

# Chemical Subdomains Within the Kinetochore Domain of Isolated CHO Mitotic Chromosomes

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**Abstract.** We have used indirect immunofluorescence in combination with correlative EM to subdivide the mammalian kinetochore into two domains based on the localization of specific antigens. We demonstrate here that the fibrous corona on the distal face of the kinetochore plate contains tubulin (previously shown by Mitchison, T. J., and M. W. Kirschner. 1985. *J. Cell Biol.* 101:755-765) and the minus end-directed, ATP-dependent microtubule motor protein, dynein; whereas a 50-kD CREST antigen is located internal to

these components in the kinetochore. Tubulin and dynein can be extracted from the kinetochore by 150 mM KI, leaving other, as yet uncharacterized, components of the kinetochore corona intact. Microtubules and tubulin subunits will associate with kinetochores in vitro after extraction with 150 mM KI, suggesting that other functionally significant, corona-associated molecules remain unextracted. Our results suggest that the corona region of the kinetochore contains the machinery for chromosome translocation along microtubules.

**T**HE partitioning of genetic material to each daughter cell is a crucial event in the propagation and growth of all living organisms. During cell division in tissue culture cells, chromosomes attach to the microtubules that comprise the mitotic spindle and move rapidly back and forth until they come to rest in the spindle midzone. At anaphase, the sister chromatids separate and sweep toward the opposite poles of the mitotic spindle (2). High resolution video and EM (18, 25, 29), fluorescence photobleaching (16), and studies in vitro (23, 24) all suggest that the movement of chromosomes during mitosis is mediated by the interaction of spindle microtubules with a specialized structure known as the kinetochore, located at the primary constriction on mitotic chromosomes.

Earnshaw and co-workers have divided the primary constriction or centromere region of mitotic chromosomes into two parts based on immunocytochemical localization of centromere proteins (11). The outer centromere region consists of the kinetochore plate and underlying chromatin. In this region one finds immunocytochemical localization of the CREST autoimmune sera antigens, CENP-A (17 kD), CENP-B (80 kD), and CENP-C (140 kD) (10, 12, 13). The proteins of the inner centromere region, the INCENP antigens, are associated with regions of close contact between sister chromatids of mitotic chromosomes (9). The outer centromere region can be further subdivided (based on early electron microscopic studies and reviewed in Rieder [28]) into the kinetochore domain, consisting of the highly organized trilaminar plate (7) and fibrous corona (19), and the central region consisting of centromere-associated repetitive DNA which, in primates,

consists of alphoid DNA-containing nucleoprotein and some CREST antigens (3, 5, 6 [reviews], 10).

Ultimately, structural studies of kinetochore components will be enhanced by functional and biochemical characterization of these components. This is because organisms such as roundworms, certain plants, and arthropods do not contain discrete kinetochores, but instead appear to contain diffuse kinetochores that are evenly distributed along the length of the chromosomes (8, 15, 33). It is likely, for such a fundamental process as chromosomal segregation, that these "holocentric" kinetochores use a similar mechanism as the more conventional mammalian kinetochore. However, just as cilia and flagella provide a model for the analysis of microtubule-based motility in a variety of systems, the high degree of structural order in mammalian kinetochores and the ability to assay their activity in vitro (23, 24), provides us with a useful paradigm for the study of kinetochore function during cell division in all organisms.

Using indirect immunofluorescence and image analysis in combination with correlative EM we have subdivided the mammalian kinetochore into two potentially functionally significant domains based on antigen localization. We have demonstrated here that the fibrous corona on the distal face of the kinetochore plate contains tubulin (previously shown by Mitchison and Kirschner [23]) and the minus end-directed, ATP-dependent microtubule motor protein, cytoplasmic dynein. This suggests that the outer corona region of the kinetochore contains the machinery for chromosome translocation along microtubules as originally predicted by Rieder et al. (18) based on real-time video microscopy. A 50-kD CREST

sera antigen is located internal to the fibrous corona. Furthermore, we present evidence that the protein composition of the fibrous corona is complex, and that tubulin and dynein are, in turn, associated with other peptide components of the fibrous corona that are distinct from the internally located CREST serum antigen.

## Materials and Methods

### Chromosome Isolation

CHO cells were grown in MEM alpha medium with 10% supplemented calf serum, L-glutamine, Pen-Strep, until just subconfluent. The medium was exchanged with fresh medium containing 10  $\mu$ g/ml vinblastine sulfate (Sigma Chemical Co., St. Louis, MO) for 10–12 h. Mitotic cells were collected by dislodging the cells with a stream of media. Cells were collected at 1,000 rpm, and resuspended in 37°C swelling buffer (5 mM Pipes, pH 6.8, 5 mM NaCl, 5 mM MgCl<sub>2</sub>, 1 mM EGTA) and collected. Mitotic cells are then quickly lysed in ice-cold PMED (10 mM Pipes [pH 6.8], 5 mM MgSO<sub>4</sub>, 1 mM EGTA, 0.1% Digitonin (Sigma Chemical Co.) plus proteolytic inhibitors (1  $\mu$ g/ml aprotinin, pepstatin, leupeptin). Mitotic chromosomes are then collected in a 20–60% sucrose gradient (10 min, 10,000 rpm, 4°C, HB-4 rotor, model RC-5, Sorvall Instruments Div., Newton, CT) in PME plus proteolytic inhibitors. Chromosomes are collected and frozen in aliquots in liquid N<sub>2</sub> until further use.

HeLa chromosomes were isolated in the same manner except that the cells were grown in suspension culture, and drugged for 16 h with 1  $\mu$ g/ml colcemid. A low speed (300 rpm, 5 min, HB-4 rotor) centrifugation was included in order to remove contaminating interphase nuclei. The cells were provided by Ibrahim Tuet, University of California at San Francisco Cell Culture facility.

### Immunofluorescence Measurements

Isolated mitotic chromosomes were fixed for 10 min in 0.5% paraformaldehyde in PME at 20°C, then centrifuged through a 33% glycerol in PME cushion onto coverslips. The coverslips are then postfixed in –20°C methanol to increase the signal of some of the antigens. However, the same results can be obtained with paraformaldehyde alone. The chromosomes were then labeled with DM1 $\alpha$  (an mAb against alpha-tubulin), monoclonal 70.1 (an mAb against the intermediate chains of cytoplasmic dynein [32]), Texas red-conjugated streptavidin, anti-thio-P (an mAb against thio-phosphate groups [14]), and human CREST sera. Two mAbs to the heavy chains of chick cytoplasmic dynein that do not crossreact with endogenous CHO chromosomal dynein were also used in this study, mAb 440.1, which crossreacts only with chick cytoplasmic dynein heavy chain, and mAb 440.4, which crossreacts with HeLa kinetochores but not with CHO kinetochores.

The mAb (mAb052) against all histone species was purchased from Chemicon (El Segundo, CA).

Preimmune sera from 10 rabbits was screened against CHO chromosomes, 8 of those 10 rabbits contained antibodies that recognized kinetochores at a dilution of 1:100. Based on these results it seemed reasonable to assume that purified rabbit IgG from unimmunized rabbits, purchased commercially, would recognize kinetochores. This proved to be the case. Therefore, in order to standardize the experiments and make it easier for other researchers to duplicate this data, all the experiments were performed using DEAE-purified rabbit IgG at a concentration of 0.2 mg/ml (cat #JOZ0003, Lot E8804; Accurate Chemical Corp., Westbury, NY), hereafter described as rabbit IgG. Goat antimouse, antirabbit, and antihuman secondary antibodies conjugated to fluorescein or Texas red were obtained from Accurate Chemical Corp. and used at a concentration of 0.1 mg/ml.

Biocytin-conjugated N-ethyl maleimide (Molecular Probes Inc., Eugene, Oregon) was incubated at a concentration of 1  $\mu$ M with isolated CHO chromosomes in PME for 5 min at 37°C. Chromosomes were fixed as described above and labeled with Texas red-conjugated streptavidin (Accurate Chemical Corp.).

Double-labeled mitotic CHO chromosomes were recorded on a Zeiss universal microscope with an SIT camera (Cohu Inc., San Diego, CA) through a Maxvision ATI image processor (Datacube) using a Panasonic OMDR. Distances between labeled kinetochores were measured using JAVA software (Jandel Video System Analysis, Jandel Scientific, Corta Medera, CA). Photomicrographs were photographed on a Zeiss Photomicroscope III using hypersensitized Kodak Technical Pan Film 2415 (Lumicon, Livermore, CA).

### Salt Extractions and Gel Electrophoresis

Isolated CHO chromosomes were incubated in PME plus varying levels of KI, MgSO<sub>4</sub>, or KCl at 4°C for 5 min, then diluted into 0.5% paraformaldehyde in PME and processed for immunofluorescence as described above.

Larger aliquots of chromosomes were washed in 150 mM KI in PME plus proteolytic inhibitors and collected at 4°C in an HB-4 rotor in a Dupont-Sorvall RC-5 at 5,000 rpm for 10 min. The chromosome pellet was washed 2 $\times$  in PME to remove residual salt and boiled in gel sample buffer. The KI super was dialyzed overnight against 100 vol of PME. Some of the salt-extracted protein precipitated out and the precipitate was collected by centrifugation (10 min, 10,000 rpm, SS-34 rotor) and boiled in gel sample buffer. The remaining KI super was concentrated in a Centriprep-10 concentrator (Amicon Corp., Danvers, MA) and boiled in sample buffer. SDS-PAGE was performed according to Laemmli (21) using 7 or 8% single concentration reducing gels. Western immunoblots were performed essentially according to Towbin et al. (34). A 7 or 8% SDS-PAGE gel was blotted onto 0.2  $\mu$ m nitrocellulose for 1 h at 1.5 A. Nitrocellulose was then air-dried, Ponceau S (Sigma Chemical Co.) stained, destained, and blocked for 1 h in 0.3% Tween-20 in TBS, pH 7.4. Nitrocellulose was then washed in TBS, incubated in primary antibody for 2 h, 20°C, and alkaline phosphatase-conjugated goat antimouse, antihuman, or antirabbit (Promega) for 1 h at 20°C, at a dilution of 1:7,000. Immunoblots were visualized using BCIP/NBT (Sigma Chemical Co.) color detection and photographed on Kodak Technical Pan film.

### Immunoelectron Microscopy

Mitotic CHO chromosomes were fixed for 10 min at 20°C in 0.5% paraformaldehyde (Ted Pella, Inc., Irvine, CA) in PME and centrifuged onto glass coverslips through a 33% glycerol cushion in PME. The chromosomes were then labeled with monoclonal 70.1, rinsed in PME, and fixed for 10 min at 20°C in 6% glutaraldehyde, 1% OsO<sub>4</sub>, in 62 mM S-collidine buffer (Ted Pella, Inc.), rinsed in ddH<sub>2</sub>O, dehydrated in ethanol, and embedded in LR White resin (Polysciences, Inc., Warrington, PA) as in reference 17. Sections on grids were labeled with goat antimouse conjugated to 5-nm colloidal gold particles (E. Y. Laboratories, San Mateo, CA). Sections were then stained and observed in a Phillips EM 400 electron microscope.

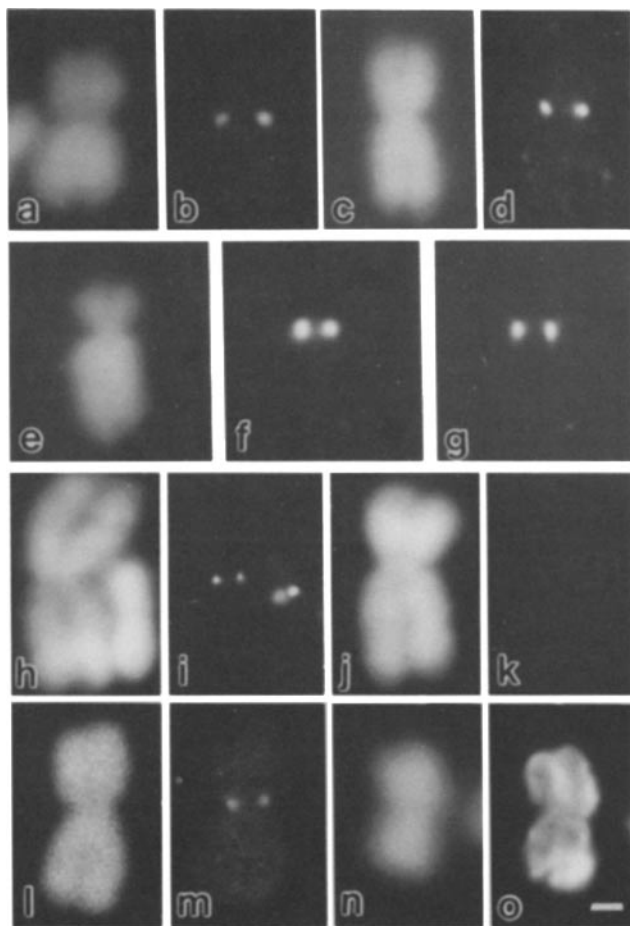
### Microtubule Binding Assays

Mitotic CHO chromosomes were passed into a thin flow chamber consisting of double-stick plastic tape spacers under a coverslip. Flowthrough volume was 20  $\mu$ l. Chromosomes adhered to the glass coverslip. Either 150 mM KI, 5 mM NEM, or 0.1 mg/ml chick brain dynein were flowed through for 5 min. All chromosomes were washed once with PME and then rhodamine-labeled, taxol-stabilized microtubules were flowed in and incubated 3 min at 37°C. Chick cytoplasmic dynein was purified from 10 adult chick brains as described for bovine brain in reference 26. The association of dynein with the kinetochores could be assayed using monoclonal antidynein 440.1, an mAb raised against the heavy chain of chick cytoplasmic dynein (32) that does not crossreact with endogenous CHO chromosomal dynein. The dilution of taxol-stabilized microtubules was chosen such that the number of microtubules bound to the kinetochore could be unambiguously counted. This is due to the fact that the greater the number of microtubules added to the chromosomes, the greater the number that bind to the kinetochore. The unbound microtubules were washed away with PME containing 10  $\mu$ M taxol, and the number of microtubules bound to the kinetochore were counted on the live chromosomes using dim phase illumination with the Zeiss rhodamine filter set.

## Results

### Domains within the Kinetochore Are Labeled by Several Antisera and Reagents that Chemically Modify Proteins

Mitchison and Kirschner (23) have previously demonstrated that CHO chromosomes isolated from vinblastine-treated cells have little or no endogenous tubulin bound to the kinetochore. However, they showed that such chromosomes will readily bind exogenous tubulin monomers specifically at the kinetochore. Bound tubulin can then be detected by antitubulin an-



**Figure 1.** A variety of reagents label kinetochores of isolated CHO mitotic chromosomes. Hoechst and immunofluorescence micrographs of an isolated CHO chromosome that has been incubated in tubulin and labeled with an antitubulin mAb (*a* and *b*). CHO chromosome labeled with pure rabbit antisera (*c* and *d*). Double-label indirect immunofluorescence of CHO mitotic chromosome (*e*–*g*) labeled with CREST antisera (*f*) and antidynein antisera (*g*). Mitotic CHO chromosome incubated with 50  $\mu$ M ATP $\gamma$ S, fixed and labeled with an antisera against thiophosphate groups (*h* and *i*). Control label of unlabeled CHO chromosomes probed with the anti-thio phosphate antisera (*j* and *k*). CHO chromosome incubated 5 min in 1  $\mu$ M Biotin-NEM, fixed and labeled with Texas red-streptavidin (*l* and *m*). Hoechst and immunofluorescence micrographs of mitotic CHO chromosome labeled with an mAb against all histone subclasses (*n* and *o*). Bar, 1.0  $\mu$ m.

tibodies. This is shown in Fig. 1, *a* and *b*. Curiously, rabbit IgG (purified rabbit IgG) at a concentration of 0.2 mg/ml will also label kinetochores of isolated chromosomes (Fig. 1, *c* and *d*, also see Materials and Methods). In whole CHO cells, although the rabbit IgG labels many structures, such as centrosomes, there is no crossreactivity with microtubules (not shown) or with tubulin on immunoblots (Fig. 2). Furthermore, the rabbit IgG strongly labels kinetochores in the absence of bound tubulin. Therefore, the rabbit IgG provides a useful marker for kinetochore-associated proteins other than tubulin.

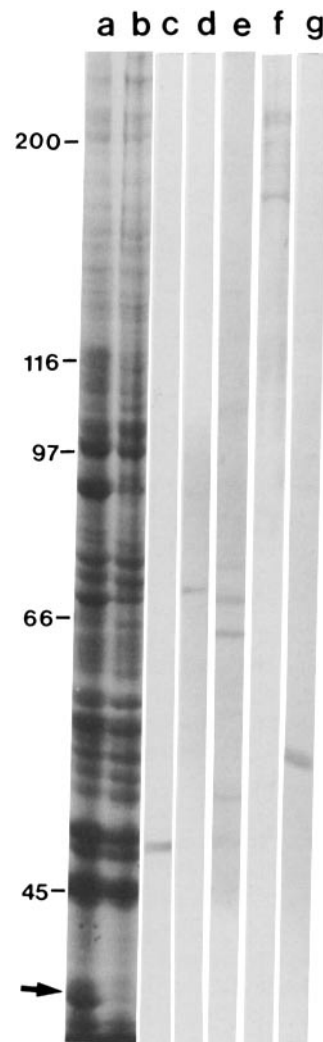
The kinetochores of isolated CHO chromosomes can be specifically labeled with CREST autoimmune sera (1, 12, 13, 20) and with antibodies against the intermediate chains of chick cytoplasmic dynein (32) (Fig. 1, *e*–*g*), and also with

polyclonal antibodies affinity purified against cytoplasmic dynein (27).

Kinetochores also become thiophosphorylated in the presence of low levels of ATP $\gamma$ S, after which they are recognized by an antisera against thiophosphate groups (14) (Fig. 1, *h*–*k*).

Because dynein is exquisitely sensitive to NEM (4, 31), and also because chromosome scaffolds are disrupted by sulfhydryl reducing agents (22), we were interested in the sulfhydryl composition of kinetochores. We found that extremely low levels (1  $\mu$ M, 5 min, 37°C) of NEM conjugated to biotin will label the kinetochore region of isolated CHO chromosomes. Fig. 1, *l* and *m* show a CHO chromosome labeled with NEM-biotin. The biotin moiety allows visualization by fluorescence microscopy using Texas red-streptavidin. Although the labeling appears to be specific to the kinetochore, immunoblots of labeled CHO chromosomes probed with alkaline phosphatase-conjugated streptavidin show cross-reactivity with over 100 peptides, making the identification of specific kinetochore peptides difficult (not shown).

Finally, because some of the kinetochore markers are nonspecific, Fig. 1, *n* and *o* show an isolated CHO chromosome labeled with an mAb against all histone subclasses.



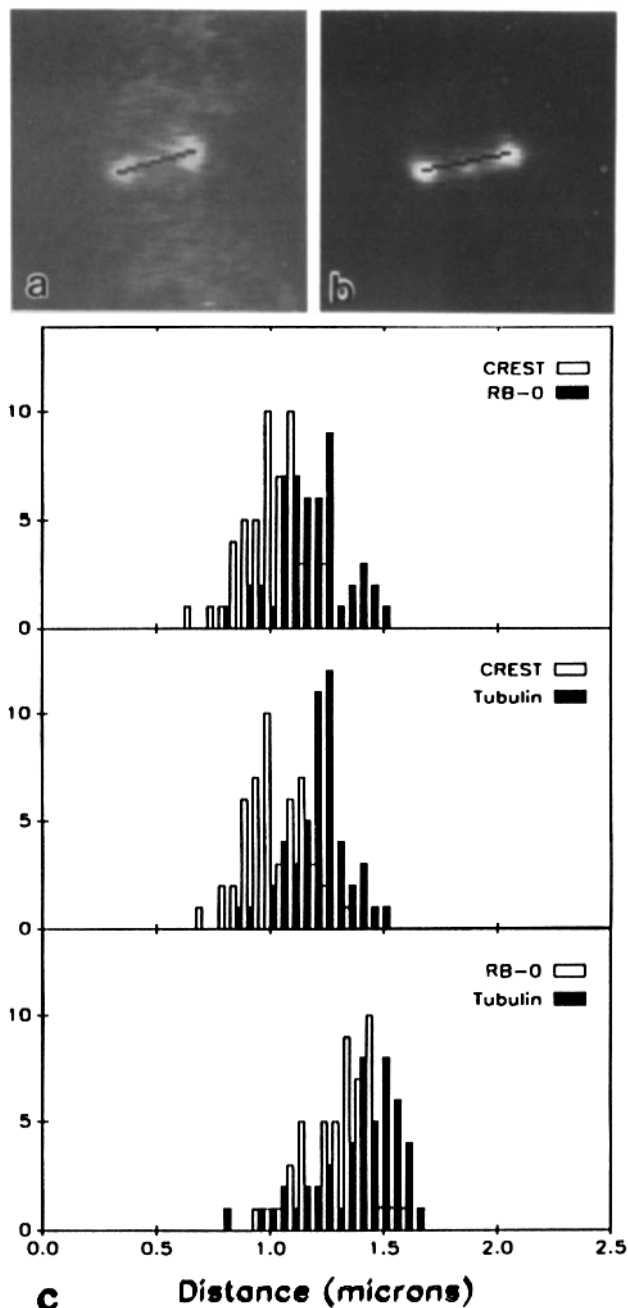
**Figure 2.** Immunoblots of isolated mitotic CHO chromosomes. Coomassie stained 8% SDS-PAGE of isolated mitotic CHO chromosomes (*a*) and whole CHO cells (*b*). Arrow shows histone H1. Immunoblot of mitotic CHO chromosomes probed with CREST autoimmune sera (*c*), antidynein intermediate chains (*d*), anti-thio-P antisera (probed against thiophosphorylated chromosomes) (*e*), pure rabbit antisera (*f*), antitubulin mAb (*g*). All lanes were from the same immunoblot but the Coomassie lanes were from a second gel.

No specific accumulations of label are seen in the kinetochore region. This shows an example in which proteins that are major components of mitotic chromosomes are not specifically accumulated at the kinetochore.

Fig. 2 shows a Western immunoblot of isolated CHO chromosomes probed with the antisera. Fig. 2, *a* and *b* show Coomassie-stained gel lanes comparing mitotic CHO chromosomes (*a*) and whole CHO cells (*b*). The arrow indicates histone H1. Some of the antisera are specific to particular peptides, such as the CREST sera (Fig. 2 *c*), which is strongly specific for a single 50-kD species. This same CREST sera also reacts with the 80-kD CENP-B (12) protein in HeLa chromosomes (not shown). The anti-70.1 (Fig. 2 *d*) is specific to the intermediate chains of cytoplasmic dynein (32). The Western immunoblots of the anti-thio-P antisera against thiophosphorylated chromosomal proteins (Fig. 2 *e*) and the rabbit IgG (Fig. 2 *f*) are more complex, and it is not possible at this time to determine which of the recognized peptides is associated specifically with the kinetochore. The immunoblots are included, however, to show that there are no proteins recognized by the CREST or antidynein sera that are also recognized by the anti-thio-P or rabbit IgG. Therefore, we are using these antisera as markers for as yet unidentified kinetochore-specific proteins. The antitubulin immunoblot is shown in Fig. 2 *g*.

When the staining patterns of some of the kinetochore labels were compared using double-label indirect immunofluorescence microscopy, subtle differences were detectable in the location of the different labeled components. Certain components consistently localized more distally (toward the outer face of the kinetochore) than other components. The location of the labeled components in the kinetochore was independent of the order in which the antisera were applied and independent of the fluorophore used for detection. This phenomenon can be observed qualitatively in the double-labeled CHO chromosome in Fig. 1, *e-g*. CREST-labeled kinetochores (Fig. 1 *f*) appear to be closer together than antidynein-labeled kinetochores (Fig. 1 *e*).

To obtain a statistically significant number of labeled chromosomes, double-labeled kinetochores were recorded on an optical disk recorder and a JAVA image processing program was employed to measure the distance between the center of the label for the two kinetochores for each labeling pattern. An example of this is shown in Fig. 3 along with histograms illustrating the length distributions for the distances between the kinetochores for exogenously bound tubulin, CREST, and rabbit IgG (RB-O). Fig. 3, *a* and *b* show the JAVA image with a calibrated line that measures the distance between the center of each labeled kinetochore for CREST sera and exogenous added tubulin, respectively. Kinetochore-associated tubulin label was consistently located more toward the distal face of the kinetochore, relative to CREST sera label, where interaction with spindle microtubules would be expected to take place. Furthermore, both endogenous tubulin (measured using colcemid-blocked CHO chromosomes that have tubulin bound to the kinetochores when isolated [measurements not shown]) and tubulin exogenously added to the kinetochores of vinblastine-blocked chromosomes, colocalize with the preimmune rabbit IgG even though the rabbit IgG does not react with tubulin on blots (Fig. 2 *e*) or immunofluorescently (not shown). This



**Figure 3.** Measurements of the distance between the center of immunofluorescently double-labeled kinetochores using a JAVA image analysis system. Computer image of CREST-labeled CHO chromosomes with calibrated line measurement (*a*). The same chromosome showing antitubulin label and calibrated line measurement (*b*). Histograms showing the measurements obtained for double-labeled chromosomes. Each histogram shows the paired measurements obtained for 50 chromosomes. CREST sera vs. rabbit IgG (RB-O), CREST vs. added tubulin, and rabbit IgG vs. added tubulin are shown.

suggests that the tubulin-binding zone of isolated kinetochores also contains other proteins.

Tables I and II show the measurements obtained for the different kinetochore labels. The absolute measurements, in microns, obtained for the different labels are variable de-

**Table I. Locations of Kinetochores Proteins**

Antisera	CREST	Anti-tubulin	CREST	Anti-dynein	Anti-tubulin	Anti-dynein
Mean distance ( $\mu\text{m}$ )	1.00	1.18	0.79	1.03	1.03	1.02
SD	0.14	0.13	0.19	0.26	0.21	0.20
Paired t-value	-2.8		-14.2		-0.9	
Conclusion	Significantly different		Significantly different		No difference	

pending on the fixation conditions and the chromosome stabilizing buffers used, but in all cases the location of the label relative to other labels was consistent from preparation to preparation. These results show that dynein, tubulin, and components recognized by preimmune rabbit IgG consistently colocalize distally on isolated kinetochores, whereas thiophosphoproteins and CREST antigens are relatively internal.

These results are summarized in the diagram in Fig. 4. CREST sera antigens were consistently found most internally; biotin-NEM labeling exhibited statistically significant localization external to the CREST sera label, but internal to the tubulin and dynein staining. Thiophosphorylated proteins were located only very slightly internal to the tubulin and dynein staining, but external to the CREST and NEM-biotin label.

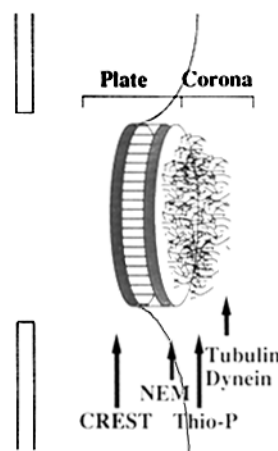
### EM Shows Labeling of the Fibrous Corona

We labeled isolated CHO chromosomes with primary antisera against the intermediate chains of cytoplasmic dynein (32). The chromosomes were then triple-fixed, embedded in L. R. White, and sectioned (17). The sections were labeled with goat antimouse conjugated to 5-nm gold particles. This method was used in order to eliminate the possibility of artifactual exclusion of gold particles from the kinetochores plate. Fig. 5 *a* shows a control isolated CHO chromosome embedded and sectioned for EM. The trilaminar plate and fibrous corona are visible. Fig. 5 *b* shows immunogold labeling of dynein bound to the kinetochores. The gold label is restricted to the fibrous corona. In addition, Mitchison and Kirschner used immunogold labeling to show that the corona is the location of bound tubulin (23). This result can be extended to suggest that antigens that colocalize with tubulin or dynein, as measured by indirect immunofluorescence, are components of the fibrous corona.

**Table II. Locations of Kinetochores Proteins**

Antisera	Biotin-NEM	Anti-dynein	CREST	Anti-thio-P	Anti-thio-P	Anti-dynein
Mean distance ( $\mu\text{m}$ )	1.03	1.13	0.98	1.12	1.10	1.11
SD	0.15	0.14	0.18	0.22	0.11	0.11
Paired t-value	7.9		-15.4		1.71	
Conclusion	Significantly different		Significantly different		Different	

### Kinetochores Subdomains



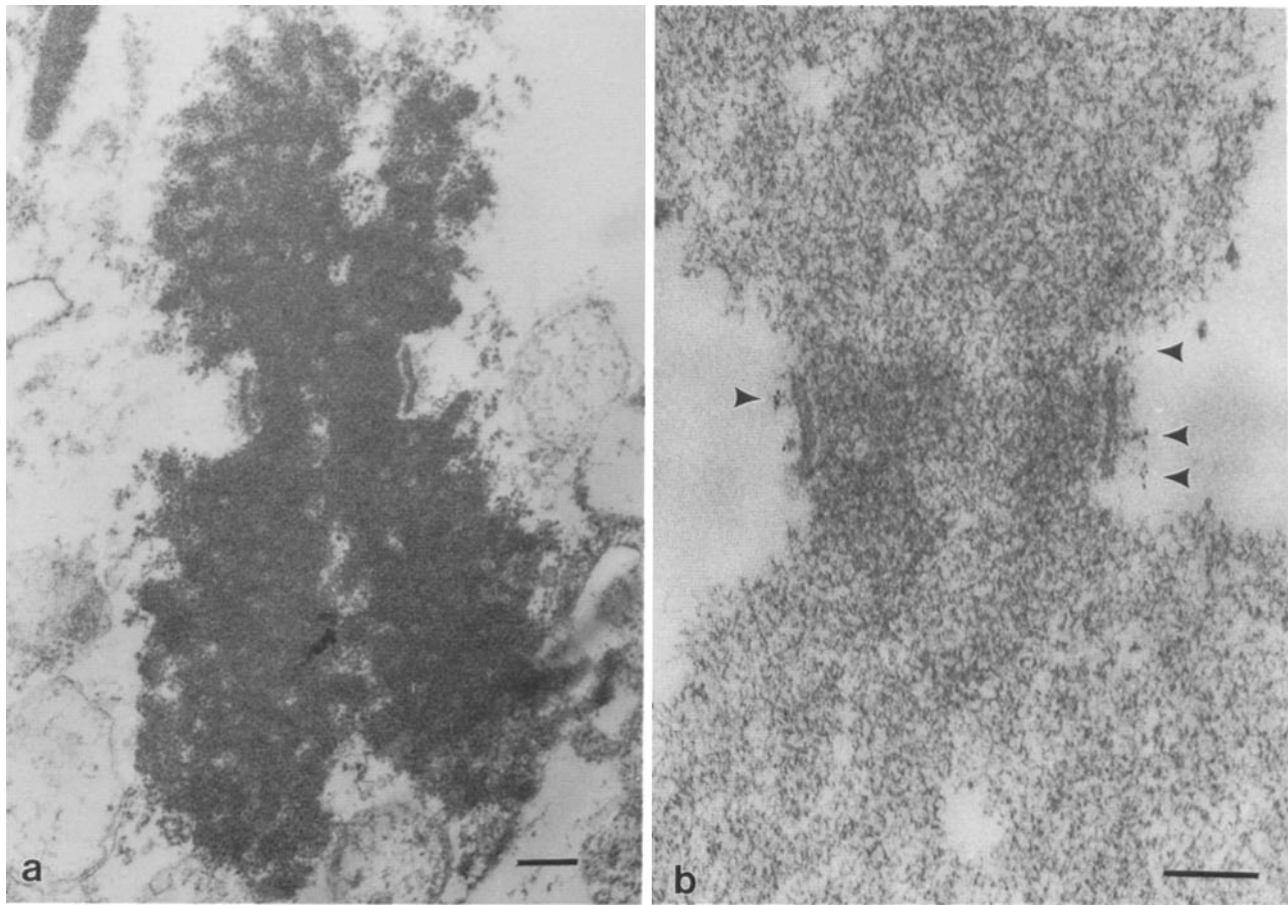
**Figure 4.** Relative locations of biochemically distinct kinetochores components. CREST sera were located most internally, whereas tubulin and dynein (which colocalize) were located most distally to the trilaminar kinetochores plate. NEM-biotin and thiophosphorylated proteins exhibited intermediate locations relative to these other components.

### Salt Extractions of Labeled Components from Isolated Kinetochores

Table III shows the salt extractability of the components that are found on kinetochores of isolated CHO chromosomes. Some of the extractions were done in a flow chamber after which the chromosomes were fixed and processed for immunofluorescence. Endogenous dynein, endogenous tubulin, rabbit IgG antigens, and CREST sera antigens were compared. All of the components of the kinetochores were stable to detergent extraction as assayed by immunofluorescence. However, the components exhibited differential sensitivity to salt extraction. Endogenous dynein was most sensitive to salt extraction. Surprisingly, endogenous tubulin was less sensitive to salt extraction than endogenous dynein. Thiophosphorylated proteins were not detectably extracted by 150 mM KI (not shown). The protein(s) recognized by rabbit IgG were the most difficult to extract, suggesting that the rabbit IgG is recognizing other, as yet unidentified, components of the fibrous corona that are distinct from dynein and tubulin. We found that none of these conditions extracted CREST sera antigens from the kinetochores. CREST sera antigens are very resistant to extraction by salt and urea and are in fact highly enriched in chromosomes after these treatments (1, 35). Finally, we also found that tubulin could be rebound to the kinetochores of 150 mM KI-washed chromosomes even after indirect immunofluorescence indicates that the endogenous dynein is gone.

Coomassie-stained gel lanes of CHO chromosomes before and after extraction with 150 mM KI are shown in Fig. 6 *A*, along with immunoblots probed with the antisera against the 70-kD intermediate chains of chick cytoplasmic dynein (Fig. 6 *C*) and with antitubulin (Fig. 6 *B*). As was predicted by the immunofluorescence results, the endogenous tubulin and endogenous dynein were extracted with 150 mM KI.

Antidynein mAbs against the heavy chains of chick cytoplasmic dynein (440.1, 440.4), (32) do not crossreact with CHO chromosomal dynein. However, in addition to the antidynein intermediate chains (70.1), one of the mAbs (440.4) does crossreact with cytoplasmic dynein found on isolated HeLa chromosomes. We have found that the 150-mM KI ex-



**Figure 5.** Immunoelectron microscopy of isolated mitotic CHO chromosomes. Unlabeled CHO chromosome showing trilaminar plate and fibrous corona (a). Antidynein localization using 5-nm gold-conjugated protein A. No gold label was visible on the chromosome arms or LR White background (b). Bar, 0.2  $\mu\text{m}$ .

traction will also extract dynein from HeLa chromosomes. Fig. 7 a shows an isolated HeLa chromosome labeled with mAb 440.4 against the heavy chain of chick cytoplasmic dynein. After extraction with 150 mM KI, the antidynein heavy chain staining is gone (Fig. 7 d), although CREST sera label (Fig. 7, b and e) is still evident. Identical results

are obtained when dynein extraction is monitored with the antidynein intermediate chain's mAb.

### *Cytoplasmic Dynein Binds to Kinetochores of Isolated CHO Chromosomes*

Previous work has shown that tubulin monomers will bind specifically to the kinetochore of isolated CHO chromosomes (23). We tested whether purified chick cytoplasmic dynein will bind to kinetochores of isolated CHO chromosomes by incubating chromosomes in chick cytoplasmic dynein and then probing with an mAb to chick dynein heavy chain that does not crossreact with endogenous CHO dynein. Exogenous dynein label was detectable associated with kinetochores at concentrations as low as 0.1 mg/ml exogenous dynein (Fig. 8). Concentrations of BSA (0.1 mg/ml) or monomeric actin (1 mg/ml) did not label kinetochores.

### *Kinetochore-associated Cytoplasmic Dynein Is Not Essential for Tubulin or Microtubule Binding to Isolated Kinetochores*

Fig. 9, a-c shows an unfixed CHO chromosome with a microtubule bound to the kinetochore composed of rhodamine-conjugated tubulin. In Fig. 9 a, the phase image of the chromosome is visible along with the fluorescent image of the microtubule. This illustrates how the bound microtubules

**Table III. Salt Extractions of Kinetochore Proteins**

Treatment	Antisera		
	Antidynein	Antitubulin	Antirabbit
PME Buffer	+++	+++	+++
150 mM KI	-	-	+++
250 mM KI*	-	-	-
1.0% TX-100*	+++	+++	+++
150 mM MgSO <sub>4</sub> *	-	-	+
150 mM KCl	+++	+++	+++
450 mM KAc*	+	+++	+++
150 mM KI →	-	+++	+++
Tubulin‡			

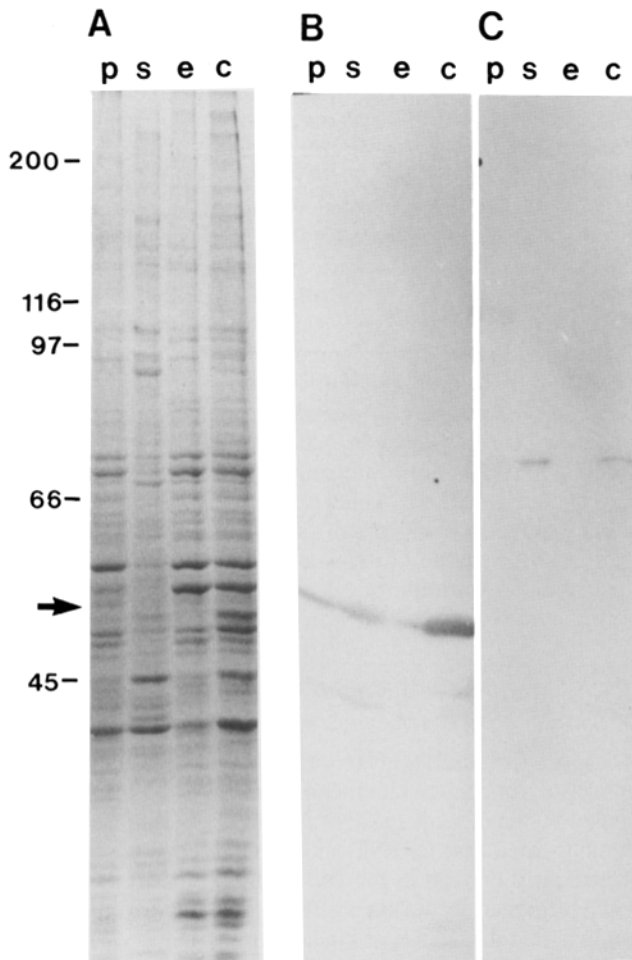
+++ refers to immunofluorescent label of maximum brightness as seen in chromosomes in control (PME) buffer.

- refers to immunofluorescent label not above background.

\* Chromosome structure begins to suffer.

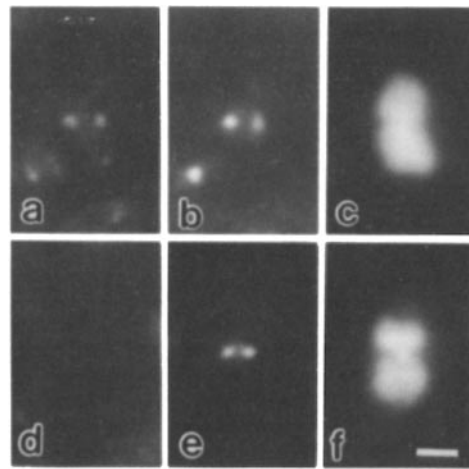
‡ Chromosomes for this experiment were vinblastine treated and had no endogenous tubulin on the kinetochores. Tubulin was added after salt wash.





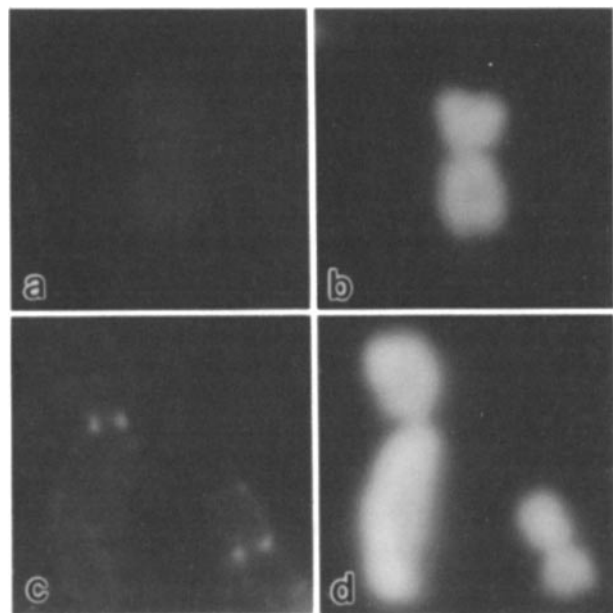
**Figure 6.** Immunoblots of salt-extracted chromosomes. (A) Coomassie stained 7% SDS-PAGE gel showing (c) control CHO chromosomes, (e) 150 mM KI-extracted chromosomes, (s) 150 mM KI supernatant after dialysis against PME, (p) 150 mM KI pellet after dialysis against PME; this lane represents protein that was extracted from chromosomes but irreversibly denatured after dialysis. Tubulin is visible in Coomassie-stained gels of isolated chromosomes (arrowhead). (B) Antitubulin immunoblot of salt-extracted chromosomes. Tubulin is extracted from isolated chromosomes and found in both salt extract lanes. (C) Antidynein immunoblot of isolated chromosomes. Dynein is extracted from chromosomes and is found in the salt extract. Extraction of chromosomes with 150 mM KI results in no disruption of mitotic chromosome structure.

were scored for the different treatments. Because the primary constriction (in phase) and the bound microtubules (in fluorescence) are visible at the same time, the attachment of the microtubules to the kinetochore region is unambiguous, within the limits of the light microscope. In Fig. 9, *b* and *c*, the Hoechst image of another chromosome and the fluorescent image of a bound microtubule are shown, respectively. Unambiguous association of this microtubule with the kinetochore region was checked by phase/fluorescence microscopy. We tested the effect of 150 mM KI, NEM incubation, and dynein incubation on the binding of short microtubule segments at the kinetochore. The dynein used was chick cytoplasmic dynein isolated as in reference 26 and assayed for activity by its ability to move microtubules on glass. The isolated chick cytoplasmic dynein bound microtubules in a dose-

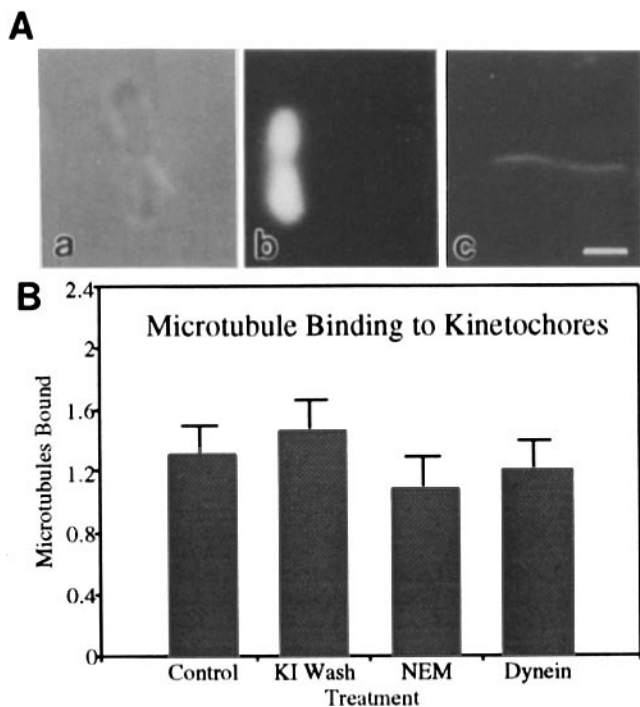


**Figure 7.** Extraction of dynein heavy chain from isolated HeLa chromosomes. Indirect immunofluorescence of isolated HeLa chromosomes double labeled with mAb 440.4 (*a* and *d*), CREST sera (*b* and *e*), or Hoechst (*c* and *f*) before (*a-c*) and after (*d-f*) extraction with 150 mM KI. Bar, 2  $\mu$ m.

dependent manner and translocated microtubules in the presence of ATP at a speed of 40  $\mu$ m/min. When we assayed CHO chromosomes for microtubule-binding activity, within the limits of the light microscope, we found no statistical difference in the number of microtubules bound, relative to control chromosomes, after these treatments. This is interesting because it suggests that, although purified dynein adsorbed onto glass has been shown to be able to bind microtu-



**Figure 8.** Isolated chick cytoplasmic dynein binds to isolated CHO kinetochores. Hoechst (*b*) and indirect immunofluorescence (*a*) micrographs of CHO chromosomes labeled with an mAb against the heavy chain of chick cytoplasmic dynein. No crossreactivity is observed between the antibody and endogenous CHO dynein. After incubation with 0.1 mg/ml chick cytoplasmic dynein, kinetochores are labeled by the antisera against the heavy chain of chick cytoplasmic dynein (mAb 440.1) (*c* and *d*).



**Figure 9.** Effect of various treatments on the binding of microtubules to kinetochores in vitro. (A) Phase/fluorescence image of an unfixed isolated CHO chromosome with a rhodamine-tubulin-labeled microtubule bound at the kinetochore. Counts were done on images like this where both the kinetochore region and the labeled microtubule were clearly visible (a). Hoechst-labeled live isolated CHO chromosome (b). Rhodamine fluorescence image of microtubule bound to the chromosome in b (c). (B) Graph of microtubules bound at the kinetochore per chromosome after various treatments. No statistically significant difference was apparent in microtubule binding activity at the kinetochore after the various treatments. Bar, 4.0  $\mu\text{m}$ .

bules, there are probably other molecules associated with the kinetochore that can bind microtubules and tubulin monomers.

## Discussion

### *Certain Antigens Consistently Colocalize to Subdomains within the Kinetochore*

We have shown that antigens that are specific to the kinetochore of isolated CHO chromosomes actually localize to subdomains within the very small but highly structured kinetochore. We were able to compare several different kinetochore-specific proteins because of the ease with which statistically significant numbers of measurements could be made at the level of the light microscope. Several antigens show consistent patterns of distribution relative to each other. Furthermore, we have used this technique to identify as yet uncharacterized kinetochore proteins that may have an important role in chromosome movement during mitosis.

The relative ease and reproducibility of immunofluorescence in comparison to immunoelectron microscopy, and the facility with which the former can be used to screen large amounts of labeled material makes it a preferable technique for the analysis of subcellular localization whenever possible. We have used light microscopy, in combination with cor-

relative EM, to reveal subtle differences in the location of a number of proteins that are associated specifically with the kinetochore of isolated mitotic CHO chromosomes. We have found that tubulin, dynein, and certain other as yet uncharacterized antigens recognized by pure rabbit IgG are components of the fibrous corona on the distal face of the kinetochore. The 50-kD CREST antigen is located internally relative to the fibrous corona. We used CHO chromosomes for the measurements because they have fairly large kinetochores that facilitate the measurements and they have been used traditionally for in vitro studies of kinetochore function. However, we have tested all our labeling techniques on HeLa and Muntjac chromosomes and we have found that our results are supported qualitatively in these systems. We believe that the measurements reflect a functional segregation of kinetochore proteins in which tubulin and microtubule motor molecules are principally associated with the distal face of the kinetochore, which is the region responsible for the initial interactions with the mitotic spindle (18, 19); whereas kinetochore-specific proteins associated primarily with the structural organization of kinetochores are located internal to the corona proteins.

### *The Limitations of a Functional Dissection of Kinetochore Biochemistry*

There are two limitations to our model for the spatial segregation of functional kinetochore proteins. The first is revealed by the elegant study of Bernat et al. 1990 (3), which demonstrated that CREST autoimmune antisera, when injected early enough in the cell cycle, will disrupt chromosome segregation during mitosis. The implication of this study is that disruption of kinetochore structure early in the cell cycle by CREST sera prevents the organization of a functional mitotic kinetochore. This work is not incompatible with our theory, however, it suggests that the assignment of functions to specific kinetochore proteins will be complicated by their interactions with each other, especially when the only testable kinetochore activities are downstream events. The second consideration is that the interaction of tubulin and microtubules with kinetochores is more complex than is suggested here. Electron micrographs of kinetochores in metaphase and anaphase cells show microtubules firmly imbedded in the kinetochore (7, 28, 30) and, in some cases, microtubules appear to pass right through the kinetochore (24, 29). Furthermore, the crosslinking studies of Balczon and Brinkley (1) suggest that the major tubulin-binding protein in CHO chromosomes is an 80-kD CREST autoimmune antigen that is resistant to stringent purification procedures. This 80-kD protein has been described as CHO CENP-B (1). Our antisera reacts very specifically with a 50-kD protein in CHO chromosomes. Anticentromere sera affinity purified from the human 80-kD centromere protein has been shown to crossreact with a 50-kD protein in rat chromosomes (12). Also, a 50-kD centromere protein has been described in muntjac chromosomes as CENP-D (20). Therefore, it is likely that the 50-kD CREST antigen in our studies is a different protein from the 80-kD tubulin-crosslinkable CREST antigen identified by Balczon and Brinkley (1) also identified in CHO chromosomes. Lastly, we have shown that the removal or chemical inactivation of at least one known microtubule-binding motor protein, dynein, does not affect the association of



microtubules with isolated kinetochores. Although our results suggest that the corona is the principal site of tubulin and microtubule binding activity, and real time video and electron microscopic studies (18, 29) suggest that the corona is the major site of microtubule-based motility during mitosis, it is probably true that the interaction of microtubules with the kinetochore involves additional levels of complexity and further functional assays are required before final conclusions can be drawn.

### Selective Extraction of Kinetochore Components

It is important to consider the possibility that many proteins essential for kinetochore function may be relatively loosely associated with the kinetochore. Nuclear envelope breakdown at mitosis may liberate kinetochores to opportunistically bind cytoplasmic proteins, such as motor molecules, in order to effect microtubule-dependent translocation, after which time such proteins may be returned to the cytoplasmic milieu. For this reason, our method for isolating mitotic chromosomes is extremely gentle and is designed to maximize protein-protein interactions. The disadvantage of our procedure is that the chromosomes are biochemically complex and contain many potential contaminants. By acquiring as many markers for kinetochore components as are available and using salt extractions in combination with indirect immunofluorescence, we have been able to progressively strip away components of the complex kinetochore corona. This technique can be used for the partial purification of kinetochore proteins and also in vitro functional assays to selectively extract and add back kinetochore components in order to determine what components are crucial for the movement of chromosomes on microtubules.

The selective extraction of kinetochore proteins by relatively low levels of salt suggest that one should reevaluate the previously published methods of isolating purified kinetochores, at least from the standpoint of studying kinetochore function. Previously, purified kinetochores (or scaffold preparations) were prepared using long DNA digests and stringent salt and urea washes. The enrichment of CREST sera antigens was used as a marker to monitor the successive steps in the purification procedure (35). Such techniques provided invaluable information about the biochemical composition of kinetochores. However, our studies and others (1, 35) suggest that the CREST sera antigens are the most tenaciously bound kinetochore-associated proteins. Furthermore, we believe that many functionally significant, kinetochore-associated proteins, especially motor proteins like cytoplasmic dynein, are lost during harsh purification steps. As discussed earlier, the case of tubulin-binding proteins is more complex. Balczon and co-workers monitored tubulin-binding and microtubule nucleation activity throughout the course of their stringent purification procedure, and found that such activity persisted in their final scaffold preparation (2). Therefore, although our study and previous studies (22) suggest that tubulin binding is a function of the corona, there may be multiple tubulin-binding activities within the kinetochore. This is not an unreasonable statement considering the importance of chromosome attachment to the mitotic spindle for the proper segregation of genetic material during cell division.

### Further Characterization of Kinetochore Proteins

Presently, we are analyzing the biochemical composition of

the kinetochore corona in greater detail. The complex behavior of chromosomes in mitotic cells (2, 25) and in vitro (23, 24) cannot be explained by tubulin, microtubule dynamics, and dynein alone. In the above study, we have used the rabbit IgG as a marker for as yet unidentified proteins that comprise the corona. We have used differential salt extractions and Western immunoblots as evidence that we are looking at different protein species and not at shared antigenic determinants recognized by different antisera. However, the purified rabbit IgG recognizes a complex mixture of proteins, of which the kinetochore protein may be only a minor component. Consequently, we are generating more defined antisera against components of the corona in the hope of identifying more microtubule motor molecules, cytoplasmic dynein-binding molecules, and further tubulin- and microtubule-binding molecules.

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