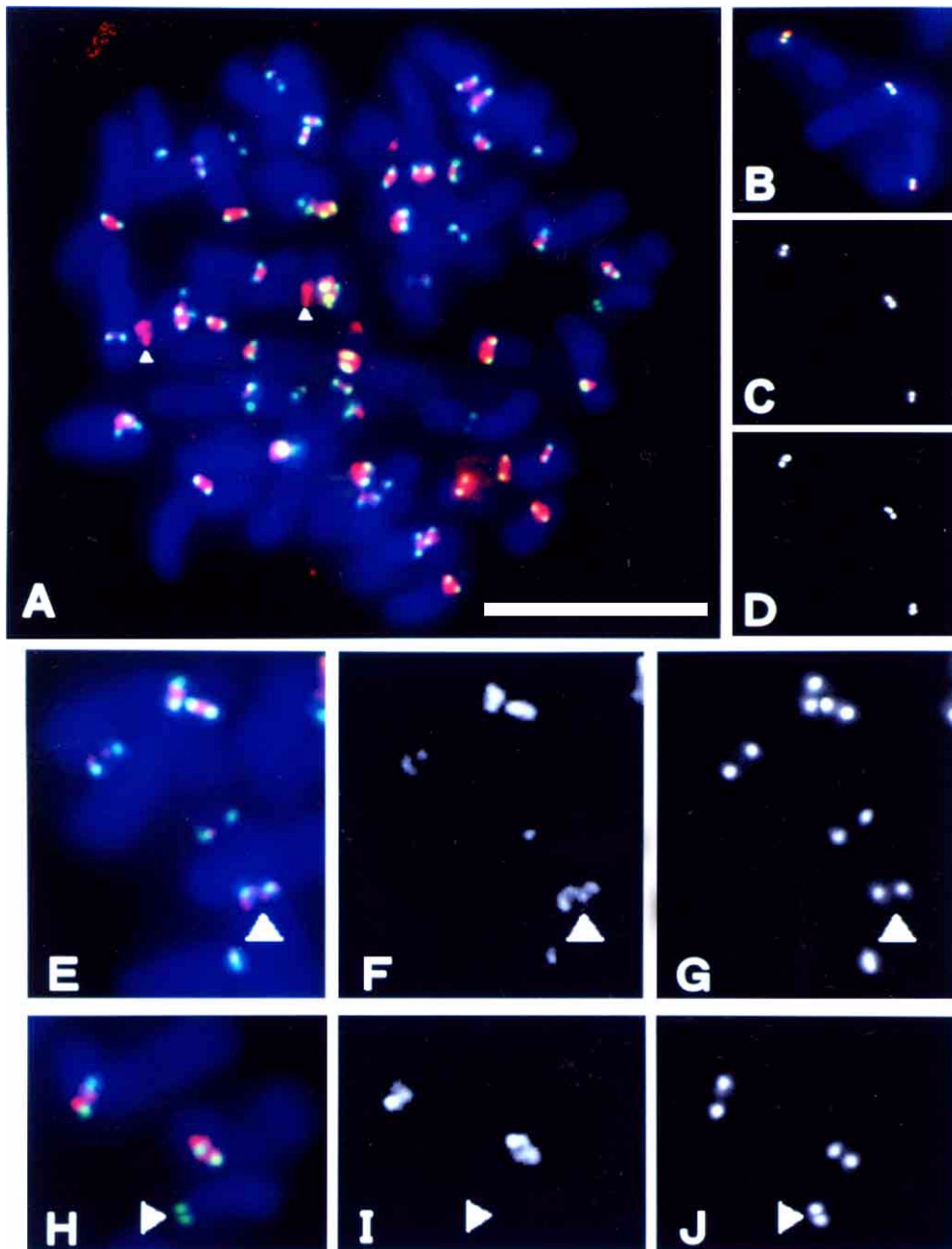


Fig. 3. Simultaneous detection of CENP-A and CENP-B on mitotic chromosomes. Mitotic MDA-AF8-A2 cells were cytocentrifuged and stained with anti-CENP-B antibodies, as described in the legend of Figure 2. **A.** A whole metaphase chromosome spread. GFP-CENP-A (*green*), CENP-B (*red*), chromosomes stained with DAPI (*blue*). Several chromosomes containing relatively small amounts of CENP-B heterochromatin (**B**, **E**) and Y chromosome (**H**). Separate images of CENP-A (**C**, **G**, **J**) and CENP-B (**D**, **F**, **I**) are also shown in black and white. Note a spiral fiber across a pair of CENP-A dots (**E**) and the absence of CENP-B signal on chromosome Y (**H**). Bar: 10 μ m.

The relative position of CENP-C to CENP-B was comparable to our previous observation (Sugimoto *et al.*, 1999).

To precisely compare the distribution of these antigens, MDA-AF8-A2 cells were arrested in G1/S phase by adding hydroxyurea to the medium. As shown in Figures 4A–4D,

we observed that CENP-B heterochromatin sometimes aggregated to form the larger amorphous mass covering multiple CENP-A/CENP-C dots in the neighbor. However, colocalization of CENP-A with CENP-C was not affected at all and the discrete dots consisting of CENP-A and CENP-C

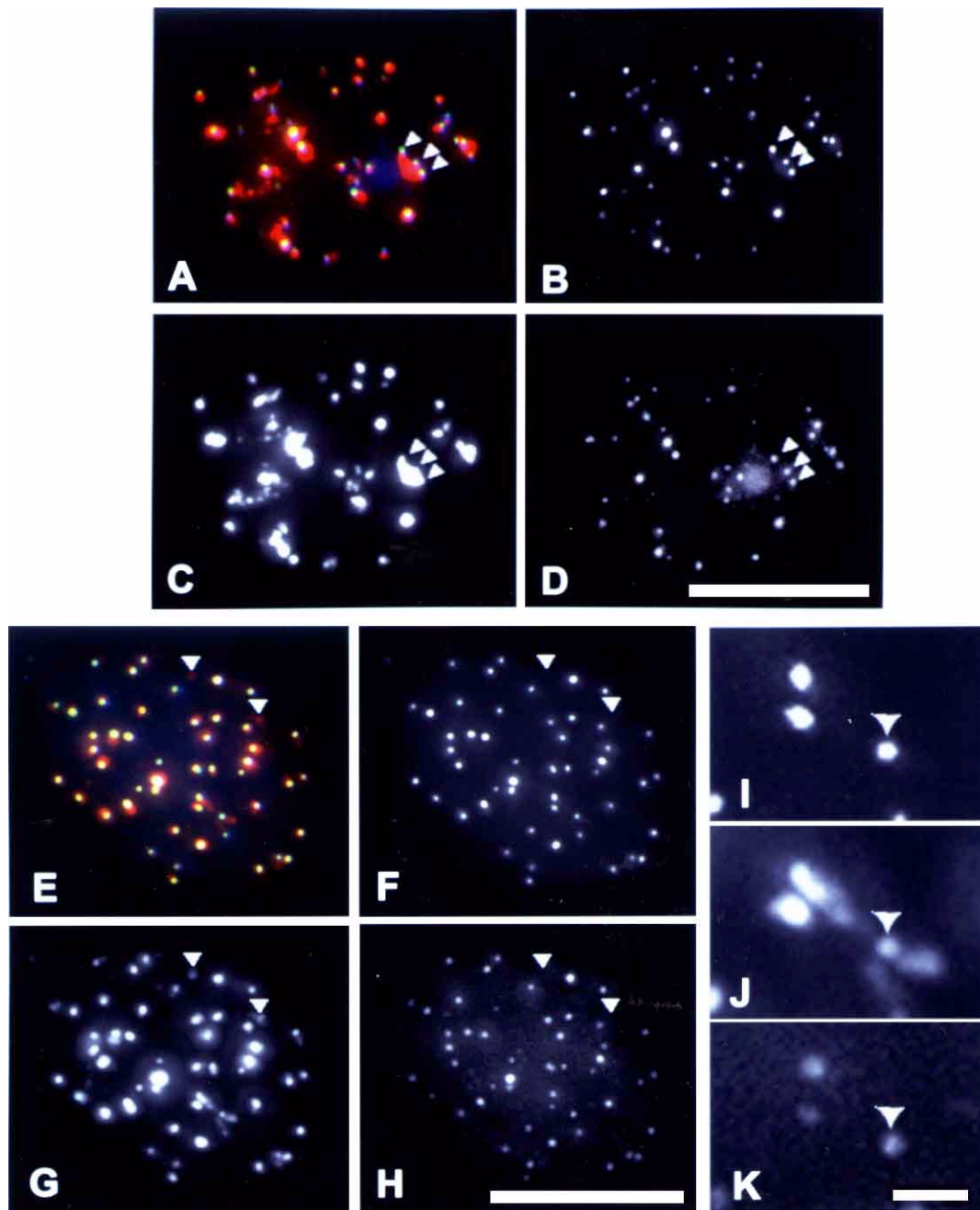


Fig. 4. Simultaneous visualization of CENP-A, CENP-B and CENP-C in human interphase nuclei. MDA-AF8-A2 cells were treated with 2 mM hydroxy-urea for 12 hr to arrest in G1/S phase. After fixation, the cells were serially incubated with rabbit anti-CENP-C and mouse anti-CENP-B antibodies, visualized by Cy5-conjugated anti-rabbit and Cy3-conjugated anti-mouse antibodies, respectively, and counterstained with DAPI. **A, E.** The overlapped images of CENP-A (*green*), CENP-B (*red*) and CENP-C (*blue*) shown in colors. **I–K.** The enlarged image of highly extended centromere heterochromatin. Separate images of GFP-fused CENP-A (**B, F, I**), CENP-B (**C, G, J**), and CENP-C (**D, H, K**) are shown in black and white. Bar: 10 μm for (**A–H**) and 1 μm for (**I–K**).

located at the edge of the larger CENP-B mass (see three arrowheads in Fig. 4B and compare to Fig. 4D). Figures 4E–4H show another type of staining pattern in which certain CENP-B heterochromatin were highly extended, while the

distribution of CENP-A was identical to that of CENP-C again (compare Fig. 4F to Fig. 4H). As shown in the enlarged image of Figures 4I–4K, both antigens remained as discrete dots and were located at the edge of the extended

CENP-B chromatin. In contrast, as indicated by the arrowhead in Figures 4F and 4H, we detected no CENP-A nor CENP-C signal on the CENP-B heterochromatin. This kind of heterochromatin may constitute the inactive centromere of marker chromosomes that have been found in this cell line (Vig *et al.*, 1996, see arrowheads in Fig. 3A), although we did not characterize it further here.

We also examined highly extended CENP-B chromatin formed in the presence of actinomycin D. Both GFP-fused CENP-A and CENP-C remained as discrete dots and were located at or near the end of the extended CENP-B chromatin (data not shown). These results indicated that CENP-A colocalized with CENP-C and located at the distinct site of CENP-B heterochromatin in the prekinetochore structure.

Centromere localization of GFP-fused human CENP-A in other mammalian cell lines

Homologs of human CENP-A gene have been identified in bovine and mouse genomes (Sullivan *et al.*, 1994; Howman *et al.*, 2000). It was expected that human CENP-A also functions in these cells, since the C-terminal histone-fold domain is highly conserved. To further visualize centromere/kinetochore in mammals, GFP-fused human CENP-A was exogenously expressed in mouse A9, bovine MDBK and porcine PK15 cells. Discrete fluorescent dots typical for

centromere staining were observed in interphase nuclei of transiently expressed cells as well as stable transformants (data not shown). When metaphase chromosomes were spread, the fluorescent double dots were shown to localize at the primary constriction of every mitotic chromosome of these cell lines (Fig. 5). The results indicated that the GFP-fused human CENP-A can be used for the visualization of the centromere/kinetochore locus in other mammalian living cells as well.

Discussion

The structure and morphogenesis of prekinetochore of mammalian cells have already been investigated by probing with anticentromere autoimmune sera (Brenner *et al.*, 1981; He and Brinkley, 1996). The antigenic structure detected in interphase nuclei has been shown to correlate with stages in the cell cycle. However, the relative position of each centromere antigen has yet to be precisely determined, because of the difficulty in identifying prekinetochore structures in interphase nuclei and the absence of an appropriate set of monospecific antibodies against each antigen. Instead of producing anti-CENP-A antibodies, we obtained mammalian cell lines stably expressing human CENP-A as a fusion to the green fluorescent protein. GFP-fused CENP-A was

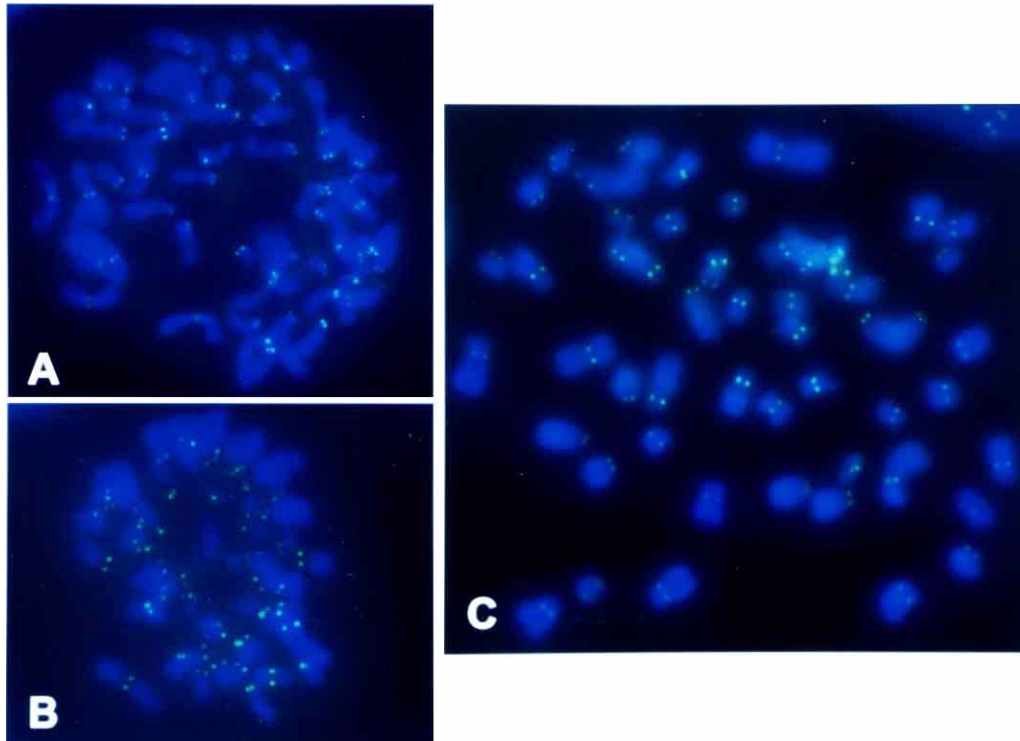


Fig. 5. Centromere localization of GFP-fused human CENP-A in mammalian cells. Mouse A9 (A), bovine MDBK (B) and porcine PK15 cells (C) were transfected with pEGFP-AF8. Mitotic cells were obtained from each of G418-resistant stable transformants, fixed with 80% ethanol, counterstained with DAPI and observed under a fluorescent microscope.

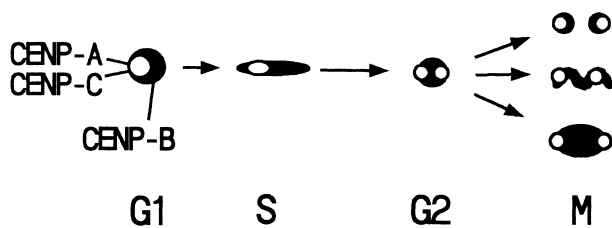


Fig. 6. Schematic illustration of morphological changes of human prekinetochores. CENP-A and CENP-C are colocalized with each other and constitute a ball-like structure that duplicates in G2 (Brenner, *et al.*, 1981). A putative “kinetochore chromatin” consisting of CENP-A and CENP-C overlaps with or locates at the edge of larger CENP-B heterochromatin.

shown to localize to centromere/kinetochore throughout the cell cycle. The availability of human fluorescent cell line (MDA-AF8-A2) enabled us to visualize three centromere antigens simultaneously. We found that GFP-fused CENP-A colocalizes with the endogenous CENP-C in interphase nuclei and occupies almost the same position of CENP-B heterochromatin or located at the edge of the larger CENP-B heterochromatin. Figure 6 illustrates a schematic organization of prekinetochore and its morphogenesis of human chromosomes except for Y chromosome. CENP-B binds a 15–17 bp motif which is present in certain repeats of alphoid DNA and constitutes centromere heterochromatin (Masumoto *et al.*, 1989; Sugimoto *et al.*, 1998). Both CENP-A and CENP-C are, directly or indirectly, associated with each other and constitute a ball-like structure, as observed by electron microscopy (Heneen, 1975). A putative “kinetochore chromatin,” identified only by the presence of CENP-A and CENP-C so far, is located at the edge of, or partly overlapped with CENP-B heterochromatin. A recent study suggested that CENP-A is essential for kinetochore targeting of CENP-C and plays an early role in organizing kinetochore chromatin at interphase (Howman *et al.*, 2000). This chromatin is likely to be duplicated during S phase and finally splits into two small discrete dots in G2 phase.

For the centromere/kinetochore localization of CENP-A, the importance of its cell cycle-dependent expression has been suggested (Shelby *et al.*, 1997). Nevertheless, our result indicated that the exogenous CENP-A faithfully localized to the centromere under a cytomegalovirus (CMV) immediate early promoter in human cells as well as several other mammalian cells (see Fig. 5). The C-terminal conserved domain of human CENP-A is likely to possess enough activity for the centromere targeting (Sullivan *et al.*, 1994). Alternatively, the endogenous promoter of CENP-A gene may be substituted by the virus promoter, as observed in their report. Interestingly, the additional expression of human CENP-A in these cell lines did not seem to interfere with their growth or to form an additional kinetochore in dicentric chromosomes (see arrowheads in Figs. 3A and 4E and a pair of dots of every chromosome in Fig. 5).

Cells with such a disadvantage, if any, might have been eliminated from the population of transformants during the G418 selection.

In this report, we constructed several stable mammalian cell lines in which the kinetochore locus was fluorescent throughout the cell cycle. It is of interest to determine which other molecules are incorporated into this structure and how its maturation process is regulated in the cell cycle. Our stable cell lines will be used in the future to analyze kinetochore dynamics of the living cells.

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