CENP-B: A Major Human Centromere Protein Located beneath the Kinetochore

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Abstract. The family of three structurally related autoantigens CENP-A (17 kD), CENP-B (80 kD), and CENP-C (140 kD) are the best characterized components of the human centromere, and they have been widely assumed to be components of the kinetochore. Kinetochore components are currently of great interest since this structure, which has long been known to be the site of microtubule attachment to the chromosome, is now believed to be a site of force production for anaphase chromosome movement. In the present study we have mapped the distribution of CENP-B in mitotic chromosomes by immunoelectron microscopy using two monospecific polyclonal antibodies together with a newly developed series of ultra-small 1-nm colloidal gold probes. We were surprised to find that >95% of CENP-B is distributed throughout the centromeric heterochromatin beneath the kinetochore. This strongly supports other emerging evidence that CENP-B is specifically associated with α-satellite heterochromatin. Although in certain instances CENP-B can be seen to be concentrated immediately adjacent to the lower surface of the kinetochore, the outer plate remains virtually unlabeled. Similar analysis with a human autoimmune serum that recognizes all three CENP antigens reveals an additional unsuspected feature of kinetochore structure. In addition to recognizing antigens in the centromeric heterochromatin, the autoantiserum recognizes a concentration of antigens lateral to the kinetochore. This difference in staining pattern may reflect the presence of a "collar" of chromatin rich in CENP-C and/or CENP-A encircling the kinetochore plates.

THE centromere of mitotic chromosomes serves at least three functions. (*a*) It provides the point of attachment for the spindle microtubules. (*b*) The regions of the centromere lateral to the kinetochore are the last points at which the sister chromatids adhere to one another. Thus, the centromere is the target for the cellular signal that triggers the separation of sister chromatids at the onset of anaphase. (*c*) The mechanochemical motor responsible for the movement of the chromatids to the spindle poles during anaphase A may be located at the centromere, perhaps as a component of the kinetochore (18, 22, 27, 33).

When mitotic chromosomes are examined by thin-section EM, the centromere appears as a dense region of chromatin with a single visible structural specialization, the kinetochore, at its surface (reviewed in reference 37). The kinetochore has been studied by EM since the mid 1960s (6, 20, 24, 37). It is composed of four layers: an inner dense plate that is closely apposed to the centromeric heterochromatin, an electron-lucent ("clear") zone, an outer dense plate, and an outermost fibrous corona. (See Fig. 10 for a map of the kinetochore.) The kinetochore plates are disk-shaped (20), thus, the structure resembles a multilayered button on the surface of the centromeric heterochromatin. The kinetochore has proven to be difficult to isolate, and relatively little is known about its protein and nucleic acid constituents.

Studies of the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* have provided virtually all we know about the specific DNA sequences that are required for centromere function. The centromere domain in *S. cerevisiae* is relatively small, corresponding to ~125 bp of CEN DNA (reviewed in 8, 17). In vivo this CEN DNA is found in a protected chromatin structure encompassing 250 bp of DNA flanked by nuclease hypersensitive sites (4). In contrast, the centromere of *S. pombe* is much larger, encompassing 40–100 kb of DNA (7, 19). In addition, *S. pombe* centromeres contain large repeated sequence elements that are present in 2–15 copies per centromere (7, 9, 16, 30, 31).

The centromeric DNA organization in higher eukaryotes differs significantly from that of the yeasts. Primate centromeres contain large amounts of repetitive DNA, the most widespread of which is the α-satellite family (38). α-satellite DNA is a complex superfamily of hierarchical repeated elements whose consensus is based on a monomer of ~171 bp (41, 43, 46). α-satellite DNA and its associated proteins are thought to be major components of the large blocks of centromere-associated constitutive heterochromatin.

The characterization of human centromere proteins has been made possible by the fortuitous discovery that certain patients with rheumatic diseases produce antibodies that recognize centromeres (28). In a pioneering study, such a patient serum was used to map the distribution of the autoantigens in chromosomes of the rat kangaroo by the immuno-

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peroxidase technique (5). The antigens were found to be restricted to the kinetochore and the chromatin immediately subjacent to it.

Sera from these patients recognize three polypeptides in immunoblots of chromosomal proteins from human cells (12). These antigens, which we designated CENP-A (17 kD), CENP-B (80 kD), and CENP-C (14 kD), were shown by immunological criteria to be structurally related (12). The results of the original immunoelectron microscopy study have been widely interpreted to indicate that all three of the CENP antigens are components of the kinetochore.

Given the recent interest in the role of the kinetochore in chromosome movements, it is clearly important to compile a definitive map of the protein components of this structure. Two technical advances have combined to enable us to begin to map the distribution of individual centromere proteins in human mitotic cells. First, we have developed high-titer monospecific antibodies for CENP-B (14). In addition, a recently developed series of ultra-small gold probes has enabled us to perform immunogold EM on chromosomal antigens in permeabilized cells for the first time.

Our study has yielded two unexpected results. First, we have observed strong binding of the anti-CENP-B antibodies to the centromeric heterochromatin beneath the kinetochore, and virtually no binding to the kinetochore itself. Second, staining of cells with autoimmune serum reveals the presence of a concentration of the centromere antigens in the chromatin that closely surrounds the kinetochore plates.

Materials and Methods

Buffers

The buffers used were: D-PBS (8.06 mM $Na_2HPO_4 \times 7H_2O$, 1.47 mM KH_2HPO_4, 137 mM NaCl, 2.7 mM KCl, 0.68 mM $CaCl_2$, 0.492 mM $MgCl_2$); KB (10 mM Tris: HCl pH 7.7, 150 mM NaCl, 0.1% BSA, 0.1% Triton X-100); KB^- (buffer KB minus Triton X-100); and TEEN (1.0 mM triethanolamine: HCl, pH 8.5, 0.2 mM NaEDTA, 25 mM NaCl).

Antisera

Anticentromere serum from scleroderma patient G. S. contains antibodies against the three major centromere autoantigens: CENP-A (17 kD); CENP-B (80 kD); and CENP-C (140 kD) (reference 12). Two rabbit polyclonal antibodies monospecific for CENP-B were also used. Antibody ra-ACA-1 was obtained by injection with a cloned fusion protein comprising 113 kD of β-galactosidase fused to the carboxy-terminal 147 amino acid residues of CENP-B (14). Antibody ra-ACA-2 was obtained by injection with a fusion protein comprising 32 kD of tryptophane synthetase fused to residues 6–599 of CENP-B. Immunoblotting of subcloned regions of CENP-B confirms that ra-ACA-2 recognizes determinants distributed along the entire length of the molecule (A. Pluta, unpublished observations). We do not yet have available high-titer monospecific antibodies against CENP-A or CENP-C.

Comparison of the binding of autoimmune serum and rabbit anti-CENP-B reveals that the autoimmune serum recognizes a number of additional determinants. These include, as expected, any unique epitopes on CENPs A and C, but also include epitopes on CENP-B. Autoantibodies affinity purified from CENP-B fusion proteins expressed in bacteria and experimental antibodies raised to them, are all monospecific for CENP-B. Thus, ra-ACA-1 and ra-ACA-2 recognize no other chromosomal proteins in immunoblots. (See, for example, reference 15.) A different result is obtained if autoantibodies are affinity purified from chromosomal CENP-B. In the case of serum G. S., such antibodies cross react strongly with CENP-A and weakly with CENP-C. (All autoantibodies that we have affinity purified from CENP-A rebind with apparent equal affinity to CENPs A and B.) We interpret this difference between the antibodies purified from the cloned and chromosomal proteins to indicate that one of the determinants recognized by autoimmune serum may involve a posttranslational modification of the CENP antigens. Such modifications are not typically performed in *E. coli*, and would thus be absent from CENP-B fusion proteins.

Immunoelectron Microscopy

Mitotic HeLa cells were shaken from tissue culture flasks and centrifuged at 450 *g* for 10 s onto glass coverslips. Interphase cells were grown on the coverslips and processed in situ. Cells were fixed in 4% formaldehyde (from paraformaldehyde) in D-PBS for 20 min and then placed in 50 mM NH_4Cl in D-PBS for 10 min. They were washed twice in KB for 5 min. Subsequent incubations in antibody (patient autoimmune serum G. S. 1:1,000 or ra-ACA-1 1:250) were diluted in KB. After 30 min at 37°C in antibody, the cells were washed twice in KB, placed in the appropriate biotinylated secondary antibody (Vector Laboratories, Burlingame, CA) for another 30 min at 37°C, and finally washed with KB^-. For immunolabeling of swollen chromosomes, cells were fixed, permeabilized with detergent, and incubated with first antibody in TEEN buffer. Subsequent washes and antibody incubations were performed in KB^- as above.

For immunogold labeling the cells were incubated in streptavidin or goat anti-rabbit IgG conjugated to 1 nm gold (Streptavidin Auroprobe 1; Janssen Life Sciences Products, Piscataway, NJ) diluted 1:100 in KB^- for 4 h at room temperature or 16 h at 4°C. After washes in KB^- and D-PBS, the cells were fixed in 2% glutaraldehyde with 0.02% tannic acid in D-PBS and silver enhanced using either IntenSe Silver Enhancement kit (Janssen Life Sciences Products) or the gum arabic/silver lactate method (11). Enhancement (enlargement) of gold particles with the IntenSe kit is apparently extremely cooperative. Certain grains become quite large, while many grains are apparently untouched, remaining undetectable. This technique was used for the images of Figs. 1, 2, and 7 *A*. When the gum arabic/silver lactate method (11) is used, many more enhanced gold grains of more uniform size are detected (our unpublished observations). The gum arabic/silver lactate method was used for the images of Figs. 3–6, 7, *B–D*, 8, and 9.

This procedure yielded good preservation of the metaphase plate, although microtubules were difficult to visualize in the final material. Poor visualization of tubules in the electron microscope probably results from the omission of OsO_4 as a stain, since the method yielded excellent microtubule morphology when streptavidin:gold was replaced by streptavidin:Texas red and cells were examined by immunofluorescence. (OsO_4 had to be avoided, as it removes the metallic silver deposited around the gold grains during enhancement.)

All coverslips were dehydrated through graded ethanol and embedded in Polybed/812. Thin (silver) sections were cut and placed on copper grids. Cells labeled with gold were stained with uranyl acetate and lead citrate. Those labeled with peroxidase were examined without further staining.

We believe that the labeling of the outer kinetochore plate occasionally observed by the immunoperoxidase method with anti-CENP-B (data not shown) may be in part due to diffusion of reaction product (3, 34). For comparison of gold and peroxidase labeling, the cells were processed as a single batch with primary antibody and biotinylated secondary antibody. At the final step, streptavidin:gold was added to some coverslips and streptavidin:peroxidase to the others. Thus, if the occasional labeling of the outer kinetochore plate observed by the immunoperoxidase method is real, this would require that binding of streptavidin:peroxidase to a primary antibody:biotinylated secondary antibody "sandwich" at the outer kinetochore plate occurs while binding of streptavidin:1 nm gold is inhibited. While there are many examples of epitope masking in immunolocalization studies, to our knowledge these have all involved inhibition of binding of the primary antibody. It seems unlikely that the binding of the third layer of such a pyramid of reagents would be specifically inhibited. We therefore favor the alternative explanation that while immunoperoxidase labeling is in general a reliable method, in this case a large amount of reaction product is produced in the centromeric heterochromatin, some of which may diffuse across the narrow lucent zone and be trapped in the outer plate.

Quantitation of Results

The relative levels of antigen detected in various regions throughout the centromere were determined by counting gold grains on the electron micrographs. The criteria applied for selection of centromeres to be scored has a significant impact on the results. We initially counted only gold grains from chromosomes with kinetochores clearly recognizable in the plane of section (method 1 in Table I). We adopted this strategy to ensure that we were only counting grains from well-fixed and sectioned cells where kinetochores could be visualized if present. Because gold grains present in planes

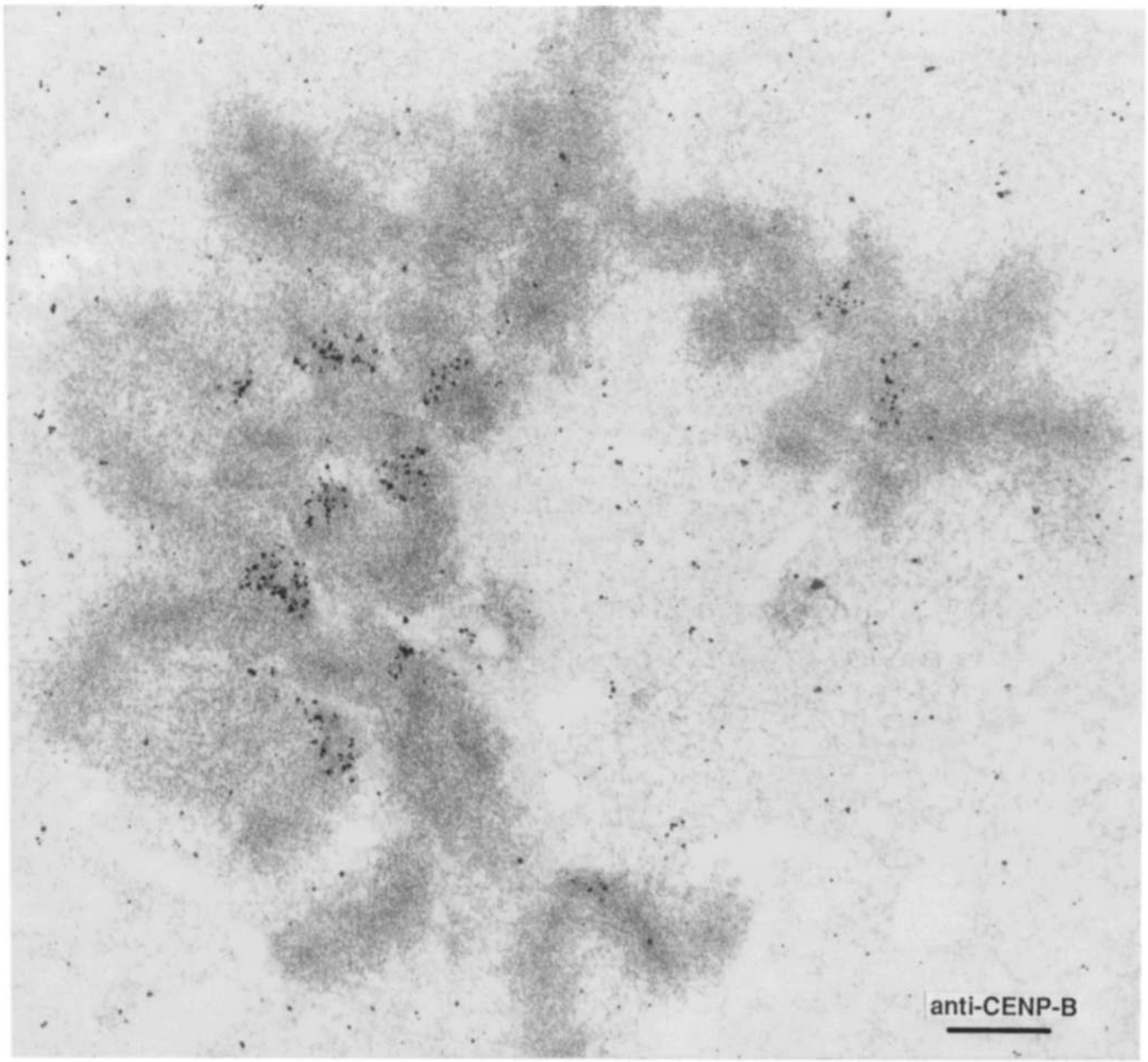

Figure 1. Localization of CENP-B in mitotic chromosomes in situ. Low magnification view of mitotic HeLa cell stained with rabbit serum ra-ACA-1 (monospecific for CENP-B [14]), followed by biotinylated antirabbit secondary antibody and streptavidin conjugated to 1 nm colloidal gold (which was enhanced [enlarged] as described in Materials and Methods). Note the extremely low level of nonspecific gold labeling of the chromosome arms. In this and all subsequent figures the bar indicates 1 μm.

of section that miss the kinetochore are not counted, and because at least 95% of CENP-B lies beneath the kinetochore, such an algorithm systematically excludes a significant portion of the CENP-B. We therefore adopted an alternative strategy to decrease this bias. We selected micrographs where at least one well-preserved kinetochore is visible and then counted all centromere-associated gold grains on all chromosomes (method 2 in Table I).

Results

CENP-B Is Located beneath the Kinetochore

When mitotic cells are processed with CENP-B-specific polyclonal antibodies, the centromere regions are heavily labeled with gold particles (Fig. 1). The background of gold particles over the chromosome arms is extremely low, while somewhat higher nonspecific background is observed over the cytoplasm.

The distribution of CENP-B relative to the kinetochore was determined in sections where the kinetochore plates were resolved in transverse section. Results obtained with two different polyclonal antibodies indicate that CENP-B is located in the heterochromatin beneath and around the kinetochore (Figs. 2 and 3). There is little or no labeling of the kinetochore plates. Previous studies demonstrated that these polyclonal antibodies recognize several sites on CENP-B and do not cross react with any other chromosomal proteins (14). The degree of staining varies on different centromeres, as it does when chromosomes stained with the antibodies are examined by immunofluorescence (14). The quantitative analysis of these labeling experiments is summarized in Table I.

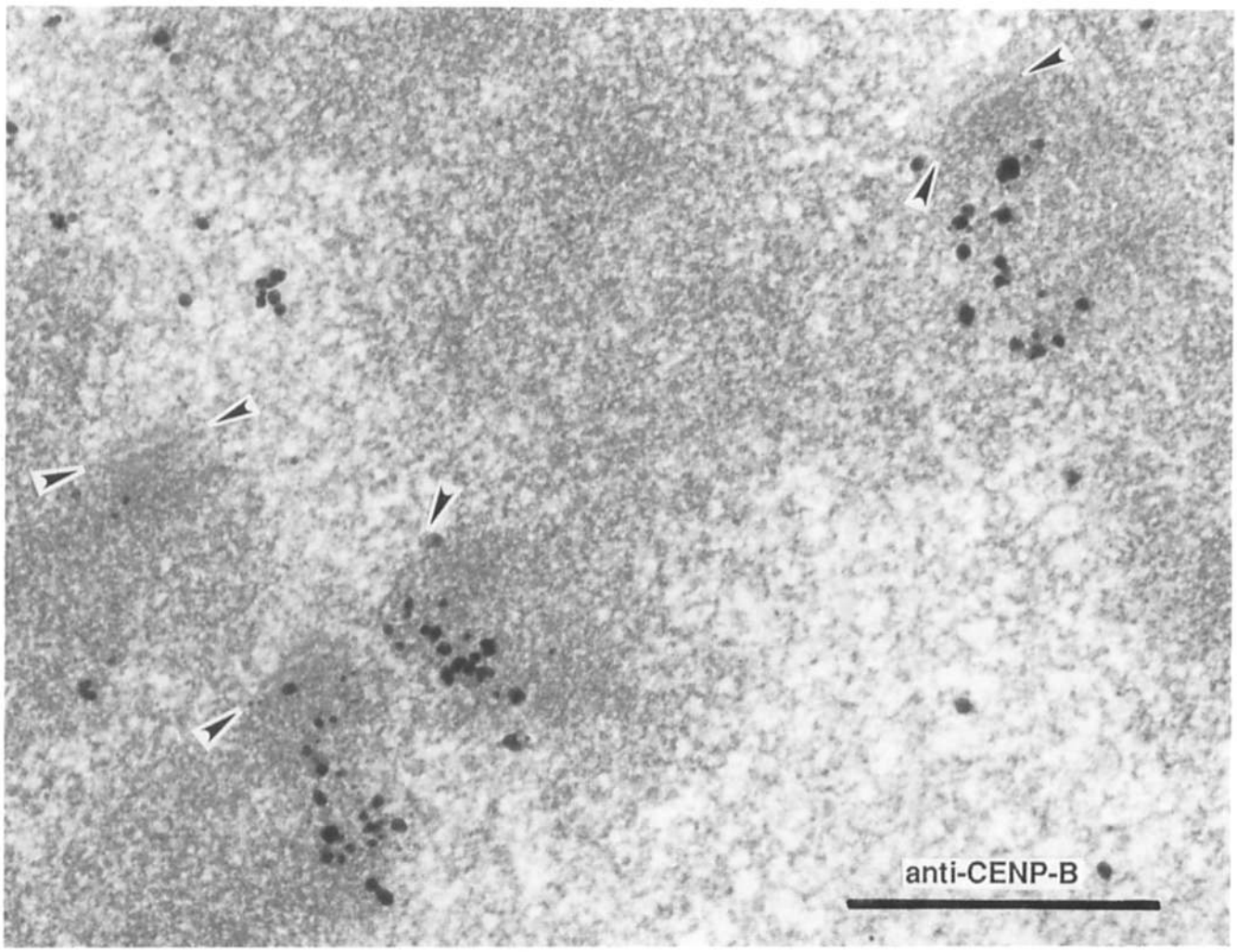

Figure 2. Higher magnification view of several kinetochores stained with rabbit anti-CENP-B (ra-ACA-1). This antibody recognizes epitopes distributed throughout the carboxyl-terminal 147 amino acids of CENP-B. Kinetochores are indicated by arrowheads, and are best recognized by noting the light stripe of the electron-lucent zone. Virtually all gold grains are localized beneath the kinetochore plates.

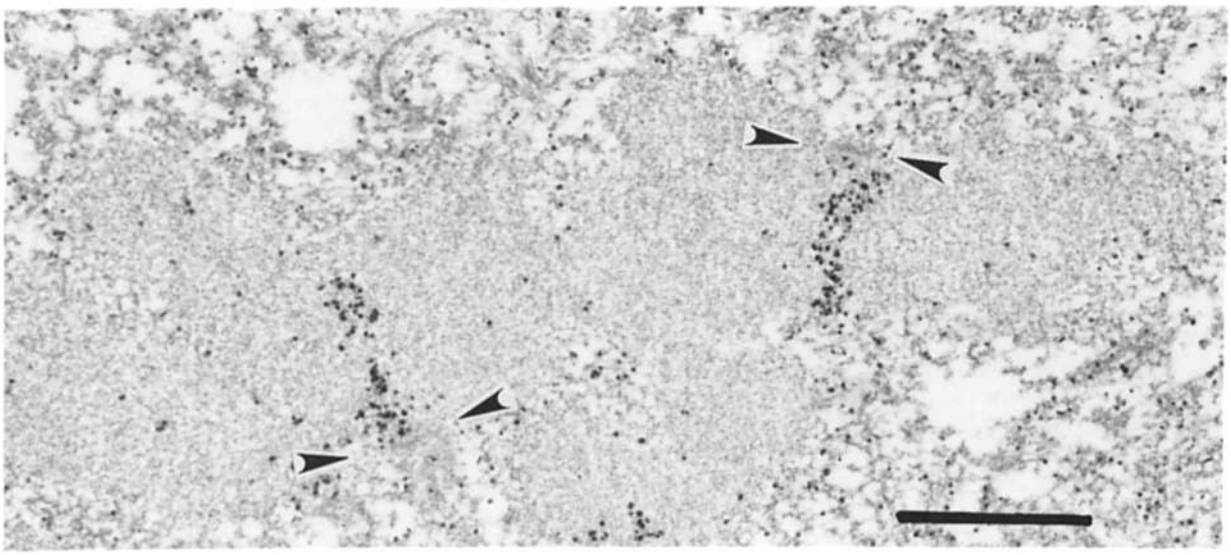

Figure 3. Mitotic cell stained with rabbit anti-CENP-B (ra-ACA-2). This antibody recognizes epitopes distributed throughout the region encompassing residues 5–599 of CENP-B. As in Fig. 2, the great majority of particles are located beneath the kinetochore. While there is a significant level of background of gold particles in the cytoplasm, background over the chromosomes is extremely low.

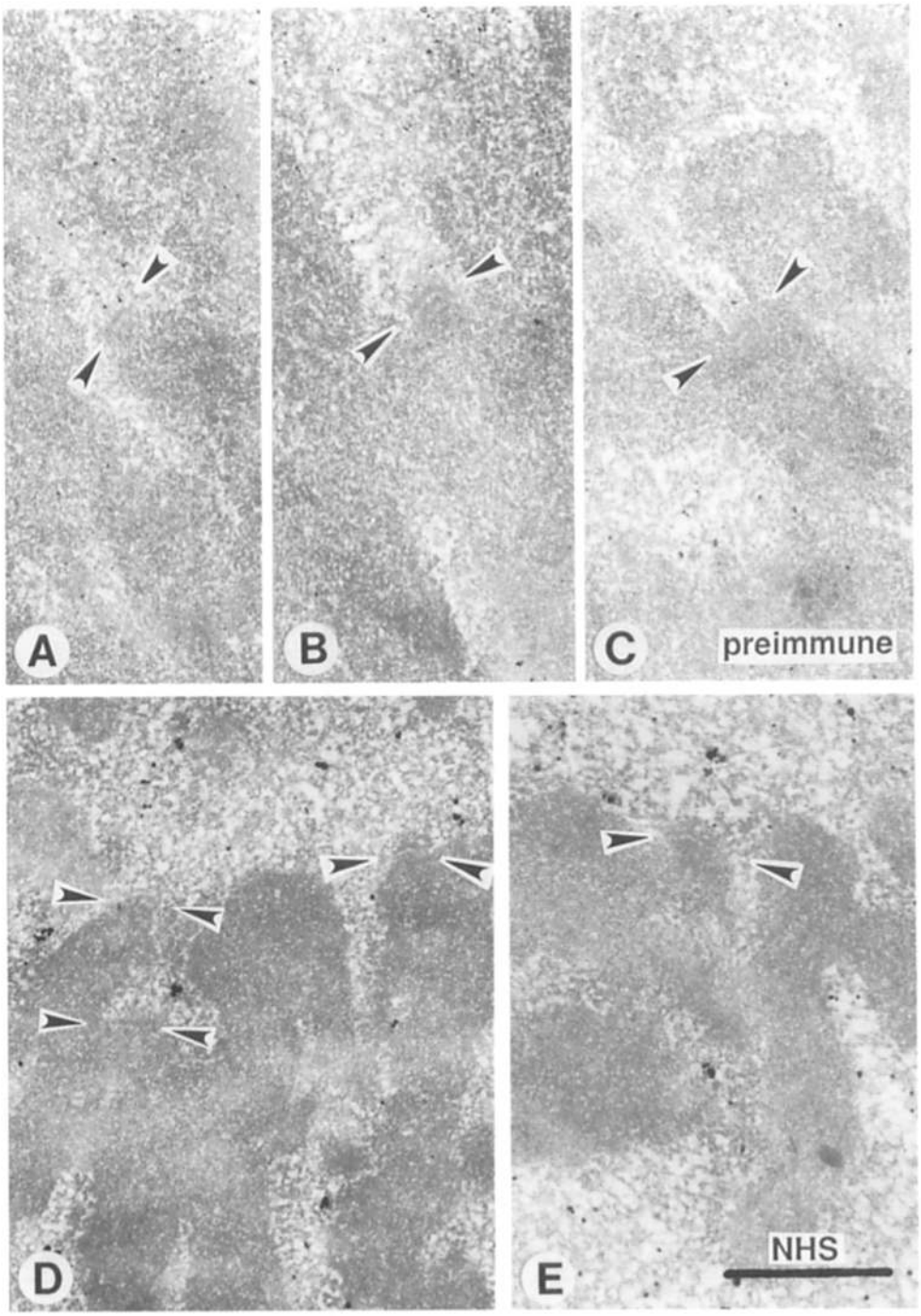

Figure 4. Control experiments. *A–C* show kinetochores from cells stained with preimmune serum from the rabbit that later produced ra-ACA-1. *D* and *E* show kinetochores from cells stained with normal human serum. In no case is specific labeling of the kinetochore region observed. All panels were from experiments performed in parallel with others using immune serum under conditions where specific staining was obtained.

Immunolocalization experiments performed in parallel using preimmune sera confirm that the specific staining observed with the rabbit serum is due to anti-CENP-B antibodies. Views of three chromosomes stained with preimmune serum are shown in Fig. 4, *A–C*. As expected, the centromeres were unlabeled.

The most straightforward interpretation of these results is that CENP-B is localized in the centromeric heterochromatin, and is absent from the kinetochore proper. However, it is also possible that some CENP-B is located in the kinetochore, but that access of antibodies to the outer plate is limited either by its condensed chromatin structure, or by the tightly clustered bundle of microtubules that terminates in it. (Immunofluorescence microscopy indicates that the spindles are well preserved in these cells even though microtubules are not visible in our electron micrographs. See Materials and Methods.) We have therefore performed two control experiments to increase the accessibility of the kinetochore to antibodies.

First, we have performed the immunolocalization on cells exposed in vivo to concentrations of colcemid sufficient to disassemble the cellular microtubules (0.6 μg/ml colcemid for 1 h). As expected, the chromosomes were distributed randomly throughout these cells, confirming that the spindle

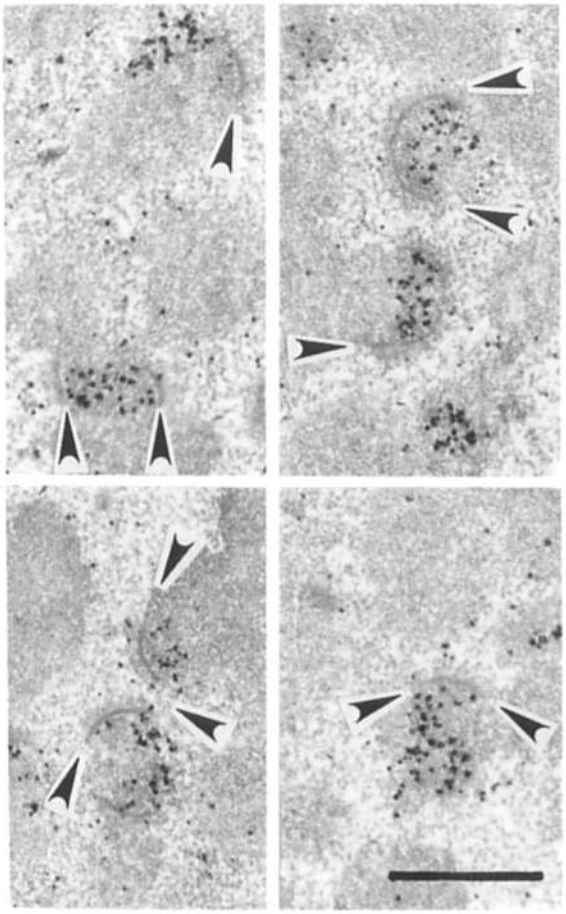

Figure 5. Colcemid-blocked mitotic cells stained with anti-CENP-B. Three alterations of the kinetochore are apparent in cells treated with high levels of colcemid (37). First, the inner plate is no longer detected. Second, the fibrous corona is much more highly developed. Third, the kinetochores bulge outward from the surface of the chromosome. Regardless of these alterations in the kinetochore morphology, the distribution of CENP-B is identical to that in untreated cells. The anti-CENP-B (in this case, ra-ACA-2) binds predominantly to the chromatin beneath the kinetochore. Similar results were obtained with autoantibody G.S., except that more gold particles were found lateral to the kinetochore plate (not shown).

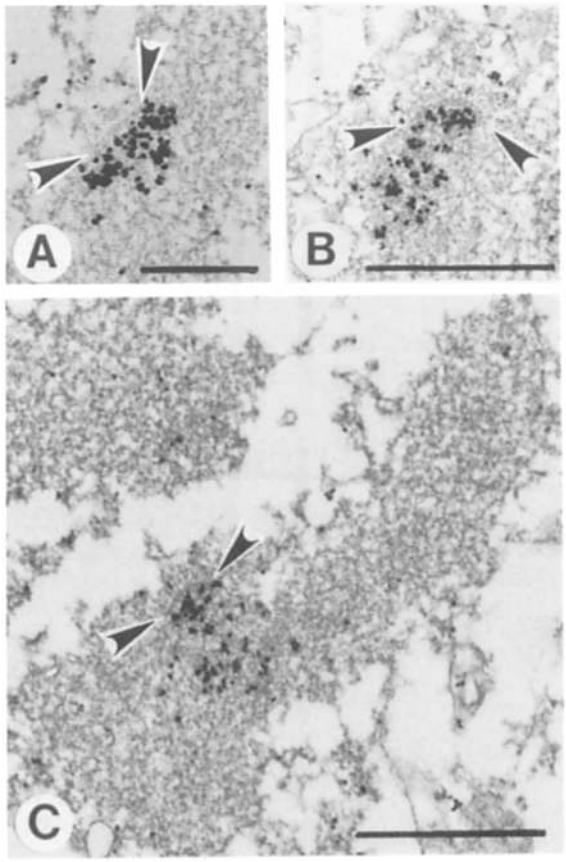

Figure 6. Chromosomes from colcemid-arrested cells fixed and probed with anti-CENP-B under conditions where the higher-order folding of the chromatin fiber is disrupted. Under these conditions the kinetochore plate is much attenuated and is difficult to distinguish from the (highly dispersed) centromeric chromatin. The plate typically appears to consist of two thin laminae. Under these conditions of fixation and labeling the CENP-B-containing chromatin appears to be in direct contact with the undersurface of the plate. The bar in *A* represents 0.5 μm. Those in *B* and *C* represent 1.0 μm.

was completely disassembled. The kinetochore morphology is altered in such colcemid-blocked cells (37). The inner plate is no longer visible, the fibrous corona becomes a prominent feature, and the kinetochore often seems to bulge outward from the condensed chromatin.

The chromosomal distribution of CENP-B determined in the absence of microtubules was essentially identical to that determined in their presence. Several representative views are shown in Fig. 5. The outer kinetochore plate is clearly seen in these cells, as is the gold label, which is concentrated beneath it. Depending on the method of scoring employed, 95–99% of the centromere-associated gold grains were located in the centromeric heterochromatin subjacent to the kinetochore (Table I). Of the small number of gold grains associated with the kinetochore plate, 85% were associated with the periphery of the structure.

A second series of control experiments was intended to address the possibility that the condensed chromatin structure of the outer kinetochore plate prevents binding of the anti-CENP-B antibodies. Such effects should be minimized by processing the cells for immunolocalization in a buffer (TEEN) that causes chromatin higher-order structure to unravel into the "beads-on-a-string" 10-nm fiber (23). We have previously shown that processing of chromosome spreads in such a buffer permits binding of anticentromere antibodies, even though the same sera fail to recognize centromeres in such spreads when processing is carried out under standard buffer conditions (15).

When colcemid-arrested cells are processed for immunolabeling in TEEN buffer, the chromosomes appear greatly swollen and the kinetochore plate is barely visible as an attenuated structure composed of two thin laminae (or layers of filaments). Examples of such kinetochores from three cells stained with anti-CENP-B are shown in Fig. 6. These swollen chromosomes are intensely labeled with gold grains. The grains extend to the lower surface of the kinetochore plate, which itself remains unlabeled. These images suggest that the kinetochore plate is in intimate contact with heterochromatin rich in CENP-B.

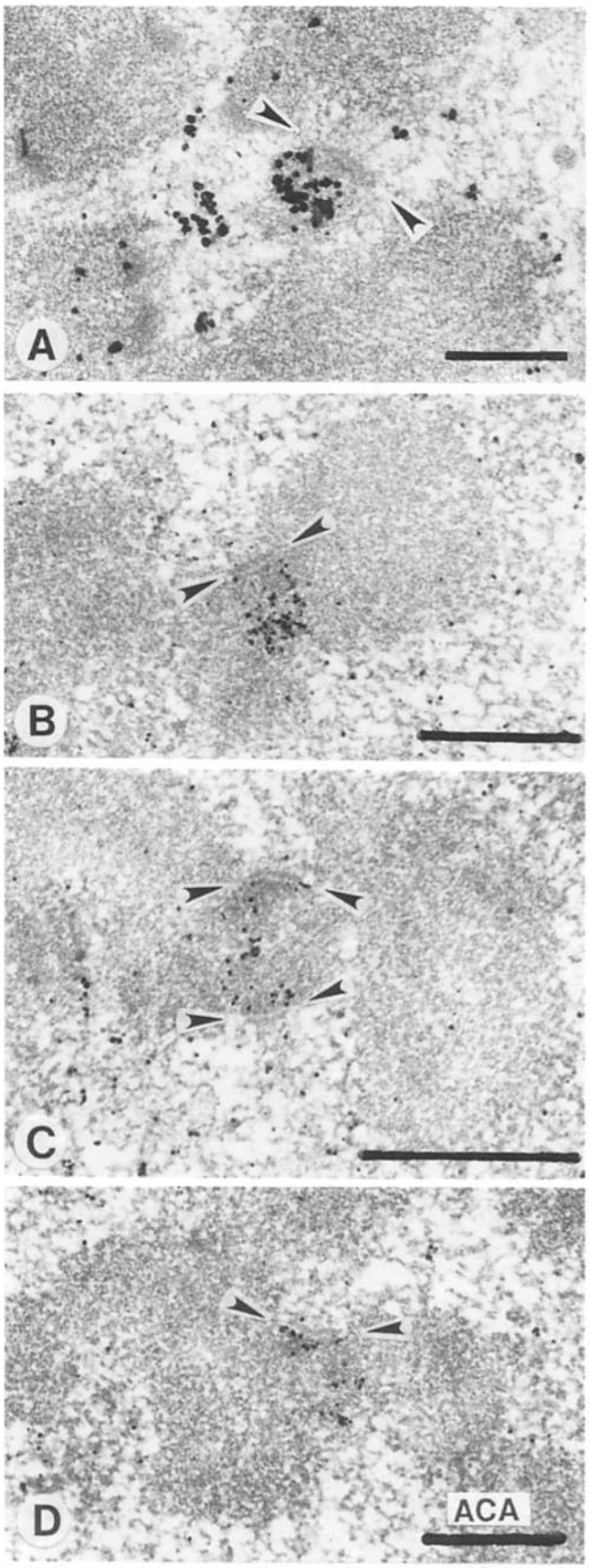

Figure 7. Gallery of higher magnification views of kinetochores stained with autoimmune anticentromere serum G. S. This serum recognizes a number of independent determinants on all three CENP antigens. Note that the majority of the gold particles are located subjacent to the kinetochore plates, although some staining of the periphery of the plates (evident in all panels) and of the inner plate (most obvious in *C*) is observed.

The Kinetochore Is Surrounded by a Region Enriched in CENP Antigens

Autoimmune serum G. S. recognizes CENP-A (17 kD) and CENP-C (140 kD) in addition to CENP-B (12). Use of this serum thus permits simultaneous location of the three CENP antigens by immunoelectron microscopy. If the staining pattern obtained with serum G. S. is compared to that obtained with anti-CENP-B, any differences should be due to the additional presence of antibodies recognizing CENP-A and CENP-C, as well as any antibodies recognizing posttranslational modifications on the three antigens (see Materials and Methods). Past immunoblotting analyses have shown that this serum recognizes only the three CENP antigens, and no other chromosomal proteins (12).

The distribution of gold grains observed after staining with autoimmune serum resembles that seen after staining with anti-CENP-B (Table I, Fig. 7). This is not surprising, given our previous observation that the titer of anti-CENP-B in serum G. S. is ~200-fold higher than that of anti-CENP-C, and that anti-CENP-A cross reacts with CENP-B (13). The bulk of the gold grains is located in the chromatin beneath the kinetochore (Fig. 7). As before, background elsewhere on the chromosome arms is low. In control experiments using normal human serum, no specific labeling of the centromere was observed (Fig. 4, *D* and *E*).

Despite the apparent similarities, a detailed statistical analysis reveals significant differences between the labeling patterns seen with autoantibodies and anti-CENP-B. If the non-kinetochore gold grains are divided into those lateral to the structure (that is, in the plane of the kinetochore plates) and those beneath the kinetochore (see Fig. 10), a higher percentage of lateral grains is observed with the autoimmune serum than with anti-CENP-B. The relative difference between the binding of autoimmune serum and anti-CENP-B is greatest for colcemid treated cells, where 7% of the grains seen with autoimmune serum are located lateral to the plate, compared to only 0.8% of those seen with rabbit anti-CENP-B (Table I).

The difference is not as dramatic for untreated cells, but again significantly more binding to the lateral region of the kinetochore is observed with autoimmune serum (18% of grains) than with rabbit anti-CENP-B (5%—Table II). This difference in grain distribution is unlikely to be due to differential penetration of the centromeric heterochromatin by the two antibodies, since the average grain count beneath the kinetochore is similar for both (35 ± 32 [45 kinetochores scored] for autoimmune serum, 40 ± 44 [55 kinetochores scored] for rabbit anti CENP-B).

Generalization from such transverse sections to a three-dimensional view of the centromere predicts that the kinetochore is closely surrounded by a ring of gold particles (see Fig. 11). This ring should be observed directly if labeled kinetochores are cut in tangential section. Despite the difficulty of recognizing kinetochores in such sections, we have ob-

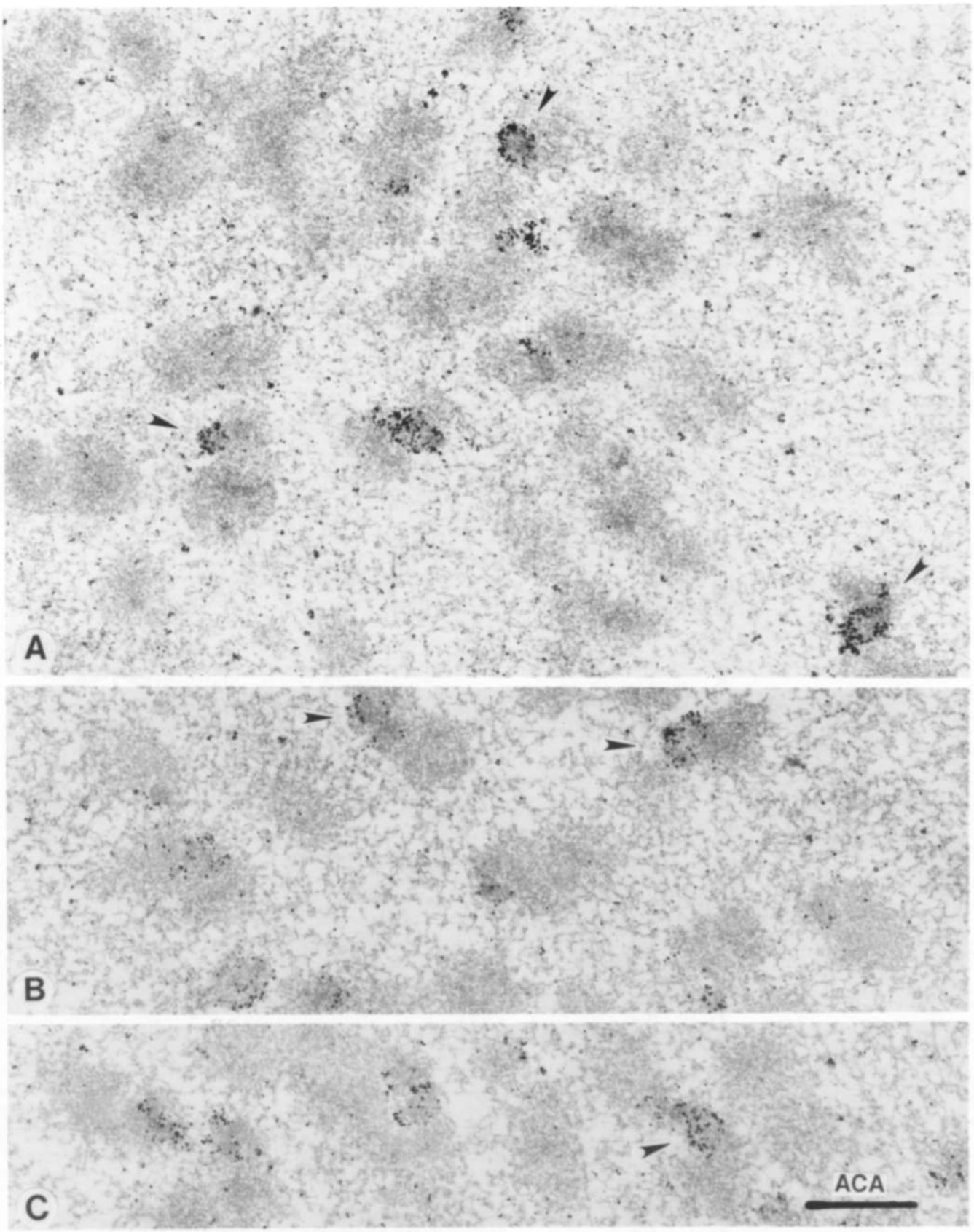

Figure 8. Tangential sections of centromeres stained with autoimmune anticentromere serum G. S. Such micrographs show sections cut parallel to the plane of the metaphase plate (i.e., perpendicular to the spindle axis). Note the prominent rings of gold grains, which often surround regions of chromatin that are darker than the bulk of the chromosomal arms (here seen in cross section).

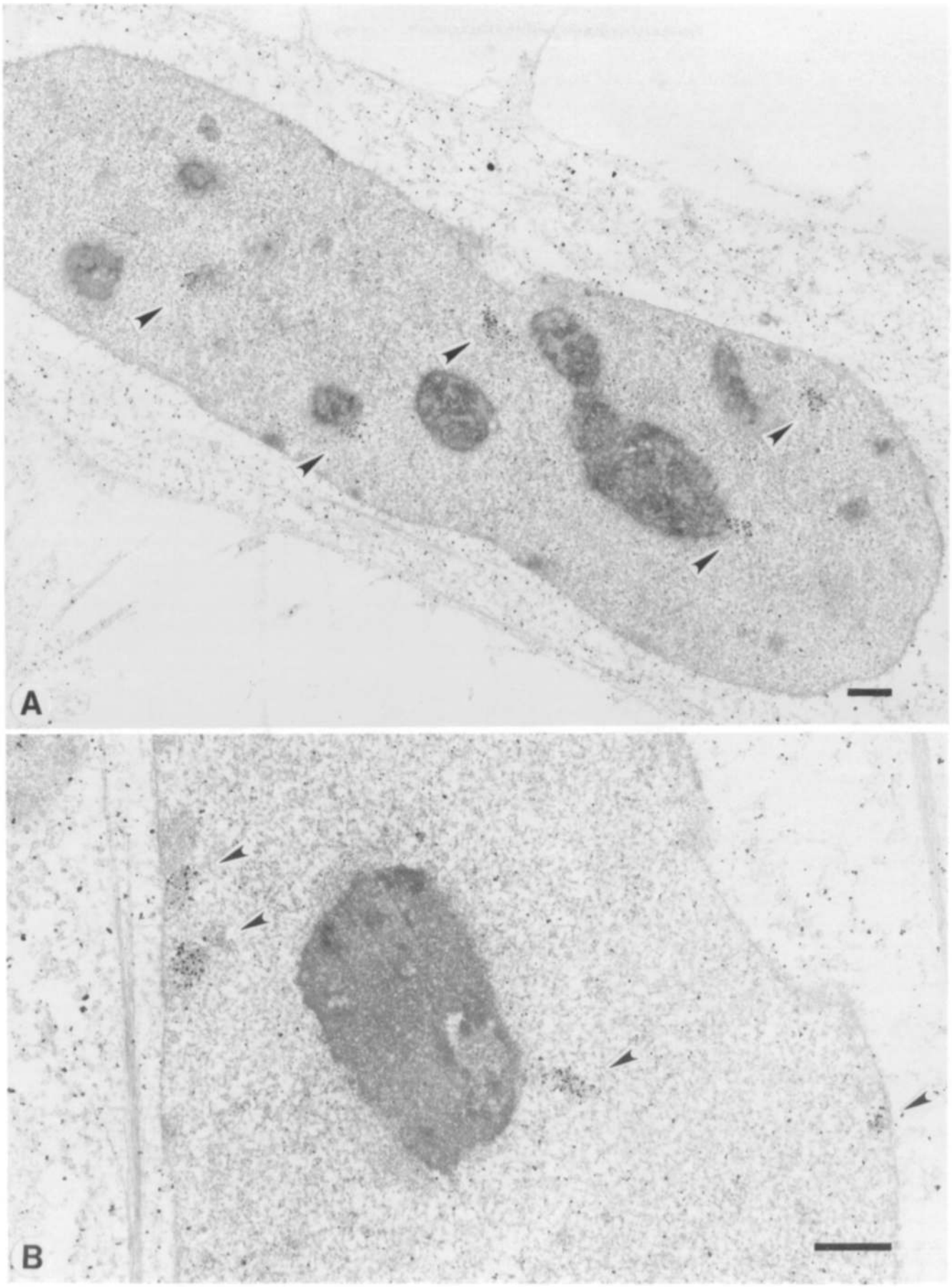

Figure 9. Interphase nuclei stained for CENP-B with ra-ACA-1. Discrete foci of gold grains are associated with small patches of condensed chromatin located throughout the nucleus. *B* is a higher magnification view of a different cell from that shown in *A*.

tained numerous views that we interpret as tangential sections through the surface of the centromere (Fig. 8). Such views are obtained when the plane of section passes through the metaphase plate perpendicular to the spindle axis. In these micrographs the chromosomes are distributed across a wide area of the cytoplasm, and the chromatid arms are cut in cross section, appearing as round or ovoid profiles. (Compare with Fig. 1 where the plate, cut in transverse section, looks like a compact mass of chromosomes with the chromatid arms seen in longitudinal section.)

The gold labeling of tangential sections through the centromere is quite distinctive (Fig. 8, *arrowheads*), with the gold grains often appearing in curved lines or complete rings surrounding less labeled areas of chromatin. The density of gold particles in these rings is greater than that seen throughout the rest of the centromere, suggesting that the rings correspond to a locally elevated concentration of the antigens. The unlabeled chromatin within the rings is frequently slightly darker (i.e., more condensed) than the peripheral chromatin. We believe that in some cases this corresponds to an en face view of the kinetochore.

Interphase Centromeres Are Often Associated with Discrete Patches of Heterochromatin

We have also used the CENP-B-specific rabbit polyclonal antibody to examine the distribution of CENP-B in interphase nuclei by immunogold labeling. Use of this procedure enabled us to readily localize CENP-B while maintaining the ultrastructure of the underlying chromatin. (In previous studies using the immunoperoxidase method, the underlying chromatin morphology was obscured by the dark reaction product.) CENP-B is found in discrete patches scattered throughout the nucleus. Two such nuclei are shown in Fig. 9. When 61 interphase centromeres were scored, 41% were juxtanucleolar, 28% were associated with the inner surface of the nuclear envelope, and 31% were found in isolated areas of the nucleoplasm. Individual nucleoli have been found to be associated with up to six CENP-B clusters.

Close examination of the higher magnification view of Fig. 9 *B* reveals that the interphase centromeres remain as localized regions of condensed heterochromatin. In many cases the patch of labeled heterochromatin is entirely covered with gold grains, indicating that centromeric heterochromatin occasionally occurs as isolated islands. Often, however, the labeled patch of centromeric heterochromatin is associated with other, unlabeled condensed chromatin (Fig. 9 *B*, *left two arrowheads*). Interestingly, the range of maximum diameters of the interphase patches of CENP-B overlaps the range of the CENP-B patches in mitotic chromosomes, implying that this region of the chromosome remains nearly completely condensed during interphase.

Discussion

Much recent discussion of the role of the centromere in mitosis has focused on the kinetochore (reviewed in reference 32). This is not only because the kinetochore is the point of attachment of the spindle microtubules to the chromosome, but also reflects recent results suggesting that the mechanochemical motor responsible for movement of the chromatids to the spindle poles during anaphase (anaphase A) is located within 1 μm of the kinetochore (33). The identification of kinetochore components, and particularly those of the outer plate where the chromosome–spindle interaction occurs, is thus of great importance. At the same time, the list of specific kinetochore proteins is remarkably short. To date, the CENP autoantigens (12) are considered to be the only specific protein markers for the kinetochore (5). Some tubulin is associated with the fibrous corona of the kinetochore (27, 36), but the vast majority of the tubulin is located elsewhere in the cell, so that tubulin cannot be regarded as a specific marker for the kinetochore.

The previous mapping of the CENP antigens to the kinetochore used cells from the rat kangaroo and Chinese hamster, species with large chromosomes having well-developed kinetochores. While this approach has many practical advantages, it rests on the tacit assumption that all mammalian centromeres are structurally similar. The validity of this assumption is open to question. For example, α-satellite DNA, the major DNA component of human centromeres, is found only in primates, and is unrelated to mouse major satellite DNA, the major DNA component of mouse centromeres (38). Likewise, when immunoblots of human and rat nuclei were performed in parallel using an autoimmune serum that recognizes CENPs A–C (17, 80, and 140 kD) in humans, only a single antigen of ~50 kD was recognized in the rat (12). Finally, it is well known that human chromosomes are preferentially lost from interspecies hybrids with mouse and hamster. Although there are many possible reasons for this, one obvious explanation is the failure of the human centromeres to function fully in the rodent cell background.

We have therefore undertaken to analyze the distribution of the human centromeric autoantigens in human mitotic chromosomes. Our data lead us to conclude that no specific marker protein for the human kinetochore has yet been identified.

CENP-B Is Located beneath the Kinetochore

The methods used in this study give an excellent signal-to-noise ratio for the detection of CENP-B, a rare antigen that we estimate to be present in ~375 copies per chromatid (R. L. Bernat, data not shown). We scored an average of 38 $\pm$ 35 gold grains on 122 centromeres with recognizable kinetochores. The average background of nonspecific labeling with these reagents was <1 grain per centromere.

CENP-B is distributed broadly throughout the heterochromatin beneath the kinetochore (Table I). The level of CENP-B detected in the kinetochore proper was consistently low. In 55 micrographs containing recognizable kinetochores, we counted 28 gold particles in the outer plate and 6,219 gold particles in the centromeric heterochromatin. (Fig. 10 presents a map of the kinetochore that identifies the major subdivisions analyzed in the present study.) The apparent absence of CENP-B from the outer plate was not due to interference with antibody binding by the dense bundle of kinetochore microtubules, since a similar distribution of the antigen was observed in the presence or absence of colcemid (Table I). Furthermore, essentially identical results were obtained with two different antibodies, one of which (ra-ACA-1) recognizes several epitopes in the carboxy-terminal 25% of CENP-B (Fig. 2), while the other (ra-ACA-2) recognizes epitopes distributed along the entire length of the molecule (Fig. 3).

While it is possible to envision complex scenarios in which some CENP-B is present in the outer plate of the

Table I. Quantitation of the Immunogold Labeling Results

Antibody	Counting Method*	Corona‡	Outer plate		Lucent zone		Inner Plate		Beneath	Background§	
			Lateral	Central	Lateral	Central	Lateral	Central		Chrom	Cyto
ACA	1	34 (1.9%)	22 (1.3%)	0	2 (0.5%)	4 (0.2%)	79 (4.5%)	17 (1%)	1587 (91%)	ND	ND
ra-ACA-1	1	20 (1.6%)	12 (0.9%)	1 (0.08%)	3 (0.2%)	1 (0.08%)	28 (2.2%)	3 (0.2%)	1213 (95%)	ND	ND
ra-ACA-2	1	7 (0.7%)	12 (1%)	3 (0.2%)	0	1 (0.1%)	3 (0.2%)	1 (0.1%)	1002 (97%)	0.88 (1.8%)	11.4 (24%)
ra-ACA-2	2	7 (0.1%)	12 (0.2%)	3 (0.05%)	0	1 (0.02%)	3 (0.05%)	1 (0.02%)	5006 (99%)	0.88 (0.3%)	11.4 (4.7%)
ACA (colcemid)	2	12 (2.7%)	32 (7.1%)	1 (0.02%)	ND	ND	NA	NA	405 (90%)	1.8 (5%)	8 (23%)
ra-ACA-1 (colcemid)	1	21 (2.5%)	9 (1%)	0	5 (0.6%)	7 (0.8%)	NA	NA	798 (95%)	ND	ND
ra-ACA-2 (colcemid)	2	37 (0.9%)	30 (0.7%)	6 (0.1%)	ND	ND	NA	NA	4080 (98%)	ND	ND

* Method 1 only counts gold grains from centromeres whose kinetochores are visible in the plane of section. Counting methods that score gold grains only from chromosomes with recognizable kinetochores, overestimate the level of CENP-B in the kinetochore plates (see Materials and Methods). We therefore adopted method 2 as an alternative. Micrographs were selected for scoring on the basis that they contained at least one well-preserved kinetochore. All centromere-associated gold grains were then counted. This gives a more accurate estimate of the total distribution.

‡ All figures are expressed as the total number of grains counted at that location together with the percentage these compose of the total grains counted in each experiment.

§ Background grain counts were determined for an area equivalent to the total over which centromeric gold grains were distributed. (Centromere and background measurements were performed as matched pairs, with the area of the centromere traced on a clear plastic overlay, which was placed at random over chromosomes and cytoplasm.) The values in these last two columns are different from those in the columns to their left. In each case, the first figure represents the average background for a single centromere. The percent figure relates this background to the total grain count for an average centromere in each experiment. Note that the cytoplasmic background is only relevant to the measurements for the corona region, and in this case, the background is likely to represent at least a 10-fold overestimate of the true background in the corona region, since this region is ~10-fold smaller than the rest of the centromere. The chromosomal background is effectively zero for regions the size of the kinetochore plates.

kinetochore in a form that is masked from our polyclonal antibodies, the most straightforward interpretation of these experiments is that the great majority of CENP-B is located in the heterochromatin beneath the kinetochore.

Relationship to Previous Work

Our results are in apparent contradiction to those of two previous studies, which indicated that all of the centromeric autoantigens were confined to the kinetochore (5), and suggested that CENP-B interacts directly with tubulin (2). These experiments are the basis for the generally held belief that the CENP antigens are kinetochore proteins. In this section we will consider possible explanations for the different results obtained in our experiments.

We first consider the previous use of the immunoperoxidase mapping method to show that a crude autoimmune serum labeled only the kinetochore of Chinese hamster and rat kangaroo chromosomes (5). We propose the following four potential explanations for the discrepancy between the earlier results and our own.

(*a*) The antibodies may differ in their specificities. Our mapping data are dominated by anti-CENP-B antibodies, even when using autoimmune serum G. S., since we have previously shown that by far the highest titer of autoantibodies present in this serum is directed against CENP-B (13). The crude patient serum used in the original immunoperoxidase mapping study was not characterized by immunoblotting (5), and it is possible that the titer of anti-CENP-B in this serum was low. Alternatively, the antibody used in the original study may have reacted only with the rat kangaroo homologues of CENP-C and/or CENP-A, and might not cross react with the CENP-B homologue from this species (if, indeed, one exists).

(*b*) The distribution of the centromeric autoantigens may exhibit some variability between species. When the immunoperoxidase procedure was used to map the distribution of the autoantigens in human cells, the antigens were found to be distributed throughout the entire centromere region, and were not just concentrated in the vicinity of the kinetochore (29). The possibility of such interspecies differences is also suggested by the recent evidence that CENP-B may bind α-satellite DNA (25), a class of repetitive DNA found only in primates (38).

Table II. Antibody Binding to the Periphery of the Kinetochore

Antibody	Corona	Central kinetochore	Lateral chromatin	Beneath
ACA (G. S.)	19 (1.1%)	14 (0.8%)	303 (18.3%)	1,333 (80%)
raACA	24 (0.8%)	4 (0.13%)	157 (5.3%)	2,802 (94%)

The micrographs scored for Table I were rescored with the regions surrounding the kinetochore divided into lateral and beneath as indicated in Fig. 10.

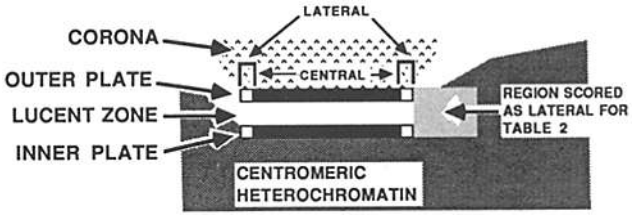

Figure 10. Map of the human kinetochore showing the subdomains in which gold particles were quantitated for Tables I and II.

(*c*) The immunoperoxidase results may be misleading. We have confirmed that significant levels of staining are seen throughout the centromeric heterochromatin in human cells stained using the immunoperoxidase reaction (data not shown). In some cases the outer kinetochore plate was stained with anti-CENP-B antibodies (in apparent confirmation of the earlier study [5]); however, we believe that this is probably due to diffusion of peroxidase reaction product (34). While the peroxidase method has been used extensively and has generally proven to be reliable, we feel that when examining structures separated by distances of 0.053 ± 0.025 μm (the average spacing between the centromeric chromatin and the outer kinetochore plate in these figures) the possibility of some localized diffusion of the reaction product must be entertained. Other reasons for arrival at this conclusion are detailed in Materials and Methods.

It should be noted that this is not the only instance of a discrepancy of localization of chromatin proteins when immunoperoxidase and immunogold mapping techniques have been compared. A similar contradiction has also arisen during determination of the localization of protein C23 in the nucleolus. An initial study using the immunoperoxidase method concluded that C23 was present in both the fibrillar centers and dense fibrillar component (39). In contrast, a more recent analysis using immunogold labeling found C23 located in the dense fibrillar component immediately bordering the fibrillar center, but failed to detect binding of the specific antibodies to the fibrillar center itself (3). The latter authors conclude that one likely explanation for this discrepancy is localized diffusion of the peroxidase reaction product.

(*d*) Alternatively, the immunogold results may be misleading due to inability of the gold-conjugated reagents to bind to CENP-B present in the kinetochore. This is unlikely for the following reasons. First, the "masking," if it occurs is not of the primary or secondary antibody, but of the third layer of the immunological detection network (discussed at greater length in Materials and Methods). Second, three different antibodies that each recognize several independent epitopes on CENP-B all give a similar result. Thus not one, but at least four different epitopes distributed along the length of CENP-B would need to be masked (13). Finally, we do not have a problem detecting CENP-B or the other autoantigens per se. There is an excellent signal for detection of these antigens in the heterochromatin beneath and around the kinetochore. Furthermore, this localization result makes excellent sense, given the DNA-binding data of Masumoto et al. (25).

We now consider a second previous study, in which it was concluded that exogenous tubulin added to Chinese hamster chromosomes could be cross-linked to an 80-kD protein (2). It was accordingly suggested that CENP-B is a microtubule-binding component of the kinetochore.

A variety of evidence appears to be inconsistent with this conclusion. First, the results of the present study indicate that a significant percentage of CENP-B is located outside of the kinetochore in human chromosomes. While it is possible that the kinetochore might contain small amounts of CENP-B that interact with tubulin, it is unlikely that the >95% of CENP-B that is distributed widely throughout the centromeric heterochromatin is involved in microtubule binding. Second, we have previously shown that CENP-B is present at the inactive centromeres of two different stable dicentric chromosomes in vivo ([15]; W. C. Earnshaw and E. W. Jabs, unpublished observations). Where examined by electron microscopy, inactive centromeres have been shown to lack a visible kinetochore (42). Thus the presence of CENP-B at a centromere is not sufficient to dictate the presence of a kinetochore or the ability of that centromere to interact with microtubules. Finally, the Y chromosome, which has neither detectable CENP-B (W. C. Earnshaw and G. Stetten, unpublished observations) nor the "CENP-B box" in any of its α-satellite DNA arrays cloned to date (25), segregates perfectly well in mitosis.

We suggest three likely explanations for the crosslinking result. (*a*) The cross-linking was performed with Chinese hamster, and the distribution of CENP-B in this species may differ from that in humans, since the hamster lacks α-satellite DNA. (*b*) The 80-kD CHO protein that is cross-linked to exogenous tubulin (2) may not be CENP-B, but may instead be a Chinese hamster microtubule-associated protein. High molecular weight microtubule-associated proteins from porcine brain have previously been shown to bind preferentially to mouse satellite DNA (45), and to subsequently bind tubulin (10). (*c*) Even though chromosome-associated microtubules were shown to be present in the cross-linking experiment (2), the tubulin cross-linked to the 80-kD protein may have been in the monomeric form. High concentrations of exogenous monomer tubulin might bind to proteins throughout the centromere and not just at the kinetochore.

The recent observation that CENP-B shares limited amino acid homology with the two microtubule-binding proteins MAP-2 and tau (21), should not be interpreted as indicating that CENP-B itself binds microtubules. The microtubule assembly-promoting peptide of MAP-2 has been identified (21) and found to lack homology with CENP-B. Instead, the region of shared homology contains four characteristically spaced proline residues, and is apparently a protease-accessible hinge region. Furthermore, a search of the MicroGenie data bank reveals that this sequence motif occurs in other proteins that have no known association with microtubules. These include glycoprotein B of Herpes Simplex virus (35), the hypothetical BHLF-1 protein of Epstein-Barr virus (1), and N-*myc* (40). The common function served by this motif in this disparate group of proteins remains to be determined.

Thus, while we cannot exclude that small amounts of CENP-B may be found in the kinetochore outer plate, we feel it most unlikely that the predominant function of CENP-B in the human is microtubule binding. Instead, as summarized below, the function of this protein appears to be linked with the (as yet unknown) function of α-satellite DNA.

CENP-B Is a Protein of the α-Satellite-rich Heterochromatin

We observed earlier that when chromosome spreads were stained with a variety of antibodies specific for CENP-B, the level of detectable antigen varied by at least an order of magnitude between chromosomes (14). Such variability is difficult to explain if CENP-B is a component of a conserved structure such as the kinetochore, but is readily explained if the binding of the protein depends on the presence of DNA sequences that vary from chromosome to chromosome. One example of a family of centromere-associated DNA sequences that exhibits such variability is α-satellite DNA (38).

Three observations suggest that CENP-B is associated with α-satellite chromatin in vivo. First, our monospecific

anti-CENP-B antibodies do not stain the human Y chromosome (W. C. Earnshaw and G. Stetten, unpublished observations). The Y has the lowest content of α-satellite DNA of any of the human chromosomes (0.3–1.2 MB [44]).

Second, we have obtained evidence suggesting that CENP-B is associated in vivo with the α-satellite chromatin on chromosome 17. This comes from examination of the binding of anti-CENP-B antibodies to a marker chromosome derived from the centromere of chromosome 17. In situ hybridization experiments have shown that this small marker chromosome retains at least 80% of the chromosome 17 α-satellite DNA (Wevrick, R., W. C. Earnshaw, P. N. Howard-Peebles, and H. F. Willard, manuscript submitted for publication). We have shown that the marker chromosome also binds ~5-fold more anti-CENP-B-specific polyclonal antibody than does the 17p-chromosome from which it was derived, suggesting that CENP-B is associated with the chromosome 17-specific α-satellite chromatin (44).

Finally, centromeric substructures that bind crude CREST (calcinosis, Raynaud's phenomenon, esophageal dysmotility, sclerodactyly, telangiectasia) patient serum have been shown to contain α-satellite DNA after subcellular fractionation (26). In addition, colocalization of α-satellite DNA and CENP antigens has been observed in both mitotic chromosomes and interphase nuclei by double-label in situ hybridization and immunofluorescence (26).

That this colocalization is due to sequence-specific binding of CENP-B to α-satellite DNA has recently been suggested by direct in vitro experiments, including precipitation of cloned α-satellite DNA from chromosomal extracts with anticentromere autoantibodies (26). These authors also demonstrated that DNA could bind to an 80-kD protein that was immunoprecipitated with patient serum, subjected to SDS-PAGE, and transferred to a filter membrane. A 17-bp putative "CENP-B box" required for the recognition of the DNA by the autoantigen was also identified (26). Interestingly, the CENP-B box has been found to be absent from all known Y chromosome-specific α-satellite arrays. It is, however, present in the minor satellite of the mouse (26).

Since α-satellite DNA is distributed throughout the centromere region, it is not surprising that CENP-B is also distributed broadly. It should be noted, however, that our data should not be extrapolated to conclude that α-satellite DNA is absent from the kinetochore. Our immunolocalization data apply only to the minority of the α-satellite monomers cloned to date that have the CENP-B box (26). The monomers lacking the CENP-B binding site could be anywhere in the centromere, including in the kinetochore plate.

Structure of the Centromere during Interphase

Use of the immunogold procedure permits the simultaneous detection of CENP-B and examination of the chromatin that contains it. In earlier studies this chromatin was obscured by dense deposits of peroxidase reaction product (5, 29). All concentrations of gold grains seen after labeling with anti-CENP-B were found over patches of heterochromatin, confirming earlier conclusions that the centromere remains relatively condensed during interphase. In fact, measurement of the average diameter of clusters of gold grains seen at interphase yielded a value indistinguishable from that observed on mitotic chromosomes. This suggests that the centromeric heterochromatin remains nearly as condensed during interphase as it is during mitosis. It is therefore tempting to speculate that heterochromatin in general may retain a comparable level of condensation throughout the cell cycle.

Conclusions

The kinetochore is surrounded on all sides by chromatin rich in the CENP antigens. In particular, the studies of swollen chromosomes suggest that the kinetochore plate may be in intimate contact with a subjacent layer of chromatin rich in CENP-B. CENP-B is also distributed more widely throughout the centromeric heterochromatin.

In addition to the CENP-B-containing chromatin upon which it rests, the kinetochore is also surrounded laterally by chromatin rich in CENP antigens. Gold grains frequently seem to "cap" the edges of the plates laterally when the kinetochores are viewed in transverse section (Fig. 6). We do not know whether the edges of the plates contain CENP antigens, or whether the antigens are localized to an adjacent, but distinct, region of specialized chromatin that surrounds the kinetochore like a collar. The relationship between the observed distributions of gold grains in the transverse and tangential sections, and the three-dimensional distribution of the CENP antigens is diagrammed in Fig. 11.

Because the lateral concentration of antigens is most prominent when chromosomes are stained with autoimmune serum G. S. (which recognizes all three CENP antigens), our current working hypothesis is that the "collar" region contains much or all of the CENP-C. (We cannot speculate further about the distribution of CENP-A since all antibodies that we have affinity purified from this antigen cross react with CENP-B.) In this respect it is interesting to note that the distribution of CENP-C exhibits two significant differences from that of CENP-B. First, indirect immunofluorescence experiments suggest that CENP-C is present in roughly equal amounts at all human centromeres, suggesting that it is a part of a conserved structure. Unlike CENP-B, CENP-C was not detectable at the inactive centromere of a dicentric autosome (15). Thus, the presence of CENP-C correlates with the presence of a functional kinetochore. This leads us to speculate that CENP-C may be involved in some aspect of chromosome–spindle interactions during mitosis.

The role of CENP-B remains, like that of satellite DNA, unknown. It seems likely, however, that the battery of specific antibodies and clones now available should permit us to address this problem directly in the future.

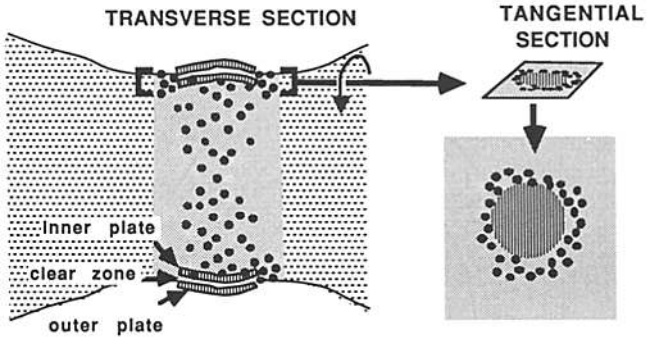

Figure 11. Diagram showing the relationship between the distributions of gold particles seen in transverse and tangential sections through the centromere.

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