

Mapping DNA within the Mammalian Kinetochores

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Abstract. The location of the *cis*-acting DNA sequences that direct the assembly of the mammalian kinetochore is not known. A variety of circumstantial evidence, however, has led to the widespread belief that they are present throughout the kinetochore including the kinetochore outer plate. To investigate this question directly, we have used two independent methods to localize DNA in and around the mammalian kinetochore. Both methods fail to reveal DNA in the

outer kinetochore plate, finding instead that the outermost detectable DNA in the centromere is located in the inner kinetochore plate. Our results imply that the outer kinetochore plate is primarily a proteinaceous structure. It is thus unlikely that fibers observed in the outer plate correspond to chromatin, as previously assumed. Our observations suggest that current models of kinetochore structure may need to be reconsidered.

MAMMALIAN mitotic chromosomes attach to the spindle at the kinetochore, a surface specialization along the outer margin of the centromere. The kinetochore is often described as being trilamellar, with an outer dense plate separated from an inner plate by an electron lucent middle "plate," each plate being 15–35 nm thick (Rieder, 1982). The middle plate is considered by many workers to represent a space within the kinetochore (Rattner and Bazett-Jones, 1989; Zinkowski et al., 1991), whereas the inner plate appears to be composed of a highly condensed layer of centromeric heterochromatin. If microtubules are absent, an ill-defined fibrous corona is observed to extend beyond the outer plate (Rieder, 1982).

It has long been believed that the location of the kinetochore on the chromosome is determined by specialized *cis*-acting DNA sequences (called CEN sequences). These sequences have been isolated from fungi, and their structural and functional characterization has led to the development of a new paradigm for the kinetochore as a protein:DNA complex (Schulman and Bloom, 1991). Recent experiments performed *in vitro* demonstrate that the 125-bp *Saccharomyces cerevisiae* CEN DNA sequence directs the assembly of a nucleoprotein complex that is capable of binding microtubules (Kingsbury and Koshland, 1991) and translocating along them in an ATP-dependent manner *in vitro* (Hyman et al., 1992).

Although the mammalian equivalent to the yeast CEN DNA sequences has not yet been identified, workers in the field assume that such sequences must exist and will likely be located either in or immediately adjacent to the kinetochore. Beginning in 1980, studies from a number of laboratories led to the development of a widespread view that DNA in the form of chromatin fibers is an integral component of the outer kinetochore plate (see Discussion). This view was

recently codified in a model for the organization of the human centromere based on the assumption that repeating satellite DNA monomers fold in a highly organized fashion to make up the outer plate of the kinetochore (Zinkowski et al., 1991).

One potential weakness of this emerging view of the mammalian kinetochore is the lack of detailed information confirming the distribution of DNA in the trilamellar structure. For example, the majority of evidence for the presence of DNA in the outer kinetochore plate has been indirect, primarily as a result of the lack of readily accessible high resolution techniques that can specifically label DNA for detection at the EM level. Recent technical developments, however, now make such studies possible.

In the present study, we have examined the overall distribution of DNA in the mammalian kinetochore using two independent techniques. The first involves immunoelectron microscopy with a commercially available mAb that recognizes DNA and RNA, coupled with detection using 1-nm gold probes. The second involves the application of a sensitive and specific stain for DNA, osmium ammine-B, which has only recently become readily available (Olins et al., 1989), and which we have detected using the sensitive method of electron spectroscopic imaging (ESI) (Bazett-Jones, 1992). Contrary to currently accepted models of kinetochore structure, we have been unable to detect DNA in the outer kinetochore plate. In contrast, DNA is readily detected in the inner plate. These observations suggest that the outer kinetochore plate is unlikely to be composed of chromatin fibers, and may instead be primarily a proteinaceous structure. The results of nuclease digestion of chromosomes *in situ* are consistent with this view. We find that if chromosomes associated with taxol-stabilized microtubules are extensively digested with nucleases, the entire body of the

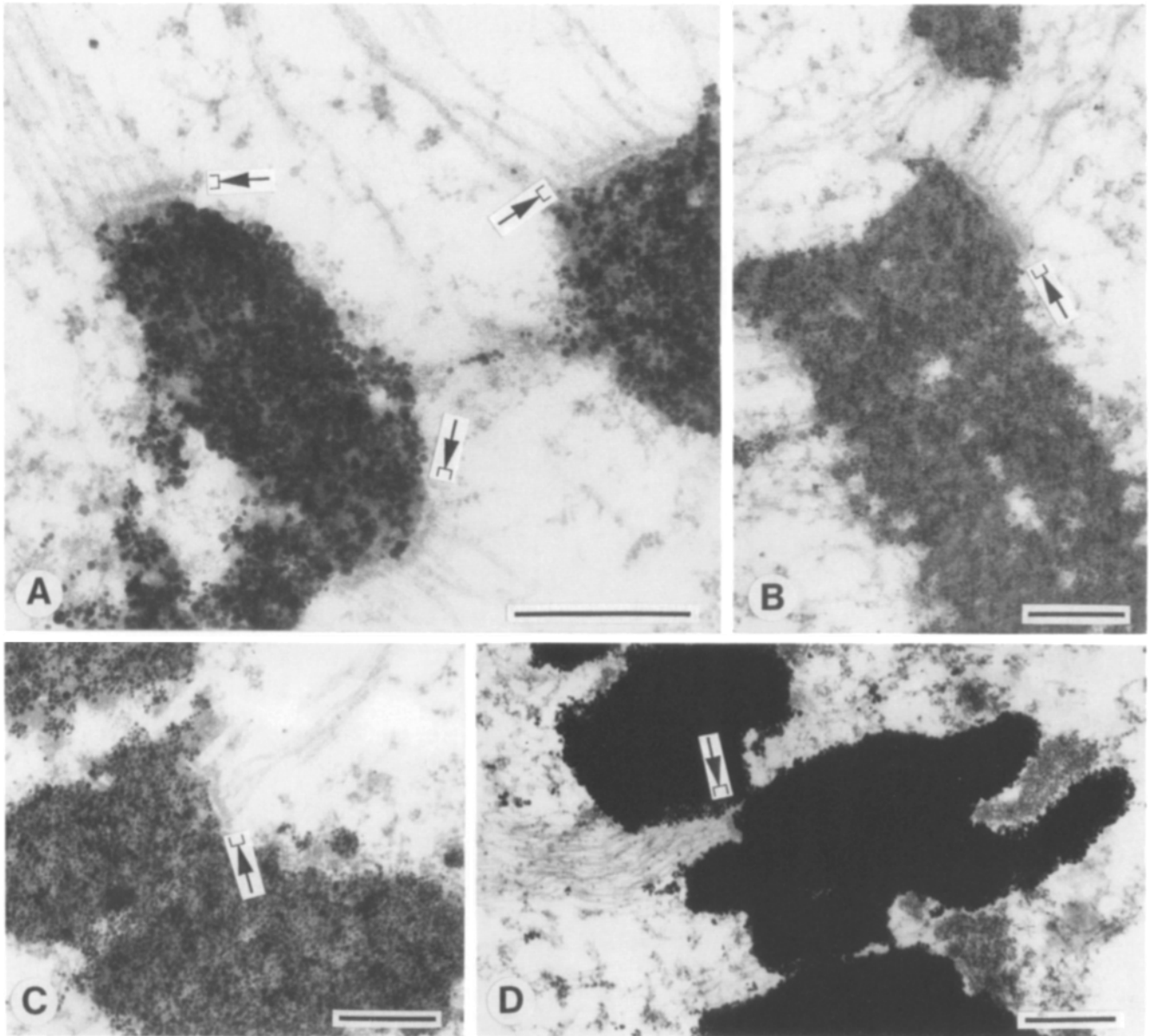


Figure 1. Immunolocalization of DNA in the centromere using a mAb. Thin sections of mitotic Indian muntjac (*A–C*) and HeLa (*D*) were reacted with a mAb to DNA. Bound antibody was subsequently detected with anti-mouse conjugated to 1-nm colloidal gold. In all cases the chromosomes were very heavily labeled, but no label was observed on the outer kinetochore plate (indicated by *arrows*). Immunogold labeling of the inner kinetochore plate is seen particularly clearly in *C* and *D*. Bars, 0.5 μm .

chromosome disappears, leaving behind bundles of microtubules that terminate in what appear to be normal trilaminar plates.

Materials and Methods

Immunoelectron Microscopy

Mitotic cells were obtained by selective detachment, centrifuged, and the resultant cell pellet fixed with 1% glutaraldehyde in D-PBS (Earnshaw et al., 1984) for 30 min at room temperature. The cell pellet was then dehydrated to 70% ethanol and embedded in L.R. White. After baking at 45°C for 14 h, thin sections were cut and picked up with nickel or gold grids. The grids were incubated first with blocking solution (10 mM Na_2HPO_4 ; 120 mM NaCl; 3 mM KH_2PO_4 ; 120 mM NaN_3 ; 1% BSA, pH 7.4) for 30 min, and then with primary antibody (mouse anti-DNA,

MAB030, Chemicon International, Inc., Temecula, CA), diluted in blocking solution for 2 h. This antibody recognizes both dsDNA and ssDNA. After washing with blocking solution plus 0.05% Tween-20, grids were incubated on drops of secondary antibody (conjugated to 1 nm gold, Janssen) at a dilution of 1:50 in blocking solution plus 0.05% Tween-20 overnight at 4°C (Johnson and Rosenbaum, 1990). The grids were then washed with ddH₂O and silver enhanced using the Danscher Method (Danscher, 1981). The enhancement reaction was stopped by rinsing with ddH₂O, and the grids were counterstained with uranyl acetate and lead citrate before viewing in the electron microscope.

Osmium Ammine-B Staining

Mitotic Indian muntjac cells were collected by selective mitotic detachment from logarithmically growing cultures and fixed in 1% paraformaldehyde, 2% glutaraldehyde, and embedded in Lowicryl K4M at -35°C . Consecutive sections, 100–150 nm thick, were collected on 400 mesh gold grids and stained with either uranyl acetate or osmium ammine-B (Olins et al., 1989).

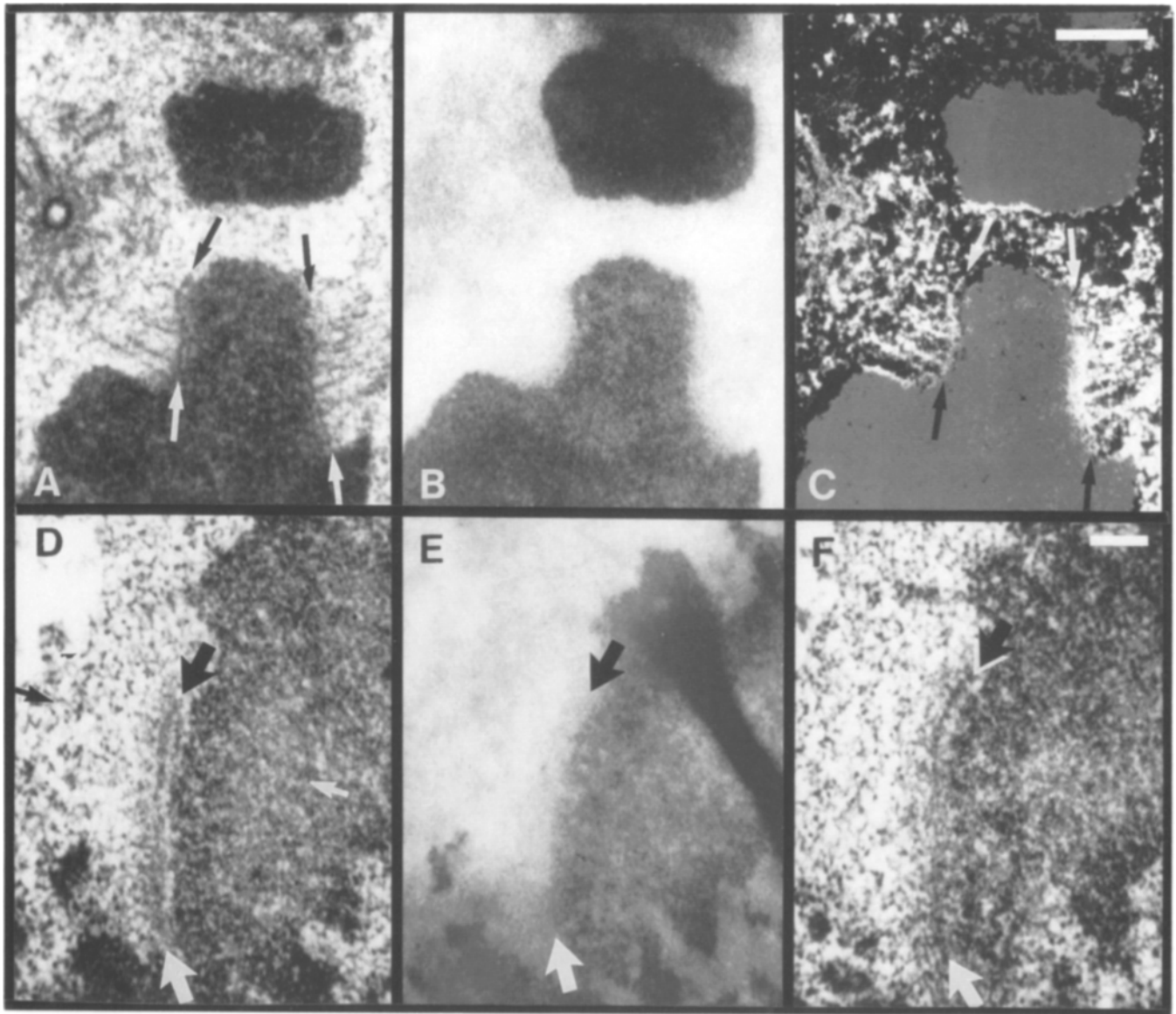


Figure 2. Electron micrographs of two Indian muntjac chromosomes (*A–B* two adjacent sections and *D–F* three consecutive sections) stained with either uranyl acetate and PTA (*A*, *D*, and *F*) or osmium ammine-B (*B* and *E*). Large arrows indicate the region occupied by the kinetochore outer plate. *C* is a computer subtraction of *A* from *B*. The structures appearing white, including the kinetochore outer plate, were not present in the osmium ammine-B-stained image of *B*.

Digested whole mount preparations of Indian muntjac chromosomes were prepared as described in Rattner (Rattner, 1986) and stained with either osmium ammine-B or ethanolic uranyl acetate followed by rotary shadowing with Platinum/Palladium 60:40. The sections and whole mounts were observed with an electron microscope equipped with an imaging electron spectrometer (EM-902; Carl Zeiss, Inc., Thornwood, NY). It was operated at 80 kV with a 700- μ m condenser aperture, a 60- μ m objective aperture, and a 15-eV energy selecting aperture. Sections stained with uranyl acetate and PTA or osmium ammine-B were imaged at an energy loss of 250 eV, a region of the spectrum where images of very high structure-sensitive contrast can be obtained (Bazett-Jones, 1992). Electron micrographs were digitized with a linear CCD camera to produce the difference image (*C*) and to obtain the optical density tracings. The tracings were obtained along the indicated line with a window five pixels wide, corresponding approximately to 30 nm. The average value of the five pixels at each point in the scan was plotted.

Nuclease Digestion of Chromosomes Attached to the Mitotic Spindle

Mitotic Indian muntjac cells were obtained by selective detachment, cen-

trifuged, and resuspended in media containing 5 μ g/ml taxol and 0.1% NP-40. 2 U of micrococcal nuclease (Pharmacia Fine Chemicals, Piscataway, NJ) were added to each 0.5-ml sample (containing 2×10^6 cells). Digestion was monitored by light microscopy. In most experiments, chromosomes in 90% of the mitotic cells were no longer visible after 3–5 min. For electron microscopy, preparations were fixed in 3% glutaraldehyde in Millonig's phosphate buffer. After a brief wash in buffer, the cells were postfixed for 1 h in 1% OsO₄ 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 of the resin, sections were cut in the silver range, stained with uranyl acetate and lead citrate, and examined in a Zeiss EM-902 at zero energy loss, operated at 80 kV.

Results

Immunoelectron Microscopy

In a first set of experiments, immunoelectron microscopy was used to detect DNA within the kinetochore of human

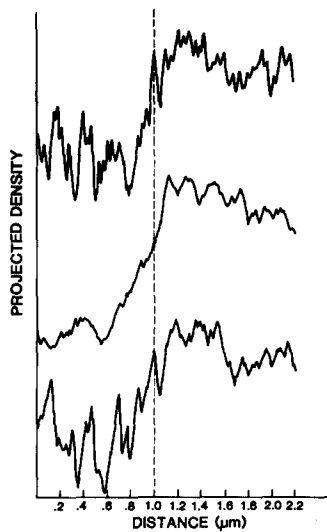


Figure 3. Density tracings of translationally aligned micrographs taken along the line indicated by the small arrows in Fig. 2 *D*. Projected density is plotted as a function of the distance from the point indicated by the left arrow in Fig. 2 *D*. The upper and lower traces correspond to the regions from Fig. 2, *D* and *F* (uranyl acetate-stained sections) respectively, and the middle trace is from Fig. 2 *E* (osmium ammine-B-stained section). The dotted line indicates the density peak corresponding to the kinetochore outer plate, identified in the upper and lower scans, but not apparent in the middle scan from the image of the osmium ammine-B stained section.

and Indian muntjac chromosomes using a procedure designed to minimize conditions that might lead to masking of the epitopes on DNA from the antibody. This particular antibody and method were previously used to demonstrate that DNA is not a prominent component of basal bodies in *Chlamydomonas*. In that study mitochondrial DNA was readily detected (Johnson and Rosenbaum, 1990).

The procedure gave a substantial level of labeling of the chromosome, with closely packed gold particles often completely obscuring the underlying chromatin (Fig. 1). Nonetheless, in every case where the outer kinetochore plate was observed, this structure was completely unlabeled (Fig. 1). Similarly, the region occupied by the middle layer of the kinetochore was also unlabeled. In contrast, we typically detected antibody binding to the region of the inner kinetochore plate (Fig. 1 *C*). The gold particles bound to this region lined the outer margin of the inner plate and closely followed the contour of the kinetochore as reflected by the outer kinetochore plate profile (Fig. 1 *C*). Identical results were obtained using both Indian muntjac (Figs. 1, *A-C*) and human (Fig. 1 *D*) chromosomes.

Detection of DNA with Osmium Ammine-B

In a second set of experiments a DNA specific stain, osmium ammine-B, and energy loss imaging were used to determine the distribution of DNA in thin serial sections of mitotic Indian muntjac cells. Fig. 2 *A* illustrates a section through the centromere region of a Y1 chromosome stained for both DNA and protein with uranyl acetate followed by phosphotungstic acid (PTA). Microtubules are seen inserting into each of the sister kinetochores with their prominent outer plates. In an adjacent serial section (Fig. 2 *B*) stained instead with osmium ammine-B, the body of the chromosome including the region occupied by the kinetochore inner plate is darkly stained, but the region containing the kinetochore outer plates shows no detectable reaction with the stain. Computer subtraction of Fig. 2 *A* from *B* (shown in Fig. 2 *C*) illustrates that only regions interior to the kinetochore outer and middle plates react with the osmium ammine-B

stain. Regions unstained by osmium ammine-B appear white in Fig. 2 *C*, while regions stained by the DNA-specific stain appear as middle gray levels in the difference image. In the cytoplasm adjacent to the Y1 centromere is a centrosome displaying profiles of both the parent and daughter centrioles as well as an abundance of pericentriolar material (Fig. 2 *A*). The corresponding osmium ammine-B image (Fig. 2 *B*) illustrates that none of the components of this region included within this section specifically react with the stain.

Three consecutive serial sections through a kinetochore of a prometaphase X+3 chromosome of the Indian muntjac that had yet to attach to the spindle are illustrated in a second example, Fig. 2, *D-F*. As in the previous example, the kinetochore outer plates are visible only in sections stained for both DNA and protein with uranyl acetate and PTA (compare Fig. 2, *D* and *F* with *E*). The surface of the chromosome in the region of the inner plate has a solid, well defined profile in the osmium ammine-B stained image (Fig. 2 *E*). Linear density tracings were made from Fig. 2, *D-F* and are presented in Fig. 3. These tracings permit the precise alignment of the images in Fig. 2, *D-F*, and confirm that staining is confined to the inner plate of the kinetochore (as well as the body of the chromosome).

Examination of Nuclease-digested Chromosomes

To study the relationship of DNA to the kinetochore and its component fibers in more detail, we prepared both thin section and whole mount preparations of muntjac metaphase cells and chromosomes after varying degrees of nuclease treatment. When mitotic cells were permeabilized with NP-40 and subjected to mild nuclease digestion, variable amounts of centromeric heterochromatin could be detected in association with the kinetochore, which retained its trilaminar structure (Fig. 4 *A*). Surprisingly, the trilaminar kinetochore morphology persisted after more extensive digestion of the DNA, which eliminated all detectable chromosomal DNA. In these samples, the spindle microtubules (stabilized with taxol) were clearly seen to end in trilaminar structures despite the fact that no other visible trace of the chromosomes remained (Fig. 4 *B*). Thus the kinetochore plate structures were resistant to these nuclease treatments, provided that spindle microtubules were present.

We showed previously that fragments derived from the centromere regions could be released from muntjac chromosomes after mild nuclease digestion under conditions where microtubules were absent (Rattner, 1986). When examined by whole mount microscopy in the absence of stain, these fragments have a homogeneous appearance (Fig. 5 *A*). In contrast, specimens stained with uranyl acetate and rotary shadowed with platinum: palladium showed well defined darkly staining kinetochore regions with a fibrous substructure (Fig. 5 *B*). It has been suggested that this fibrous area is a remnant of the kinetochore outer plate, a proposal supported by both whole mount (Rattner, 1986) and scanning EM studies (Rattner, 1987), and consistent with the architecture of the outer plate seen in thin sections of intact kinetochores (Ris and Witt, 1981; McEwen et al., 1993).

After osmium ammine-B staining, the region corresponding to the kinetochore remnant appears as a roughly circular unstained "ghost" with well defined margins, surrounded and superimposed on intensely staining heterochromatin (Fig. 5

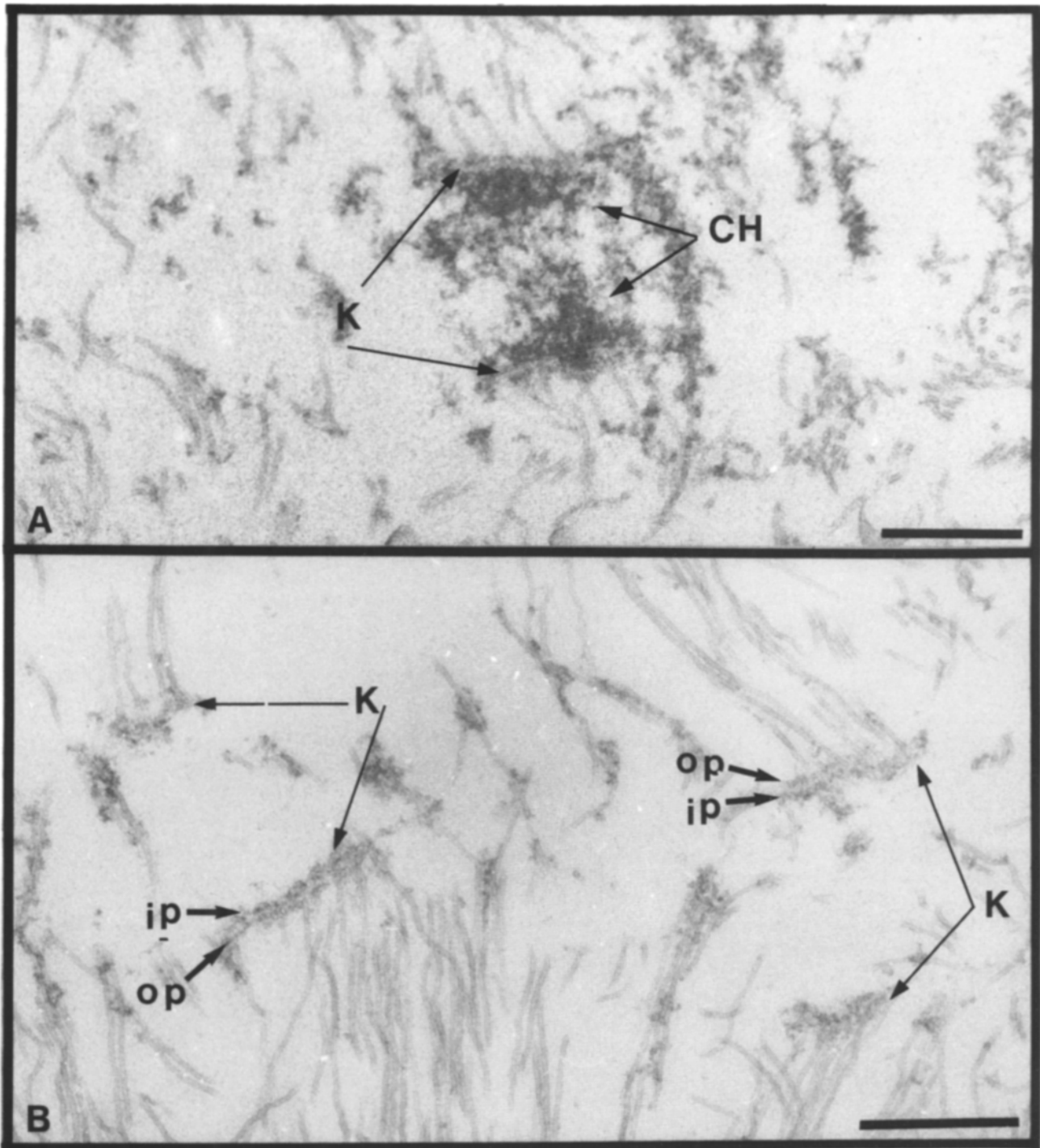


Figure 4. Nuclease-digested chromosomes associated with microtubules retain trilaminar kinetochores. (A) An electron micrograph of a chromosome fragment seen within a Taxol-stabilized Indian muntjac spindle following brief nuclease digestion. The fragment contains sister kinetochores (*K*) with a characteristic trilaminar morphology, associated with a small amount of residual centromeric heterochromatin (*CH*). (B) An electron micrograph of an extensively digested Taxol-stabilized Indian muntjac spindle depleted of detectable chromosomal DNA. Two sets of sister kinetochores (*K*) are illustrated. Each kinetochore is composed of both an outer (*op*) and inner (*ip*) plate. Bars, 0.5 μm .

C). This figure also illustrates that the osmium ammine-B stain is capable of revealing individual nucleosomes and extended 10-nm chromatin fibers. A diagrammatic representation of the image shown in Fig. 5 C, showing the relationship of the DNA to the kinetochore area, is presented in Fig. 5 E. The centromere region from a chromosome comparable

with the one shown in Fig. 5 C, stained with uranyl acetate and rotary shadowed, is illustrated in Fig. 5 D. Again, it is clear that under these conditions of nuclease digestion the kinetochore with its fibrous substructure is present on these centromere fragments. After more extensive digestion, the region occupied by the kinetochore outer plate still appears

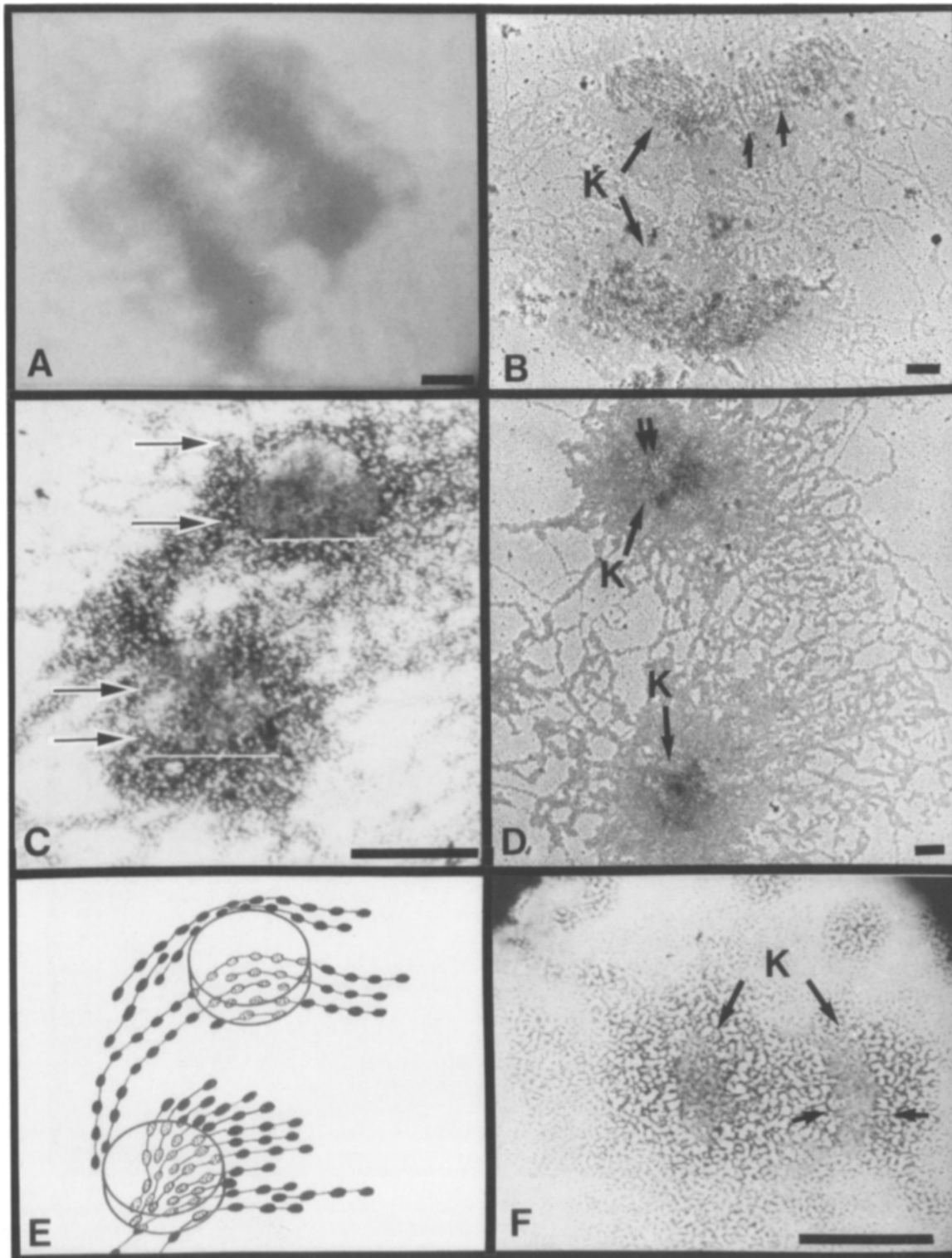


Figure 5. Osmium ammine-B does not detect DNA in kinetochores detached from chromosomes by mild nuclease treatment. (A) Unstained whole mount preparation of the centromere region of an Indian muntjac X+3 chromosome. (B) Centromere and Kinetochore 'K' regions from a nuclease digested Indian muntjac X+3 chromosome comparable to that illustrated in A, stained with uranyl acetate and rotary shadowed with Platinum:Palladium (see Rattner, 1986). (C) Comparable image stained with osmium ammine-B without rotary shadowing. Arrows and underlining denote the areas occupied by the kinetochore outer plate which appears as a well defined osmium ammine-negative roughly spherical "ghost" surrounded by densely stained DNA fibers. Our interpretation of this image (diagrammed in E) is that the underlying heterochromatin looks different because it is overlaid with the remnant of the outer plate that is not stained with osmium ammine-B. (D) A centromere region containing kinetochores (K), comparable with that shown in C, stained with uranyl acetate and rotary shadowed with Platinum:Palladium. (E) A diagrammatic representation of Fig. 4 C showing the position of the kinetochore outer plate on top of the residual chromosomal DNA. (F) As in C, extensively digested centromeric fragment stained with osmium ammine-B showing unstained sister kinetochore outer plates (K) overlaid with positively staining heterochromatin, small arrows. Bars, 0.3 μ m.

as an osmium ammine-B-negative patch with well defined margins that could only be identified because of the inherent electron density of the structure (Fig. 5 F). Residual fragments of osmium ammine-B-stained DNA are detected passing under this structure and lateral to the kinetochore area (Fig. 5 F, *small arrows*), indicating that they are not the fibrous components of the kinetochore seen in uranyl acetate-stained images.

Thus, the whole mount preparations confirm that the kinetochore domain contains a prominent component that does not react with osmium ammine-B even under conditions in which the structure is directly exposed to the stain. In addition, when the various images are compared, it is clear that the fibrous component of the kinetochore seen in conventional images (Fig. 5, B and D) is not detected in comparable osmium ammine-B-stained preparations (Fig. 5, C and F). This argues that the major fibrous component of the kinetochore outer plate is unlike the chromatin seen in the remainder of the chromosome.

Discussion

DNA Is an Integral Component of Yeast Kinetochores

Perhaps the most compelling argument that the kinetochore should contain DNA comes from analysis of the kinetochores of the budding yeast, *S. cerevisiae*. The cloning of functionally autonomous centromeric (CEN) DNA sequences from this organism (Clarke and Carbon, 1980) confirmed that the ability of chromosomes to interact with the mitotic spindle could be directed by a relatively short (125 bp) region of DNA (reviewed in Schulman and Bloom, 1991). CEN DNA in budding yeast does not appear to direct the assembly of a morphologically distinct kinetochore like that seen in mammalian cells: when yeast spindles were examined by EM, the microtubules appeared to terminate directly on chromatin fibers (Peterson and Ris, 1976).

By analogy with the structure of yeast centromeres, it is generally assumed that mammalian centromeres also contain functional CEN DNA sequences that determine the sites of chromosome-spindle interaction. Mammalian CEN DNA, however, has not yet been identified, perhaps both because of the current lack of a clear cut assay for such sequences and because of the vastly greater complexity of the mammalian centromere. Mammalian centromeres are considerably larger than those of either the budding or fission yeast, spanning regions of DNA of up to several megabases in size. Functional CEN DNA analogous to that studied in the yeasts presumably comprises only a small proportion of this centromeric DNA. Furthermore, the dissection of these large centromeres is complicated by the presence of large amounts of highly reiterated DNA sequences, known as satellite DNA (Singer, 1982; Willard, 1990). This satellite DNA could function in interactions with the spindle (see Haaf et al., 1992), or its role might be primarily in establishing the specialized constricted structure observed at centromeres of higher eukaryotic chromosomes. Satellite DNA is known to be largely comprised of nucleosomal chromatin (reviewed in Simpson, 1990), although at least one specialized satellite DNA binding protein has been identified (Masumoto et al., 1989).

Studies of the yeast kinetochore have progressed to a level

where they are beginning to offer detailed information about the interaction of CEN DNA and microtubules. The problem in applying this information to the analysis of mammalian kinetochore function is in determining wherein the structural and functional homologies lie. Because budding yeast lack morphologically distinct trilaminar kinetochores, it is unclear whether the genetically and biochemically defined CEN nucleoprotein complex corresponds to one of the layers of the mammalian kinetochore (presumably the outer plate, since this is predominantly where microtubules attach), or whether the yeast kinetochore is a miniaturized version of the entire trilaminar structure. In any case, the functional analogy between yeast and mammalian kinetochores provides intellectual impetus for the concept that the outer kinetochore plate might be a specialized chromatin structure containing CEN DNA.

DNA May Not Be a Major Component of the Outer Kinetochore Plate of Mammalian Chromosomes

In the present study, both immunoelectron microscopy and osmium ammine-B staining have failed to reveal DNA within the outer layer of the mammalian kinetochore while clearly showing that the inner plate of the kinetochore does contain DNA. We do not interpret these results as conclusive proof that there is no DNA in the outer kinetochore plate, but rather as a strong indication that DNA, if present, is a minor component.

This view of the outer kinetochore plate is consistent with the finding that the outer plate persists after extensive nuclease digestion of taxol-stabilized mitotic spindles, a treatment that appears to eliminate the entire body of the chromosome. The apparent preservation of the inner plate in these residual kinetochores indicates that, in addition to DNA, this layer of the kinetochore may also contain an organized protein framework. The ability of kinetochores to retain their association with microtubules after mild nuclease digestion has been noted in an earlier study (Rattner et al., 1975). The persistence of this interaction after extensive digestion implies that intact chromatin fibers are not essential for kinetochore-microtubule interactions.

Two experimental difficulties could in theory prevent us from detecting DNA in the outer kinetochore plate. First, our staining methods might lack the necessary sensitivity. This is unlikely if, as proposed in recent models for kinetochore structure (Ris and Witt, 1981; Rattner, 1986; Zinkowski et al., 1991), the outer plate is assembled from close-packed 10–30-nm chromatin fibers. In this case, a volume equivalent to that of the outer plate in human would contain as much as 100 kb DNA, with correspondingly much greater amounts of DNA in the muntjac. Both the antibody and osmium ammine-B methods have been used to visualize mitochondrial DNAs of 16 kb (Johnson and Rosenbaum, 1990; Liu et al., 1991). In fact, osmium ammine-B is much more sensitive, being able to detect the DNA of single nucleosomes as shown in our work (Fig. 5 C) and that of others (Woodcock et al., 1990).

A second potential experimental difficulty might be the masking of the DNA by tightly bound proteins. This is always a potential problem with antibody studies, although we note that under our conditions the antibody readily recognizes DNA in the highly condensed chromatin throughout the body of the chromosome, including the centromere. Such

masking arguments are much less likely to apply to the staining of DNA by a small molecule like osmium ammine-B, particularly since the staining protocol that we have used involves floating the sections face down on a solution of 5 N HCl for 25 min to hydrolyze the DNA and expose active aldehyde groups (Olins et al., 1989).

Relationship with Previous Work

The only direct evidence for the presence of DNA in the outer kinetochore plate is the observation that the outer kinetochore plate of chromosomes from hypotonically treated chromosomes was stained with the Aggarwal Feulgen-Pt thymine method for DNA (which uses the same chemistry as the osmium ammine B procedure) (Aggarwal, 1976; Ris and Witt, 1981). However, such hypotonic pretreatments cause a substantial dispersion of the chromatin fibers. If these dispersed chromatin fibers were to adhere to the kinetochore plate, this could give rise to the observed staining. Alternatively, we cannot exclude that some aspect of the experimental protocol used by Ris and Witt (1981) enabled them to detect low levels of DNA in the kinetochore under conditions where we have been unable to do so.

All other evidence for the presence of DNA in the kinetochore is indirect. For example, the first evidence that the kinetochore outer plate might contain DNA came from studies in which chromosomes were exposed to nucleases: prolonged nuclease treatment caused the disruption of the outer plate (Pepper and Brinkley, 1980). This experiment does not provide direct evidence for the presence of DNA in the plate itself. The outer plate might lack DNA but still require specific interactions with underlying chromatin to maintain its structure. This would be consistent with the results of recent microinjection studies, which indicate that interference with assembly of the chromatin in the region subjacent to the kinetochore *in vivo* disrupts kinetochore structure (Bernat et al., 1991).

Numerous studies have revealed that the outer kinetochore plate has a fibrous substructure (Brinkley and Stubblefield, 1966; Comings and Okada, 1971; Ris and Witt, 1981; Rattner, 1986; McEwen et al., 1993). This was first seen clearly when chromosome structure was perturbed by exposure to hypotonic solutions, which unraveled the kinetochore region, revealing a fibrous substructure (Ris and Witt, 1981). In certain instances microtubules were observed to end on filaments that resembled chromatin, providing a powerful analogy with the previous EM of yeast spindles (Peterson and Ris, 1976). Subsequent whole amount EM of kinetochore remnants generated by nuclease treatment of chromosomes revealed the presence of folded filaments 25–30 nm in diameter (Rattner, 1986).

From these studies it was suggested that these fibers in the outer plate were chromatin fibers. At the same time, the authors noted significant differences between the kinetochore fibers and those of the centromeric heterochromatin. Although the kinetochore fibers have a diameter of 10–30 nm, similar to that of chromatin, they respond differently to alterations in ionic strength (Ris and Witt, 1981), appear more electron dense, and show a heightened resistance to nuclease treatment (Rattner, 1986), as demonstrated dramatically in Fig. 4.

A final argument for the presence of DNA in the outer kinetochore plate came from a previous study in which we

used electron spectroscopic imaging (ESI) to demonstrate that this structure contains significant levels of phosphorus (Rattner and Bazett-Jones, 1989). The outer plate does not appear to contain significant levels of RNA, as detected by the EDTA regressive staining procedure (Rieder, 1979). Thus, this result appeared to implicate the presence of DNA, although we argued that the staining could reflect the presence of phosphoproteins (Rattner and Bazett-Jones, 1989). ESI can detect phosphate in proteins: for example, microtubules in the vicinity of the kinetochore also produced a significant phosphate signal (Rattner and Bazett-Jones, 1989). We therefore favor the interpretation that the phosphorus signal in the outer plate is due to phosphoprotein. This suggestion is in agreement with the finding that the anti-phosphoprotein antibody MPM-2 reacts with the kinetochore region (Vandre et al., 1984).

What Is the Composition of the Outer Kinetochore Plate?

Since the discovery of the trilaminar kinetochore, this structure has been the focus of repeated morphological investigations. These studies have for the most part agreed that the most prominent feature of the kinetochore is an outer plate composed of fibers 10–30 nm in diameter. This outer plate forms the major site of microtubule attachment to the chromosome, an interaction about which little is known, apart from a single study which indicated that microtubules bind directly to the kinetochore fiber (Ris and Witt, 1981). Over the years, it has become widely accepted that these kinetochore fibers represent chromatin. Our present study now indicates that this is unlikely to be the case. DNA, if present at all in the outer plate, is likely to be only a very minor component. Thus, our results are most consistent with a model where the outer kinetochore plate is primarily a proteinaceous structure that assembles on the surface of the centromere, perhaps in a DNA-dependent fashion.

Our results do not rule out an alternative model where loops of DNA corresponding to the very abbreviated CEN sequence of budding yeast extend out from the inner plate into the outer plate and form the sites of microtubule attachment. Indeed, connections between the inner and outer plates have been seen in several morphological investigations of the kinetochore (Ris and Witt, 1981; Rattner and Bazett-Jones, 1989; McEwen et al., 1993). In the human, where each kinetochore only binds ~20 microtubules (Rieder, 1982), it is conceivable that in such a model the entire outer plate would contain as little as 2 kb of DNA. We cannot rule out the possibility that such a low level of DNA might have escaped detection in the present study.

Nonetheless, the bulk of the mass of the outer plate, including the prominent fibers, must be composed primarily of protein, and presumably serves to hold the microtubule binding sites in a proper quaternary structure. The importance of this higher-order structure for kinetochore function has been demonstrated by antibody microinjection experiments, where assembly of the characteristic trilaminar structure was disrupted (Bernat et al., 1991). Such kinetochores appeared to bind normal numbers of microtubules, but were unable to move chromosomes along them (Bernat et al., 1990, 1991).

Paradoxically, although the outer plate is morphologically the most prominent component of the kinetochore, it re-

mains the least characterized component of this structure biochemically. At the present time no component of the outer plate has been identified unambiguously. For example, none of the known centromere proteins has been shown by immunoelectron microscopy to localize in this region. At the same time, components of both the fibrous corona (Wordeman et al., 1991) and inner plate (Saitoh et al., 1992) have been identified and localized. Thus, there is a real need to identify bona fide protein components of the outer kinetochore plate. It may be that such components will be found to bind DNA and, if so, the question of the distribution of DNA in the kinetochore may then be approached from a new perspective.

Whatever the solution to the problem of kinetochore structure eventually turns out to be, our results indicate that current models of kinetochore organization need to be re-evaluated. In particular, we can conclude with confidence that the outer plate is unlikely to be composed of packed 10–30-nm chromatin fibers, as has been proposed in recent models of kinetochore structure (Ris and Witt, 1981; Rattner, 1986; Zinkowski et al., 1991).

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References

- Aggarwal, S. K. 1976. Platinum-pyrimidine complexes for electron microscopic cytochemistry of deoxyribonucleic acid. *J. Histochem. Cytochem.* 24:984–992.
- Bazett-Jones, D. P. 1992. Electron spectroscopic imaging of chromatin and other nucleoprotein complexes. *Electron Microsc. Rev.* 5:37–58.
- Bernat, R. L., G. G. Borisy, N. F. Rothfield, and W. C. Earnshaw. 1990. Injection of anticentromere antibodies in interphase disrupts events required for chromosome movement at mitosis. *J. Cell Biol.* 111:1519–1533.
- 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.
- Brinkley, B. R., and E. Stubblefield. 1966. The fine structure of the kinetochore of a mammalian cell in vitro. *Chromosoma (Berl.)* 19:28–43.
- Clarke, L., and J. Carbon. 1980. Isolation of a yeast centromere and construction of functional small circular chromosomes. *Nature (Lond.)* 287:504–509.
- Comings, D. E., and T. A. Okada. 1971. Fine structure of kinetochore in Indian muntjac. *Exp. Cell Res.* 67:97–110.
- Dansch, G. 1981. Localization of gold in biological tissue: a photochemical method for light and electron microscopy. *Histochemistry.* 71:81–88.
- Earnshaw, W. C., N. Halligan, C. Cooke, and N. Rothfield. 1984. The kinetochore is part of the chromosome scaffold. *J. Cell Biol.* 98:352–357.
- Haaf, T., P. E. Warburton, and H. F. Willard. 1992. Integration of human α -satellite DNA into simian chromosomes: centromere protein binding and disruption of normal chromosome segregation. *Cell.* 70:1–20.
- Hyman, A. A., K. Middleton, M. Centola, T. J. Mitchison, and J. Carbon. 1992. Microtubule-motor activity of a yeast centromere-binding protein complex. *Nature (Lond.)* 359:533–536.
- Johnson, J. A., and J. L. Rosenbaum. 1990. The basal bodies of *Chlamydomonas reinhardtii* do not contain immunologically detectable DNA. *Cell.* 62:615–619.
- Kingsbury, J., and D. Koshland. 1991. Centromere-dependent binding of yeast minichromosomes to microtubules in vitro. *Cell.* 66:483–495.
- Liu, D. F., M. El-Alfy, and C. P. Leblond. 1991. Visualization of DNA within mitochondria by osmium-amine staining of mouse duodenal crypt cells. *J. Cell Sci.* 101:785–793.
- Masumoto, H., H. Masukata, Y. Muro, N. Nozaki, and T. Okazaki. 1989. A human centromere antigen (CENP-B) interacts with a short specific sequence in alphoid DNA, a human centromeric satellite. *J. Cell Biol.* 109:1963–1973.
- McEwen, B. F., J. T. Arena, J. Frank, and C. L. Rieder. 1993. Structure of the colcemid treated PtK1 kinetochore outer plate as determined by high voltage electron microscopic tomography. *J. Cell Biol.* 120:301–312.
- Olins, A. L., B. A. Moyer, S.-H. Kim, and D. P. Allison. 1989. Synthesis of a more stable osmium ammine electron-dense DNA stain. *J. Histochem. Cytochem.* 37:395–398.
- Pepper, D. A., and B. R. Brinkley. 1980. Tubulin nucleation and assembly in mitotic cells: evidence for nucleic acids in kinetochores and centrosomes. *Cell Motil. Cytoskeleton.* 1:1–15.
- Peterson, J. B., and H. Ris. 1976. Electron microscopic study of the spindle and chromosome movement in the yeast *Saccharomyces cerevisiae*. *J. Cell Sci.* 22:219–242.
- Rattner, J. B. 1986. Organization within the mammalian kinetochore. *Chromosoma (Berl.)* 93:515–520.
- Rattner, J. B. 1987. The organization of the mammalian kinetochore: a scanning electron microscope study. *Chromosoma (Berl.)* 95:175–181.
- Rattner, J. B., and D. P. Bazett-Jones. 1989. Kinetochore structure: electron spectroscopic imaging of the kinetochore. *J. Cell Biol.* 108:1209–1219.
- Rattner, J. B., A. Branch, and B. A. Hamkalo. 1975. Electron microscopy of whole mount metaphase chromosomes. *Chromosoma (Berl.)* 52:329–338.
- Rieder, C. L. 1979. Localization of ribonucleoprotein in the trilaminar kinetochore of PtK1. *J. Ultrastruct. Res.* 66:109–119.
- Rieder, C. L. 1982. The formation, structure and composition of the mammalian kinetochore and kinetochore fiber. *Int. Rev. Cytol.* 79:1–58.
- Ris, H., and P. L. Witt. 1981. Structure of the mammalian kinetochore. *Chromosoma (Berl.)* 82:153–170.
- Saitoh, H., J. E. Tomkiel, C. A. Cooke, H. R. Ratrie, M. Maurer, N. F. Rothfield, and W. C. Earnshaw. 1992. CENP-C, an autoantigen in scleroderma, is a component of the human inner kinetochore plate. *Cell.* 70:115–125.
- Schulman, I., and K. S. Bloom. 1991. Centromeres: an integrated protein/DNA complex required for chromosome movement. *Annu. Rev. Cell Biol.* 7:311–336.
- Simpson, R. T. 1990. Nucleosome positioning: occurrence, mechanisms, and functional consequences. *Prog. in Nucleic Acid Res. and Mol. Biol.* 40:143–184.
- Singer, M. F. 1982. Highly repeated sequences in mammalian genomes. *Int. Rev. Cytol.* 76:67–112.
- Vandre, D. D., F. M. Davis, P. N. Rao, and G. G. Borisy. 1984. Phosphoproteins are components of mitotic microtubule organizing centers. *Proc. Natl. Acad. Sci. USA.* 81:4439–4443.
- Willard, H. F. 1990. Centromeres of mammalian chromosomes. *Trends Genet.* 6:410–416.
- Woodcock, C. L., R. A. Horowitz, D. P. Bazett-Jones, and A. L. Olins. 1990. Localization of DNA in chromatin using electron spectroscopic imaging and osmium ammine staining. *In Proc. of XII Int. Congr. for Electron Microscopy.* L. V. Peachey and D. D. Williams, editors. San Francisco Press Inc., San Francisco, CA. 3:116–117.
- Wordeman, L., E. Steurer, M. Sheetz, and T. Mitchison. 1991. Chemical subdomains within the Kinetochore domain of isolated CHO mitotic chromosomes. *J. Cell Biol.* 114:285–294.
- Zinkowski, R. P., J. Meyne, and B. R. Brinkley. 1991. The centromere-kinetochore complex: a repeat subunit model. *J. Cell Biol.* 113:1091–1110.