Structure and dynamic organization of centromeres/prekinetochores in the nucleus of mammalian cells

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SUMMARY

Although considerable research has been focused on understanding the structure and molecular organization of the centromere-kinetochore complex of mitotic chromosomes, few reports have dealt with the centromere (prekinetochore) in the interphase nucleus. In the present study, we utilized anti-centromere antibodies from the serum of patients with the autoimmune disease, scleroderma CREST (calcinosis, Raynaud's phenomenon, esophageal dismotility, sclerodactyly, telangiectasia), as probes to investigate the structure and morphogenesis of the centromere in interphase nuclei of three cell lines using laser scanning confocal microscopy and immunoelectron microscopy. Of particular interest were the chromosomes of the Indian muntjac ($2n=6$ in females and $2n=7$ in males), whose large centromeres are thought to have evolved through the tandem fusion of smaller centromeres of a Chinese muntjac-like progenitor species ($2n=46$). The various forms and patterns of centromeres observed in the nucleus correlated with stages in the cell cycle as determined by bromodeoxyuridine labeling and apparently represent stages in prereplication, replication and matura-tion. Immunoelectron microscopic studies using CREST antisera indicated that the high order structure of chromatin associated with each prekinetochore undergoes a regular unfolding-refolding cycle, displaying small bead-like subunits tandemly arranged along a linear thread of centromeric DNA, much like that reported for mitotic chromosomes. Individual centromeres/prekinetochores form a stable association with the 9-13 nm core filaments of the nucleoskeletal network in the nucleus that later become the chromosome scaffold of mitotic chromosomes. Our findings provide morphological support for the hypothesis that the spatial arrangements of individual centromeres within the nucleus may have influenced centromeric translocations and fusions during chromosome evolution. Therefore, the centromere-kinetochore complex, best known for its essential role in partitioning chromosomes in mitosis and meiosis, may also function in chromosome movements and associations in interphase.

Key words: Centromere, Kinetochore, Prekinetochore, Nucleus, Mitosis

INTRODUCTION

The centromere, a heterochromatic region of metaphase chromosomes, located at the primary constriction functions to organize the kinetochore and brings about chromosome attachment to the mitotic spindle. A defect in centromere development and function is believed to cause chromosome loss or nondisjunction, a condition associated with genetic disorders, including Down's syndrome and genomic instability leading to malignancy. Most studies of the structure, molecular organization and function of the centromere/kinetochore complex have focused on mitotic chromosomes (for reviews see Rieder, 1982; Pluta et al., 1990; Rattner, 1991; Brinkley et al., 1992). Human autoantiserum from patients with the CREST variety of scleroderma (Moroi et al., 1980) can be used to detect the centromere-kinetochore complex of mitotic chromosomes as well as fluorescent foci in interphase nuclei. Relatively little is known, however, about centromeres during interphase when duplication and maturation occur. Earlier studies indicated that the CREST antisera detected numerous fluorescent foci throughout the nucleus of cells in the G_1-phase of the cell

cycle, and that the number of such foci exactly doubled in the G_2 nuclei (Brenner et al., 1981). Since CREST autoantibodies recognize several centromere proteins (CENPs) at or near the kinetochore of metaphase chromosomes (Earnshaw and Rothfield, 1985; Valdivia and Brinkley, 1985), it is safe to assume that fluorescent foci seen in interphase nuclei represent components of the centromere and, likely, the presumptive kinetochore (prekinetochores) as originally described by Brenner et al. (1981). Beyond these observations, relatively little is known about the structure and morphogenesis of the centromere/prekinetochore during interphase or how centromere duplication and maturation occurs prior to the onset of mitosis. Compared to the well organized trilaminar plates of the mitotic kinetochore, it is not known whether the interphase centromere displays specific higher order structure.

In the present study, we utilized scleroderma CREST anti-centromere antibodies as probes to investigate the centromere/prekinetochore structure and morphogenesis throughout the cell cycle by confocal and electron microscopy. This involved three mammalian cell lines: Indian muntjac, Chinese muntjac and HeLa. Muntjac deer cells were particularly inter-

esting because of their unique chromosome complement and intranuclear centromere association during interphase (Brinkley et al., 1984). Moreover, the chromosomes of the Indian muntjac contain a unique centromere-kinetochore organization that is thought to have evolved from the fusion of multiple smaller centromeres from the chromosomes of a progenitor species. Termed compound kinetochores (Brinkley et al., 1984), these individual centromere-kinetochore complexes are sufficiently large such that their morphological changes can be visualized in interphase nuclei by immunofluorescence microscopy, thereby providing a unique model system for investigating centromere morphogenesis throughout the cell cycle. Our electron microscopy (EM) observations of the smaller centromeres in the other two mammalian cell lines revealed that the cycle of morphogenesis seen in compound centromeres/prekinetochores of the Indian muntjac may also be common to all mammalian cells.

This study suggests that in all three cell lines, the centromere/prekinetochore has similar and characteristic 3-D organization. The varied structural patterns observed in centromeres in the interphase nucleus are apparently due to a unique and regular cycle of events that occurs during prekinetochore replication and maturation. Such morphogenic activity is accompanied by characteristic patterns of intranuclear movements and spatial repositioning of centromeres within the nucleus. The intranuclear movements result in the clustering of groups of centromeres into specific arrays prior to centromere replication. Our analysis of the ultrastructure of prekinetochores supports our earlier hypothesis that the centromere-kinetochore complex of mammalian chromosomes is composed of multiple repetitive subunits (Zinkowski et al., 1991).

We also investigated prekinetochore structure in highly extracted nuclear matrix core filament preparations (He et al., 1990) and found that prekinetochores and some associated CENPs persist after salt extraction, retaining their spatial arrangements within the nucleus.

Although the term prekinetochore is widely used in the literature, it is now known that CREST antigens are mostly located in centromeric heterochromatin subjacent to the kinetochore. It is, therefore, questionable as to whether some or all of the CREST-positive regions in the nucleus actually function as presumptive kinetochores. With this caveat, we use the terms *centromere* and *prekinetochore* interchangeably throughout this report to identify CREST-positive regions in the interphase nucleus.

MATERIALS AND METHODS

Cell culture and antiserum

HeLa cells were grown in DMEM supplemented with 10% FBS. Indian muntjac (*Muntiacus muntjac vaginalis*) cells were obtained from the American Type Culture Collection and Chinese muntjac (*Muntiacus muntjac reevesi*) were kindly provided by R. Liu, at the Zoology Institute, Kunmin, China. Muntjac cells were grown in Ham's F-10 medium supplemented with 15% FBS. CREST scleroderma anti-centromere serum S. H. was obtained from the Comprehensive Arthritis Center at the University of Alabama at Birmingham and has been characterized elsewhere (Brinkley et al., 1988). In order to arrest some cells at the G_1/S-phase boundary of the cell cycle, exponentially growing cultures of Chinese and Indian muntjac cells were

grown in culture medium containing hydroxyurea (Sigma Chemicals) at a concentration of 2 mM for 20 hours (Zinkowski et al., 1991).

Immunofluorescence

Extracted or non-treated cells were fixed on the coverslips with 3% paraformaldehyde in PBS for 30 minutes. After three PBS washes and blocking in 1% BSA, the coverslips were incubated with CREST antiserum (1:1,000) and FITC-conjugated goat anti-human antibody (1:40), sequentially. Subsequently, samples were counterstained with either DAPI or propidium iodide (PI) and mounted in antifade mounting medium. Cells were examined using a Zeiss Axiophot fluorescence microscope, and images were obtained using a high-resolution/high sensitivity three chip CCD video camera (Hamamatsu) operated with a Pentium workstation running Optimas 6.0 and Adobe Photoshop software. High resolution imaging of cells prepared for double-label immunofluorescence was obtained by collecting a Z-series of optical sections (0.3 μm steps) using a Molecular Dynamics (Sunnyvale, CA) Multi Probe 2001 laser scanning confocal microscope. Imagespace software (Molecular Dynamics) was used to perform volume rendering of the Z-series in order to project 3-D images of centromeres within nuclei. Spatial arrays of centromeres were viewed and analyzed in tilted and rotated 3-D projections.

BrdU labeling

In order to identify cells in S-phase and other stages of the cell cycle, BrdU (bromodeoxyuridine; Sigma Chemicals) was added to cell culture medium at a concentration of 10^{-5} M. For pulse-label experiments, the cells were incubated in BrdU medium for 8 minutes and then transferred to fresh medium lacking BrdU. For continuous labeling, some cells were exposed to BrdU for periods of up to 5 hours. Cells were fixed in 3% formaldehyde, 2% Triton X-100, in PBS for 30 minutes, followed by treatment with 2.5 N HCl at room temperature for 30 minutes to denature the DNA. For double labeling with BrdU and CREST to detect both DNA synthesis and centromeres, cells were incubated with a mixture of first antibodies including CREST antiserum (1:1,000) and monoclonal anti-BrdU antibody (Sigma Chemicals, 1:1,000) for 30 minutes. Subsequently, the samples were rinsed and incubated with a mixture of second antibodies including FITC-conjugated anti-human and Texas red- or Rhodamine-conjugated anti-mouse antibodies.

Nuclear matrix/core filament preparation

The nuclear matrix core filaments were prepared as previously described (He et al., 1990) by extracting cell monolayers in cytoskeleton (CSK) buffer (10 mM Pipes, pH 6.8, 100 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$, 1 mM EGTA, 4 mM vanadyl riboside complex, 1.2 mM PMSF, and 0.5% Triton X-100). Subsequently, the samples were then treated with 100 μg/ml RNase-free DNase I (Boehringer-Mannheim Biochemicals) at room temperature for 30 minutes in CSK buffer. Ammonium sulfate was added from a 1 M stock solution to a final concentration of 0.25 M. After 3 minutes at room temperature, samples were rinsed and transferred into CSK buffer and NaCl was added dropwise from a 4 M stock in CSK buffer to achieve a final concentration of 2 M. In order to preserve core filaments, very careful and gentle handling is critical during these procedures. The preparations were then fixed, processed for immunofluorescence or immunogold for examination in the light and electron microscope as described for non-extracted cells.

Immunogold labeling for light and electron microscopy on the same sample

In order to observe some samples first by light microscopy (LM) and then by EM, cells were either grown on or cytocentrifuged onto plastic coverslips and fixed with 3.7% formaldehyde plus 0.1% glutaraldehyde for 30 minutes at 4°C. After incubation with 1 mg/ml NaBH₄, the cells were blocked with gelatin/BSA. The cells were then incubated with CREST autoantiserum (1:1,000) at 37°C for 30 minutes and then

with 1 nm gold-conjugated anti-human antibody (AuroProbe One, 1:50 dilution) or 1.4 nm gold-conjugated anti-human Fab′ fragment (Nanogold, Inc., 1:50 dilution) at room temperature for 3 hours. The cells were then post-fixed with 3% glutaraldehyde. Silver enhancement was carried out for 3.5 minutes with a HQ silver enhancement kit (Nanogold, Inc.). In some experiments, 5 nm gold bead-conjugated antibody was used without silver enhancement. The samples were then dehydrated and in situ embedded with Spurr's resin. The stained samples were then observed under brightfield optics, and selected regions were marked, cut out of the plastic, mounted onto a blank block and sectioned on a Richart Ultramicrotome. The sections were collected on 200 mesh copper grids, post-stained in alcoholic uranyl acetate. The samples were observed and micrographs collected using a JEOL 100CX transmission EM operated at 75 kV.

Embedding-free immunogold-EM of the nuclear matrix

Core filament preparations involving cells grown and processed on plastic coverslips were further processed for immunogold staining with CREST antiserum and 10 nm gold-conjugated secondary antibody, as described for non-extracted samples. After post fixation with 3% glutaraldehyde, samples were dehydrated in a graded series of ethanol to absolute ethanol, and then transferred to absolute butanol for two changes. Coverslips were then immersed in liquefied dithiodiglycol (DGD) at 60°C for 3 hours. After DGD was allowed to solidify at room temperature, the flat embedded sample was examined by LM and, in some cases, photographed prior to sectioning. The desired regions were then marked, cut out and mounted on a blank block, and sections at a thickness of about 500-2,000 nm were cut as described above. Critical point drying was carried out after removal of DGD with butanol. No additional staining was needed for EM observation.

RESULTS

Spatial movement and distribution of centromeres within the nucleus

When interphase nuclei of cultured mammalian cells are stained with CREST anti-centromere autoimmune serum, numerous fluorescent spots can be seen throughout the nucleus representing prekinetochores. These may appear as single or double spots depending on the stage of the cell cycle.

The most striking arrays of prekinetochores seen within the nucleus were those of muntjac deer cells, especially the Indian muntjac. As described previously (Brinkley et al., 1984), the mitotic chromosomes of the Indian muntjac are large and have a low diploid number (2N=6♀, 7♂). The cell line used in this study carries a prominent Xp-3q translocation, resulting in centromeres with a long segmented neck. Due to its size and shape, the centromere of the X-3 chromosome can often be distinguished from other centromeres within the interphase nucleus when stained with the CREST antisera (see Fig. 2A,E). Collectively, the nuclei of Indian muntjac cells display a varied and complex array of CREST-stained centromeres as shown in Figs 1A to H. In order to identify specific stages of the cell cycle, CREST anti-centromere antiserum was combined with BrdU/anti-BrdU antibodies to correlate centromere staining patterns with stages of the cell cycle. Using a pulse label it was possible to identify the centromere staining pattern of only those cells in S-phase. Continuous labeling for 5 hours enabled us to identify cells in S- and G_2-phase, while cells in G_1-phase were expected to be unlabeled under these conditions.

At telophase and early G_1, 7 single fluorescent dots can be seen near the center of the nucleus in each daughter cell (Fig.

1A-B). Nuclei with either a series of 7 discrete single dots or 7 enlarged dots with short or elongated fluorescent tails extending from each dot (Fig. 1C) were determined, by the above criteria, to also be in G_1-phase.

A third pattern, also found in nuclei preceding S-phase, consisted of elongated fluorescent threads extending in a line almost all the way across the nucleus (Fig. 1D,E; see also Fig. 3A). Improved resolution at the LM level, afforded by using small 1.4 nm immunogold beads and cleaved Fab′ CREST fragments, enabled us to resolve the prekinetochore cycle in Indian muntjac nuclei in much greater detail (Fig. 2A-E). As the prekinetochores elongate, it is possible to observe a series of densely stained bead-like particles arranged along the highly extended prekinetochores (see arrows, Fig. 2C). In what may be a more advanced stage, the thin filaments appear to fragment into numerous punctate dots clustered throughout the nucleus (Fig. 1F). BrdU/anti-BrdU labeling (see Fig. 4A-D) in combination with CREST immunofluorescence indicated that highly extended centromeres (Figs 1D,E and 3B) first appeared in G_1 and persisted until early S-phase of the cell cycle. An apparent intermediate stage is shown in Fig. 2D, where the duplicated strands were not completely re-condensed and clearly appear to be double. In one centromere, one member of the paired strands is considerably longer than its sister strand (double arrows) and displays an array of small beads all along its length. Point-to-point pairing of beads is apparent only at one end of the strand (Fig. 2D). This staining pattern suggests that asymmetry occurs in the deposition of proteins to sister strands during replication, with CENPs being added preferentially to the end of one sister strand.

In smaller, post-replication nuclei, 7 double fluorescent spots were seen representing the fully duplicated and re-condensed prekinetochores (Fig. 1G). A striking comparison of the prekinetochores in G_1 vs G_2 nuclei can be seen in Fig. 1B and G, respectively. At the G_2-prophase stage, the CREST staining pattern is very similar to that seen on metaphase chromosomes.

Having established prekinetochore dynamics in nuclei of Indian muntjac cells, we next sought to compare them with the Chinese muntjac. The Chinese muntjac cell line used in this study contained a diploid number of 46 small acrocentric chromosomes. As in the Indian muntjac cells, the prekinetochores of the Chinese muntjac are arranged into a variety of patterns during interphase as shown in Fig. 1I to P.

In telophase and early G_1 nuclei, approximately 46 small, single fluorescent spots were seen arranged in the center of each nucleus (Fig. 1I,J). In a second pattern, the prekinetochores appear to fuse into clusters of varying sizes along the periphery of the nucleus (Fig. 1K,L). In some nuclei, the prekinetochore clusters appear to be transformed into linear arrays formed by the tandem association of prekinetochores extending from the nuclear lamina (Fig. 1M). At this stage, they resemble the extended beads-on-a-string configuration seen in nuclei of Indian muntjac cells (compare Fig. 1M with E or 2C). Like in the Indian muntjac, BrdU labeling experiments (Fig. 4C,D) indicated that nuclei such as shown in Fig. 1M and earlier, are in the G_1-phase of the cell cycle. Some Chinese muntjac nuclei display fluorescent dots arranged in elaborate loops or circles (Fig. 1N), somewhat similar to the arrangements seen in some nuclei of the Indian muntjac (compare Fig. 1N with F). In some nuclei, the linear prekinetochore arrays disperse forming
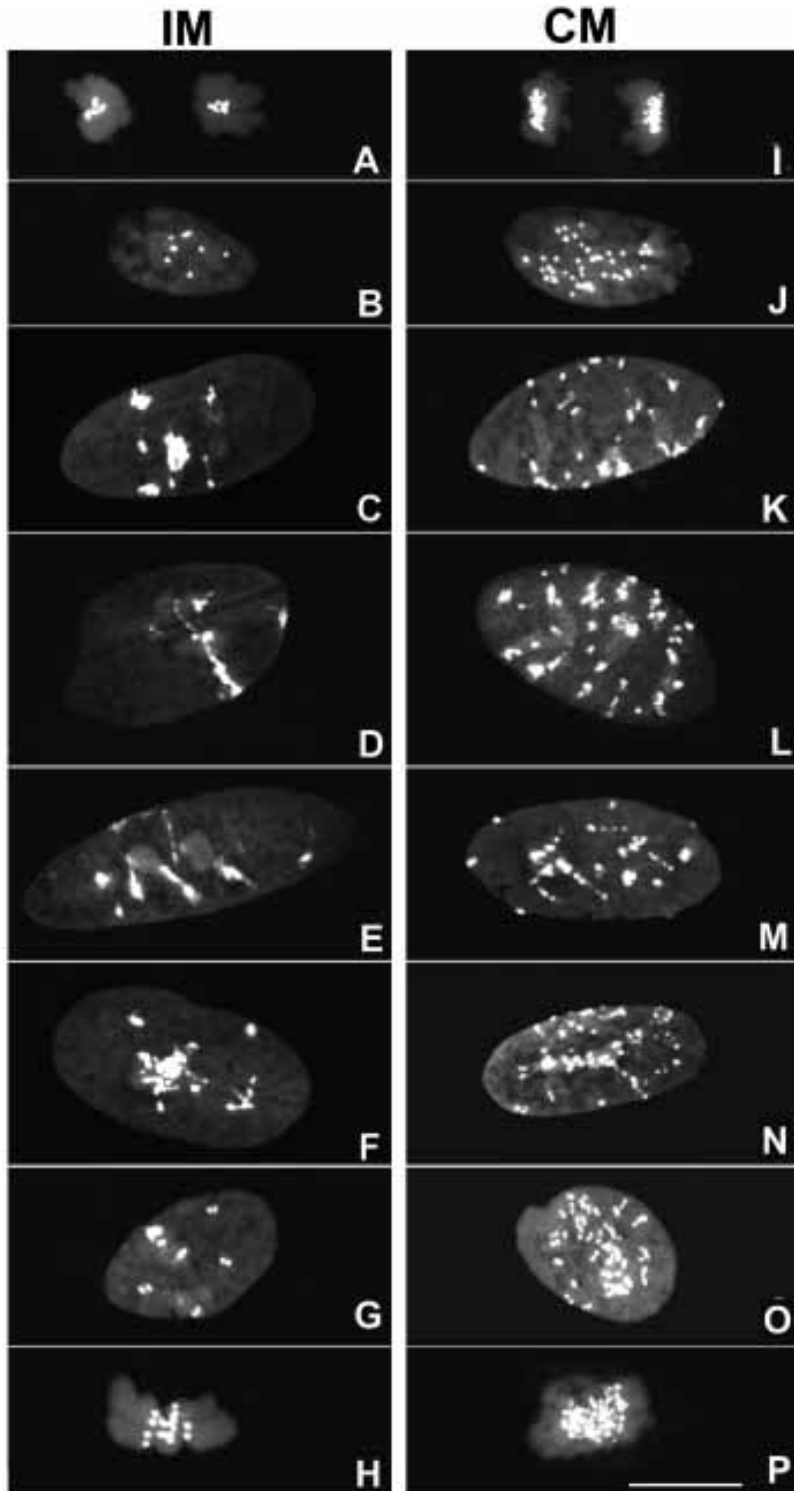
Fig. 1. Confocal microscope images showing prekinetochore arrays in nuclei of Indian muntjac (A-H) and Chinese muntjac (I-P). During early G₁-phase (B), Indian muntjac (IM) nuclei display 7 distinct prekinetochores. Later (C), each prekinetochore swells and extends out finger-like projections that continue to elongate as straight lines (D,E). Note that the volume of nuclei have increased considerably in these stages. In what is believed to be an even later stage (F) the finger-like projections appear to fragment into numerous fluorescent dots near the center of the nucleus. Subsequently, each fluorescent dot appears double (G), indicating a nucleus in G₂-phase following centromere duplication. Chromosomes aligned on the metaphase plate are shown in H̲. A somewhat similar pattern of centromere movements is seen in the nuclei of Chinese muntjac (CM) cells. During early G₁ (J) 46 single spots are seen throughout the nucleus. Later (J,K) the fluorescent dots cluster into aggregates near the nuclear lamina forming multiple linear arrays (M). At this stage, the prekinetochore arrays are similar to those seen at the same stage in Indian muntjac nuclei (compare E and M). The prekinetochore arrays continue to aggregate forming large looped arrays (N) and are finally transformed into double dots (O) representing fully duplicated prekinetochores. Bar, 10 μm.

numerous double spots representing fully duplicated prekineto-chores scattered throughout the nucleus (Fig. 1O). As reported earlier (Brenner et al., 1981), these cells were either in late S- or G₂-phase. At metaphase the double spots are aligned on the metaphase plate as shown in Fig. 1P.

## Centromere patterns in nuclei of cells arrested in G₁/S-phase

In order to further confirm that centromere patterns seen in

interphase nuclei of Indian muntjac cells related to stages of the cell cycle, we examined CREST-stained nuclei of Indian and Chinese muntjac cells that had been arrested at the beginning of S-phase after treatment with hydroxyurea (HU) for 20 hours, as described elsewhere (Brinkley et al., 1988). The nuclei of HU-treated cells displayed most of the prekinet-ochore arrays seen in the control nuclei except stages where each fluorescent spot was clearly double. The majority of the nuclei in HU-arrested cells displayed prekinetochores that
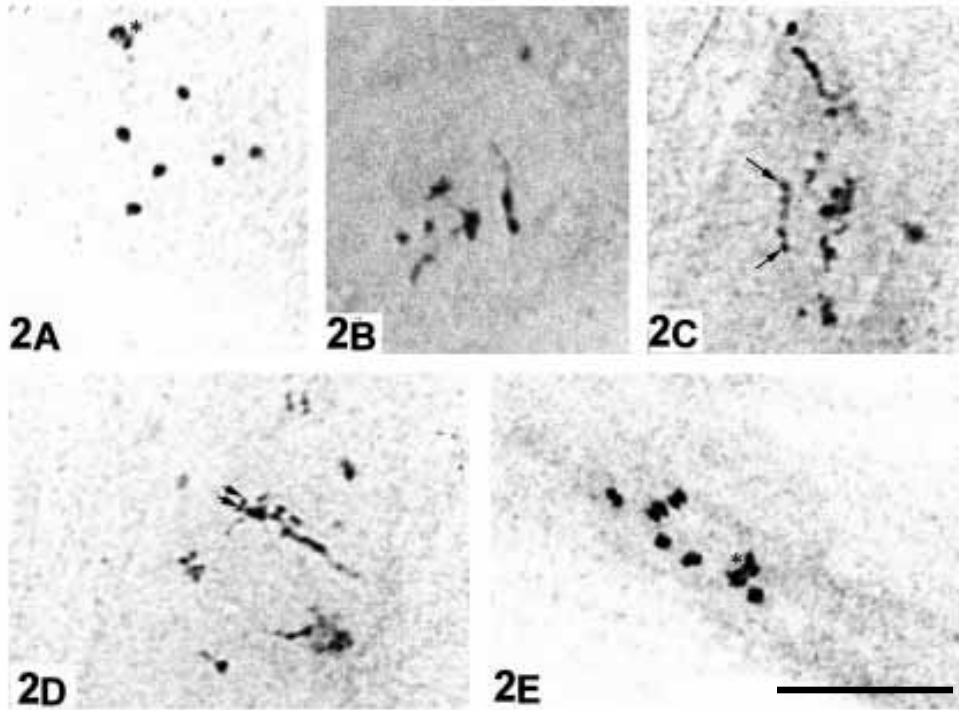
Fig. 2. CREST anti-centromere antibody, followed by gold-tagged Fab′ fragment as the second antibody was used to stain Indian muntjac nuclei at various stages of the cell cycle. (A) nucleus from a cell in early G₁, note the segmented prekinetochore (asterisk) of the X-3 chromosome. In a later stage, each of the 7 prekinetochores begin to elongate (B) and eventually show a beads-on-a-string staining pattern (arrows, C). During an apparent intermediate stage of duplication, extended strands appear to be paired, with one member of the pair considerably longer than its sister strand (arrowheads, D). Following duplication, each of the prekinetochores have doubled (E). Note the large segmented prekinetochore of the X-3 chromosome (asterisk, E). Bar, 10 μm.
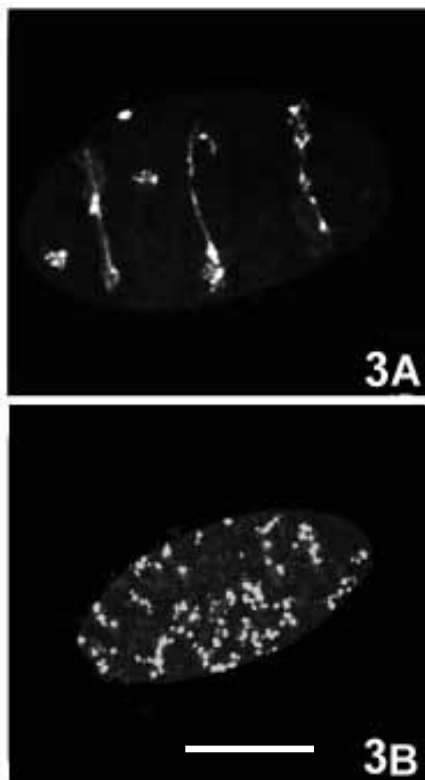
Fig. 3. Confocal images of prekinetochore arrays in nuclei of cells treated with HU to arrest growth at the beginning of S-phase. These arrays represented the most frequently observed patterns seen in the arrested cell population suggesting that considerable unfolding and elongation of centromeric heterochromatin occurs prior to or at the beginning of S-phase in Indian muntjac cells (A). The centromeres of Chinese muntjac (B) nuclei cluster or align into linear arrays prior to or at the beginning of S-phase. Bar, 10 μm.
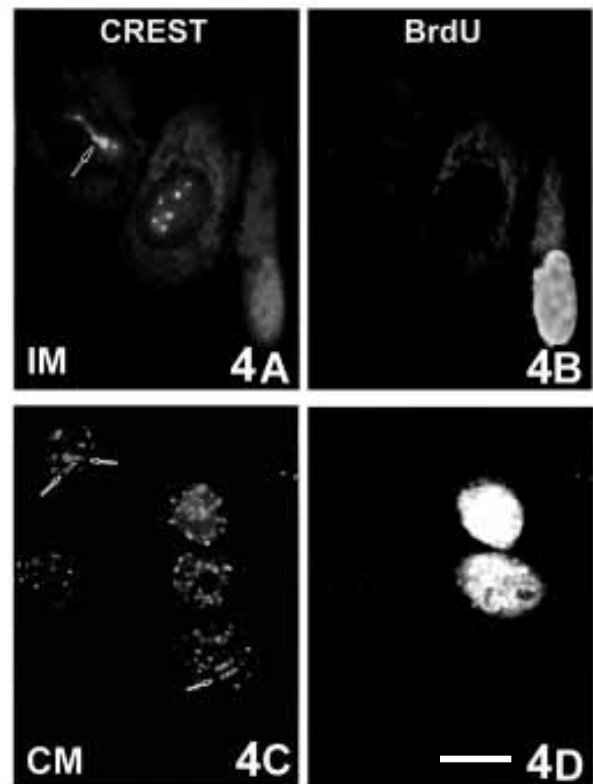
Fig. 4. Conventional immunofluorescence images of Indian muntjac (top panel) and Chinese muntjac (bottom panel) exposed to BrdU for 5 hours continuously, then double stained with CREST autoserum (left column) and anti-BrdU antibody (right panel). Although nuclei in S and G₂ cells were intensely stained with BrdU antibody, nuclei in G₁ remained unlabeled. The extended and clustered arrays of prekinetochores (arrows) were seen in BrdU-negative nuclei indicating that this configuration organized prior to S-phase. Bar, 10 μm.
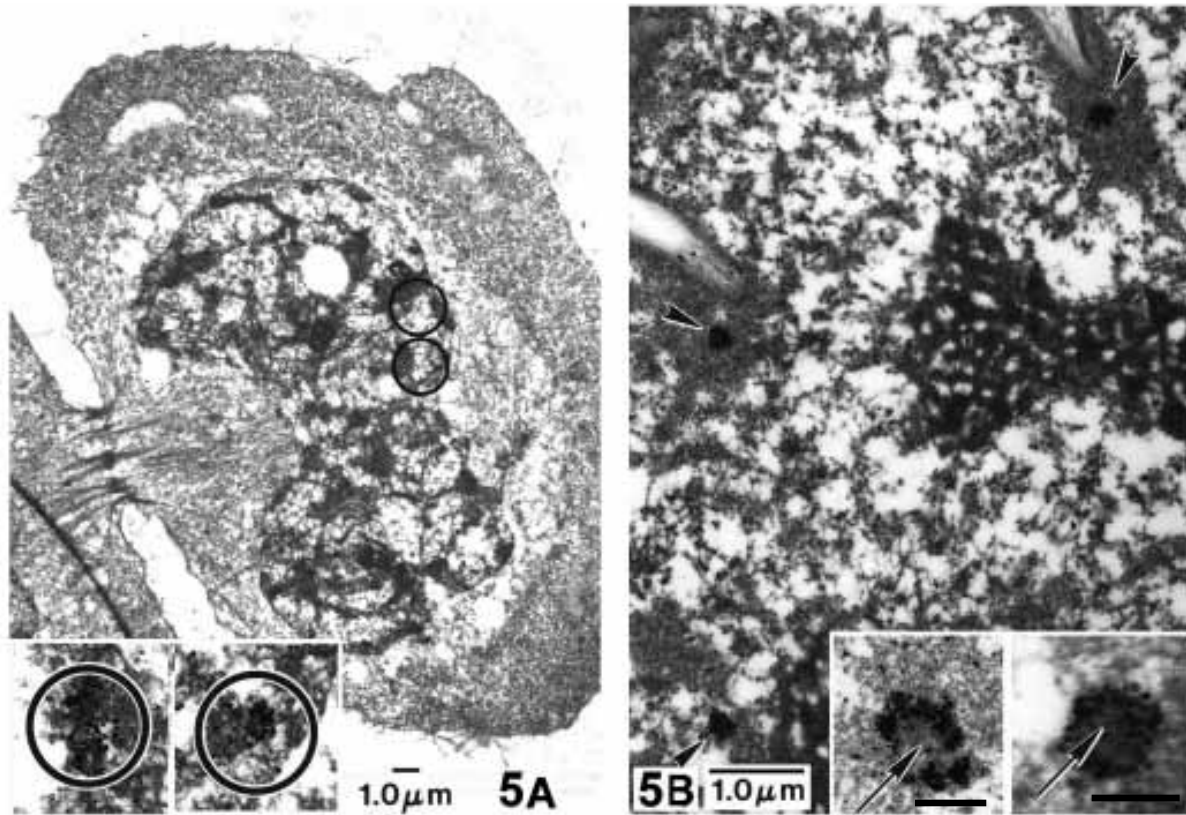
Fig. 5. Electron micrographs of immunogold labeled late telophase/early G_1 cells (A) of the Chinese muntjac showing midbody and prekinetochores (rings). Higher magnifications are shown in the two insets. In B, labeled small round foci appear to be embedded in blocks of dense heterochromatin (arrowheads). Each of the prekinetochores at this stage display a dense outer ring of gold label with a central unlabeled core (arrows, insets). Bars: 1 µm (insets, 0.5 µm).

were in a greatly extended form (Fig. 3A). Since these cells are arrested in early S-phase, the initial unfolding and elongation of the kinetochores of the Indian muntjac occurring in G_1 may be a precondition for the replication of centromeric heterochromatin in these large chromosomes. Fig. 3B shows nuclei from Chinese muntjac cells blocked with HU showing that centromeres are tandemly arranged in linear arrays.

We also observed various arrays of prekinetochores in the nuclei of HeLa cells (data not shown). Since prekinetochore arrays in human nuclei have been described in previous publications (Bartholdi, 1991; Ochs and Press, 1992), these sources should be consulted for details.

Ultrastructure of prekinetochores

Having defined morphological changes of prekinetochores at the light microscopic level, comparable stages were examined by electron microscopy using cleaved Fab′ immunogold-tagged fragments as the second antibody to detect CREST anti-centromere antibody.

Fig. 5A shows a thin-section cut through a Chinese muntjac cell at late telophase/early G_1 nucleus with the midbody still attached and where the chromosomes were not fully decondensed. Two gold-labeled foci representing CREST-positive sites can be seen at low magnification (circles, Fig. 5A), but otherwise, no discernible morphological features exist to indicate that these sites represent anything other than patches of heterochromatin. At higher magnification, they showed a completely different pattern from centromeres on mitotic chromo-

somes (insets, Fig. 5A). Some labeled prekinetochores appear either as dispersed or spherical bodies (Fig. 5B), with the CREST immunogold concentrated within condensed heterochromatin in the outer cortex of the nucleus. In other G_1 nuclei, the CREST label was localized in discrete foci approximately 0.5 µm in diameter with most of the label confined to an outer ring surrounding an unlabeled dense core (Fig. 5B, insets). The central unlabeled core (arrows, Fig. 5B, insets) was consistently seen in G_1 nuclei in all of the mammalian cells studied in this report and was either due to the absence of CREST antigens in this area or to lack of antibody penetration.

Most CREST-labeled sites were nestled within a larger patch of heterochromatin in which the gold-labeled 25-30 nm fibers of the prekinetochores appeared almost identical to the surrounding heterochromatin fibers. Greatly extended prekinetochores were observed by EM in nuclei of Indian muntjac cells (Fig. 6A,B). These appeared to correspond to a similar form seen at the light microscopic level (compare with Fig. 1D,E). As described earlier, prekinetochores at this stage swell and develop thin finger-like projections up to 20 µm in length. Continuous and discontinuous arrays of gold-labeled fibers were distributed along the fingers of heterochromatin, extending out from a central core located at the base of the prekinetochore near the nuclear lamina (Fig. 6B).

Examination of prekinetochore arrays in the nuclei of the Chinese muntjac revealed a similar beads-on-a-string arrangement of prekinetochores as shown in Fig. 6C. It is remarkable that individual centromeres of Chinese muntjac chromosomes,
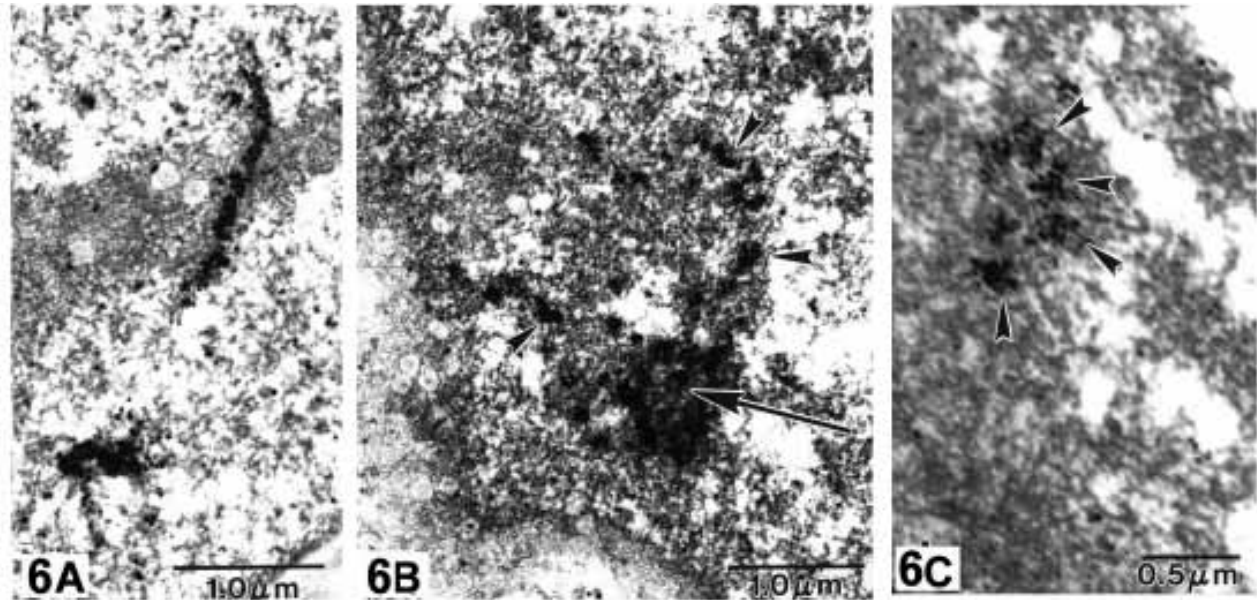
Fig. 6. Immunogold labeled prekinetochores of Indian muntjac (A,B) and Chinese muntjac (C) at various stages of unfolding, elongation and replication. Long finger-like projections extending from a central core (arrow, B) break up into a linear series of densely labeled beads (arrowheads, B,C) embedded in surrounding heterochromatin. The arrowheads in C point to individual subunits of a single, slightly unfolded prekinetochore in the nucleus of a Chinese muntjac cell. See Fig. 1K for comparable image of the centromere at this stage. Bars: A,B, 1 μm; C, 0.5 μm.
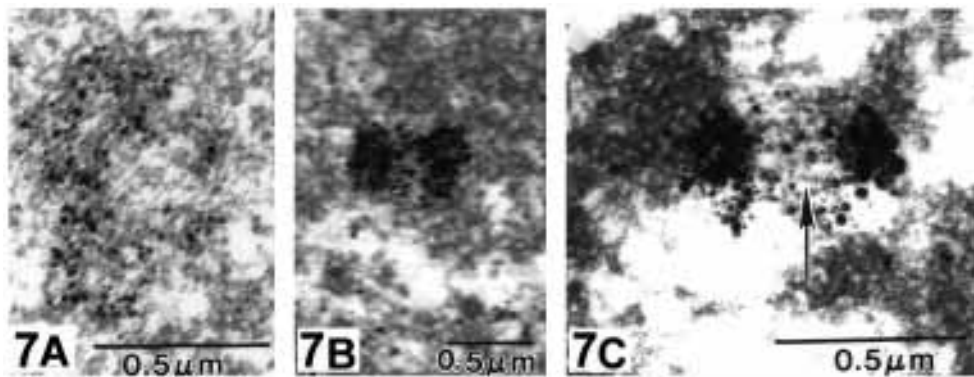
Fig. 7. Just prior to or during duplication, prekinetochores display a loose arrangement of labeled fibers (A). Fully duplicated prekinetochores (B) display labeled thin filaments extending across the interzone connecting the two sister halves (arrow, C). Bar, 0.5 μm.

although much smaller than those of the Indian muntjac, display the same beaded pattern. Although individual beads seen by EM were the same size in the two species, the beads in Indian muntjac centromeres numbered 30 or more in a single section, while only 10-15 were seen in the centromeres of Chinese muntjac. Therefore, the individual bead seen by EM probably represents the basic subunit in the centromere. As would be expected, therefore, the small centromeres in Chinese muntjac nuclei, seen by immunofluorescence, contain fewer subunits than the larger centromeres seen in nuclei of the Indian muntjac, but the subunits are approximately equal in size.

In what appears to be a slightly advanced stage, the beads-on-a-string configuration condenses into a loose mass of fibers as shown in Fig. 7A. These loosened fibers eventually condense into a tightly packed mass that is clearly bipartite (Fig. 7B). The two densely-labeled masses, each about 0.5 μm, in diameter, are surrounded by heterochromatin. In some sections, the region between the duplicated prekinetochores was connected by thin,

sparsely-labeled fibers (arrow, Fig. 7C). Examination of serial sections cut through the doublet prekinetochores often revealed a small unlabeled core in the center of each member of the doublet, much like that seen in G_1 prekinetochores. Thus, the doublet seen by EM immunogold in G_2 nuclei appears to represent a morphological duplicate of the singlet prekineto-chore seen in the G_1 nucleus.

As the nuclei entered early prophase, pairs of densely-labeled sister kinetochores were observed within condensed patches of heterochromatin (Fig. 8A,B). The unlabeled cores were no longer observed and the sister perkinetochores were positioned about 0.8 μm apart. The prophase centromeres and surrounding heterochromatin appear to be among the first sites along the chromosome to become fully condensed, suggesting that the prophase centromere may be a principal site for initi-ating chromosome condensation. Indeed, as prophase continued, chromosome condensation appeared to advance outward from the dense centromeres to include the arms.

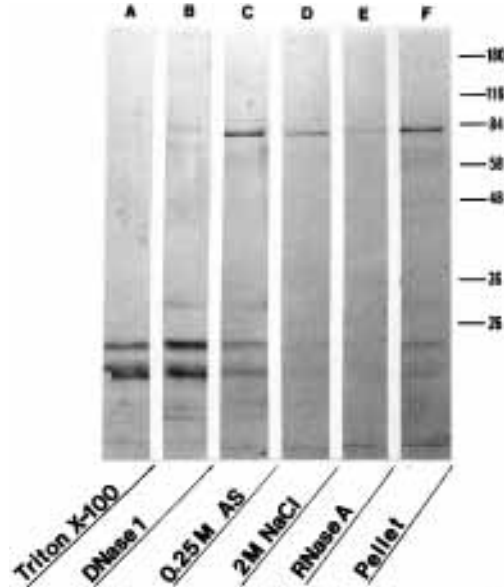At early prophase, each sister prekinetochore was composed
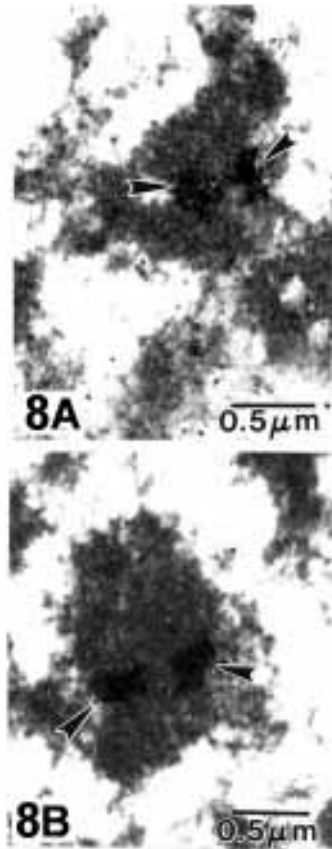
Fig. 8. Early stages of condensation at the centromere of two prophase chromosomes. The two sister kinetochores can be seen on each face of the chromatid separated by an unlabeled central domain (arrowheads, A,B). The outer kinetochore plates have not yet formed at this stage. Bar, 0.5 μm.

Fig. 9. Western blot using CREST antibodies to detect CENP-A (17 kDa) band and CENP-B (80 kDa) in various washes from nuclear extracts. CENP-A (lanes A,B,C), a histone-like protein, is extracted in 2 M NaCl, while CENP-B (lanes C-F) persists after salt extraction and remains in the nuclear matrix pellet.

of a dense spherical mass of gold-labeled fibers positioned on opposite sides of the centromere (Fig. 8). Except for the conspicuous absence of kinetochore plates, these paired structures resembled those seen on mitotic chromosomes. The prekinetochores were often positioned near the nuclear lamina throughout late G2/early prophase.

Prekinetochores and the nuclear matrix

When muntjac cells were treated with Triton X-100, DNase I, 0.25 M ammonium sulfate and 2 M NaCl to produce a nuclear matrix and core filament fraction, only a small subset (5%) of the cells' total proteins remained in the fraction. Fig. 9 shows western blots of various extracts obtained during the preparation of the nuclear matrix. Most of the CENP-A was released initially by extraction simply with detergent, or with DNase I plus salt while a small amount remained in the final pellet. In contrast, CENP-B (apparent molecular mass, 80 kDa) completely resisted detergent extraction and was differentially extracted by salt; about 50% remained in the pellet suggesting that this portion is a stable component of the nuclear matrix. It should be noted that CENP-C, also a differentially stable component in the nuclear matrix of HeLa cells (He et al., 1995), was not detected in the muntjac cells by the CREST antisera used in this study.

Examination of the extracted nuclei with CREST antibodies revealed prekinetochores arranged in arrays very much like those seen in intact nuclei (Fig. 10A-D and F). In order to show that DNA was largely extracted in the nuclear matrix preparations, a DAPI stained nucleus of an unextracted cell is shown in Fig. 10E (inset) and in Fig. 10F (inset) after extraction. Thus, removal of chromatin and other soluble nuclear components has little effect on the anchorage of prekinetochores to the nuclear matrix.

When resinless sections were examined by EM after labeling with CREST immunogold labeled antibodies, it was possible to detect individual prekinetochores arranged in a 3-D anastomosing array of fibers identical to core filaments (Fig. 10G). Each prekinetochore consisted of a discrete amorphous mass of material decorated with CREST immunogold label. When compared to prekinetochores seen by EM in unextracted nuclei, the extracted structures were less fibrous and resembled a particulate mass of material surrounded by core filaments. Otherwise, the extracted prekinetochores retained approximately the same shape, size and nuclear localization as those found in normal intact nuclei.

DISCUSSION

The centromere cycle

Although large heterochromatin blocks are visible throughout the nucleus the centromere, per se, is not discernible by conventional light and electron microscopic procedures. It was not until the discovery of anti-centromere autoantibodies in the serum of patients with the CREST variety of scleroderma (Moroi et al., 1980) that centromeres could be detected in the nucleus. Using indirect immunofluorescence staining with the
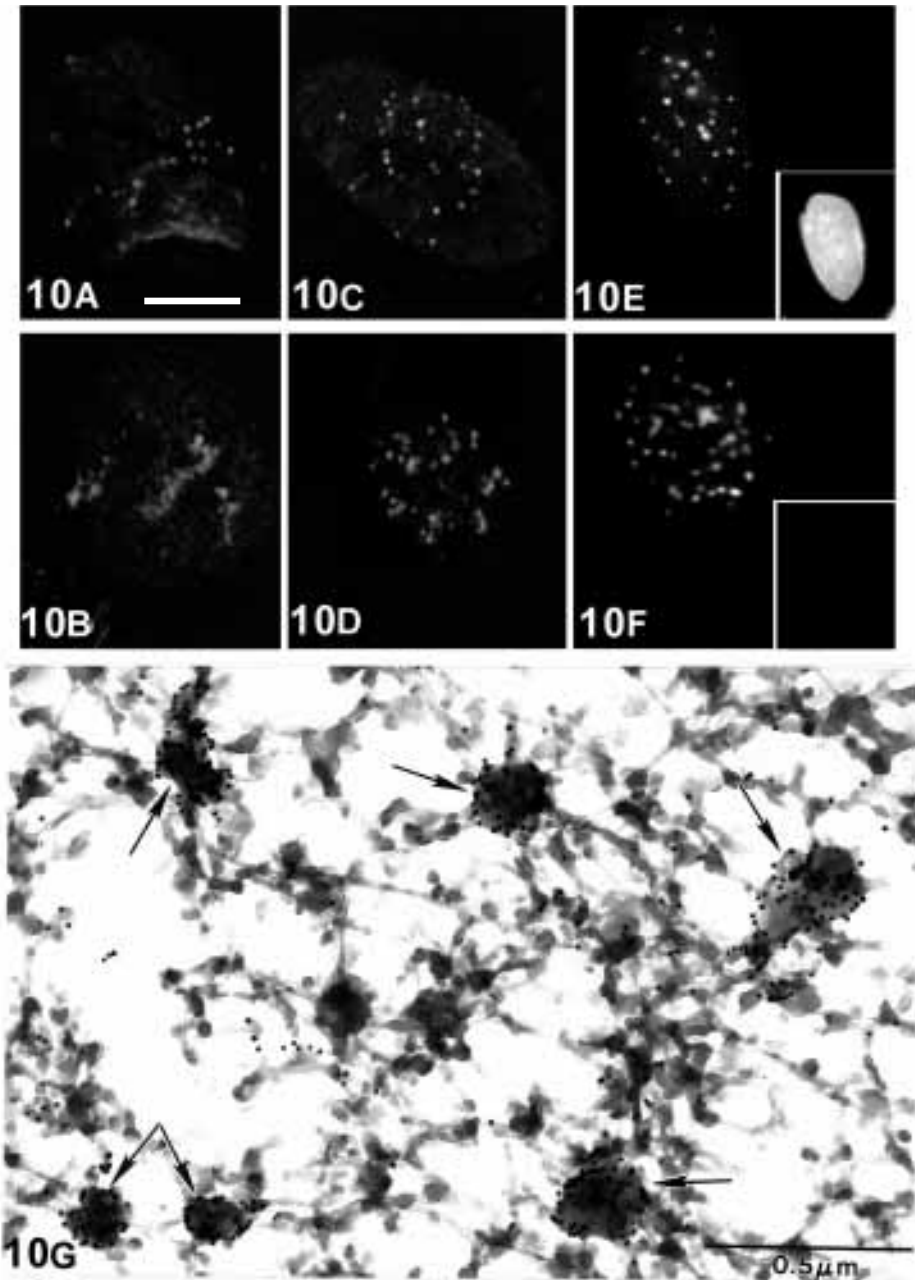
Fig. 10. The prekinetochores persist in nuclei of Indian muntjac (A,B) and Chinese muntjac (C,D) shown by immunfluorescence after extraction of chromatin leaving nuclear matrix. Unextracted and extracted cells are shown in E and F, respectively. DAPI staining (E, inset) shows that most of the DNA was removed after extraction (F, inset). The electron micrograph of a resinless-embedded nucleus shows immunogold labeled prekinetochores (arrows) associated with an anastomosing array of core filaments (G). Bars: A-F, 10 μm; G, 0.5 μm.

CREST antisera, Brenner et al. (1981) determined that centromeres appeared as single fluorescent spots in G_1 nuclei but became double after replication. These investigators concluded that the interphase spots were presumptive kinetochores and coined the term *prekinetochore* to properly identify them. As mentioned earlier in this report, the term prekinetochore may be inappropriate because most CREST antisera stain a region of centromeric heterochromatin that is subjacent to the kinetochore (Cooke et al., 1990). Whether all or part of the CREST-positive foci in the interphase nucleus actually qualifies as a presumptive kinetochore remains unknown. Given this caveat, we know that prekinetochores are not randomly distributed within the nuclei of mammalian cells but are often arranged in groups or clusters and may display considerable movement and rearrangement during interphase (Brinkley et al., 1984, 1986). In muntjac cells for example, prekinetochores are dispersed throughout the nucleus in early G_1 but form into tandem pairs and linear arrays later in the cell cycle (Brinkley et al., 1984). During the maturation of mouse spermatids, prekinetochores of nonhomologous chromosomes were clustered into distinct chromocenters (Brinkley et al., 1986). Zalensky et al. (1993) identified linear arrays of prekinetochores in mature human sperm. Nonrandom arrays of satellite DNA sequences have also been reported in nuclei of mouse neurons (Manuelidis, 1985). Centromere autoantigens associate with the nucleolus in a variety of mammalian cells in vitro (Ochs and Press, 1992) and interact with nucleoli and the nuclear envelope of dorsal root ganglion neurons (Park and De Boni, 1992). In human diploid fibroblasts, prekinetochores fuse into chromocenters and form patterns of rings and lines during S-phase (Bartholdi, 1991). Such rearrangements suggest that centromeres are not permanently anchored to a specific region of the nucleus but

may undergo considerable movement and redistribution throughout the interphase cycle.

Collectively, these studies indicate that centromeres retain properties of motility even within the nucleus. In the present study, we used confocal microscopy and immunogold EM to fully document structural changes and spatial distribution of prekinetochores within the nuclei of three species of mammalian cells. All species displayed prekinetochores that assumed striking morphological and spatial forms throughout the cell cycle; but by far, the most dynamic cycle of prekinetochore movement and morphogenesis was seen in muntjac cells. Muntjacs are small Asian deer that display almost identical phenotypes but contain strikingly different chromosome numbers. The large mitotic chromosomes of the Indian muntjac ($2N=6♀,7♂$) contain 'compound kinetochores' composed of a tandem array of smaller repetitive subunits (Brinkley et al., 1984). Chinese muntjacs, believed to be evolutionary progenitors of all other muntjac species, have chromosomes that are much smaller and more numerous ($2N=46$) and display a pair of centromeres at the end of each telocentric chromosome. Although the chromosomal evolution of the muntjacs has been discussed elsewhere (Wurster and Atkin, 1972; Matthey, 1973; Shi et al., 1980), our results with confocal microscopy lend considerable support to the argument that the evolution of the compound kinetochore may have been influenced by the spatial arrangements of prekinetochores within the nuclei of ancestral species. Compound kinetochores may have evolved through multiple centric fusions facilitated by the spatial juxtaposition of clustered prekinetochores (Brinkley et al., 1984; Lin et al., 1991).

The movement and redistribution of prekinetochores in interphase likely reflects nuclear metabolism and dynamic structural activity occurring within the genome. Centromeric DNA replication takes place in late S-phase (O'Keefe et al., 1992), and the movement of prekinetochores to specific locations within the nucleus may be required to accommodate the replication machinery anchored to the nuclear matrix as proposed for DNA replication in general (Hozak et al., 1993, 1994; Nakayasu and Berezney, 1989). If so, our observations that prekinetochores achieve nonrandom patterns even in nuclei arrested at the G_1/S-phase boundary of the cell cycle with hydroxyurea, argue that movement and unfolding precedes centromeric DNA synthesis and the duplication process.

Prekinetochore ultrastructure before and after duplication

Our confocal microscopy and immunogold EM observations have provided insight into the dynamic structural changes that occur within the prekinetochore before and after duplication as summarized in the illustration shown in Fig. 11. In early G_1, the prekinetochore is a single condensed spherical body approximately 300-600 nm in diameter embedded in a patch of heterochromatin. At this stage, the CREST antigens are segregated within a narrow ring or 'doughnut' surrounding an unstained central core. This CREST-positive doughnut stage appears to be transformed by unfolding into an extended strand in which CREST-positive 'beads' are arranged in tandem along the centromeric strand. The clear demonstration of these uniformly sized beads strongly suggested a structurally defined subunit within the muntjac prekinetochores because: (1) they
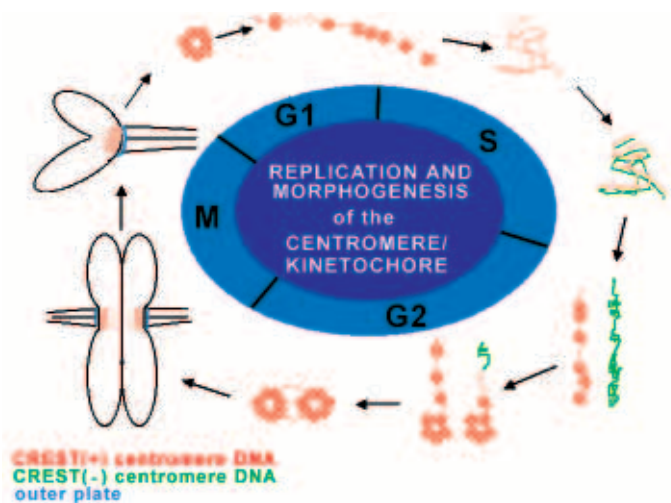
Fig. 11. Diagram derived from the EM and immunofluorescence observations, showing the fate of kinetochores and centromere/prekinetochores during various stages of morphogenesis and replication in the cell cycle. During M-phase, the centromere-kinetochore complex is organized on the chromosomes at the primary constriction with a kinetochore plate (blue) that attaches to microtubules. In early G_1, the kinetochore plates disappear and the centromere is folded into a condensed structure with CREST labeling at the outer zone (red) surrounding an unlabeled central core. Later in G_1, the condensed centromere and associated centromeric heterochromatin unfolds revealing linear, bead-like organization of the CREST-stained region. The beads-on-a-string arrangement persists well into S-phase where it is transformed into a loose fibrous bundle. Daughter strands (green) form after centromere DNA replication and doubling of the prekinetochore occurs in late S-phase. The CREST antigens remain with one of the parent strands (red beads) while new CREST antigens are deposited along the daughter strand. Upon refolding in late G_2, two condensed structures are formed, each displaying CREST labeling in the outer zone and an unlabeled central core. During prophase, these duplicated prekinetochores further differentiate at the primary constriction and give rise to the kinetochore plates at the time of nuclear envelope dissolution.

were consistently observed during many phases of normal morphogenesis; (2) they were detected in all cell lines; and (3) they could be resolved in prekinetochores in both G_1 and G_2 nuclei. We propose, therefore, that they are the interphase equivalents of the subunit structure previously described in the centromere-kinetochore complex of mitotic chromosomes (Zinkowski et al., 1991). The tandem arrangement of individual prekinetochores forming long strings such as those seen in Chinese muntjac nuclei, may have biased centric fusions and translocations favoring the evolution of the large compound kinetochores such as those seen in the Indian muntjacs (Brinkley et al., 1984). The unfolding of individual centromeric chromatin as described herein would likely facilitate such fusions.

Centromere DNA replication, presumably occurring in late S-phase (O'Keefe et al., 1992), would be facilitated by the maximal unfolding of centromeric chromatin. In this regard, Haaf and Ward (1994) investigated the relationship of α-satellite DNA and centromere replication using a combination of fluorescence in situ hybridization (FISH) and CREST immunofluorescence. These investigators presented evidence that the duplication of centromere proteins as detected by

CREST is 'temporally and/or mechanistically' separated from centromeric DNA replication. Thus, fully replicated α-satellite doublets could be observed with only a single CREST signal suggesting that assembly of a mature centromere requires two independent events: replication of centromeric DNA followed by the assembly of CENPs on the replicated strands.

The present study confirms at both the light and EM levels, that centromeric heterochromatin unfolds into a long 'beads-on-a-string' arrangement prior to and during replication as previously reported (Haaf and Ward, 1994). Moreover, we detected a specific stage at which the centromere gains new CREST antigens. Our images suggest that the CREST antigens remain with one of the parent strands in a conserved arrangement and are added initially to one end of the other newly replicated strand.

Thus our images suggest that CENPs are added to the sister strand beginning at one end of the centromere and advancing, point-to-point, toward the opposite end (see Fig. 2D and our interpretation in Fig. 11). Our EM immunogold images suggest that the assembly of CREST occurs when centromeric chromatin is in a loosened, fully extended state (see Fig. 7A and our interpretation in Fig. 11). As further morphogenesis occurs, the two strands apparently fold and condense forming two compact daughters of approximately equal size. The extension of fine gold labeled filaments between the densely labeled daughter suggests that CENPs may span the inner centromere region immediately after duplication.

Prophase transition

The ultrastructural development of the kinetochore from prophase to metaphase has been documented in earlier studies (Brinkley and Stubblefield, 1970; Roos, 1973; reviewed by Rieder, 1982). During early prophase, CENPs are localized within each half of the duplicated centromeres occupying a dense cluster of heterochromatin. The entire centromere, localized near the nuclear lamina, appears to represent a focal point for the initiation of chromosome condensation in early prophase nuclei of the Chinese muntjac cells. Kinetochore plates are absent in prophase centromeres and appear at about the time the nuclear envelope breaks down.

Relationship to the nuclear matrix

Interphase chromatin is thought to be supported throughout the nucleus by a latticework of insoluble proteins known as the nuclear matrix, of which the 9-13 nm filaments seen by EM in resinless sections represent core filaments of the nucleoskeletal network (He et al., 1990). This nucleoskeleton is thought to maintain 3-D spatial order within the nucleus and provide a scaffold on which DNA replication, RNA synthesis and processing, and nuclear transport takes place (for review see He et al., 1995). Components of the prekinetochore, including CENP-B and CENP-C, are detected by western blotting in solubilized nuclear matrix proteins run on SDS gels (He et al., 1995). When we examined CREST immunogold-labeled nuclei in resinless-section by EM, distinct prekinetochores could be seen within the matrix core filaments. Although highly extracted and free of associated heterochromatin, the prekinetochores retained their approximate normal sizes and shapes appearing as single amorphous structures in G_1 nuclei and double structures in G_2. Since clusters and linear arrays of prekinetochores remain firmly attached to the core filaments in

the highly extracted nuclei, we presume they are, indeed, firmly anchored to the matrix. The retention of considerable order within the arrays of prekinetochores in highly extracted nuclei argue that residual nuclear organization is not seriously disrupted by chromatin extraction.

Our observations of matrix-bound centromeres and CREST antigens suggest that much of the movement and unfolding and refolding of prekinetochores during the cell cycle may occur in close proximity to the core nuclear matrix. Moreover, it is logical that the striking movements and particularly the tandem association of prekinetochores occurs in association with the matrix, possibly along the core filaments (He et al., 1995). Although some nuclear movements may be passively related to chromatin condensation and decondensation, the alignment of multiple prekinetochores into linear arrays such as those seen in muntjac appear too directed and cycle-specific to be explained as a passive event. The rates of chromosome movements within prophase nuclei are greater than those on the mitotic spindle (Rickards, 1981). The mechanism of intranuclear movements and the extent of its involvement with the matrix remains unknown. Further studies are needed to determine how prekinetochore movements relate to the nucloskeleton and whether such movements and associations are energy driven and require molecular motors.

Our results also raise the possibility that the centromere, known best for its essential role in mitotic chromosome movement, may also serve a more subtle, non-mitotic function in interphase. Because the prekinetochore appears to be associated with the nuclear matrix, yet remains motile, the latter may influence the positioning and direction of intranuclear movement of chromosomes within the nucleus. The centromere may also help to facilitate chromosomal associations within the interphase nucleus and may function as a principal site for the initiation of chromatin condensation and decondensation. Such an active role for the centromere in nuclear movement and spatial organization of chromatin would likely require a complex molecular mechanism including regulatory molecules and nuclear motors, none of which have, as yet, been identified.

The persistence of CENPs and prekinetochores within the nuclear matrix, and their further development into functional kinetochores in mitosis, is another example of conserved multifunctional roles of nuclear matrix proteins in the cell cycle. This paradigm involves a growing number of nuclear matrix-mitotic apparatus proteins including NuMA, CENP-F, topo II, MPM2, BiC8 and the retinoblastoma (RB) protein that departs the nucleus at mitosis and becomes associated, either structurally or functionally, with the mitotic apparatus during the M-phase (for reviews see He et al., 1995; Mancini et al., 1996). The centromere-kinetochore, known best for its essential role in chromosome movement, also appears to be involved in dynamic movement and positioning of centromeric chromatin within the interphase nucleus.

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