Kinetochoore formation and behaviour following premature chromosome condensation

J. B. RATTNER* and T. WANG

Department of Anatomy, Faculty of Medicine, The University of Calgary, 3330 Hospital Drive N.W., Calgary, Alberta, Canada T2N 4N1

*To whom all correspondence should be addressed

Summary

The potential for interphase centromeres to support kinetochore formation following premature chromosome condensation (PCC) has been investigated. We show that the centromere remains competent to initiate kinetochore formation throughout the cell cycle. PCC-kinetochores display a typical trilaminar morphology, associate with microtubules and show movement towards the centrosome. Indirect immunofluorescence studies illustrate that the centromere/kinetochore region of prematurely condensed chromosomes associates with proteins that are normally found within this region in both a cell cycle-dependent and an independent manner.

Key words: kinetochore, premature chromosome condensation.

Introduction

The mammalian kinetochore is a specialized structure, found at the surface of the primary constriction of metaphase chromosomes, that plays a role in both the capture of spindle microtubules and movements associated with anaphase A (for a review, see Rattner, 1991). The kinetochore is first detected as an aggregate of fine fibrillar material associated with condensing chromatin in mid- to late-prophase nuclei (Roos, 1973). This material coalesces to form the mature kinetochore, which in electron micrographs appears as a plate-like structure composed of three distinct layers. A fibrous corona is associated with the outer layer of the kinetochore when microtubules are absent. While the protein composition of the outer kinetochore plate is largely unknown, at least one protein, CENP-C, has been localized to the kinetochore inner plate (Saitoh et al., 1992). Although the factors which are responsible for the formation of the kinetochore are still undefined, microinjection and DNAase digestion studies indicate that the integrity of the underlying chromatin is essential for proper kinetochore organization (Pepper and Brinkley, 1980; Rattner, 1986; Bernat et al., 1991).

Functional kinetochores have been documented in cells arrested at the G1/S boundary of the cell cycle with 2 mM hydroxyurea and induced into premature mitosis with 5 mM caffeine (Brinkley et al., 1988). Kinetochore positions in these cells form after a brief period of DNA and protein synthesis, which is essential for the appearance of mitotic cells (Zinkowski et al., 1989). This type of observation raises questions concerning the factors which are required for kinetochore formation. For example, is kinetochore formation linked to the execution of a specific sequence of cellular events, or can the centromere of interphase chromosomes support the formation of the kinetochore given the proper environment and, if so, are these kinetochores capable of proper kinetochore function? To address these questions we took advantage of the ability of metaphase cells to induce nuclear envelope breakdown and premature chromatin condensation (PCC) in interphase nuclei following cell fusion (Rao and Johnson, 1972). Whereas this phenomenon has been carefully documented, reports concerning the behaviour of the centromere/kinetochore during PCC are brief (Matsui et al., 1972). We show that the centromeres of prematurely condensed chromosomes retain the ability to support kinetochore formation throughout the cell cycle. Further, these kinetochores contain proteins typically found in association with metaphase centromeres/kinetochores, associate with microtubules and show movement towards the centrosome.

Materials and methods

Induction of premature chromosome condensation

Premature chromosome condensation (PCC) was induced in HeLa cells using polyethylene glycol-mediated fusion of lectin-bound cells (Hanks et al., 1982). To obtain populations enriched for G1 and S-phase cells, cultures were treated with nocodazole (20 μg/ml) for 12 h and mitotic cells were collected by selective mitotic detachment. The cells were then allowed to reverse for 3-5 h (G1) or 8-12 h (S-phase) in fresh medium prior to fusion. The cell cycle in this cell line has been established: G1 - 6 h, S - 12 h, G2 - 2 h, M - 2 h (J. B. Rattner, unpublished observations). In some experiments, random interphase populations from logarithm-
mically growing cultures were used. Following the induction of PCC, cells were processed for light or electron microscopy.

**Indirect immunofluorescence**

To prepare cells for indirect immunofluorescence (IIF), cells fused on coverslips were fixed in 100% methanol at −20°C for 20 min and air dried. In some experiments, formaldehyde fixation and Triton X-100 permeabilization was used. The IIF pattern observed in these preparations was comparable to that observed with methanol fixation (data not shown). In experiments designed to detect p34\(^{cdc2}\), HeLa cells were grown on coverslips and fixed directly, or after growth in the presence of colcemid (0.1 µg/ml) for 3–12 h. Following rehydration in PBS, the coverslips were incubated in the appropriate serum for 1 h at 37°C. The sera used in this study included (1) an ACA serum (Patient designation MF), used at a dilution of 1:250, (2) a monoclonal antibody to β-tubulin (Boehringer Mannheim) used at a dilution of 1:50, (3) a monoclonal antibody to CENP-E (a gift from Tim Yen) used at a dilution of 1:100, (4) a human autoimmune serum reactive with a 400 kDa protein CENP-F (patient designation DA), used at a dilution of 1:250 (Rattner et al., 1991b), (5) an autoimmune serum reactive with the centromere/kinetochore proteins of G1-PCC, used at a dilution of 1:100. Following three washes in PBS, the coverslips were incubated with either a fluorescein conjugated anti-human IgG (H+L), anti-mouse IgG (H+L) or anti-rabbit IgG (H+L) secondary antibody (Dimension Labs) at a dilution of 1:20 for 1 h at 37°C. The coverslips were then washed three times in PBS, mounted in 90% glycerol containing paraphenylenediamine and observed using a Nikon Optiphot fluorescence microscope. Some samples were counterstained with DAPI (4',6-diamidino-2-phenylindole, 0.4 µg/ml, Sigma Co.). Images were recorded on Ilford HP-5 film.

**Electron microscopy**

Following induction of PCC on the surface of 35 cm Petri dishes, the cells were fixed for 1 h in 3% glutaraldehyde in Millonig’s phosphate buffer. After a brief wash the cells were post-fixed for 1 h in a 1% OsO\(_4\) solution buffered in a similar manner. The specimens were then washed in water, passed through a graded ethanol series and embedded in Spurr’s resin. After polymerization, sections in the silver range were collected, stained with uranyl acetate and lead citrate and examined in an Zeiss EM-902 at zero energy loss operated at 80 kV.

**Results**

In order to establish whether the centromeres of interphase chromosomes could support the formation of kinetochores, we fused mitotic HeLa cells obtained by colcemid arrest with populations of interphase cells taken from different points during the cell cycle. Fused cells were first analyzed by electron microscopy. Fig. 1A shows a cell exhibiting G1-PCC. Two distinct chromosome groups (denoted by a dotted line in Fig. 1A) are identifiable, one belonging to the mitotic cell and the other derived from the G1 cell. The mitotic chromosomes are clustered and display a broad profile characteristic of chromosomes with paired chromatids. In contrast, the G1 chromosomes display a thinner profile consistent with single chromatid morphology. Along the G1 chromosomes, it is possible to observe several prominent kinetochores displaying a typical trilaminar structure (Fig. 1B). Kinetochores of similar structure are also observed within the metaphase chromosome group (Fig. 1C). In cells incubated for 45–90 minutes after fusion, kinetochores are found that show association with microtubules (Fig. 1B). A fibrous corona was found on kinetochores that lacked microtubule association (Fig. 1B). This general morphology was also seen in G2-PCC (data not shown).

When S-phase cells were fused with mitotic cells, two prominent groups of condensed chromatin were again apparent (Fig. 1D). However, in contrast to the highly condensed nature of the G1-PCC, the S-phase chromatin displayed a pulverized appearance. Examination of the surface of the clusters of condensed chromatin revealed the presence of trilaminar kinetochores. In some instances the kinetochores appeared to be dislocated from the underlying heterochromatin so that the chromatin of the lower plate became clearly visible (Fig. 1E). As with G1-PCC, kinetochores of S-PCC showed association with microtubules after prolonged incubation following fusion (data not shown).

In many images showing cross sections through the kinetochores of prematurely condensed chromosomes, the kinetochores displayed a cup-shaped appearance. The curvature associated with this morphology was either subtle (Fig. 1F) and comparable to that found in kinetochores of mitotic chromosomes (Fig. 1C), or quite pronounced (Fig. 1G), the latter being most prominent in cells containing S-PCC. When the kinetochores displaying this “U”-shaped morphology were sectioned in a plane parallel to the surface of the chromosome, ovoid bull’s eye structures with an electron dense chromatin core surrounded by the outer kinetochore plates were observed (Fig. 1H). The outermost plate was particularly prominent in these images and the outer and inner margins of this kinetochore layer appeared more electron dense than its interior. In some regions the outer plate profile had a braid appearance (Fig. 1G and H).

To further characterize the centromere and kinetochore regions of interphase chromosomes, we prepared PCCs for examination by indirect immunofluorescence. In these studies several types of antibody probes were employed: (1) human autoantibodies against centromere antigens present throughout the cell cycle (anti-centromere antibody (ACA) serum reactive with CENP-A, B and C), (2) antibodies against antigens found at the centromere/kinetochore only during the prophase-metaphase period (CENP-E, CENP-F), (3) antibodies against β-tubulin, (4) autoantibodies that react with the centromere in a cell cycle-independent manner and (5) p34\(^{cdc2}\), which specifically reacts with several spindle domains including the centrosomes, spindle fibers and kinetochores of G2/M cells.

When interphase HeLa cells at G1, S or G2 phase of the cell cycle were fused with mitotic HeLa cells and reacted with ACA serum, staining could be detected on both the mitotic chromosomes and the interphase PCCs. An example of a cell displaying S-PCC and stained in this manner is shown in Fig. 2A, B. The area reactive with the ACA serum within the prematurely condensed chromatin appeared slightly larger and more intense than the reactive area found in the mitotic centromeres (Fig. 2A, B). When
Fig. 1. (A) Electron micrograph of a fused cell displaying G1-PCC. The mitotic chromosomes are at the top of the dotted line and the G1-PCCs are positioned at the bottom of the dotted line. Three kinetochores (K1, K2, K3) are indicated by arrows and are shown at higher magnification in (B) and (C). Microtubules (mt) are found in association with K2 from the PCC set. (D) Electron micrograph of a fused cell displaying S-PCC. The mitotic chromosomes are at the top of the dotted line and the S-PCCs are at the bottom of the dotted line. Two kinetochores (small arrows) within the S-PCC are shown at higher magnification in (E). The inner plate (ip) and outer plate (op) are denoted in the partially displaced kinetochore shown in the lower portion of the figure. (F, G and H) illustrate three profiles of kinetochores found in association with S-PCCs. Arrows denote outer plates. Bars, 0.3 µm (B, C, E-H), 0.15 µm (A, D).
Fig. 2. Indirect immunofluorescent images of fused HeLa cells reacted with DAPI (A, C, E, G, I, K) or specific antibody probes (B, D, F, H, J, L). (A, B) S-PCC reacted with anti-ACA serum (small arrows) denote centromere reactivity in the S-PCCs. (C, D) S-PCC reacted with anti-ACA serum, illustrating staining at the centromere of the mitotic chromosomes (small arrows) and at the centromeres of the PCCs which have clustered into a single focus (large arrow). A cell showing two foci is illustrated in (E and F). (G, H) G1-PCC reacted with anti-tubulin antibodies. Prominent reactivity is detected at the centrosomes (c) and along microtubules extending from the centrosome (large arrows). Short microtubules and kinetochore reactivity are detected in the region of the mitotic chromosomes (small arrows). (I, J) S-PCC double stained with antibodies to both CENP-F and the centrosome. Large arrows denote centrosome reactivity, small arrows in the lower portion of the micrograph denote PCC centromeres clustering about one of the centrosomes and stained for CENP-F. Small arrows in the upper portion of the micrograph denote some of the mitotic chromosomes stained with CENP-F. The majority of kinetochores are out of the focal plane. (K, L) G2-PCC reacted with antibodies to CENP-E. Reactivity is detected on both chromosome sets (small arrows). Bar, 10 µm.
the pattern of reactivity was compared in cells displaying PCC, collected at various times after fusion, it became apparent that there was a change in the distribution of the PCC centromeres following fusion. Initially the centromeres of both the mitotic chromosomes and the PCC chromosomes were found throughout the cytoplasm of the fused cell (Fig. 2A, B). However, upon further incubation, the PCC centromeres began to cluster until they were found in roughly spherical bundles (compare Fig. 2A, B and C-F). This clustering was observed in G1- and S-PCC, but examples of G2-PCC showing this phenomena were not observed within the time period studied. A centrosome was detected in association with these centromere clusters when cells were reacted with anti-tubulin or anti-centrosome antibodies (Fig. 2G-J). Further microtubule bundles were detected within the centromere/centrosome region (Fig. 2H). Clustering of mitotic chromosomes was not observed, although centromere/kinetochore reactivity and short microtubule bundles were often observed in association with these chromosomes (Fig. 2G-H). Tubulin association with colcemid treated metaphase chromosomes is well documented (Mitchison and Kirschner, 1985; Rattner et al., 1990). When this antibody was reacted with HeLa cells, prominent staining was observed within the mitotic apparatus including the spindle and centrosomes (Fig. 3A, B). Punctate staining in the region of the chromosomes, detected just above background, reflects kinetochore reactivity.

When our HeLa cell line was grown in the presence of colcemid prior to fixation, spindle fiber and centrosome reactivity was abolished although the punctate staining associated with kinetochore reactivity persisted (Fig. 3C, D). In fused cells double stained with antibodies to p34cdc2 and a serum reactive with the centrosome, p34cdc2 was not detected in sites stained by the anti-centrosomal antibody within the regions occupied by either the mitotic chromosomes or prematurely condensed chromosomes (Fig. 3E-G). The general cytoplasmic reactivity found in fused cells was comparable to that reported for mitotic cells using this antibody (Rattner et al., 1990). The absence of p34cdc2 suggests that the protein composition of all the centrosomes in fused cells is not comparable to those found in actively dividing cells.

Discussion

We have taken advantage of the ability of mitotic cells to induce premature chromatin condensation in interphase cells to explore some of the parameters that affect the formation of the mammalian kinetochore. Our studies indicate that the centromere retains the ability to support kinetochore formation throughout the cell cycle and that the exposure of the centromere to the proper cellular environment is apparently sufficient to induce kinetochore formation.

The appearance of kinetochores on G1-PCCs indicates that DNA replication is not a prerequisite for kinetochore formation and that the centromere retains the ability to form a kinetochore immediately following mitosis. Thus, processes which occur at telophase to disassemble the trilaminar kinetochore do not affect the ability of the centromere to re-initiate kinetochore formation. Further, the centromere does not appear to have to “mature” by passing through all or part of the cell cycle to support kinetochore formation.

A number of proteins have been mapped to the centromere/kinetochore, many of which are normally detected throughout the cell cycle within either the centromere (CENP-A, B, D; Rattner, 1991) or kinetochore region (CENP-C, Saitoh et al., 1992). Our antibody studies illustrate that members of this protein class are constituents of PCC-kinetochores. The presence of these proteins may provide the basis for the assembly of the kinetochore on prematurely condensed chromosome.

Temporally associated centromere/kinetochore proteins have also been identified. In this study we have shown that at least some of these proteins are found in association with the kinetochores of prematurely condensed chromosomes. This indicates that the PCC-kinetochores have the ability

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to associate with proteins that normally appear at the kine-
tochores of mitotic chromosomes as they become func-
tional, and implies that PCC-kinetochores undergo a matu-
ration process similar to that associated with normal cell
division. While little is known about CENP-F, CENP-E
appears to be a member of the kinesin family of motor pro-
teins (Tim Yen, personal communication). It may be that
many of the transiently associated proteins detected in asso-
ciation with the kinetochore are related to the kinetochore
function, the capture of microtubules and the regulation of
chromosome movement. The presence of this class of pro-
teins may be related to the apparent ability of the PCC-
kinetochores to associate with microtubules and migrate
toward the centrosome.

One striking feature of HeLa cells exhibiting PCC is the
absence of a mitotic spindle. In our study, the mitotic cells
are obtained from cultures treated with colcemid. The HeLa
cell line used in the present study does not have the abil-
ity to reverse from the colcemid block. Thus, it is not sur-
prising to see that in the fused cells the mitotic chromo-
somes do not participate in attempts to establish a spindle.
The interphase chromosomes which have not been exposed
to colcemid readily establish a monopolar orientation, but
bi-polar spindle formation was not observed. This is in con-
trast to cell fusions carried out with mitotic cells that have
not been exposed to colcemid and have already established
a spindle. After fusion involving these cells, evidence for
chromosome orientation, movement and segregation
involving prematurely condensed chromosomes has been
noted (Heneen and Rohme, 1982). We have been unable to
induce PCC in our HeLa cells with unarrested mitotic cells.

Our studies suggest that the centrosomes from colcemid-
treated HeLa cells and fused cells, which are interphase in
origin, do not contain detectable p34^cdc2. Thus, the protein
composition of these centrosomes is not comparable to
those found in actively dividing cells. This may, in part,
explain why spindle formation is not observed in fused cells
and HeLa cells following reversal from colcemid arrest. The
functionality of kinetochores from the mitotic cells may
also be perturbed by exposure to colcemid. In addition, it
is possible that the cytoplasm of the interphase cell con-
tains factors which inhibit processes associated with spin-
dle formation. The centrosomes and kinetochores may be
sites affected by these interphase factors.

Prematurely condensed chromosomes induced by fusion
of interphase cells with mitotic cells show some striking
similarities to and differences from condensed chromo-
somes induced by caffeine treatment (Zinkowski et al.,
1989). While kinetochore formation occurs in the absence
of DNA synthesis in PCC, a small amount of DNA syn-
thesis is required for chromatin condensation following caf-
feine treatment. Caffeine treatment apparently induces chro-
matin and kinetochore fragmentation, an event which does
not occur in PCC. The bull’s eye morphology of the kine-
tochore is found with both caffeine treatment and PCCs.
One possible explanation for the appearance of this mor-
phology may lie in the state of the underlying chromatin.
In both S-PCC and caffeine-induced chromosomes, the
organization of the chromatin underlying the kinetochore is
disrupted. In caffeine treatment this is due to chromatin
fragmentation, while in PCC this is the result of differen-

Fig. 3. A HeLa cell reacted with either DAPI (A, C, E), an
antibody to the C-terminal region of p34^cdc2 (B, D, G) or an anti-
centrosomal antibody (F). (A-B) illustrate a HeLa cell showing
p34^cdc2 reactivity within the mitotic apparatus including the
centrosome (arrows). (C-D) illustrate a HeLa cell after growth in
the presence of colcemid. Note the absence of detectable
centrosomal reactivity. (E-G) illustrate a fused cell stained for
centrosomes (F) or p34^cdc2 (G). Bar, 10 µm.
tial chromatin condensation. It may be that, in the absence of physical constraints provided by the underlying chromatin, the kinetochore bows inward. This implies that the organization of the kinetochore may result in either the application of some force towards the surface of the chromosome or that the underlying heterochromatin is necessary for plate stability. The importance of the integrity of the underlying heterochromatin to kinetochore organization has also been suggested by microinjection studies using anti-centromere antibodies (Bernat et al., 1991). The detection of the bull’s eye morphology in PCCs illustrates that this morphology is not a necessary diagnostic feature of kinetochore detachment or fragmentation as suggested by the caffeine studies.

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