CELL STRUCTURE AND FUNCTION 26: 705–718 (2001)

Molecular Behavior in Living Mitotic Cells of Human Centromere Heterochromatin Protein HP1α Ectopically Expressed as a Fusion to Red Fluorescent Protein

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ABSTRACT. **We constructed stable mammalian cell lines in which human heterochromatin protein HP1α and kinetochore protein CENP-A were differentially expressed as fusions to red (RFP-HP1) and green fluorescent proteins (GFP-CENP-A). Heterochromatin localization of RFP-HP1 was clearly shown in mouse and Indian muntjac cells. By preparing mitotic chromosome spreads, the inner centromere localization of RFP-HP1 was observed in human and Indian muntjac cells. To characterize its molecular behavior in living mitotic cells, time-lapse images of RFP-HP1 were obtained by computer-assisted image analyzing system, mainly with mouse cells. In G2 phase, a significant portion of RFP-HP1 diffused homogeneously in the nucleus and further dispersed into the cytoplasm soon after the nuclear membrane breakdown, while some remained in the centromeric region. Simultaneous observations with GFP-CENP-A in human cells showed that RFP-HP1 was located just between the sister kinetochores and then aligned to the spindle midzone. With the onset of anaphase, once it was released from there, it moved to the centromeres of segregating chromosomes or returned to the spindle equator. As cytokinesis proceeded, HP1α was predominantly found in the newly formed daughter nuclei and again displayed a heterochromatin-like distribution. These results suggested that, although the majority of HP1α diffuses into the cytoplasm, some populations are retained in the centromeric region and involved in the association and segregation of sister kinetochores during mitosis.**

Key words: CENP-A/centromere/heterochromatin/HP1/timelapse

The centromere is morphologically defined as a microtubule attachment site of metaphase chromosomes. It consists of repetitive DNA sequences and specific proteins that are directly or indirectly associated with it. Although a number of centromere proteins have been identified, they can be classified into two categories according to their molecular behavior during the cell cycle: centromere-chromatin proteins and centromere-associated proteins (Craig *et al*., 1999). The former proteins are likely to possess certain DNA-binding activity and form centromere-specific chromatin throughout the cell cycle. In contrast, the latter proteins transiently associate with the centromere/kinetochore only during mitosis, probably via protein-protein interactions dependent on the cell cycle. Immunostaining with monospecific anti-centromere antibodies showed that the metaphase centromere consists of at least three structural units: kinetochore, central and pairing domains. The central domain is defined as a central region that lies between the kinetochore and pairing domains. This area is rich in centromeric repetitive sequences, such as alphoid DNA in human and minor satellite in mouse (Wong and Rattner, 1988; Masumoto *et al*., 1989a). Centromere protein B (CENP-B), first identified as one of three major centromere autoantigens often recognized by anticentromere autoimmune sera of scleroderma patients (Earnshaw and Rothfield, 1985; Muro *et al*., 1990), specifically binds to a 15–17bp motif found in these satellite DNA and constitutes a major portion of centromere heterochromatin (Masumoto *et al*., 1989b;

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Abbreviations: CENP, centromere protein; GFP, green fluorescent protein; HP1, heterochromatin protein 1; RFP, red fluorescent protein.

Sugimoto *et al*., 1992, Sugimoto *et al*., 1998).

The kinetochore domain is the outer centromeric region where the microtubules directly attach. CENP-A and CENP-C, the remaining two centromere autoantigens, localize to the kinetochore domain (Saitoh *et al*., 1992; Warburton *et al*., 1997). CENP-A is a centromere-specific histone whose C-terminal half is related to histone H3 (Sullivan *et al*., 1994), while CENP-C is a basic 140kDa protein that is homologous to budding yeast kinetochore protein MIF2 at the C-terminal region (Brown, 1995) and is involved in kinetochore assembly (Lanini and McKeon, 1995; Sugimoto *et al*., 1997). These two proteins possess DNA-binding property (Sugimoto *et al*., 1994; Vafa *et al*., 1997) and constitute a kinetochore chromatin throughout the cell cycle (Sugimoto *et al*., 2000). Interestingly, the antigenic structure, called prekinetochore, is present in interphase nuclei (Brenner *et al*., 1981) and functions as a kinetochore organizing center, the site where the kinetochore structure is assembled (Sugimoto *et al*., 1999; Sugimoto *et al*., 2000). Recent reports suggest that CENP-G and CENP-H also constitute this structure (He *et al*., 1998; Sugata *et al*., 2000). During mitosis, a number of centromere-associated proteins further associate with the prekinetochore, and differentiate into the fully maturated kinetochore seen on metaphase chromosomes (Craig *et al*., 1999).

The pairing domain is the inner centromeric region where the sister chromatids are connected with each other. This region is the anchor portion of the centromere heterochromatin that will finally separate in anaphase. The inner centromere protein (INCENP) is the one most characterized among those mapped to this region (Cooke *et al*., 1987). In *Drosophila*, it is known that certain cohesion proteins such as Mei322 localize to condensed centromere until the sister chromatids separate (Moore *et al*., 1998). However, less known is about the molecular mechanism of sister-kinetochore association and separation in mammalian cells.

Heterochromatin protein HP1 is a highly conserved non-histone protein. *Drosophila* HP1 localizes to the centromere and telomere heterochromatin and is known to be one of the modifiers of heterochromatin-induced position effect variegation (PEV), encoded by Su(var)2–5 gene (James and Elgin, 1986; Eissenberg *et al*., 1990). Interestingly, it is also required for correct chromosome segregation in *Drosophila* embryos (Kellum *et al*., 1995). In mammalian cells, three related homologs, HP1α, HP1β and HP1γ, have been identified. Human homologs were first identified as 22–25kDa autoantigens recognized by a subset of anticentromere autoantibodies (Saunders *et al*., 1993; Nicol and Jeppesen, 1994; Iwai *et al*., 1996). Although these proteins are assumed to be involved in the epigenetic control of gene expression, their role in chromosome segregation has yet to be examined in detail. They have two conserved motifs at the N- and C-termini, called chromo and chromoshadow domains, respectively (Eissenberg and Elgin, 2000) and thus share antigenicity to some extent (Nicol and Jeppesen, 1994). This property has made it difficult to specifically characterize each peptide. The distinct subcellular localization of each peptide has been clarified by producing monospecific antibodies against each or expressing it as a fusion to a certain molecular tag (Minc *et al*., 1999; Yamada *et al*., 1999; Nielsen *et al*., 2001). Interestingly, only HP1α localizes to centromere heterochromatin in human cells, while HP1α and β do so in mouse cells. Recently, the molecular interaction with INCENP was reported by a yeast two-hybrid screen system (Ainsztein *et al*., 1998), suggesting that HP1α may constitute the inner centromeric region and have some epigenetic function in chromosome segregation.

Here, to elucidate the biological significance of HP1α *in vivo*, we expressed it in mammalian cells as a fusion to red fluorescent protein (RFP, or DsRed) and carefully observed its molecular behavior in living mitotic cells. To determine its precise position in the centromere structure, kinetochore protein CENP-A was simultaneously expressed as a fusion to green fluorescent protein (GFP). Live images of HP1α from G2 to G1 phase were captured by a highly sensitive CCD camera equipped with a computer-assisted image analyzing system.

Materials and Methods

Construction of pDsRed-HP1α

pEGFP-HP1 (Yamada *et al*., 1999) was digested with *Sac*I and *Xho*I and the inserted 430 bp fragment was recloned into the *Sac*I-*Sal*I site of pDsRed-C1 (Clontech, Palo Alto, CA, USA), resulting in pDsRed-HP1α. pEGFP-AF8 has been described elsewhere (Sugimoto *et al*., 2000).

Construction of stable cell lines

Human MDA-AF8 (Sugimoto *et al*., 2000), mouse C3H, Indian muntjac cells were grown in DMEM (Nissui Pharmaceutical, Tokyo, Japan) containing 10% FCS at 37°C in 5% CO_2 atmosphere. Plasmid DNAs (16 μg for pEGFP- or pDsRed-derived plasmid and 7 μg for pTK-Hyg, if necessary) were mixed and introduced into cells by electroporation, as described (Sugimoto *et al*., 2000). Stable transformants were selected in the presence of G418 (Nacalai Tesque, Kyoto, Japan) or hygromycin B (Roche Molecular Biochemicals, Mannheim, Germany).

Immunofluorescence

Aliquot of cells ($1–3\times10^5$) 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. Mitotically dividing cells were obtained by gently tapping them off from a randomly growing culture in a 90 mm dish. After treating them with a hypotonic buffer (10 mM Tris-HCl, pH 7.4, 40 mM glycerol, 20 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$), the cells were sedimented onto glass slides

using Cytospin 2 (Shandon Southern Instruments, Sewickley, PA, USA) and fixed with 80% ethanol. Monolayer cells and mitotic chromosome spreads were counterstained with DAPI (1 μg/ml) and observed under fluorescent microscope (Eclipse E600, Nikon, Tokyo, Japan) equipped with a PlanApo 60x objective (NA 1.40, Nikon) and a MicroMAX 1300Y cooled CCD camera (Princeton Instruments, Princeton, NJ, USA). Images were obtained by MetaMorph software (Universal Imaging Corp., West Chester, PA, USA).

Live image analysis

Cells were grown in a 35 mm dish that was equipped with a glass coverslip on the bottom (Iwaki glass base dish, Asahi Techno Glass, Funabashi, Chiba, Japan) on the stage of a fluorescent microscope (Eclipse TE300, Nikon) with a PlanApo 60x objective (NA 1.40, Nikon), controlling the temperature and CO_2 concentration in a humid chamber using a thermostat and a CO_2 inject control timer (Kokensha Engineering Co. Ltd., Tokyo, Japan). Live images were obtained by LuminaVision software (Mitani Corp., Tokyo, Japan), controlling a cooled CCD digital camera (C4742-95, Hamamatsu Photonics, Shizuoka, Japan) and a BioPoint MAC3000 controller system for a filter wheel and a Z-axis motor (Ludl Electronic Products Ltd., Hawthorne, NY, USA), for collecting Z-series optical sections (0.2 μm intervals).

Results

Ectopical expression of RFP-HP1 in mammalian cells

To clarify their molecular behavior during the cell cycle and to precisely determine their position relative to the kinetochore locus, we differentially expressed HP1α as well as CENP-A as fusions to RFP and GFP, respectively. We obtained human stable cell lines and simultaneously observed these two proteins under fluorescent microscope. Figure 1A shows a typical interphase image of MDA-AF-HP1, a stable cell line. RFP-HP1 often exists as discrete dots in nuclei, as previously reported for GFP-HP1 (Yamada *et al.*, 1999). Although some RFP-HP1 dots overlap with the centromere/kinetochore visualized by GFP-CENP-A, others did not seem to colocalize with it. The same results were obtained when the endogenous centromere proteins were stained with anti-centromere antibodies (data not shown). In certain interphase cells, however, we noticed that RFP-HP1 actually localized to the centromeric heterochromatin attached to the nuclear membrane, although we could not determine the exact phase in the cell cycle here (Figs. 1B and 1C). Interestingly, the intensity of RFP signals seemed to be quite different among the populations, suggesting a cell cycle-dependent localization.

To further examine whether RFP-HP1 localizes to hetero-

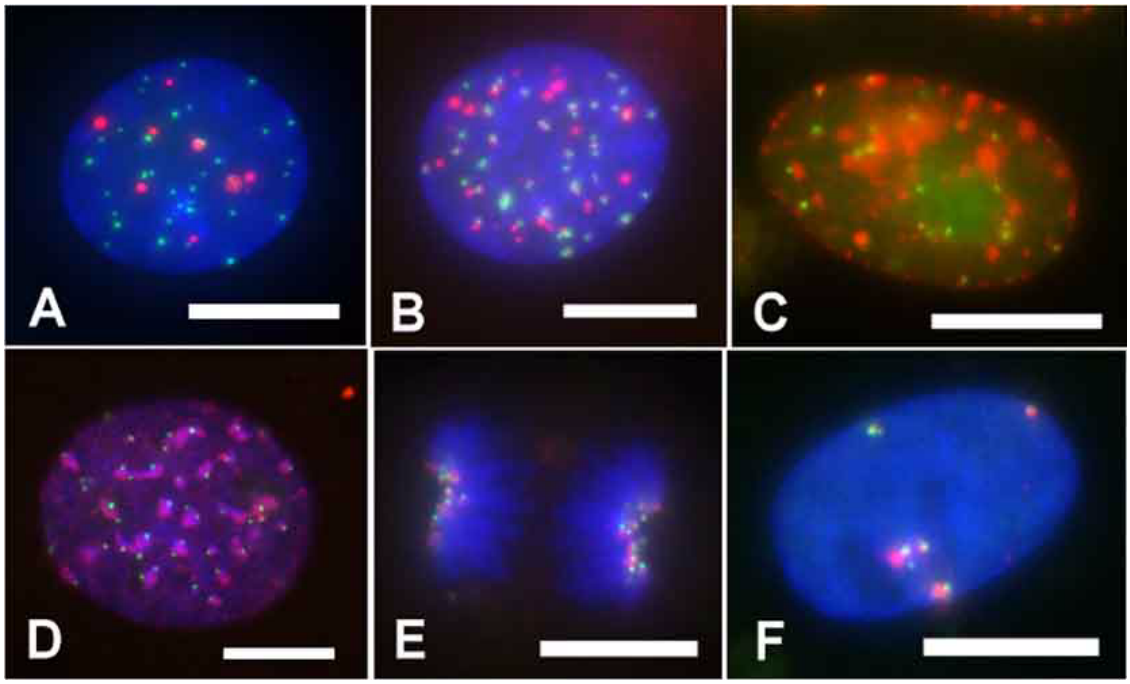

Fig. 1. Cellular localization of RFP-fused human HP1α in mammalian cells. Human MDA435, mouse C3H and Indian muntjac cells were transfected with pDsRed-HP1α and pEGFP-AF8. Stable transformants simultaneously expressing RFP-HP1 (red) and GFP-CENP-A (green) were obtained and observed under a fluorescent microscope. Interphase nuclei of MDA-AF-HP1 (A, B), C3H-AF-HP1 (D), Mun-AF-HP1 (F), and an anaphase cell of C3H-AF-HP1 (E) were fixed with paraformaldehyde and counterstained with DAPI (blue). A fluorescent image of RFP-HP1 and GFP-CENP-A in living MDA-AF-HP1 cells (C). Bar: 10 μm.

chromatin in other mammalian cells, we obtained a mouse stable cell line, C3H-AF-HP1, since the heterochromatin of this species is easily identified as a region that is extensively stained with DAPI. As shown in Figure 1D, RFP-HP1 colocalized with the DAPI-stained region in most interphase cells, consistent with a previous report on its endogenous localization (Minc *et al*., 1999). GFP-CENP-A exists as small discrete dots attached to the edge of the RFP-HP1 heterochromatin mass in interphase cells. RFP-HP1 was also observed in the centromere heterochromatin of anaphase chromosomes (Fig. 1E). Figure 1F shows the results with an Indian muntjac cell line, Mun-AF-HP1. Interestingly, RFP-HP1 colocalized with GFP-CENP-A in this species, although the number of HP1 dots was sometimes larger than seven, the chromosome number of this cell line (data not shown). These results indicated that ectopically expressed RFP-HP1 has the ability to localize to heterochromatin in these mammalian cells, although its distribution pattern in interphase nuclei seemed to be different among these species.

Inner centromere localization of RFP-HP1 in metaphase chromosome spreads

We previously showed that HP1α localized to centromere heterochromatin in human mitotic cells (Yamada *et al*., 1999). Nevertheless, its precise location in the centromere structure has yet to be examined. We prepared metaphase chromosome spreads using the above mammalian cells and compared the relative position of RFP-HP1 to the kinetochore locus simultaneously visualized by GFP-CENP-A. As shown in Figures 2A and 2B, RFP-HP1 was predominantly found in the inner centromeric region between a pair of kinetochores in human and Indian muntjac cells. In mouse cells, however, RFP-HP1 was rather widely distributed throughout the whole area of the centromere and partly in the telomere (Fig. 2C), as originally reported for *Drosophila* HP1 (James and Elgin, 1986).

Timelapse analysis of RFP-HP1 in mouse mitotic cells

We noticed that some interphase cells did not show the typical heterochromatin-like distribution anymore, and that the signal was rather faint in mitotic cells. These cells were identified to be in G2 or prophase, since the sister kinetochores, visualized by GFP-CENP-A, were under duplication (Fig. 3E) or have been duplicated (Figs. 3H and 3K). This clearly showed that heterochromatin localization of RFP-HP1 depends on the cell cycle, and that HP1α diffuses in the nucleus just before mitosis. However, the above results indicated that RFP-HP1 was mainly located to centromeric heterochromatin on mitotic chromosomes. To examine the molecular behavior of HP1α at the interphase:metaphase transition, timelapse images of mouse C3H-AF-HP1 cells were captured by computer-assisted image analyzing system.

We started with a late S/early G2-phase cell in which the prekinetochore dots were duplicating. As shown in Figure 4, RFP-HP1 no longer showed the typical heterochromatin-like distribution (compare to Fig. 3C). It took 76 min for this cell to enter prophase. During this period, HP1α gradually diffused in the nucleus and distributed rather homogeneously at 60 min, and then started to form smaller heterochromatin-like mass again at 70–75 min. We could recognize that the nuclear membrane breakdown occurred at 76 min, since some portions of RFP-HP1 in the nucleus suddenly started to disperse into the cytoplasm. Interestingly, the heterochromatin-like mass remained in the area where the nucleus had been. In another 5 min, each heterochromatin mass that had been widely distributed in the nucleus gradually linked together to form a loop-like structure at 81-85 min. This type of centromere arrangement was seen in a rosette-like structure of mitotic chromosomes in prometaphase cells (see Fig. 1C of Sugimoto *et al*., 2000).

A late G2 cell was observed at this time to follow its movement throughout the whole stage of mitosis. This cell soon entered prophase and completed mitosis after about 1 hr. As shown in Figure 5, the nuclear membrane breakdown occurred 6 min after observation (prophase). Again, most of RFP-HP1 mass in the nucleus diffused into the cytoplasm, while some remained as if the nucleus were still present (see 7 min). They linked together and formed an extended loop-like structure, requiring another 6 min (prometaphase) and finally aligned themselves along the spindle midzone at 21 min (metaphase). Interestingly, the cell had just started to shrink and its shape was not round at this stage. During this course, we could follow the movement of a small RFP-HP1 mass, as indicated by arrowhead (see 18–20.5 min), and could calculate its migration rate to be 2.1 μm/min (19–19.5 min), 4.5 μm/min (19.5–20 min) and 0.6 μm/min (20–20.5 min) (Fig. 6A). The RFP-HP1 mass moved back and forth around the spindle equator for 12 min or so, and then began to segregate to the opposite poles at 33 min (anaphase). To compare the migration rate of RFP-HP1 in anaphase to that in prometaphase and to estimate the transition time from anaphase A to B, the distance between the separating sister heterochromatins was also measured. As illustrated in Fig. 6B, they moved apart from each other at 3.8 μm/min for the initial 4 min (from 33 min to 37 min), thus at a rate of 1.9 μm/min per sister chromatid. This value was about half of that obtained in prometaphase. It took 4 min for it to reach the poles (anaphase A) and the migration rate slowed down at the time when the cleavage furrow was recognized on the cell surface (39 min, anaphase B). Coincidently, the fluorescence of RFP-HP1 in the cytoplasm rapidly diminished. Although we could not tell when the nuclear membrane reappeared here, it took 40 min for this cell to start the nuclear membrane breakdown and to complete the cytokinesis (45 min, telophase). The newly formed daughter nucleus, filled with RFP-HP1 heterochromatin, was extremely small. As it

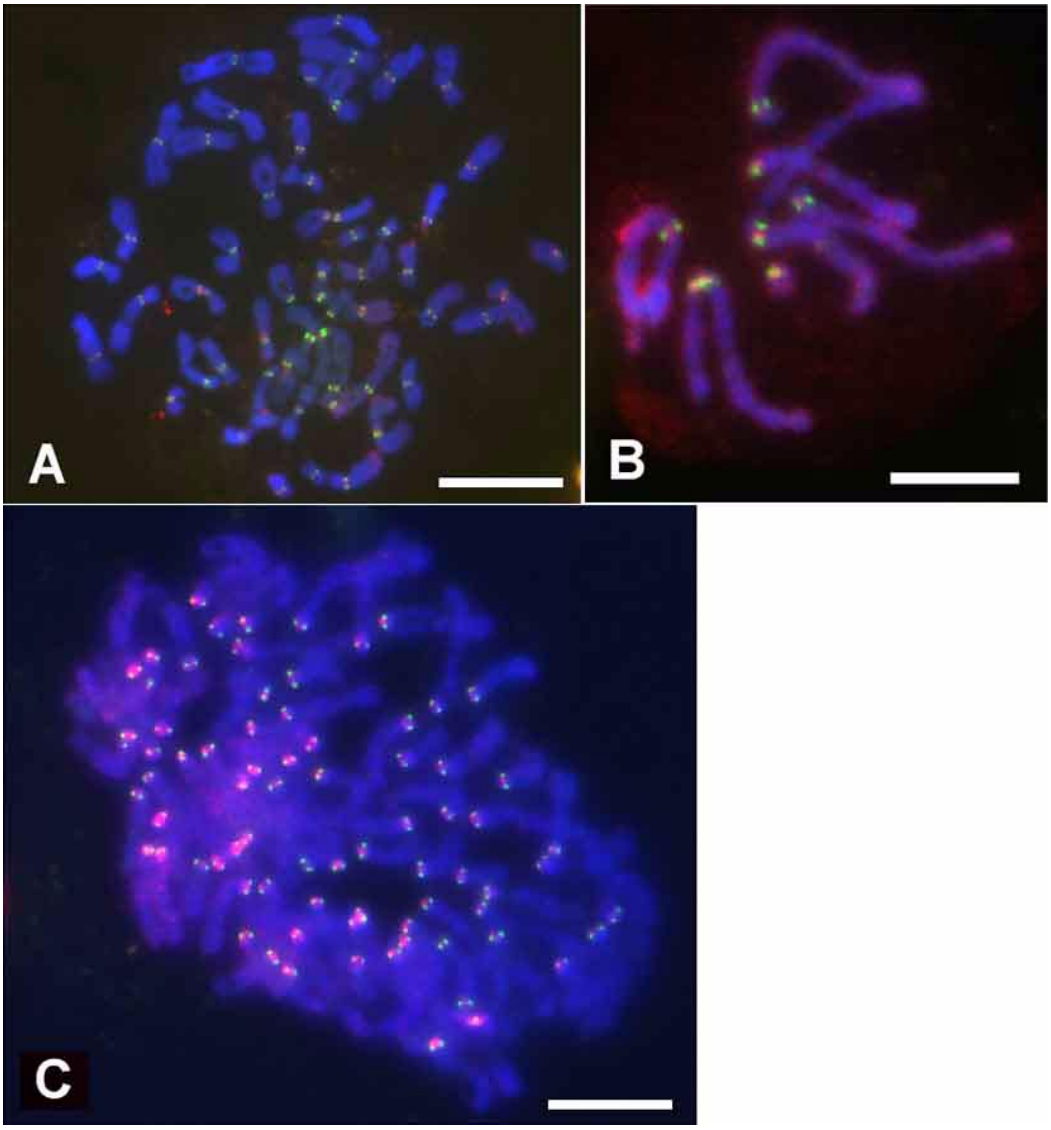

Fig. 2. Relative location of RFP-HP1 to the kinetochore locus on mitotic chromosomes. Mitotic cells were obtained from a random cell culture of MDA-AF-HP1 (A), Mun-AF-HP1 (B) and C3H-AF-HP1 cells (C), ectopically expressing GFP-CENP-A (green) and RFP-HP1 (red), treated with a hypotonic buffer and cytocentrifuged onto a slide. The mitotic chromosome spreads were fixed with 80% ethanol and counterstained with DAPI (blue). Bar: 10 μm.

gradually enlarged, RFP-HP1 displayed a heterochromatin-like distribution again (early G1).

These results clearly showed that HP1α drastically changes its localization at least at two critical points in the cell cycle, that is, at the G2:prophase transition and at the initiation of cytokinesis. Although the majority of HP1α diffused into the cytoplasm, some portion of it was retained in the centromeric region throughout mitosis.

Live images of RFP-HP1 and GFP-CENP-A in human mitotic cells

In human MDA435 cells, a significant portion of RFP-HP1 did not seem to localize to heterochromatin in typical interphase nuclei, while it was predominantly found in the inner centromeric region on metaphase chromosome spreads (compare Fig. 1 to Fig. 2). This is probably because only a small portion of HP1α was retained in centromere from G2 to prophase. Nevertheless, this does not explain its limited localization to the inner centromeric region of mitotic chromosomes. It is possible that, by treating mitotic cells with a hypotonic solution, RFP-HP1 was simply extracted from that region except for the inner area. To exclude this possibility and further confirm the inner centromere localization of RFP-HP1 in living cells, time lapse images of MDA-AF-HP1 cells were obtained again.

We first observed a prometa/metaphase cell in which a

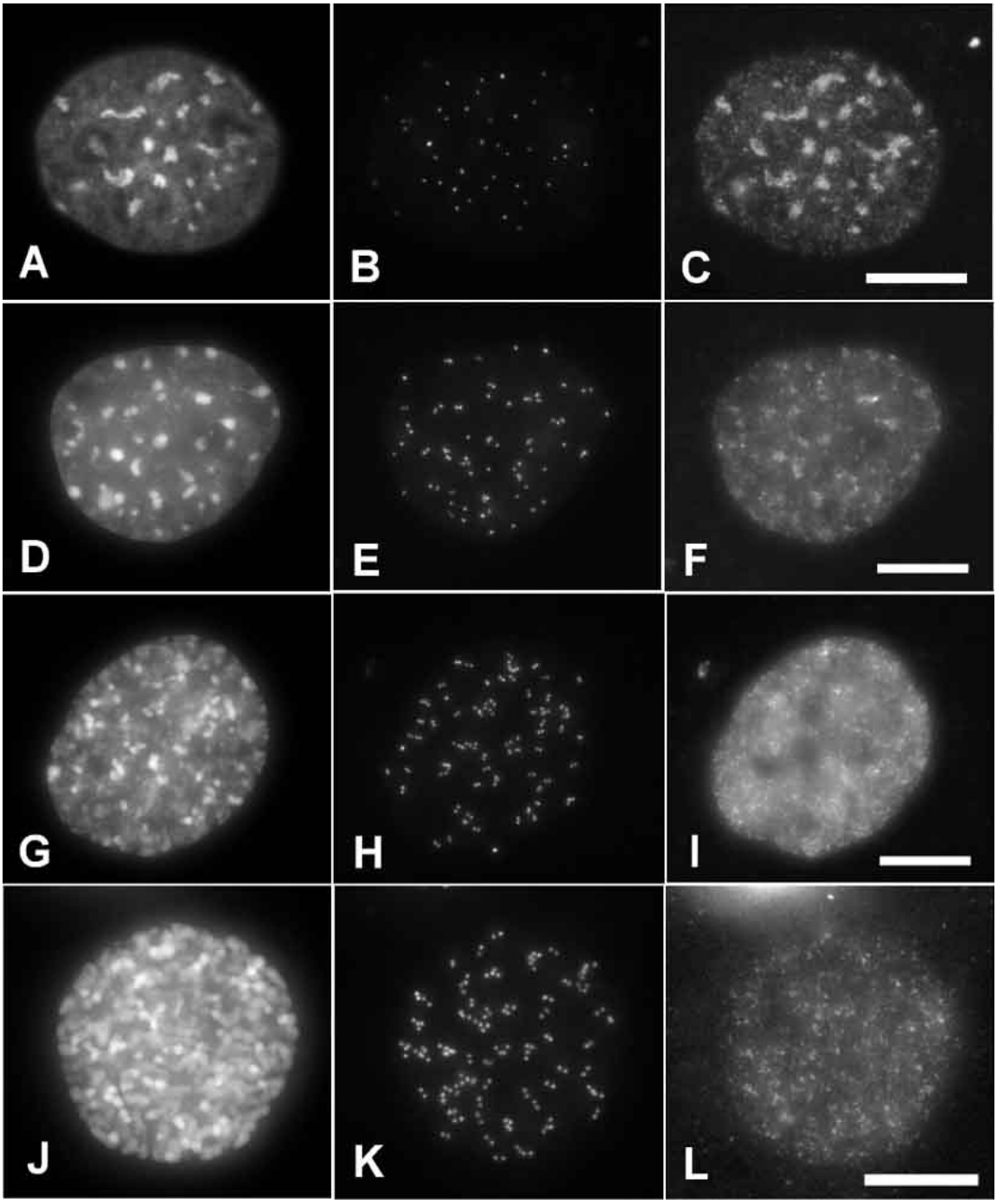

Fig. 3. Distribution patterns of RFP-HP1 observed in mouse interphase nuclei. A random culture of mouse C3H-AF-HP1 cells was fixed with paraformaldehyde and counterstained with DAPI. Fluorescent images of GFP-CENP-A (B, E, H, K) and RFP-HP1 (C, F, I, L) were compared to DAPI-stained heterochromatin (A, D, G, J). A typical interphase cell (A–C); a late S/early G2 phase cell (D–F); a G2 phase cell (G–I); a late G2/early prophase cell (J–L). Note that sister kinetochores were under duplication in late S/early G2 (E) and have been duplicated in G2/prophase (H, K). Bar: 10 μm.

pair of sister kinetochores of GFP-CENP-A were arranged parallel to the spindle equator (Fig. 7). The significant portion of RFP-HP1 has diffused homogeneously in the cytoplasm. When observed carefully, however, a faint RFP-HP1 signal is found in the centromeric region, and is located just between a pair of GFP-CENP-A dots, which is consistent with the above observation with mitotic chromosome spreads (compare to Fig. 2A).

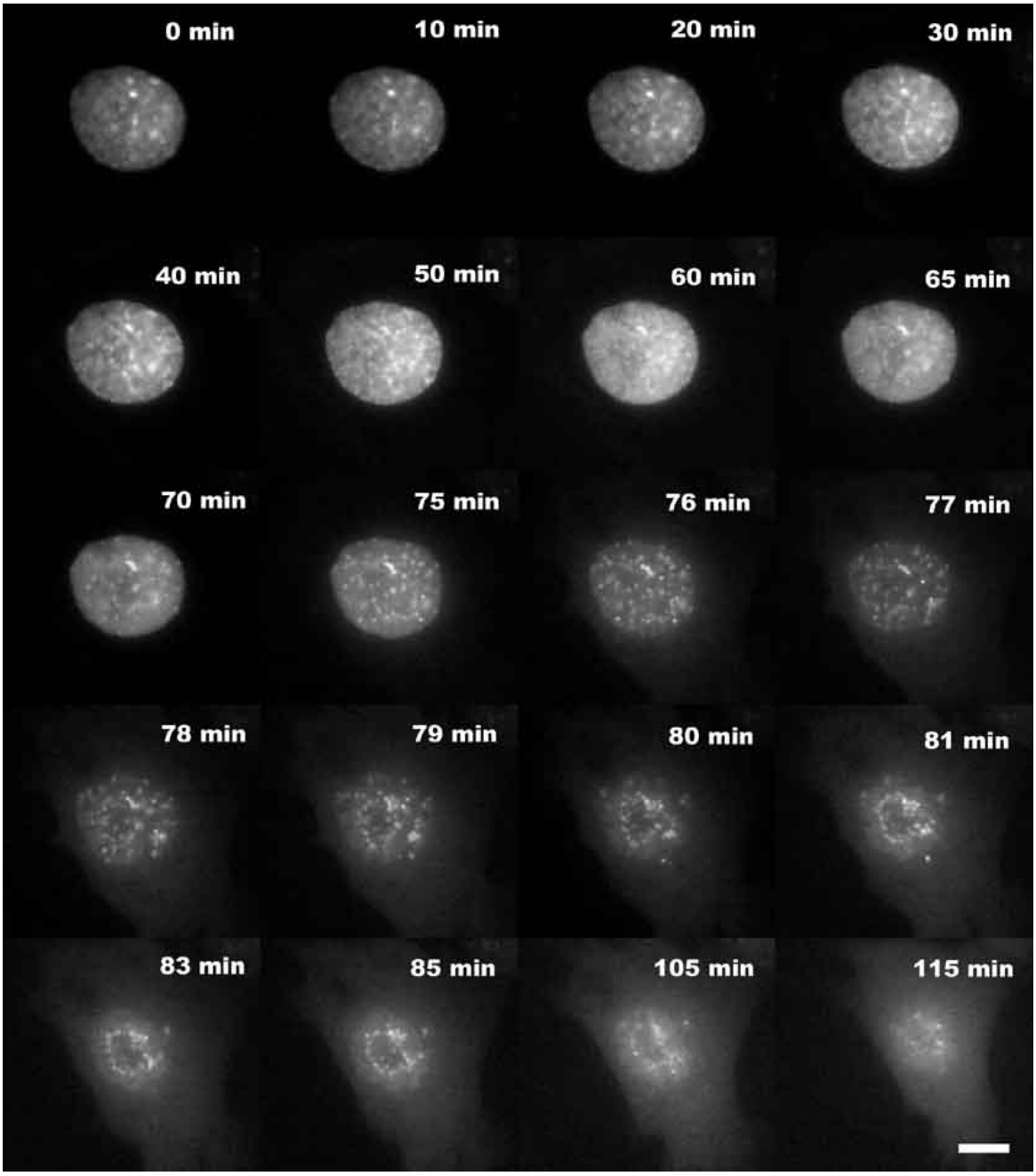

Fig. 4. Molecular localization of RFP-HP1 from G2 to prometaphase in a living mouse C3H cell. Fluorescent images of C3H-AF-HP1 cell in late S/early G2 were captured at 1 min intervals by a computer-assisted image analyzing system equipped with a cooled CCD camera. The elapsed time was shown in the upper right corner of each panel. Note that the nuclear membrane breakdown occurred at 76 min. Online supplemental videos are available at http://csf.jstage.jst.go.jp. When you click "supplementary materials" under abstract, you can see the movie. Bar: 10 μm.

We then examined the molecular behavior at the metaphase:anaphase transition. As shown in Figure 8, GFP-CENP-A dots are arranged in two lines along the spindle equator (0 min, metaphase). RFP-HP1 is present just between the paired dots of sister kinetochores, as if it were some kind of cohesive glue. Six minutes later, the sister kinetochores begins to separate and move to the opposite poles (6.5 min, anaphase). Concurrent with the onset of anaphase, RFP-HP1 is released from the spindle midzone and slightly diffused between the separating sister kinetochores. Some populations of RFP-HP1 then localize to the segregating chromsomes, but some remain around the

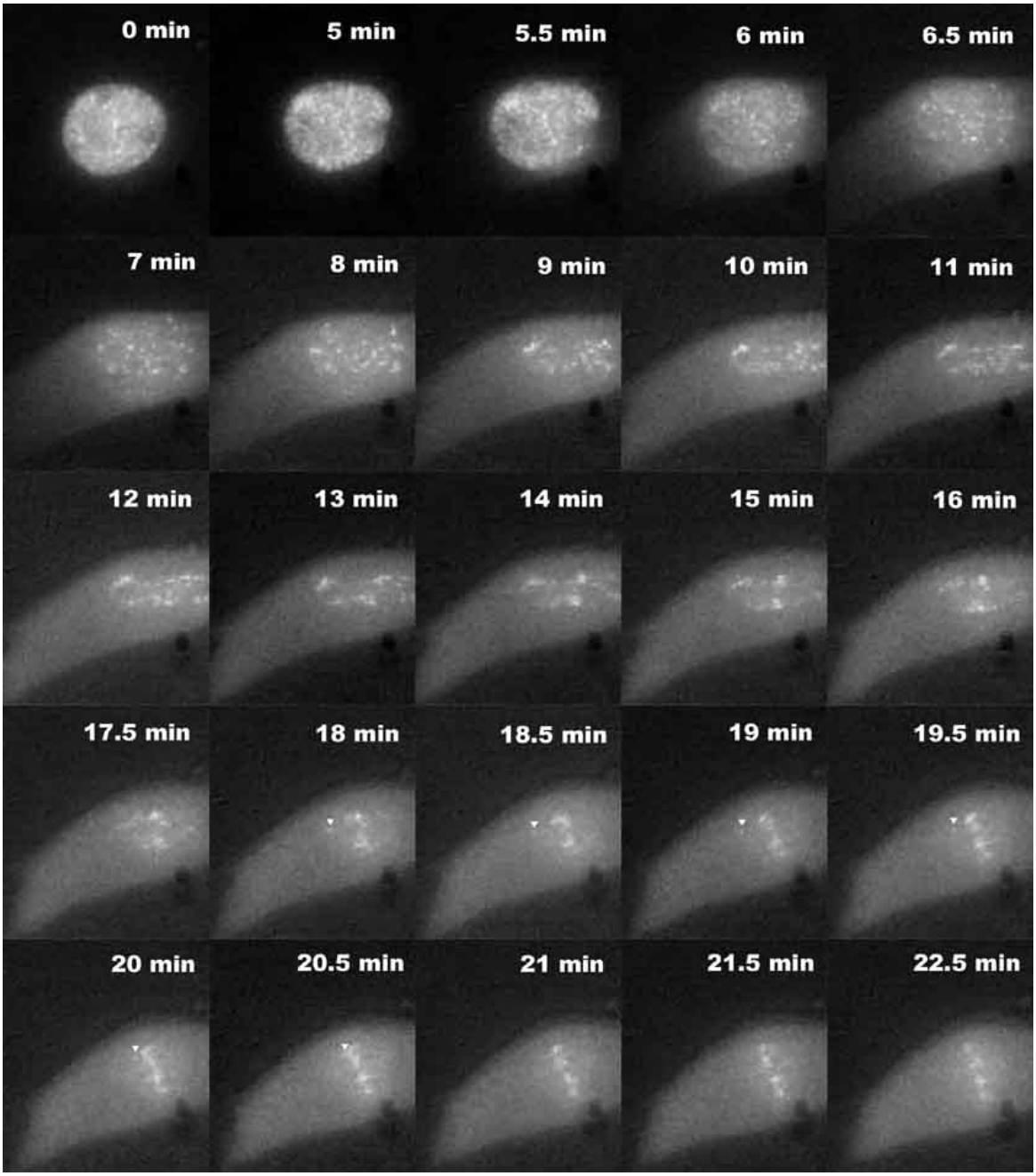

Fig. 5a. Dynamic redistribution of RFP-HP1 throughout the entire process of mitosis. Fluorescent images of C3H-AF-HP1 cell in late G2 were captured at 30 s intervals. Elapsed time is shown in the upper right corner of each panel. Note the occurrence of nuclear membrane breakdown (6 min), formation of a rosette-like structure (12 min), alignment along the spindle equator (21 min), anaphase onset (33 min) and the initiation of cytokinesis (45 min). A small heterochromatin block, the migration rate of which is calculated in Fig. 6, is indicated with arrowhead (see 18–20.5 min). Online supplemental videos are available at http://csf.jstage.jst.go.jp. When you click "supplementary materials" under abstract, you can see the movie. Bar: 10 μm.

spindle equator (12–15 min). As cytokinesis proceeds, the latter portion concentrates around the midzone and is found predominantly in the midbody region (19 min). These results indicated that certain portions of HP1α localize to the inner centromere region of mitotic chromosomes in living cells as well.

Discussion

HP1-like proteins are evolutionary conserved among

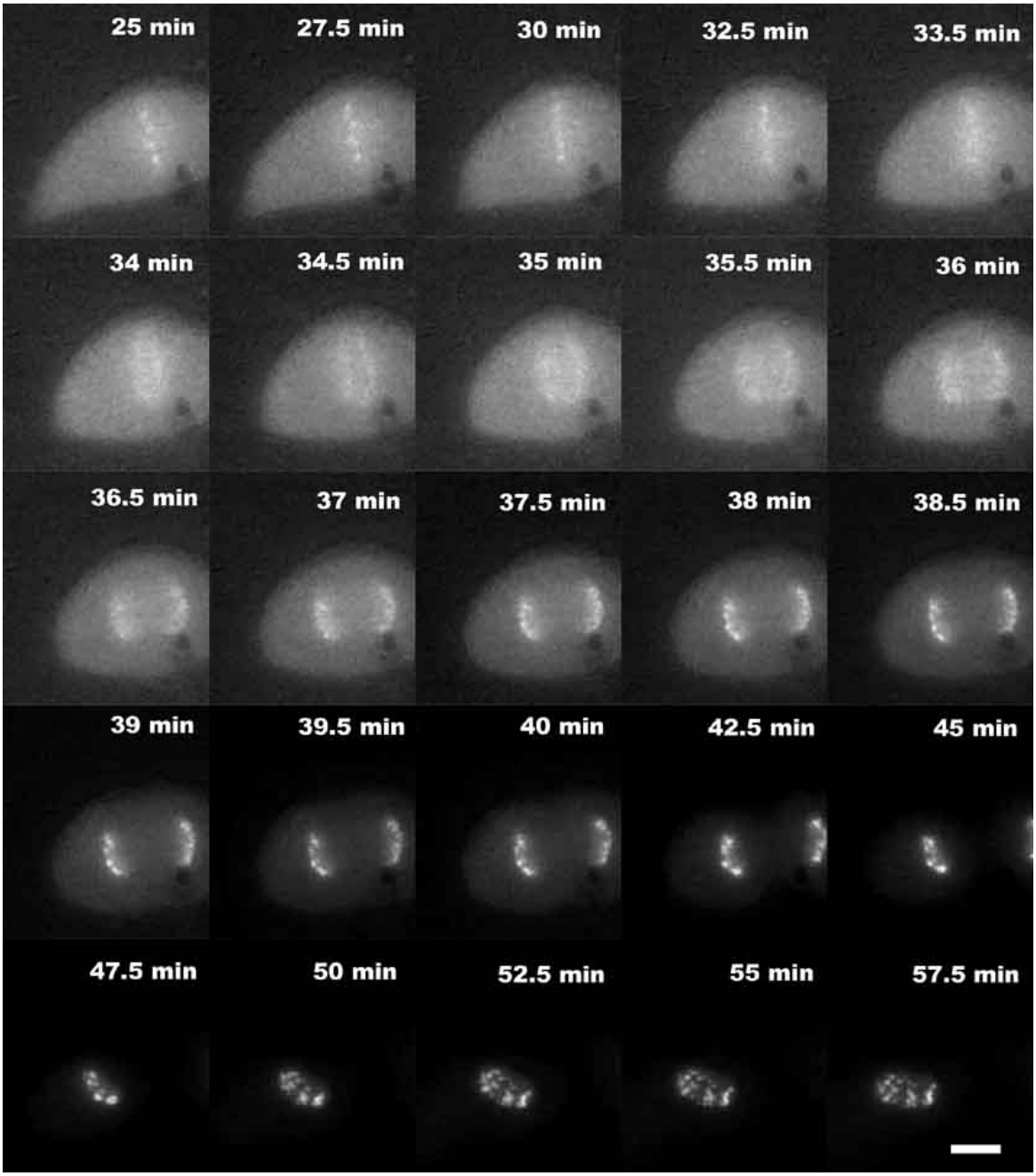

Fig. 5b

eukaryotes, from yeasts to mammals, and are known to be involved in the epigenetic control of chromatin structure (Wallrath, 1998). In addition, *Drosophila* HP1 and its fission yeast homolog, SWI6, localize to centromere heterochromatin and are essential for proper chromosome segregation *in vivo* (Kellum *et al*., 1995; Halverson *et al*., 2000). In mammalian cells, three related polypeptides have been identified as "structural" homologs of *Drosophila* HP1. This is the first report on the molecular behavior of human HP1α in mammalian living cells. Recently, new HP1-like proteins (HP1b and c) were also identified in *Drosophila*. Interestingly, HP1b localized to both heterochromatin and euchromatin and HP1c targeted euchromatin (Smothers and Henikoff, 2001). Our recent study showed that human HP1β and HP1γ that were fused to GFP did not localize to centromere in human cells at all (Sugimoto & Tachibana, unpublished results). We propose here that HP1α is the "functional" homolog of *Drosophila* HP1 (now designated

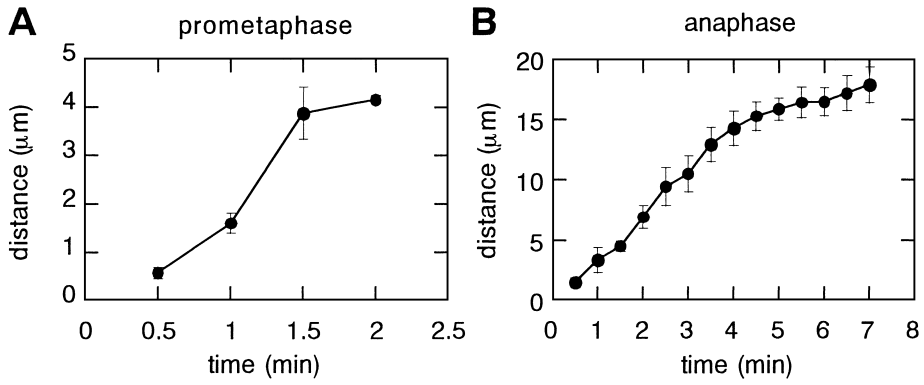

Fig. 6. Estimation of the migration rate of RFP-HP1 heterochromatin in prometaphase and anaphase. (A) Migration in prometaphase. The distance that the small heterochromatin block indicated with arrowhead in Fig. 5 moved for each 30 s interval, was measured by MacScope software (Mitani Corp.) (B) Migration in anaphase. The distance between two sister heterochromatins was measured at 30 s intervals from anaphase onset (33 min) to the initiation of cytokinesis (39 min). Note that the migration rate was relatively constant at the beginning, calculated as 1.9 μm/min per heterochromatin.

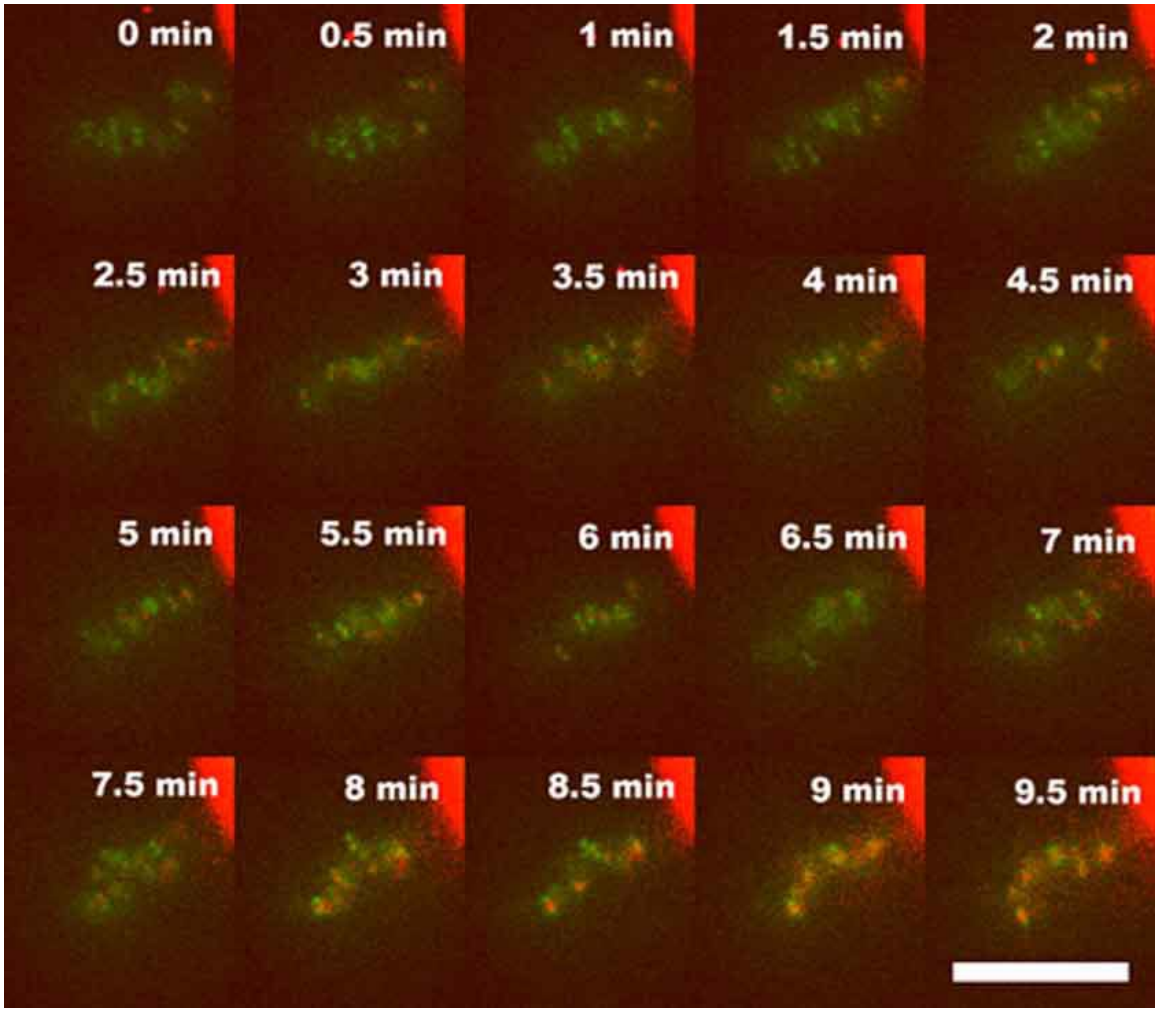

Fig. 7. Inner centromere localization of RFP-HP1 in a living mitotic cell. Fluorescent images of GFP-CENP-A and RFP-HP1 of a metaphase MDA-AF-HP1 cell were captured at 30 s intervals. Elapsed time is shown in the upper right corner of each panel. Note that RFP-HP1 mass was located just between the sister kinetochores. Online supplemental videos are available at http://csf.jstage.jst.go.jp. When you click “supplementary materials” under abstract, you can see the movie. Bar: 10 μm.

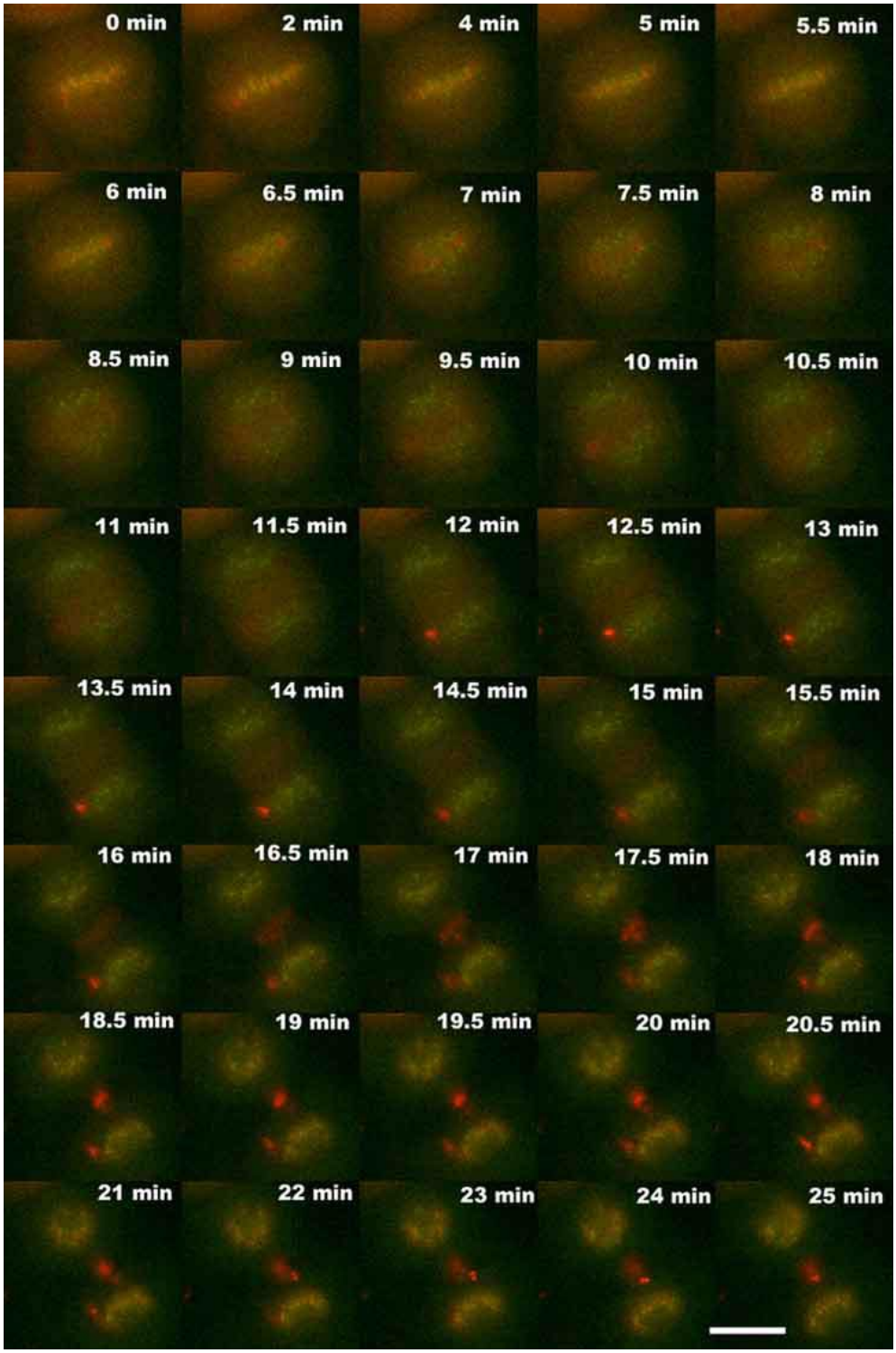

Fig. 8. Molecular dynamics of GFP-CENP-A and RFP-HP1 in a human mitotic cell. Fluorescent images of MDA-AF-HP1 cell were captured from metaphase to early G1 phase at 30 s intervals. Elapsed time is shown in the upper right corner of each panel. Note that once RFP-HP1 was released from the spindle midzone (6.5–9 min), it moved to the centromeres of segregating chromosomes or returned to the spindle equator (15–16 min). Online supplemental videos are available at http://csf.jstage.jst.go.jp. When you click “supplementary materials” under abstract, you can see the movie. Bar: 10 μm.

HP1a) or *Schizosaccharomyces* SWI6.

The present study showed that HP1α drastically changes its localization during mitosis. Figure 9 schematically illustrates the molecular behavior of HP1α during the cell cycle, in conjunction with CENP-A as a kinetochore marker. In mouse cells, HP1α localizes to centromere heterochromatin in typical interphase nuclei. A significant portion of it diffuses homogeneously in late G2 and further disperses into the cytoplasm, which coincides with the nuclear membrane breakdown in prophase. HP1α has been shown to be hyperphosphorylated in mitotic phase (Minc *et al*., 1999). Thus, it is reasonable to assume that the majority of HP1α molecules are modified by relatively abundant protein kinase(s) in mitotic cells and then become diffusible into the cytoplasm. Nevertheless, some populations of RFP-HP1 are retained in the centromeric region of mitotic chromosomes, where they associate with each other, resulting in the formation of a rosette-like structure in prometaphase. They align to the spindle midzone at metaphase, and in anaphase, once they diffuse from there, they move to the centromere heterochromatin of segregating chromosomes or return to the spindle equator. Apparently, the localization of these HP1α molecules is separately regulated from the others that had been dispersed into the cytoplasm. What kind of mechanism makes them remain in the centromeric region? They may simply be escaping general protein modifications taking place, since the inner centromeric region would not easily be accessed by such enzymes. However, recent studies indicate that specific enzymes such as SUV39H1 methylase or Aurora-B kinase are only present in the centromeric region from G2 through to metaphase and then dissociate from there after the metaphase:anaphase transition (Aagaard *et al*., 2000; Adams *et al*., 2000). Therefore, it is likely that the molecules in this limited area are being independently regulated by these protein modification systems. As cytokinesis progresses, HP1α is predominantly found in newly formed

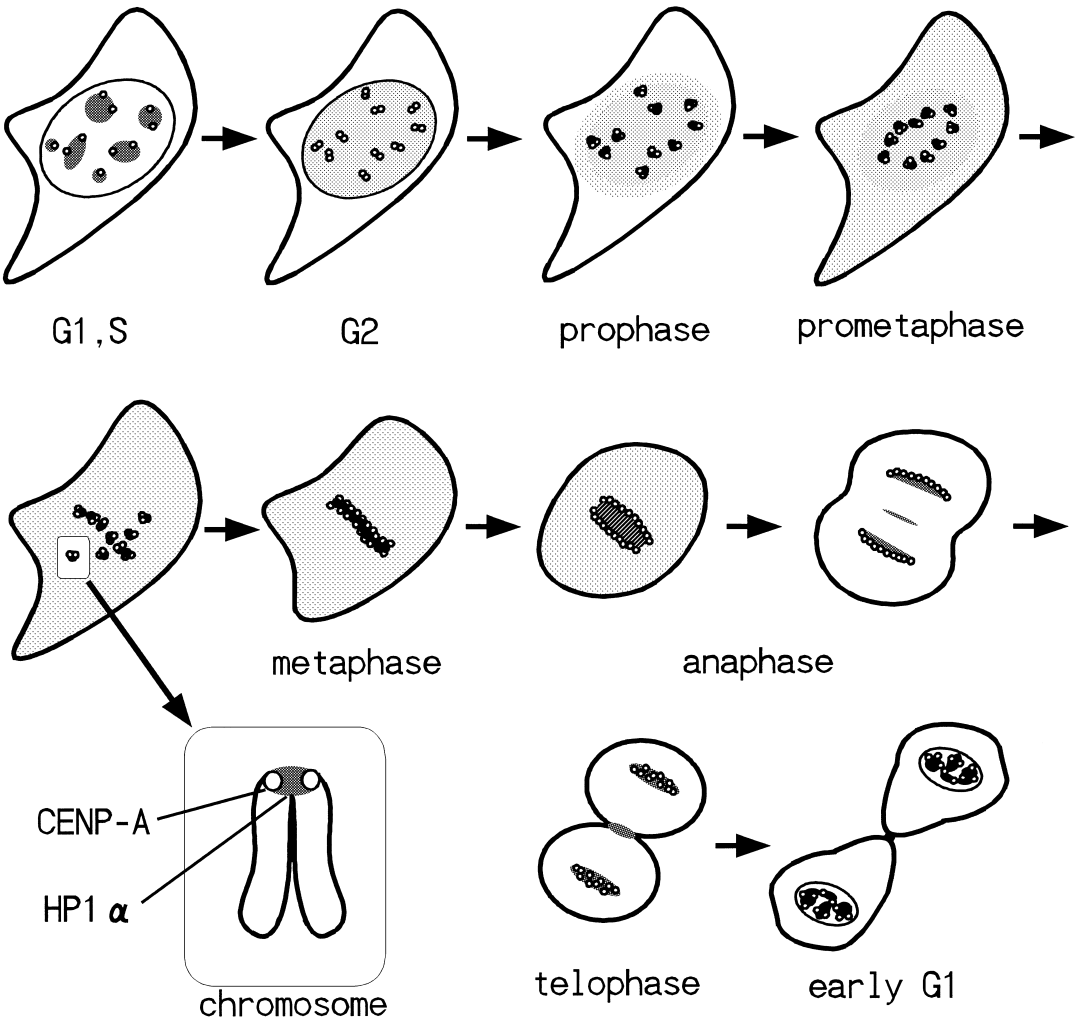

Fig. 9. Schematic illustration of molecular behavior of HP1α during the cell cycle. See text for detail.

daughter nuclei. This is probably because HP1α has the ability to associate with certain components of the inner nuclear membrane structure (see Fig. 1C, Ye *et al*., 1997). Our data suggests that the localization of HP1α is regulated spatially and temporally, at least, at four stages in the cell cycle: late G2, G2/prophase, metaphase/anaphase, and the initiation of cytokinesis.

In the present study, we prepared metaphase chromosome spreads from stable transformants of human and Indian muntjac cell lines and showed that RFP-HP1 was mainly found in the inner centromeric region. So far, we still do not know what kind of mechanism is involved in targeting HP1α to the inner centromeric region and transferring it to the spindle midzone. Inner centromere protein (INCENP) is well known to localize to the pairing domain of sister chromatids (Cooke *et al*., 1987). We noticed that the localization of HP1α was similar to that reported for INCENP. Since the physical interaction between them has been shown by a yeast two-hybrid screen system (Ainsztein *et al*., 1998), it is possible that INCENP mediates the inner centromere localization of HP1α. However, recent studies indicated that the chromo domain of HP1 is responsible for its specific binding to methylated histone H3 (Lachner *et al*., 2001). An alternative explanation is that HP1α itself is directly involved in the centromere localization. This is consistent with the fact that, in contrast to INCENP, most of RFP-HP1 relocalized to the centromere heterochromatin of segregating chromosomes in anaphase (see Figs. 5B and 8).

We previously reported the construction of mammalian stable cell lines in which human kinetochore protein CENP-A was ectopically expressed as a fusion to GFP (Sugimoto *et al*., 2000). GFP-fused CENP-A faithfully localized to the centromere/kinetochore throughout the cell cycle, indicating its usefulness as a fluorescent marker in living cells. In this study, we further obtained mammalian cell lines in which CENP-A and HP1α were simultaneously expressed as fusions to GFP and RFP, respectively, and characterized the molecular behavior of HP1α compared to the kinetochore marker. RFP-HP1 localized to heterochromatin precisely in mouse interphase cells and predominantly to centromere in Indian muntjac cells (see Fig. 2). In human cells, some portions of RFP-HP1 were found in centromere heterochromatin. Our results showed that RFP-HP1 can be used as a heterochromatin marker in living cells, although its distribution seems to differ markedly from species to species, possibly reflecting their respective heterochromatin state. We still do not know whether all of the RFP-HP1 dots observed in interphase nuclei correspond to heterochromatin. In fact, a few bright RFP-HP1 dots were sometimes observed in human mitotic cells, even after the nuclear membrane breakdown (see Fig. 8, 12–14 min). Since HP1-like proteins possess self-associating or hetero-oligomeric activity *in vitro* (Cowell and Austin, 1997; Yamada *et al*., 1999), some but not all of the RFP-HP1 dots may be an "aggregate," especially when the recombinant protein is highly expressed under a strong promoter. Nevertheless, it must be emphasized that most of the heterochromatin-like dots were diffused in the cytoplasm of mitotic cells.

Centromere proteins CENP-A, B, and C were originally identified as the major autoantigens often recognized by anticentromere autoimmune sera of scleroderma patients (Earnshaw and Rothfield, 1985; Muro *et al*., 1990). These proteins localize to the centromere/kinetochore chromatin throughout the cell cycle and constitute a prekinetochore complex even in interphase nuclei (Sugimoto *et al*., 2000). Human HP1α was also identified as a minor autoantigen for anticentromere sera (Saunders *et al*., 1993). This study showed that these four centromere autoantigens are somehow associated with each other and form a large heterochromatin complex from late G2 to metaphase. This macromolecular complex might be the common antigenic determinant for anticentromere antibodies in scleroderma patients. Further studies will be required to clarify the functional relation between autoimmune response and the chromosome segregation process *in vivo*.

Acknowledgments. Mouse C3H and Indian muntjac cells were obtained from the Health Science Research Resources Bank, Sennan, Osaka, Japan. This work was supported by a grant for Specific Research Assistance Program from the Asahi Glass Foundation, Tokyo, Japan.

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(*Received for publication, September 13, 2001*
and in revised form, October 25, 2001)