

Topoisomerase II α Is Associated with the Mammalian Centromere in a Cell Cycle- and Species-specific Manner and Is Required for Proper Centromere/Kinetochore Structure

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Abstract. A study of the distribution of Topoisomerase II α (Topo II) in cells of six tissue culture cell lines, human (HeLa), mouse (L929), rat, Indian muntjac, rat kangaroo (PTK-2), and wallaby revealed the following features: (1) There is a cell cycle association of a specific population of Topo II with the centromere. (2) The centromere is distinguished from the remainder of the chromosome by the intensity of its Topo II reactivity. (3) The first appearance of a detectable population of Topo II at the centromere varies between species but is correlated with the onset of centromeric heterochromatin condensation. (4) Detectable centromeric Topo II declines at the completion of cell division. (5) The

distribution pattern of Topo II within the centromere is species- and stage-specific and is conserved only within the kinetochore domain. In addition, we report that the Topo II inhibitor ICRF-193 can prevent the normal accumulation of Topo II at the centromere. This results in the disruption of chromatin condensation sub-adjacent to the kinetochore as well as the perturbation of kinetochore structure. Taken together, our studies indicate that the distribution of Topo II at the centromere is unlike that reported for the remainder of the chromosome and is essential for proper formation of centromere/kinetochore structure.

THE centromere is a unique chromosomal region that differs from the remainder of the chromosome in its DNA sequence composition, protein composition, and function (Pluta et al., 1995). Furthermore, within the centromere are several discrete domains each with distinct structural, compositional, and functional features (Rattner et al., 1988; Earnshaw and Rattner, 1989). These domains include the outer surface of the centromere containing the kinetochore, the point of association of the chromosome with the spindle, and the site of the mechanochemical motors for chromosome movement (Mitchison and Kirschner, 1985); the inner surface of the centromere, believed to mediate sister chromatid interaction (Earnshaw and Cooke, 1991; Bickel and Orr-Weaver, 1996) and the central region, thought to function in maintenance of centromere integrity (Rattner, 1991; Earnshaw and Rattner, 1989).

Whereas the centromere contains many unique proteins (Pluta et al., 1995), it also contains proteins that are found throughout the chromosome. One member of this latter

class of proteins is the enzyme Topoisomerase II α (Topo II)¹ which has the ability to catalyze strand passing of double-stranded DNA in an ATP-dependent fashion (for review see Wang, 1985). During cell division this enzyme has been found to be essential for chromosome condensation and sister chromatid segregation (DiNardo et al., 1984; Newport and Spann, 1987; Uemura et al., 1987; Holm et al., 1989; Wood and Earnshaw, 1990; Adachi et al., 1991; Downes et al., 1991; Shamu and Murray, 1992; Funabiki et al., 1993). Studies of the distribution of Topo II within the metaphase chromosome of higher vertebrates and birds, using isolated chromosomes subjected to hypotonic treatment, have indicated that Topo II has an axial distribution along the chromosome arms (see for example Earnshaw and Heck, 1985; Saitoh et al., 1995; Sumner, 1996). Whereas in studies of histone H1 depleted chromosomes and chromosomes from insects and amphibians, a more general distribution has been noted (Boy de la Tour and Laemmli, 1988; Swedlow et al., 1993; Buchenau et al., 1993). The degree to which Topo II plays a structural role within the centromere is still unclear (Saitoh et al., 1995).

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1. *Abbreviations used in this paper:* IIF, indirect immunofluorescence; Topo II, topoisomerase II α .

Although the centromere is a structurally unique region of the chromosome, the distribution of Topo II within the centromere has only recently begun to receive special attention. Interestingly, the relative abundance of Topo II in the centromere when compared to the remainder of the chromosome has been noted at metaphase, and it has been suggested that this distribution pattern is conserved in the mammals (Taagepera et al., 1993; Sumner, 1996). One troubling aspect of these studies is that they have been carried out on chromosome preparations that have been hypotonically treated and it is unclear to what degree this preparative protocol affects Topo II distribution. Furthermore, considerable variations in chromosomal staining patterns have been noted within an individual preparation (Sumner, 1996). To address these preparation concerns and to obtain additional information concerning the relationship between Topo II and the centromere region within the mammals, we carried out an indirect immunofluorescence (IIF) study of untreated cycling cells using six cell lines derived from diverse mammalian species representing both eutherians and metatherians. These studies were complemented by functional studies using the Topo II inhibitor ICRF-193. The results of these studies indicate that Topo II has a distinct temporal and spatial distribution within the centromere that is species-dependent. We show that, in the Indian muntjac, centromeric Topo II is required for proper centromere/kinetochore structure.

Materials and Methods

Cell Culture and Indirect Immunofluorescence

Monolayer cultures of HeLa, mouse L929, rat, Indian muntjac, PTK2, or wallaby cells (Amer. Type Tissue Collection, Rockville, MD) were grown on coverslips and fixed for 10 min in 100% methanol. Fixation in 3% paraformaldehyde in Dulbecco's phosphate buffered saline (D-PBS) or acetone were also used in separate studies. No additional pattern to that seen with 100% methanol was detected. Fixed preparations were air dried and stored at -20°C until use. Isolated chromosome preparations were prepared as described in Kingwell and Rattner (1987). Coverslips were rehydrated in D-PBS and then incubated for 1 h at 37°C in a 1:100 dilution of a rabbit antibody directed against the COOH-terminal region of Topo II (TopoGen Inc, Columbus, OH). After three washes in D-PBS, the samples were incubated for 1 h at 37°C in secondary antibody: a Cy 3-conjugated anti-rabbit IgG (H+L) (Dakopatts, Glostrup, Denmark). In some experiments the samples were double labeled with a human ACA serum previously described (Kingwell and Rattner, 1987) or a human serum reactive with the centrosome (Johnstone et al., 1992). In these experiments a FITC-conjugated anti-human IgG (H+L) (Dakopatts) was used. After incubation, the specimens were washed in D-PBS, counterstained with either DAPI (4',6-diamidino-2-phenyl-indole) or 33258 HOECHST, and mounted in 90% glycerol containing paraphenylenediamine and observed using a Nikon Optophot fluorescent microscope. Images were recorded on Ilford HP-5 film.

Immunoblotting

Immunoblotting was performed on nitrocellulose strips containing proteins separated by 12% SDS polyacrylamide gel electrophoresis (Immunoblotting, Towbin et al., 1987; SDS-PAGE, Laemmli, 1970). The proteins used in these blots were from mitotic cells harvested by selective mitotic detachment and from centromeres isolated from digested chromosomes. To obtain isolated mouse centromeres, mitotic cells obtained by selective detachment were resuspended in culture media without FCS and lysed by the addition of an equal volume of media containing 5% NP-40. 30 U of EcoRI were added to 300 μl of the chromosome suspension and incubated at 37°C for 10 min. Digestion was monitored by light microscopy. At the completion of digestion, the centromere suspension was concentrated by centrifugation for 10 min at 3,000 g. The supernatant was de-

canted and the pellet resuspended in SDS sample buffer. For correlated IIF, the centromere suspension was deposited on a coverslip following the procedure described in Kingwell and Rattner (1987). Antibody binding to nitrocellulose strips was detected using ECL (Amersham Corp., Arlington Heights, IL).

Fluorescent Microscopy and Digital Optical Sectioning

Fluorescently stained cells were examined with a Zeiss Universal microscope using a 100×1.32 N.A. Leitz lens. A $1.25\times$ optivar was generally used, although in some cases, a $1.6\times$ or $2.0\times$ optivar was used. Images were collected using a Kodak Megaplug 1.6 digital camera. Camera noise was reduced by mapping "hot" pixels within the 512×512 matrix and then applying a 3×3 median filter for each of the hot pixels. Other noise reduction filters designed for application for whole images were not used with the exception of the DAPI image of Indian muntjac prophase cells where a 3×3 median filter was applied.

Digital optical sectioning (digital "confocal" microscopy) was performed by collecting separate images at $0.5\text{-}\mu\text{m}$ intervals. The z-axis was controlled by an electronic stepper motor calibrated to have a resolution of at least $0.2\text{ }\mu\text{m}$. Optical slices were then obtained using Vaytek Microtome version 3.1. This software uses the slices immediately above and below the section of interest to calculate haze content produced by a point source. Digital optical sections were imported into Adobe Photoshop 2.51. Images were converted to indexed color and false colored red, green, and blue. The resulting images were then combined and aligned using Adobe Photoshop 2.51.

ICRF-193 Studies

Indian muntjac cells were plated onto coverslips 48 h before use; ICRF-193 was added to cultures at a final concentration of 5–100 μM for 1 h. The effect of concentrations of 5, 10, 50, and 100 μM were studied. Cells were fixed for IIF as described above or processed for electron microscopy by standard protocols. In some IIF studies, treated cultures were fixed in methanol and reacted with human ACA serum (Kingwell and Rattner, 1987) or a polyclonal rabbit serum to CENP-F (Liao et al., 1995).

Results

The association of Topo II with the centromere was studied using a commercially prepared antibody raised against the COOH-terminal portion of this protein. Western blot analysis of total mitotic cell protein from mouse (Fig. 1, lane 1), human, Indian muntjac and rat kangaroo mitotic cells (data not shown), and isolated mouse centromeres (Fig. 1, lane 2) prepared by selective digestion (Fig. 1, *a* and *b*) revealed that this antibody recognized a single 170-kD band corresponding to Topo II. The detection of a single reactive antigen in Western blots of isolated centromere proteins indicates that the following description is due to Topo II and not a centromere-specific antigen.

The Topo II antibody was used in indirect immunofluorescent microscopic studies in order to examine the distribution of Topo II with respect to the centromere in six cell types: human (HeLa), mouse (L929), rat, Indian muntjac, rat kangaroo, and wallaby. This large number of species was studied in order to establish what aspects of the relationship between Topo II and the centromere were conserved. Our studies are distinguished from a majority of previous investigations in that the chromosomes were not isolated from the cell and subjected to hypotonic conditions, conditions that could potentially induce a preparation-dependent reorganization of Topo II. A variety of fixation protocols were used including 100% methanol, 3% paraformaldehyde, and acetone. We found that all three fixatives revealed the same centromeric pattern. For simplicity, only images of methanol-fixed materials are shown in the following report.

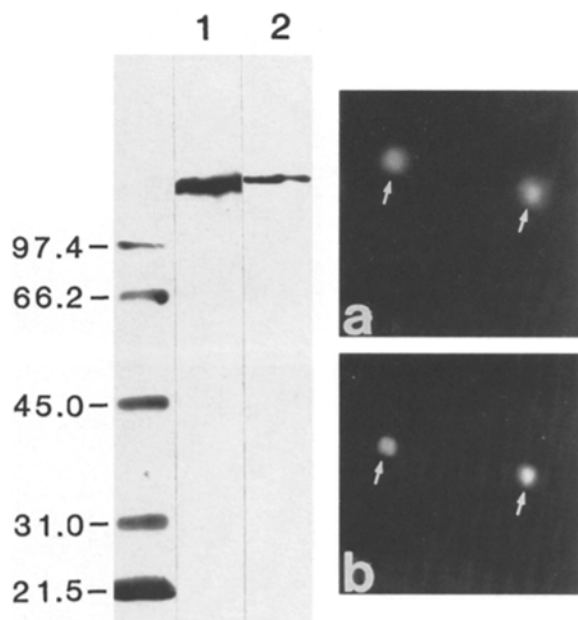


Figure 1. Western blot of mouse mitotic (lane 1) and isolated mouse centromeric protein (lane 2) reacted with the anti-Topo II antibody. A single band corresponding to Topo II is seen in both protein preparations. Isolated centromeres (arrows) stained for DNA with DAPI (a) and Topo II (b).

The application of anti-Topo II antibodies to HeLa cells from rapidly dividing cultures revealed a general punctate nuclear staining pattern in a majority of interphase cells. Only a single region of increased reactivity was detected and this correlated with the nucleolus (Fig. 2, a and b).

A small population of nonreactive interphase cells was also observed and this population likely represents non-cycling cells as previously reported (Heck et al., 1988). At prophase, Topo II was detected in association with the chromosomes and prominent sites of increased reactivity were detected (Fig. 2, c and d). Examination of images of metaphase cells or individual chromosomes indicated that these areas of high reactivity corresponded to the centromere (Fig. 2, e and f). Close examination of the staining pattern within the centromere revealed that the centromere was uniformly labeled by the antibody. Thus, the centromere appeared as a broad region of staining. This is in contrast to the axial pattern of staining seen along the chromosome arms (Fig. 2 e). Fig. 2 e also illustrates that despite the high Topo II signal at the centromere, sister centromeres are separated by a region of relatively low Topo II intensity. Comparison of the level of centromere reactivity at prophase-metaphase with cells in the later stages of cell division revealed that the intense centromere staining pattern declined during the completion of mitosis so that, in cells completing karyokinesis, the centromere region was indistinguishable from the remainder of the chromosome (Fig. 2, g and h). We have observed a similar pattern of reactivity in several additional human cell lines (data not shown).

To determine if the pattern of reactivity seen in HeLa cells was conserved in other mammalian species, anti-Topo II antibodies were applied to mouse L929 cells. Within the interphase cell population, a majority of the in-

terphase nuclei displayed a punctate staining pattern with a greater intensity at the nucleolus similar to that seen in the HeLa cell population. When the Topo II pattern was overlapped with the DNA pattern in these cells (detected with 33258 HOECHST), no preferential association of Topo II with the centromeric heterochromatin was noted (Fig. 3, a-c). In a small population of interphase nuclei, however, numerous foci of Topo II reactivity were apparent. Comparison of the Topo II and HOECHST images from this cell population revealed that Topo II reactivity was preferentially associated with the margins of each centromeric heterochromatin patch (Fig. 3, d-f). In cells showing centromeric heterochromatin with a greater degree of condensation, Topo II was homogeneously distributed throughout the centromeric DNA (Fig. 3, g-i). When a population of 100 cells containing Topo II reactive centromeric heterochromatin was analyzed, 100% were found to contain duplicated centrosomes and 89% displayed centrosomes that had begun centrosome separation in preparation for cell division (Fig. 3, j-m). Since the centrosome cycle is closely associated with the cell cycle in this cell line (Rattner and Phillips, 1973), this population can be correlated with the G2 period of the cell cycle. Thus, Topo II association with the centromere in L929 cells is correlated with the progressive condensation of this portion of the chromosome which occurs as a prelude to cell division.

In prophase mouse cells, foci of Topo II reactivity could be detected within the condensing chromosomes (Fig. 2, i and j). At metaphase it was clear that these foci corresponded to the centromere region. As was seen in the HeLa chromosomes, reactivity appeared to extend throughout the centromere while the arms displayed an axial distribution (Fig. 2, k and l). Within the mouse L929 karyotype is a marker chromosome with multiple centromere regions containing both the major and minor satellite (Wong and Rattner, 1992). Only the central centromere is known to have a functional kinetochore. Analysis of isolated chromosomes revealed the presence of this chromosome and elevated Topo II reactivity appeared at each of the centromere regions (Fig. 2, m and n). Thus, Topo II reactivity is not correlated with functional centromeres but rather appears to follow the distribution of centromeric heterochromatin. As in human cells, as the nucleus began to reform, the centromere could no longer be distinguished from the remainder of the chromosome based on Topo II reactivity (Fig. 2, o and p). The pattern of reactivity seen in both human and mouse cells was also observed in a rat cell line (data not shown).

To determine if the pattern of centromere reactivity varied in other mammalian species, anti-Topo II antibodies were applied to cycling Indian muntjac cells (data not shown). As with the human and mouse cells, the interphase nuclei displayed a general punctate pattern of reactivity and an increase in the intensity at the nucleolus (data not shown). Indian muntjac cells progressing into prophase showed highest Topo II reactivity at the centromere. However, unlike the general pattern seen in mouse and human prophase cells, the intense reactivity was confined to a well defined area within the centromere so that each centromere appeared to contain Topo II staining on its outer surface separated by a region of relatively low Topo II staining.

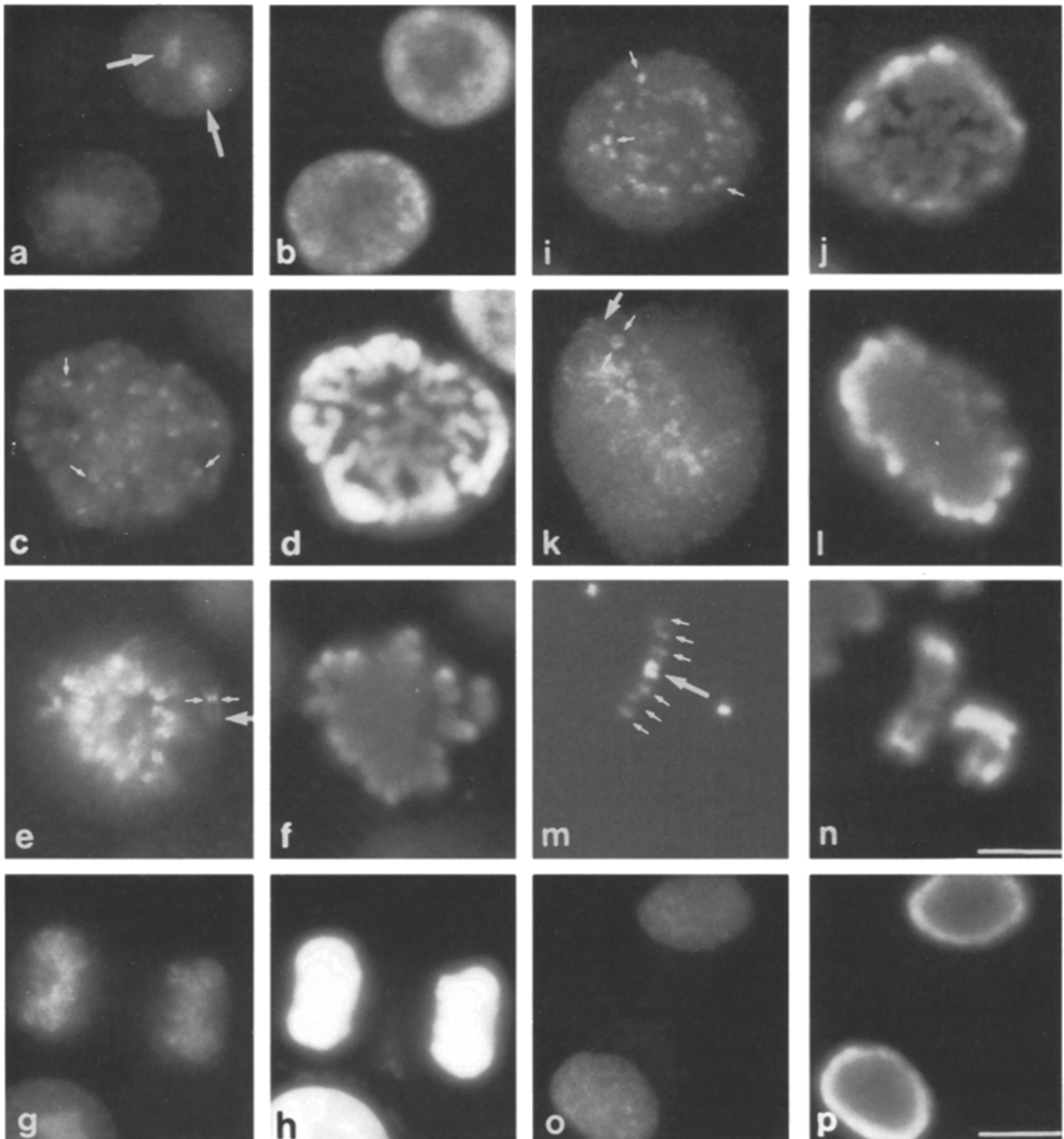


Figure 2. Centromeric Topo II in HeLa and mouse L929 cells. Topo II (*a, c, e, g, i, k, m, and o*) and DAPI (*b, d, f, h, j, l, n, and p*) images of HeLa (*a-h*) and mouse cells (*i-p*) throughout cell division. Interphase (*a* and *b*) arrows denote nucleoli; prophase (*c, d, i, and j*) arrows denote centromeres; metaphase (*e, f, k, and l*) small arrows denote centromeres, bold arrows denote axial staining in the chromosome arms; Anaphase (*g* and *h*); late telophase (*o* and *p*). Bar, 6 μ m. The marker chromosome of the L929 karyotype is shown in Fig. 2, *m* and *n*. Note regions of high Topo II staining correlates with each of the centromere regions (arrows denote centromere regions). Bar, 12 μ m.

The localization of the highest concentration of Topo II at the outer margin of the centromere, the region containing the kinetochore, was confirmed by superimposing confocal images of prophase cells reacted with both Topo II and ACA antibodies (Fig. 4, *a-d*). ACA antibodies have been shown to react selectively with the kinetochore do-

main of the centromere in this species (Brinkley et al., 1984; Rattner, 1986). The region stained by Topo II is slightly larger than that highlighted by ACA (Fig. 4, *a-d*). Thus, the major concentration of Topo II in the Indian muntjac centromere is confined to a specific domain within the centromere. The remainder of the centromere

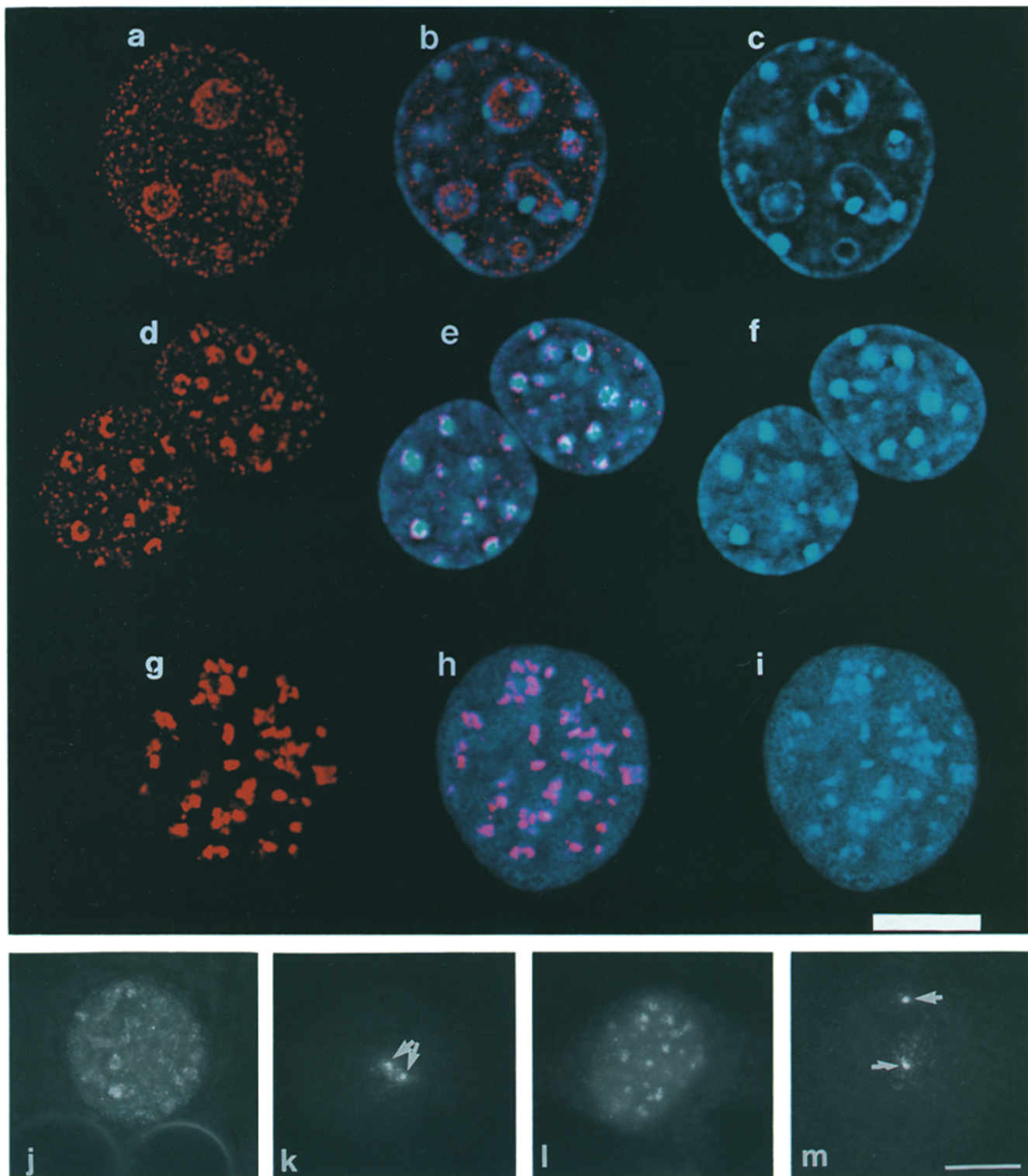


Figure 3. Relative localization of Topo II and HOECHST-rich heterochromatin within interphase L929 cells. Individual 0.5- μm digital optical slices were collected as described in Materials and Methods. The Topo II images were false colored red and the HOECHST images were false colored blue. The HOECHST image was then digitally overlaid on the Topo II image and aligned according to morphological features that obviously corresponded to both images (e.g., nucleolus in *a*, heterochromatin in *b* and *c*). (*a-c*) Pattern characteristic of a majority of interphase nuclei (*d-f*) G2 phase nucleus with early stages of condensation (*g-i*) G2 nucleus displaying condensed heterochromatin. Topo II is false-colored red, DAPI is false-colored blue, and the overlap region is pink-to-white. The bar size is 10 μm . *j-m* illustrates G-2 L929 cells double stained with Topo II (*j* and *l*) and an antibody reactive with the centrosome, arrows (*k* and *m*). Bar, 6 μm .

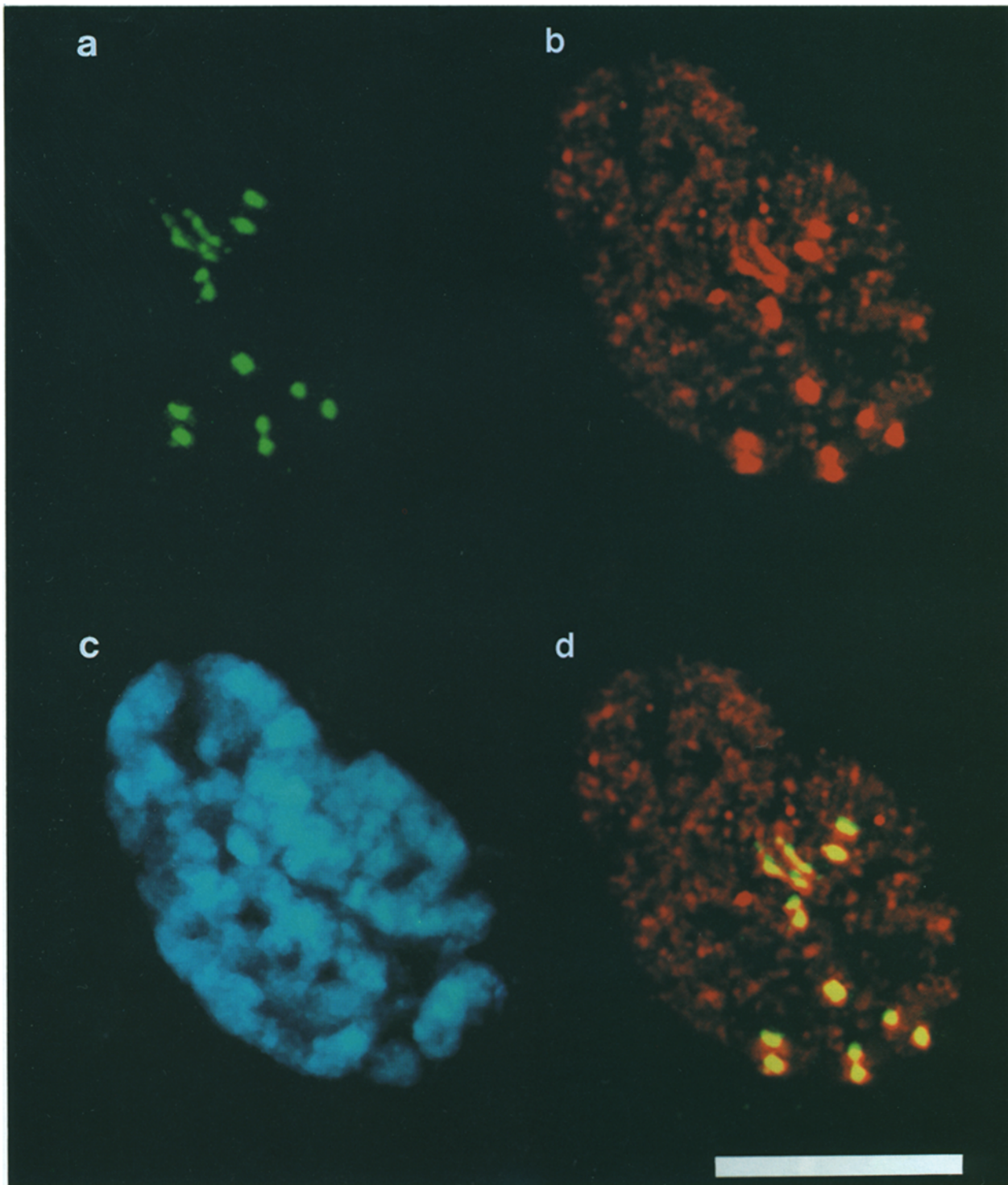


Figure 4. Colocalization of ACA and topo II antigens during prophase in Indian muntjac cells. Individual 0.5- μm digital optical slices were collected as described in Materials and Methods. Five consecutive slices were then projected into a single image for each of the DAPI, ACA, and Topo II images series. This optimized the number of individual chromosomes that could be simultaneously imaged. (a) ACA, (b) Topo II, (c) DNA (DAPI), and (d) ACA/Topo II composite image. Overlap between Topo II and ACA is yellow. Bar, 10 μm .

displayed a lower level of homogeneous staining. As Indian muntjac cells progress to metaphase, the intense staining at the centromere persisted (data not shown) but declined as the cells progressed to the completion of cell division (data not shown).

The localization of a population of Topo II at the region adjacent to the kinetochore was also observed in cycling cells of the rat kangaroo, PTK2 (Fig. 5). As in the muntjac, reactivity adjacent to the kinetochore first appeared at prophase. Confocal images of prophase cells double

stained with Topo II and ACA revealed that the region identified by these antibodies overlapped. As was found in the Indian muntjac, the region highlighted by Topo II was slightly larger than that demarcated by ACA (Fig. 5, *a–c*). However, in this species, as the cell proceeded to metaphase, an additional area of reactivity between sister kinetochore regions appeared (Fig. 5, *d–f*). We were unable to observe cells in transition between the two morphologies, suggesting that the appearance of the central Topo II population occurs rapidly. Furthermore, we could find no examples of prophase cells with general centromere reactivity prior to nuclear envelope breakdown indicating that the localization of Topo II between the kinetochore regions occurs following the completion of major chromosome condensation. The intensity of Topo II staining declined as the cell completed cell division, as seen in the other species (data not shown). The pattern of reactivity within the centromere of PTK2 cells was also observed in a wallaby cell line (data not shown).

To investigate the function of centromeric Topo II, we grew Indian muntjac cells in the presence of the Topo II inhibitor ICRF-193 for 1 h using various concentration (5, 10, 50, 75, 100 μM). We chose Indian muntjac cells because of the localized pattern of centromeric Topo II and the large size of the centrosome/kinetochores in this spe-

cies. The affect of the drug on the accumulation of Topo II at the centromere was assayed using anti-Topo II antibodies and IIF. We found that it was necessary to use high levels of ICRF-193 (100 μM) in order to abolish the selective concentration of Topo II at the centromere in all prophase and metaphase cells within treated cultures (a total of 50 cells was observed at each stage) (Fig. 6, *a–d*). Intense centromere reactivity was found in all prophase and metaphase cells (a total of 50 cells was observed at each stage) at lower concentrations of the drug. At drug concentrations of 10–100 μM , Topo II did not display a pronounced axial distribution in the arms of treated chromosomes but rather had a more general distribution (Fig. 6, *c–d*). ICRF-193 did not change the distribution of Topo II in either the chromosome arms or centromeres of chromosomes in cells that had reached metaphase at the time of drug addition.

Mitotic cells grown in the presence of ICRF-193 were examined by electron microscopy to study the morphology of the chromatin underlying the kinetochore and that of the kinetochore itself. Electron micrographs of chromosomes from untreated cells revealed centromeres with densely packed chromatin and kinetochores displaying a prominent tri-lamellar morphology (Fig. 6 *e*). At low concentrations of ICRF-193, normal centromere and kinetochore structure were observed although occasionally the

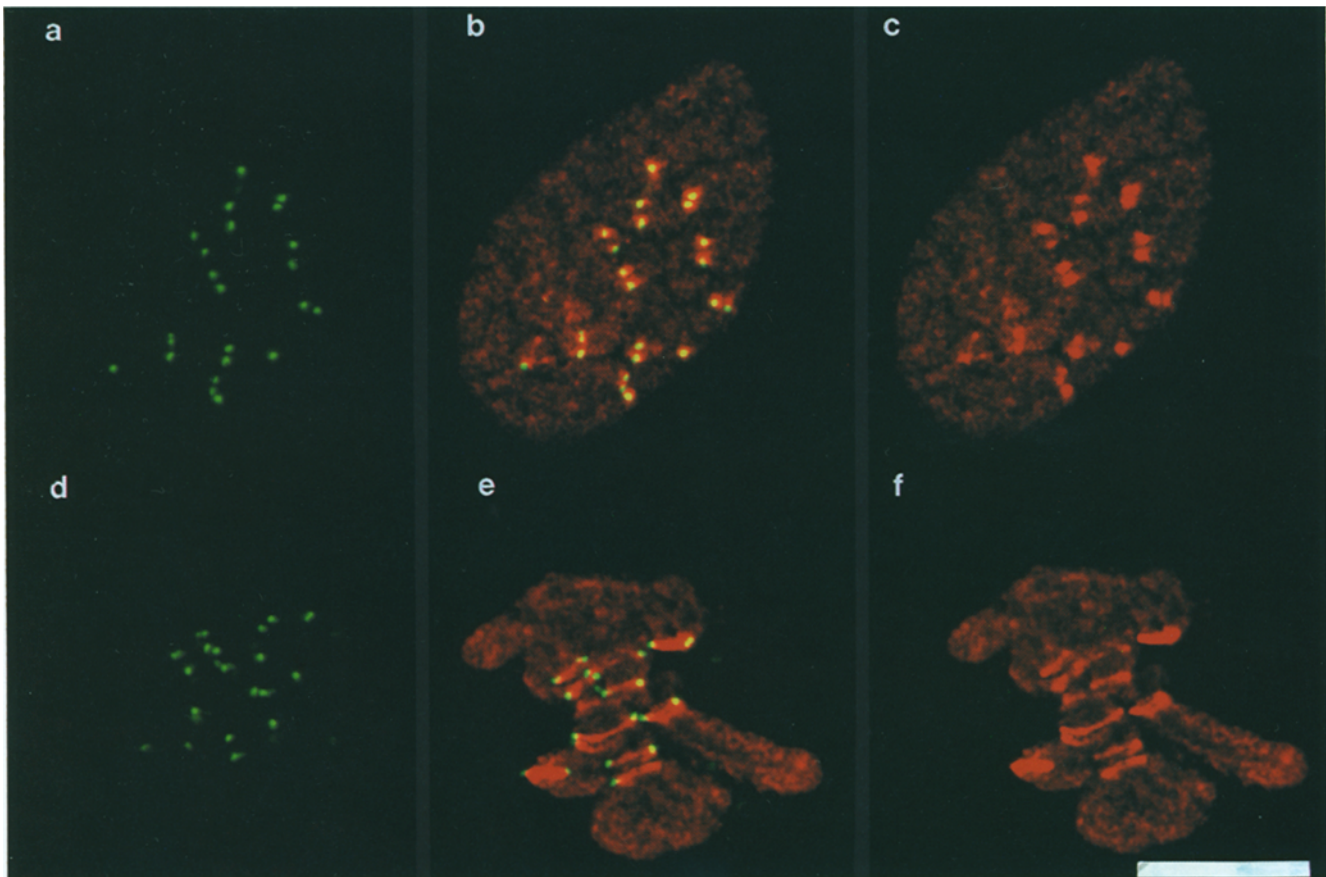
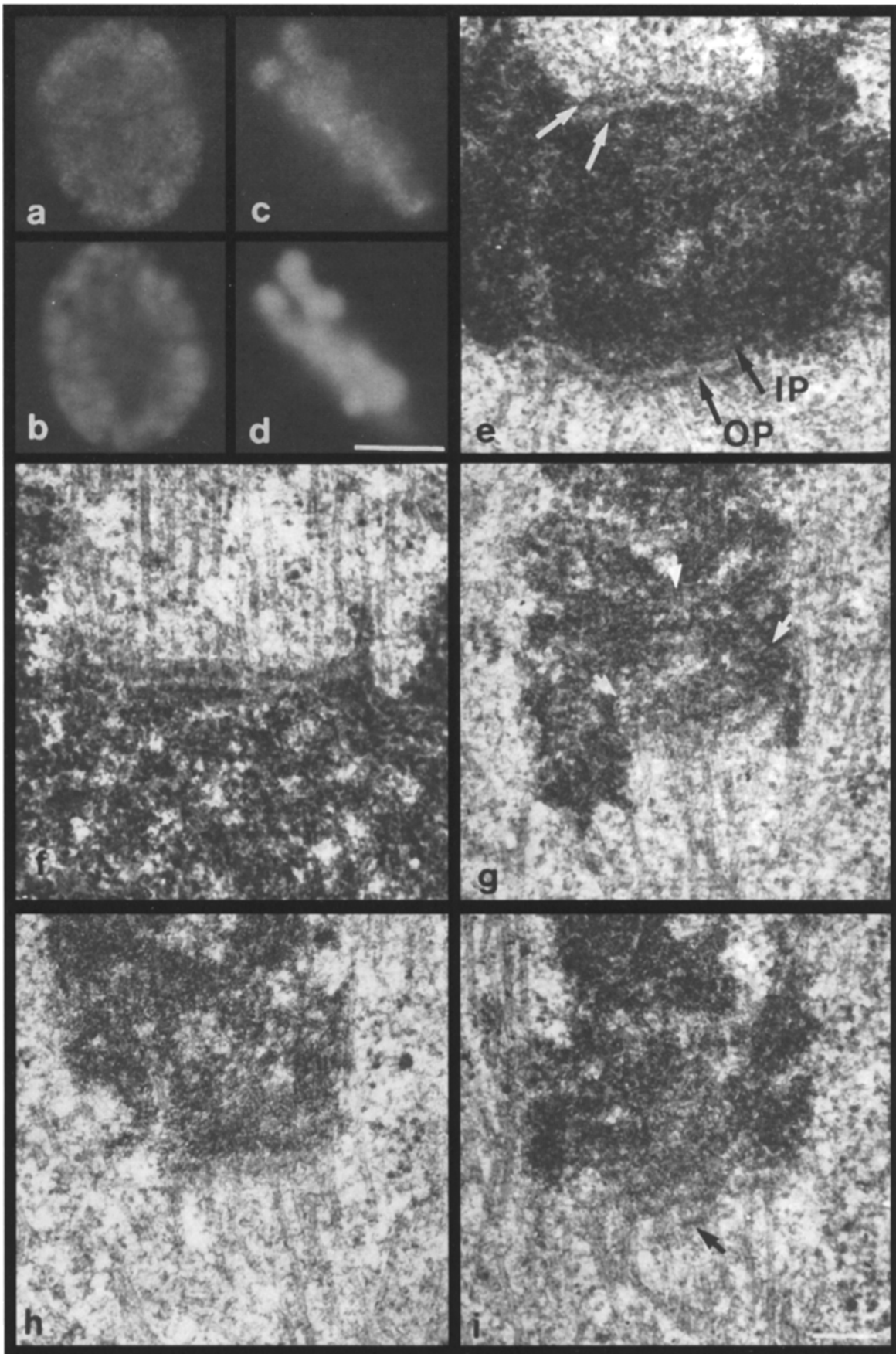


Figure 5. Comparative localization of Topo II and ACA in prophase and metaphase PTK2 cells. Individual 0.5- μm digital optical sections were collected as described in Materials and Methods. Prophase cell staining is shown in the top panels whereas metaphase staining is shown in the bottom panels. (*a* and *d*) ACA, (*c* and *f*) Topo II, composite images are shown in the middle panels (*b* and *e*) where overlap, between ACA and Topo II is shown as yellow. Bar, 10 μm .



centromeric chromatin had a less condensed appearance (Fig. 6 *f*). However, at 100 μM ICRF-193, the concentration at which centromeric Topo II was no longer detected, altered structure at the centromere was observed. Fig. 6, *g–i* illustrate a collection of images from the centromere region of chromosomes from cells treated at the higher concentration. In comparison to untreated chromosomes, these centromeres contained chromatin that displayed a spongy rather than a compact morphology (Fig. 6, *e, g, and h*). The kinetochores appeared aberrant with reduced or undetectable outer plates and the middle layer was not apparent (Fig. 6, *g–i*). In some images the kinetochore appeared fragmented (Fig. 6 *i*). Microtubules, however, were associated with the aberrant kinetochores but inserted into the kinetochore region at different levels (Fig. 6 *i*). Examination by light microscopy revealed that chromosomes in treated cultures were able to form a metaphase plate, and, in rare cases, underwent an aberrant anaphase which lacked chromatid separation (data not shown).

To investigate the effect of high concentrations of ICRF-193 on the protein composition of the centromere, we reacted mitotic cells from treated cultures with antibodies to cell cycle invariant proteins (CENP-A-C) and antibodies to the transiently associated kinetochore protein CENP-F. Fig. 7 illustrates that these proteins can still be detected in treated chromosomes indicating that ICRF-193 does not appear to affect the general protein composition of the centromere.

Discussion

This study illustrates that (1) a population of Topo II associates with the centromere in a cell cycle-specific manner. (2) The centromere is distinguished from the remainder of the chromosome by its Topo II pattern and intensity. (3) The first appearance of a detectable population of Topo II at the centromere coincides with the condensation of this region. (4) The distribution pattern of Topo II within the centromere is species- and stage-specific, although, the localization of Topo II adjacent to the kinetochore is conserved. (5) Centromeric Topo II activity is necessary for the proper condensation of the chromatin underlying the kinetochore and for the proper formation of the kinetochore. (6) Detectable centromeric Topo II declines as the cell progresses towards the completion of cell division. Thus, both the temporal interaction and spatial distribution of Topo II at the centromere differs from that reported for the remainder of the chromosome (for reviews see Gasser et al., 1986; Saitoh et al., 1995). Topo II must be considered as an important component in the formation of centromere/kinetochore structure during cell division.

It is informative to note that, in contrast to studies using hypotonically treated chromosomes (Sumner, 1996), our studies on intact cells yield more consistent Topo II pat-

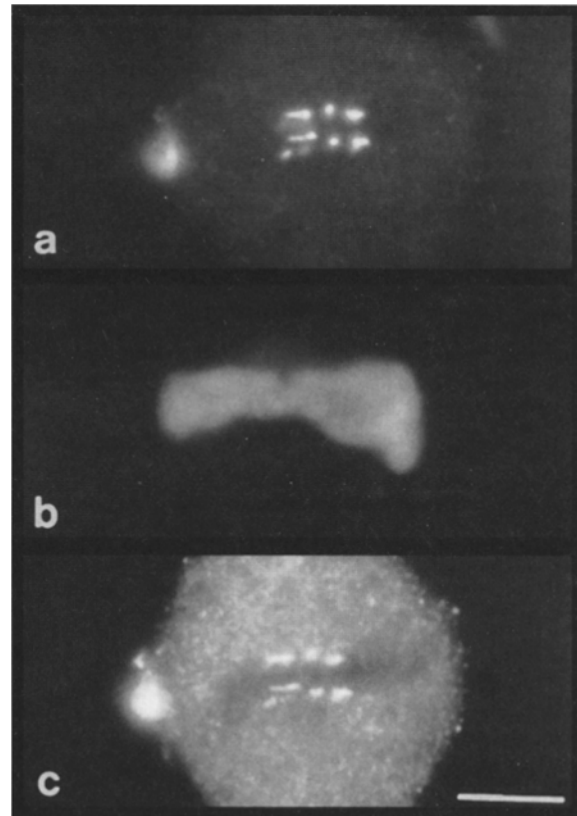


Figure 7. Indian muntjac cells treated with ICRF-193 (100 μM) and reacted with anticentromere antibodies. (a) Cell reacted with human ACA serum (b) DAPI counter stain (c) anti-CENP-F image. Bar, 10 μm .

terns from cell to cell, less variation between fixation protocols, detection of prophase centromere reactivity, a clear resolution of Topo II centromere pattern, the clear detection of axial arms staining in species such as mouse, and the clear definition of centromeric heterochromatin staining during the cell cycle. These multiple differences underscore the limitations of using hypotonically treated chromosomes to study chromosome organization.

Topo II has been ascribed a role in chromosome condensation, sister chromatid separation, and as a chromosomal scaffold component. It is unlikely that the centromeric Topo II population detected in our study plays a conserved role in moderating sister chromatid interaction at the inner surface of the centromere since this population is not present in this region in the muntjac. We cannot, however, rule out the possibility that this population may function in this capacity in species where a more general centromeric Topo II distribution has been detected. It is interesting to note in this context that, in marsupials, the

Figure 6. Indian muntjac cells treated with ICRF-193. Cells grown in the presence of 100 μM ICRF-193 for 1 h and stained with anti-Topo II revealed that this treatment prevented the accumulation of the intense centromeric Topo II population at the centromeres of both prophase (a) or metaphase (c) cells. b and d shows counterstained DAPI images. In untreated cells, Indian muntjac centromeres contain highly condensed chromatin and kinetochores with prominent inner (IP) and outer (OP) plates (e). At low concentrations (10 μM) of ICRF-193 (f) normal morphology is seen. However, centromere cells treated with 100 μM ICRF-193 (g–i) have undercondensed chromatin (denoted by white arrows in g) and fragmented plates (black arrows in i). Bars: (d) 10 μm ; (i) 3 μm .

detection of intense Topo II reactivity extending to the sister chromatid interface does not appear until prometaphase. Thus, any potential role for this subpopulation in moderating sister chromatid interaction must be confined to the post-prometaphase period in these species. It is also possible that a second, less prominent Topo II population is present within the centromere that modulates sister chromatid interaction. This population may be correlated with the weak Topo II signal detected outside the region of high Topo II intensity in some of the species examined in this study. The difference in the Topo II pattern within the centromere of some species makes it unlikely that the selective concentration of Topo II at the centromere is a simple reflection of DNA concentration.

Our studies with the drug ICRF-193 in Indian muntjac cells implicate the intensely staining population of centromeric Topo II in both the normal condensation of the chromatin underlying the kinetochore and the formation of normal kinetochore structure. This conclusion is also supported by the finding that, in all species studied, the appearance and disappearance of centromeric Topo II coincides with condensation and decondensation of the centromere. Furthermore, in the mouse, the association and the distribution of Topo II closely correlates with changes in the condensation state of the centromere that can be specifically visualized in G2.

The relationship between Topo II and the kinetochore may be complex. For example, alterations seen at the kinetochore at high ICRF-193 concentrations may occur indirectly as a result of the disruption of the underlying chromatin, or reflect a direct requirement for Topo II at the kinetochore. In this regard we cannot rule out the possibility that the forces generated by microtubule-kinetochore interaction induce or exaggerate the alteration in kinetochore morphology. In the present study we examined serial sections of eight cells by electron microscopy representing different concentrations and mitotic stages (1 cell/5 μ M, 2 cells/10 μ M, 2 cells/50 μ M and 4 cells/100 μ M). At the 100 μ M concentration all the kinetochores appeared abnormal, suggesting the uniformity of response to the drug treatment on centromere morphology.

The association of microtubules with aberrant kinetochores is not unprecedented. For example, microtubules have been found in association with abnormal kinetochores that form following the interphase injection of ACA serum (Bernat et al., 1991). It is interesting to note that this treatment not only produced abnormal kinetochores but also induced defects in the condensation of the sub-adjacent chromatin. Given the striking similarity of our results to those of Bernat et al. (1991), it would be of interest to see if the microinjection of ACA antibodies disrupts the cell cycle localization of centromeric Topo II.

The detection of a cell cycle-specific association of Topo II with the centromere underscores the dynamic nature of this chromosomal region. When the appearance of Topo II is compared with that of other transiently associated centromere proteins, Topo II can be seen to be one of the first proteins to associate with the centromere as it approaches cell division. Our study indicates that Topo II associates specifically with the centromere in mouse as early as the late S-G2 period. Only one other protein, CENP-F (Liao et al., 1995), has been shown to associate with the

centromere during this pre-mitotic period. The association of these proteins is followed by those such as MCAK at prophase and CENP-E and P34^{cdc2} at prometaphase (Wardeman and Mitchison; 1995, Yen et al., 1992; Rattner et al., 1990). The departure of Topo II from the centromere during the later stages of cell division appears to coincide with a major movement of proteins such as CENP-E and -F from this chromosomal region (Liao et al., 1995; Brown et al., 1996). While a majority of recent studies investigating centromere structure/function have focused on proteins that are found only at the centromere, our study indicates that ubiquitous chromosomal proteins such as Topo II must also be considered as participants in centromere dynamics during cell division.

Topo II has been shown to be a component of the isolated chromosome scaffold (Earnshaw et al., 1985). Although the scaffold was originally envisioned as a static axial structure within the chromosome (Earnshaw and Heck, 1985), more recent studies show that it may be more diffuse and dynamic. Our study indicates that if centromeric Topo II plays a scaffolding role it does so in a dynamic manner and that the scaffold is likely to be arranged differently in the centromere and the chromosome arms.

Recent studies of living cells have revealed the presence of multiple populations of Topo II during early *Drosophila* embryogenesis (Swedlow et al., 1993), with major movements in these populations at prophase and anaphase. Our observations support the presence of multiple Topo II populations and the temporal shift in the location of these populations. The presence of multiple Topo II populations complicates functional studies since it is not possible to completely target a specific population. However, in our studies it is likely that the alterations seen at the centromere in drug-treated cells are related to the centromeric Topo II population since drug treatment appeared to prevent the association of this population with the centromere while the remainder of the chromosome still retained Topo II. In summary, our studies confirm and extend previous studies noting the presence of Topo II at the centromere by describing, for the first time, the detailed cell cycle- and species-specific nature of the interaction between the centromere and a population of Topo II. We also demonstrate a relationship between this Topo II population and centromere/kinetochore structure.

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References

- Adachi, Y., M. Luke, and U.K. Laemmli. 1991. Chromosome assembly *in vitro*: topoisomerase II is required for condensation. *Cell*. 64:137-148.
- Bernat, R.L., M.R. Delannoy, N.F. Rothfield, and W.C. Earnshaw. 1991. Disruption of centromere assembly during interphase inhibits kinetochore morphogenesis and function in mitosis. *Cell*. 66:1229-1238.

- Bickel, S.E., and T.L. Orr-Weaver. 1996. Holding chromatids together to ensure they go their separate ways. *BioEssays*. 18:293–300.
- Boy de la Tour, E., and U.K. Laemmli. 1988. The metaphase scaffold is helically folded: sister chromatids have predominately opposite helical handedness. *Cell*. 55:937–944.
- Brinkley B.R., M.M. Valdivia, A. Tousson, and A.L. Brenner. 1984. Compound kinetochores of the Indian muntjac. Evolution by linear fusion of unit kinetochores. *Chromosoma*. 91:1–11.
- Brown, K.D., K.W. Wood, and D.W. Cleveland. 1996. The kinesin-like protein CENP-E is kinetochore-associated throughout poleward chromosome segregation during anaphase-A. *J. Cell Sci.* 109:961–969.
- Buchenau, P., H. Saumweber, and D.J. Arndt-Jovin. 1993. Consequences of topoisomerase II inhibition in early embryogenesis of *Drosophila* revealed by *in vivo* confocal microscopy. *J. Cell Sci.* 104:1175–1186.
- DiNardo, S., K. Voelkel, and R. Sternglanz. 1984. DNA Topoisomerase II mutants of *Saccharomyces cerevisiae*: topoisomerase II is required for segregation of daughter molecules at the termination of DNA replication. *Proc. Natl. Acad. Sci. USA* 81:2616–2620.
- Downes, C.S., A.M. Mullinger, and R.T. Johnson. 1991. Inhibitors of DNA Topoisomerase II prevent chromatid separation in mammalian cells but do not prevent exit from mitosis. *Proc. Natl. Acad. Sci. USA*. 88:8895–8899.
- Earnshaw, W.C., and M.M.S. Heck. 1985. Localization of Topoisomerase II in mitotic chromosomes. *J. Cell Biol.* 100:1716–1725.
- Earnshaw, W.C., and J.B. Rattner. 1989. A map of the centromere (Primary constriction) in vertebrate chromosomes at metaphase. Mechanisms of Chromosome Distribution and Aneuploidy. Baldev Vig, editor. CRC Press, New York. 33–43.
- Earnshaw, W.C., and C.A. Cooke. 1991. Analysis of the distribution of the IN-CENPs through mitosis reveals the existence of a pathway of structural changes in the chromosomes during metaphase and early events in the cleavage furrow. *J. Cell Biol.* 121:443–461.
- Earnshaw, W.C., N. Halligan, C. Cooke, M.M.S. Heck, and L.F. Liu. 1995. Topoisomerase II is a structural component of mitotic chromosome scaffolds. *J. Cell Biol.* 100:1706–1715.
- Funabiki, H., I. Hagan, S. Uzawa, and M. Yanagida. 1993. Cell cycle-dependent specific positioning and clustering of centromeres and telomeres in fission yeast. *J. Cell Biol.* 121:961–976.
- Gasser, S.M., T. Laroche, J. Falquet, E. Boy de la Tour, and U.K. Laemmli. 1986. Metaphase chromosome structure involvement of topoisomerase II. *J. Mol. Biol.* 188:613–629.
- Heck, M.S., W.N. Hittelman, and W.C. Earnshaw. 1988. Differential expression of DNA topoisomerases I and II during the eukaryotic cell cycle. *Proc. Natl. Acad. Sci. USA*. 85:1086–1090.
- Holm, C., T. Stearns, and D. Botstein. 1989. DNA Topoisomerase II must act at mitosis to prevent nondisjunction and chromosome breakage. *Mol. Cell Biol.* 9:159–168.
- Johnstone, S.A., D.M. Waisman, and J.B. Rattner. 1992. Enolase is present at the centrosome of HeLa Cells. *Exp. Cell Res.* 202:458–463.
- Kingwell, B., and J.B. Rattner. 1987. Mammalian kinetochore/centromere composition: a 50 kDa antigen is present in the mammalian kinetochore/centromere. *Chromosoma*. 95:403–407.
- Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the bacteriophage head T4. *Nature (Lond.)*. 227:680–685.
- Liao, H., R.J. Winkfein, G. Mack, J.B. Rattner, and T.J. Yen. 1995. CENP-F is a protein of the nuclear matrix that assembles onto kinetochore at late G2 and is rapidly degraded after mitosis. *J. Cell Biol.* 130:507–518.
- Mitchison, T.J., and M.W. Kirschner. 1985. Properties of the kinetochore *in vitro*. II Microtubule capture and ATP-dependent translocation. *J. Cell Biol.* 101:755–765.
- Newport, J., and T. Spann. 1987. Disassembly of the nucleus in mitotic extracts; membrane vesicularization, lamin disassembly, and chromosome condensation are independent processes. *Cell*. 48:219–230.
- Pluta, A.F., A.M. Mackay, A.M. Ainsztein, I.G. Goldberg, and W.C. Earnshaw. 1995. The centromere: hub of chromosomal activities. *Science (Wash. DC)*. 270:1591–1594.
- Rattner, J.B. 1986. Organization within the mammalian kinetochore. *Chromosoma*. 93:515–520.
- Rattner, J.B. 1991. The structure of the mammalian centromere. *BioEssays*. 13: 51–56.
- Rattner, J.B., and S.G. Phillips. 1973. Independence of centriole formation and DNA synthesis. *J. Cell Biol.* 57:359–372.
- Rattner, J.B., J. Lew, and J.H. Wang. 1990. p34^{cdc2} kinase is localized to distinct domains within the mitotic apparatus. *Cell Motil. Cytoskeleton*. 17:227–235.
- Rattner, J.B., B. Kingwell, and M.J. Fritzler. 1988. Detection of distinct structural domains within the primary constriction using autoantibodies. *Chromosoma*. 96:360–367.
- Saitoh, N., I. Goldberg, and W.C. Earnshaw. 1995. The SMC proteins and the coming of age of the chromosome scaffold hypothesis. *BioEssays*. 17:759–766.
- Shamu, C.E., and A.M. Murray. 1992. Sister chromatid separation in frog egg extracts requires DNA topoisomerase II activity during anaphase. *J. Cell Biol.* 117:921–934.
- Sumner, A.T. 1996. The distribution of Topoisomerase II on mammalian chromosomes. *Chromosome Research*. 4:5–14.
- Swedlow, J.R., J.W. Sedat, and D.A. Agard. 1993. Multiple chromosomal populations of Topoisomerase II detected *in vivo* by time-lapse, three dimensional wide-field microscopy. *Cell*. 73:97–108.
- Taagepera, S., P.N. Rao, F.H. Drake, and G.J. Gorbisky. 1993. DNA Topoisomerase II α is the major chromosome protein recognized by the mitotic phosphoprotein antibody MPM-2. *Proc. Natl. Acad. Sci. USA*. 90:8407–8411.
- Towbin, H.S., S. Staehelin, and J. Gordon. 1987. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets; procedures and some applications. *Proc. Natl. Acad. Sci. USA*. 76:4350–4355.
- Uemura, T., H. Ohkura, Y. Adachi, K. Morino, K. Shiozaki, and M. Yanagida. 1987. DNA Topoisomerase II is required for condensation and separation of mitotic chromosomes in *S. pombe*. *Cell*. 50:917–925.
- Wang, J.C. 1985. DNA Topoisomerase. *Annu. Rev. Biochem.* 54:665–697.
- Wong, A.K.C., and J.B. Rattner. 1992. The centromere. In *Advances in Molecular and Cell Biology*. JAI Press, Inc. Greenwich, CT. 1–35.
- Wood, E., and W.C. Earnshaw. 1990. Mitotic chromatin condensation *in vitro* using somatic cell extracts and nuclei with variable levels of endogenous topoisomerase II. *J. Cell Biol.* 111:2839–2850.
- Wordeman, L., and T.J. Mitchison. 1995. Identification and partial characterization of mitotic centromere-associated kinesin, a kinesin-related protein that associates with centromeres during mitosis. *J. Cell Biol.* 128:95–105.
- Yen, T.J., G. Li, B. Schaar, I. Szilak, and D.W. Cleveland. 1992. CENP-E is a putative kinetochore motor that accumulates just before mitosis. *Nature (Lond.)*. 359:536–539.