Differential Expression of a Phosphoepitope at the Kinetochores of Moving Chromosomes

Gary J. Gorbsky and William A. Ricketts

Department of Anatomy and Cell Biology and The University of Virginia Cancer Center, University of Virginia, Charlottesville, Virginia 22908

Abstract. A phosphorylated epitope is differentially expressed at the kinetochores of chromosomes in mitotic cells and may be involved in regulating chromosome movement and cell cycle progression. During prophase and early prometaphase, the phosphoepitope is expressed equally among all the kinetochores. In mid-prometaphase, some chromosomes show strong labeling on both kinetochores; others exhibit weak or no labeling; while in other chromosomes, one kinetochore is intensely labeled while its sister kinetochore is unlabeled. Chromosomes moving toward the metaphase plate express the phosphoepitope strongly on the leading kinetochore but weakly on the trailing kinetochore. This is the first demonstration of a biochemical difference between the two kinetochores of a single chromosome. During metaphase and anaphase, the kinetochores are unlabeled. At metaphase, a single misaligned chromosome can inhibit further progression into anaphase. Misaligned chromosomes express the phosphoepitope strongly on both kinetochores, even when all the other chromosomes of a cell are assembled at the metaphase plate and lack expression. This phosphoepitope may be involved in regulating chromosome movement to the metaphase plate during prometaphase and may be part of a cell cycle checkpoint by which the onset of anaphase is inhibited until complete metaphase alignment is achieved.

In higher eukaryotes prometaphase beings with the breakdown of the nuclear envelope. Normally one of the kinetochores on each chromosome captures and stabilizes microtubules emanating from one of the mitotic poles. The chromosome then orients and moves rapidly to that pole (21, 36). In due course, microtubules from the opposite pole attach to the free kinetochore. Then, with microtubule bundles extending to both poles, each chromosome undergoes a series of saltatory movements culminating in its arrival at the metaphase plate midway between the mitotic poles.

Until the past few years the dominant theory of chromosome movement in anaphase was the traction fiber hypothesis, which held that chromosomes moved because they were attached to microtubules that were "reeled in" at the poles. We (15, 16) and others (37, 40) demonstrated that the majority of chromosome motility in anaphase occurs by the translocation of the chromosomes along the spindle microtubules. Subsequently, chromosomes were also found to move along spindle microtubules in prometaphase (3, 4, 44). Recently it has been found that some poleward microtubule movement or "flux" occurs in prometaphase and metaphase cells (14, 17, 38) and to a lesser extent in anaphase cells (39). Thus while chromosome motility is due primarily to the movement of chromosomes along the microtubules, poleward microtubule translocations may also contribute.

Please address all correspondence to Dr. Gary J. Gorbsky, Box 439, University of Virginia Health Sciences Center, Charlottesville, VA 22908.

At the molecular level much interest now centers on the kinetochore, what powers its movements on spindle microtubules, and how these movements are properly guided. Evidence suggests that microtubule motors play an important part in chromosome motion. Rieder and Alexander (44) showed that prometaphase kinetochores can move rapidly along the lateral surfaces of microtubules in the absence of microtubule disassembly. This evidence indicates that kinetochores contain microtubule motors, analogous to those that move organelles in fast axonal transport. In lower eukaryotes a number of mutations in proteins containing microtubule motor domains cause defects in meiosis and mitosis (8, 9, 19, 23, 34, 35, 53) although it is not yet certain which if any of these are due to defects in chromosome motility per se. Immunolabeling studies have revealed the presence of cytoplasmic dynein (42, 47, 51) and kinesin-related proteins (45) at the kinetochore. In mammalian cells microinjection of antibodies to a putative kinetochore protein containing microtubule motor domains caused disruption of normal chromosome segregation (52). A protein complex exhibiting poleward-directed microtubule motility has been isolated from yeast extracts by affinity purification on a centromere-specific DNA sequence (27).

While evidence implicating microtubule motors is strong, some experiments indicate that microtubule assembly and disassembly may generate forces in moving chromosomes. In reconstituted systems consisting of isolated microtubules attached to chromosomes, dilution of the tubulin concentra-

tion induces microtubule disassembly at the kinetochore. This disassembly can by itself drive chromosome movements in vitro (5, 30). Whether microtubule assembly or disassembly plays a role in driving chromosome movement in living cells is uncertain.

While chromosomes in prometaphase arrive at the metaphase plate at various times, they enter anaphase synchronously. Cells containing misaligned chromosomes are delayed an unusually lengthy time before entering anaphase (54). Thus the presence of misaligned chromosomes appears to act as a checkpoint, inhibiting further progress through mitosis. Cells contain surveillance mechanisms that monitor proper assembly of the mitotic spindle. The disruption of the spindle with the use of microtubule inhibitors is a long standing method of accumulating mammalian cells in mitosis (for review see Rieder and Palazzo [43]). Recently, researchers have begun to dissect this checkpoint mechanism genetically through the isolation of mutants in yeast that fail to block mitotic progression in response to microtubule inhibitors (24, 32).

An accumulating body of evidence points to essential roles for kinases and phosphatases in many aspects of cell cycle regulation in M-phase (for review see Jacobs [28]). Some recent experiments directly implicate phosphorylation and dephosphorylation in checkpoint control pathways for mitotic progression. Larsen and Wolniak (50) reported that phosphatase inhibitors applied to plant cells in culture result in the asynchronous entry of chromosomes into anaphase. Andreason and Margolis (1) showed that cells treated with 2-aminopurine, a nonspecific kinase inhibitor, could escape mitotic arrest by microtubule inhibitors. In lower eukaryotes, several mitotic arrest mutants have been shown to contain defects in phosphatases (2, 7, 29). Fernandez et al. (10) found that antibodies to Protein Phosphatase 1 (PP1)[1] exhibit intense immunofluorescent labeling of mitotic chromosomes. They further demonstrated that mitotic progression was blocked by the microinjection of cells with antibody to PP1 that inhibits its enzyme activity. Microinjection of mammalian cells with an antibody to cdc25A phosphatase blocks cells in mitosis (11). Protein components of important structural components such as mitotic poles and kinetochores become specifically phosphorylated at mitosis. The mpm-2 antibody, a monoclonal anti-phosphoepitope antibody labels mitotic poles, kinetochore fiber microtubules, as well as kinetochores and chromosome arms (49). Results from our laboratory have recently demonstrated that topoisomerase II is the major target of this antibody in mitotic chromosomes (48).

In the present study we used 3F3/2 monoclonal antibody, originally prepared by Drs. Martha Cyert and Mark Kirschner to extracts of Xenopus eggs that were treated with ATP-γ-S (6, 13). This antibody was thought to bind exclusively to proteins that were thiophosphorylated by in vitro treatment with ATP-γ-S in the presence of an active kinase, giving rise to the idea that the thiophosphate moiety was an obligate component of the antibody epitope. As a result the 3F3/2 antibody has generally been referred to as a "thiophosphate antibody" (31, 51).

We have found that the 3F3/2 antibody binds to structures within cells that have not been exposed to ATP-γ-S. In mitotic cells, the antibody binds to kinetochores in an unprecedented and intriguing manner. Expressed on all kinetochores in prophase and early prometaphase cells, the epitope becomes differentially expressed at the kinetochores of cells in mid to late prometaphase. The epitope may identify a regulatory event that controls chromosome movement to the metaphase plate. It may also signal a checkpoint control pathway by which anaphase onset is inhibited until all chromosomes are properly aligned at the metaphase plate.

Materials and Methods

Cell Culture and Fixation

The permanent cell lines Potoroo tridactylis kidney (Ptk1) and MDBK (Maudin-Darby bovine kidney) were obtained from the American Type Culture Collection (Rockville, MD). Ptk1 cells were cultured in Ham's F12 medium (GIBCO BRL, Gaithersburg, MD) containing 10% Nu-serum (Collaborative Research, Bedford, MA) and penicillin and streptomycin. MDBK cells were grown in DMEM enriched with nonessential amino acids and supplemented with 5% bovine calf serum (Hyclone Laboratories, Logan, UT), 20 mM Hepes and antibiotics. For immunofluorescence, cells were grown on 22-mm coverslips. When ~50–75% confluent, the cells were rinsed twice with 60 mM Pipes, 25 mM Hepes at pH 6.95, 10 mM EGTA, and 2 mM $MGCl_2$ (PHEM) and lysed with 1.0% CHAPS detergent in PHEM with 40 nM microcystin (GIBCO BRL, Gaithersburg, MD). After 5 min in the lysis buffer, cells were fixed in 1.0% formaldehyde (freshly prepared from paraformaldehyde) in PHEM for 15 min. The cells were then rinsed twice in 10 mM Mops at pH 7.4, 150 mM NaCl containing 0.05% Tween 20 (MBST) and stored in the same buffer until used for immunolabeling. In some instances, after fixation, cells were treated for 30 min at 37°C with 250 U/ml PP1 (provided by Dr. Timothy Haystead, Department of Biochemistry, University of Virginia) in PHEM. To determine whether preextraction with detergent caused changes in the immunolabeling patterns, some cells were fixed before treatment with detergent.

Immunolabeling

The 3F3/2 antibody and hybridoma cell line was obtained from Drs. Linda Wordeman and Tim Mitchison at the University of California, San Francisco. Before immunolabeling, cells were treated with 20% boiled normal goat serum in 10 mM Mops at pH 7.4, 150 mM NaCl (MBS) for 30 min at room temperature to bock non-specific binding. Cells were labeled with an ascites preparation of the 3F3/2 antibody diluted 1:4,000 in 5% boiled goat serum in MBS. A human autoimmune CREST scleroderma serum (a gift from Dr. J. B. Rattner, University of Calgary) was diluted 1:2,000 and used to label all the kinetochores. After incubating with the primary antibodies for 45 min at room temperature, the cells were rinsed in MBS with 0.05% Tween 20 (MBST) and washed in the same buffer for 20 min with gentle agitation. The cells were then treated with fluorescein-conjugated, goat anti-human IgG (Pierce, Rockford, IL) at 1:200 and Texas red-conjugated goat anti-mouse IgG (Jackson Immunoresearch Laboratories, West Grove, PA) at 1:400 in MBS containing 5% boiled goat serum. After 45 min at room temperature, the cells were again rinsed and washed for 20 min in MBST. The cells were then rinsed in H_2O and labeled with 0.5 μg/ml DAPI for 5 min. After a final rinse in H_2O, the cells were mounted in Vectashield (Vector Laboratories, Burlingame, CA) that was supplemented with 10 mM $MgCl_2$ to maintain better preservation of chromatin. Finally the edges of the coverslips were sealed with clear nail polish.

For some experiments the 3F3/2 antibody was microinjected into living cells growing on coverslips. For these experiments, the 3F3/2 antibody was purified from ascites on Protein A–Sepharose (20) and dialyzed to 10 mM $NaPO_4$ at pH 7.2, 150 mM KCl. We microinjected cells to ~5% their volume as described previously (15), using 1.6 mg/ml IgG in the micropipette. After microinjection at room temperature, the cells were incubated ~10–15 min at 37°C, lysed with detergent, and fixed. The cells were immunolabeled

1. *Abbreviations used in this paper*: MDBK, Maudin-Darby bovine kidney; MBS, 10 mM Mops at pH 7.4, 150 mM NaCl; MBST, MBS with 0.05% Tween 20; PHEM, 60 mM Pipes, 25 mM Hepes at pH 6.95, 10 mM EGTA, and 2 mM $MGCl_2$; PP1, Protein Phosphatase 1; Ptk1, Potoroo tridactylis kidney; TBST, 10 mM Tris, pH 8.0, 150 mM NaCl 0.05% Tween 20.

as described above except the 3F3/2 antibody was not used in the incubation step for the primary antibodies.

Immunofluorescence observations were performed with a Nikon Diaphot microscope equipped for epifluorescence and phase contrast microscopy. Cells were imaged with a Nikon 60× 1.4 NA objective through a 6.1 × coupler (Optec Inc., Lowell, MI) into a Genisys intensifier and CCD video camera (Dage-MTI, Michigan City, IN). The images were collected digitally onto removable hard disks (Syquest, Fremont, CA) using Image 1 hardware and software (Universal Imaging, Media, PA). The gain and offset of the intensifier, camera and image processor were adjusted to maintain the image within the linear range of the system. Micrographs for publication were produced by photographing images from the image monitors or with a film recorder. In some cases false color was introduced into the black and white images via routines from the image processor.

Live Cell Recording

For optimal phase contrast observations of living cells, the bottoms of 35-mm plastic dishes were drilled with 18-mm diam holes. The holes were then covered and sealed by overlaying 22-mm glass coverslips secured with a ring of silicone grease. A scratch pattern, produced with a diamond scribe, was placed on the coverslip to act as a reference in relocating observed cells. The dishes and lids were sterilized by inversion for 5 min onto an ultraviolet transilluminator (UVP Inc., San Gabriel, CA). Ptk1 cells were plated onto these chambers and incubated until ~50% confluent. Before use the medium was replaced with fresh medium that was then overlain with light mineral oil to prevent evaporation. The chambers were placed on the stage of the Nikon Diaphot. Temperature was maintained at 33 ± 1°C by means of a hair dryer controlled by a rheostat. Live cells were observed by phase contrast microscopy using a Nikon 100× 1.3 NA objective, a 2× coupler and a Dage CCD camera. Images from the camera were averaged every four frames to reduce noise and collected onto video tape with an Extended Definition Beta video recorder (Sony, Cypress, CA). Images selected at intervals were digitally stored and photographed as described above.

Cells in mid to late prometaphase containing one or more chromosomes that were located near the poles were observed. The location of the cell in relation to the scratch mark on the coverslip was noted. When definitive movement of a chromosome toward the metaphase plate took place, the chamber was removed from the stage of the microscope, rinsed twice with PHEM buffer and lysed with 1% chaps detergent in PHEM containing 40 nM microcystin. The length of time between the final video image of the live cell until lysis with detergent was 12–15 s. After lysis, the cells were fixed for 15 min in 1% formaldehyde in PHEM. After rinsing in MBST, the coverslips were removed from the chambers and processed for double label immunofluorescence as described above.

Cell Fractionation and Immunoblotting

Chromosomes were prepared through modifications of the methods of Gasser and Laemmli (12). To accumulate mitotic cells, 10 150-mm dishes of 3/4 confluent MDBK cells were blocked 16 h with 0.15 μg/ml colcemid. The rounded mitotic cells were rinsed off the dishes and collected by centrifugation at 300 *g* for 5 min. They were washed twice with a room temperature, swelling buffer containing 10 mM Hepes at pH 7.4, 40 mM KCl, 5 mM EGTA, 4 mM $MgCl_2$, 1 mM DTT, 40 nM microcystin, and 1 μg/ml of each of the following protease inhibitors: aprotinin, leupeptin, antipain, pepstatin, and chymostatin. The cells were then chilled on ice and lysed with a Dounce homogenizer in 1% Chaps in PHEM containing 1 mM DTT, 100 nM microcystin, and 5 μg/ml of the protease inhibitors. Chromosomes were further purified on a gradient comprised of steps of 40 and 80% glycerol prepared in 0.4× PHEM buffer to which was added 60 mM KCl, 2.2 mM $MgCl_2$, 1 mM DTT, 100 nM microcystin, and 5 μg/ml of the protease inhibitors. Gradients were centrifuged in a swinging bucket rotor for 5 min at 1,000 *g* and 20 min at 4,000 *g*, and the chromosomes were collected from within the 80% glycerol. The chromosomes were then treated with DNAse (40 μg/ml for 20 min) and prepared for electrophoresis. For comparison, a crude extract of nuclei and insoluble cytoplasmic material was prepared from asynchronous cultures.

Samples were electrophoresed on 5–20% gradient polyacrylamide-SDS gels and electroblotted to nitrocellulose. The blots were blocked for 4 h in 10 mM Tris, pH 8.0, 150 mM NaCl, 0.05% Tween 20 (TBST) containing 1% BSA, 15% glycine. Blots were incubated in 3F3/2 ascites at a dilution of 1:1,000 in TBST for 1 h, washed in three changes of TBST and incubated with a 1:5,000 dilution of peroxidase-conjugated goat anti-mouse IgG (Jackson Immunoresearch Laboratories, West Grove, PA). After another wash in TBST, immunolabeled bands were visualized by enhanced chemiluminescence (Amersham Corp., Arlington Heights, IL) according to the manufacturer's directions.

Results

The 3F3/2 Antibody Recognizes an Endogenous Phosphoepitope

Detergent extraction of cells in the presence of the potent phosphatase inhibitor, microcystin, preserves the epitope recognized by the 3F3/2 antibody at the kinetochores of prophase and prometaphase PtK1 cells (Fig. 1, *A–F*). Cells extracted in the absence of microcystin exhibit little or no labeling. While the labeling of the kinetochores and centrosomes is most clear in cells that were extracted in detergent before lysis, labeling at the kinetochores is also seen in cells fixed while intact and then permeabilized (Fig. 2, *G–I*). To test whether the endogenous binding site is a phosphoepitope, cells were extracted in the presence of microcystin, fixed, and then treated with phosphatase PP1. Phosphatase treatment destroyed immunoreactivity with the 3F3/2 antibody (Fig. 3, *J–M*). However, the kinetochores in cells treated with PP1 in the presence of the phosphatase inhibitor microcystin retained their immunoreactivity.

The Epitope Recognized by the 3F3/2 Antibody Appears on Kinetochores at Prophase and Becomes Differentially Expressed during Mid to Late Prometaphase

In interphase Ptk1 cells labeled by indirect immunofluorescence, the 3F3/2 antibody showed no cytoplasmic labeling but showed some weak labeling of a number of irregular patches within the nuclei (Fig. 2). The distribution of the label within the interphase nuclei did not correspond with labeling of interphase kinetochores as detected by CREST human autoantisera. During prophase, label appeared at the kinetochores (Fig. 3). The mitotic poles also became positive at this time. In early prometaphase, just after nuclear envelope breakdown, all the kinetochores and the mitotic poles were brightly labeled by the 3F3/2 antibody. With progression through prometaphase, heterogeneity of labeling among the kinetochores became apparent. At mid prometaphase chromosomes within any one cell can exhibit three types of immunolabeling. Some chromosomes showed strong labeling on both kinetochores. Others exhibited weak or no labeling on either kinetochore, while in others, one of the kinetochores was labeled brightly while its partner kinetochore was labeled weakly or not at all. In a late prometaphase cell it is not unusual to find only one or two labeled kinetochores among the entire complement of chromosomes.

For cells in mid prometaphase the position of a chromosome within the spindle predicted how it would express the 3F3/2 antibody epitope. In general, chromosomes that were very near the poles were strongly labeled on both kinetochores. Those that were at the metaphase plate tended to be weakly labeled on one or both kinetochores. Chromosomes situated between the pole and the metaphase plate often exhibited very asymmetric labeling of their two kinetochores with greater labeling on the kinetochore toward the metaphase plate. Kinetochores in metaphase and anaphase cells exhibited no detectable label with the 3F3/2 antibody. Label-

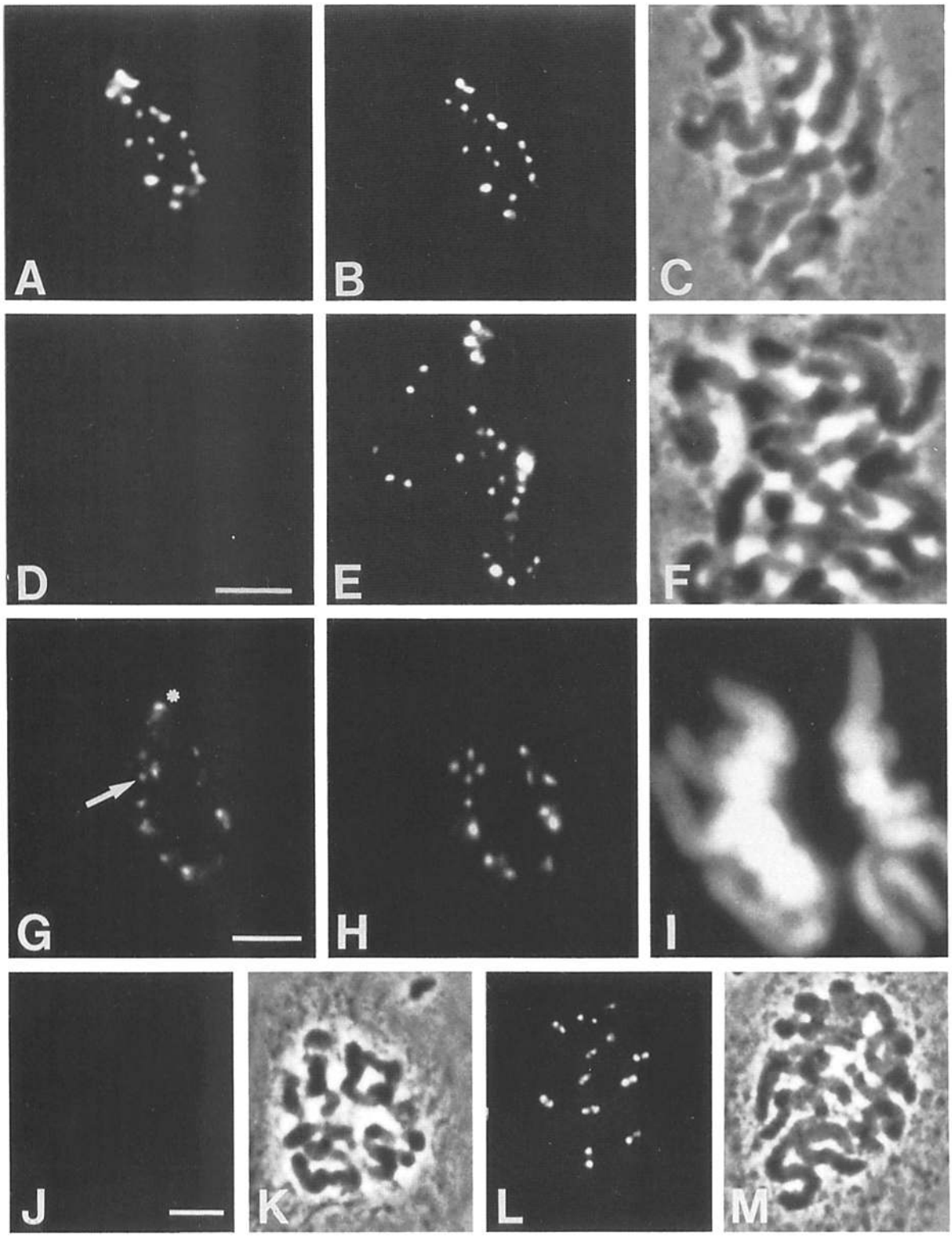

Figure 1. The 3F3/2 antibody phosphoepitope is expressed at the kinetochores of cells in prometaphase. Early prometaphase cells extracted with detergent in the presence of microcystin (*A*, *B*, and *C*) or in the absence of microcystin (*D*, *E*, and *F*) were fixed and prepared for double label immunofluorescence with 3F3/2 antibody (*A* and *D*) and CREST antikinetochore antibody (*B* and *E*) and imaged by phase contrast microscopy (*C* and *F*). Preservation of the 3F3/2 antibody epitope during detergent extraction is dependent on the phosphatase inhibitor, microcystin. Cells fixed with formaldehyde before detergent-extraction also exhibit the 3F3/2 antibody epitope. A prometaphase cell, fixed with formaldehyde and subsequently treated with detergent, was labeled with 3F3/2 antibody (*G*), CREST antikinetochore serum (*H*), and dapi (*I*). The 3F3/2 antibody epitope is expressed at the kinetochores (*arrow*) and the mitotic poles (*asterisk*). Application of exogenously protein phosphatase 1 to fixed cells destroys the 3F3/2 antibody epitope. Cells were detergent-extracted in the presence of microcystin and fixed. They were then treated with PP1 (*J* and *K*) or with PP1 in the presence of 100 nM microcystin (*L* and *M*). PP1 treatment in the absence of the microcystin completely eliminates immunoreactivity of the 3F3/2 antibody. For each experiment, corresponding control and experimental immunofluorescence images were obtained and printed under identical conditions. Bars, 5 μm.

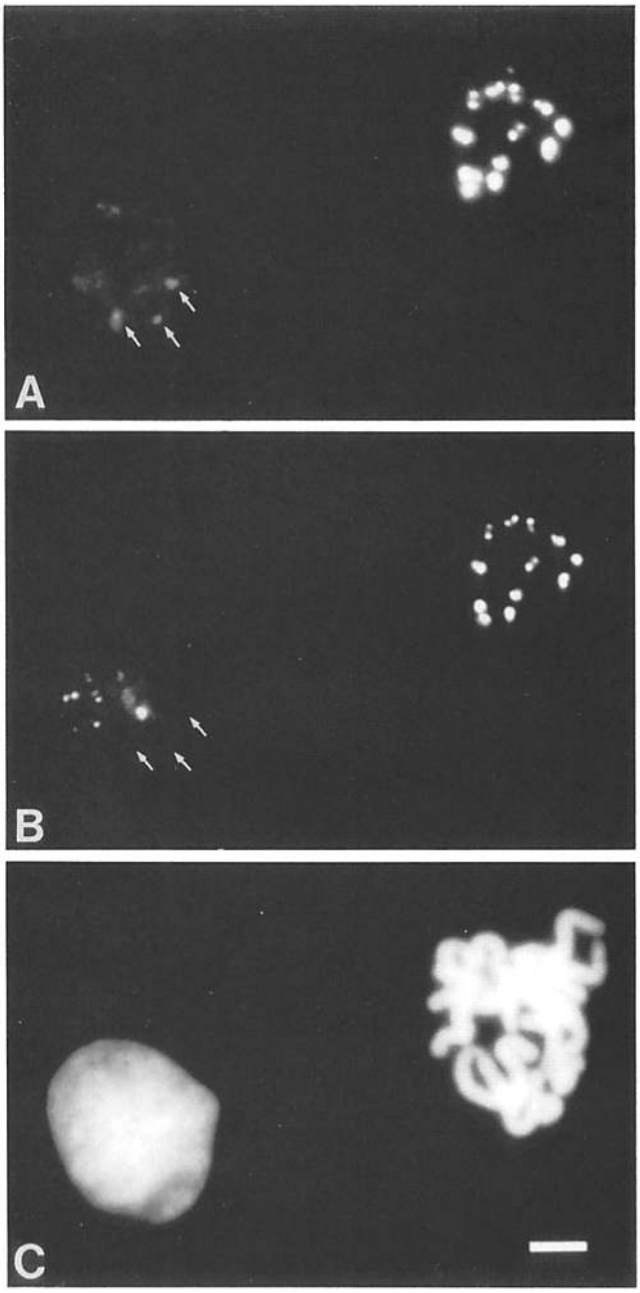

Figure 2. Comparison of labeling by 3F3/2 antibody in interphase and mitotic cells. Cells were detergent-extracted in microcystin and fixed. Neighboring interphase and prometaphase cells were imaged for 3F3/2 label (*A*), CREST antikinetochore serum (*B*), and DNA (*C*). The kinetochore labeling of the mitotic cell in *A* was deliberately overexposed to reveal the weak patchy labeling of the interphase nuclei by the 3F3/2 antibody. Unlike in the mitotic cells, the strongest areas of 3F3/2 label in the interphase nucleus (*arrows*) did not correspond to the kinetochore regions as detected by the CREST antikinetochore serum in *B*. Bar, 5 μm.

ing of the mitotic poles by the 3F3/2 antibody was first detected at prophase and became more intense during prometaphase. Unlike the labeling of the kinetochores, labeling of mitotic poles persisted through metaphase and anaphase.

Chromosomes Moving Toward the Metaphase Plate Are Labeled Preferentially on the Leading Kinetochore

We investigated whether chromosome movement is related to the differential labeling of partner kinetochores in prometaphase cells. We recorded phase contrast, video images of cells in late prometaphase cells in which most of the chromosomes had already moved to the metaphase plate, leaving one or a few chromosomes still at the poles. We then watched for movement of these chromosomes toward the metaphase plate. During this movement, the cells were rinsed with buffer, extracted in detergent, and fixed. They were then labeled with the 3F3/2 antibody and CREST anticentromere serum (Fig. 4). In most cells, the leading kinetochore, i.e., the one toward the metaphase plate, was brightly labeled while its partner was unlabeled or very weakly labeled. Of 53 chromosomes that we have recorded moving toward the metaphase plate, 47 (89%) exhibited distinctly preferential label of the leading kinetochore. Of the others, 5 chromosomes (9%) showed no label of either kinetochore, and 1 (2%) showed equal label on the two kinetochores.

We have not made a concerted effort to track nonmoving chromosomes by video recording. However, the cell depicted in Fig. 5 represents an informative example. We began recording this cell at time 0 where it contained a fully formed spindle with all but two chromosomes near the metaphase plate. One chromosome remained near each of the mitotic poles. Normally such a cell would take only a few minutes to reach full metaphase. However, this cell was abnormal. After over 20 min of video recording, the two noncongressed chromosomes remained near the poles. We extracted, fixed, and immunolabeled the cell. The chromosomes that had congressed to the metaphase plate exhibited no labeling with the 3F3/2 antibody. In each noncongressed chromosome, both kinetochores were labeled. In one of the chromosomes, the labeling was somewhat greater on the kinetochore facing away from the mitotic pole.

Kinetochores of Chromosomes Unattached to the Mitotic Spindle Exhibit Strong Expression of the 3F3/2 Antibody Epitope

Occasionally, we found examples in which one or more of the chromosomes appeared to be completely unattached to the mitotic spindle as judged by phase contrast microscopy. These "lost" chromosomes invariably exhibited brilliant labeling on both kinetochores. In some instances, the kinetochores of single unattached chromosomes showed bright labeling in a cell in which all the other chromosomes had progressed to the metaphase plate and were devoid of label (Fig. 6).

Cells Microinjected with 3F3/2 Antibody Become Differentially Labeled at the Kinetochore

To further investigate the distribution of the 3F3/2 antibody epitope in living cells, small amounts of purified 3F3/2 antibody were microinjected into living PtK1 cells in prophase. After microinjection, the cells were incubated 10–20 min at 37°C to allow them to enter prometaphase. They were extracted with detergent to release any antibody free in the cytoplasm and were then fixed. The cells were labeled with fluorescent anti-mouse IgG secondary antibody to reveal the

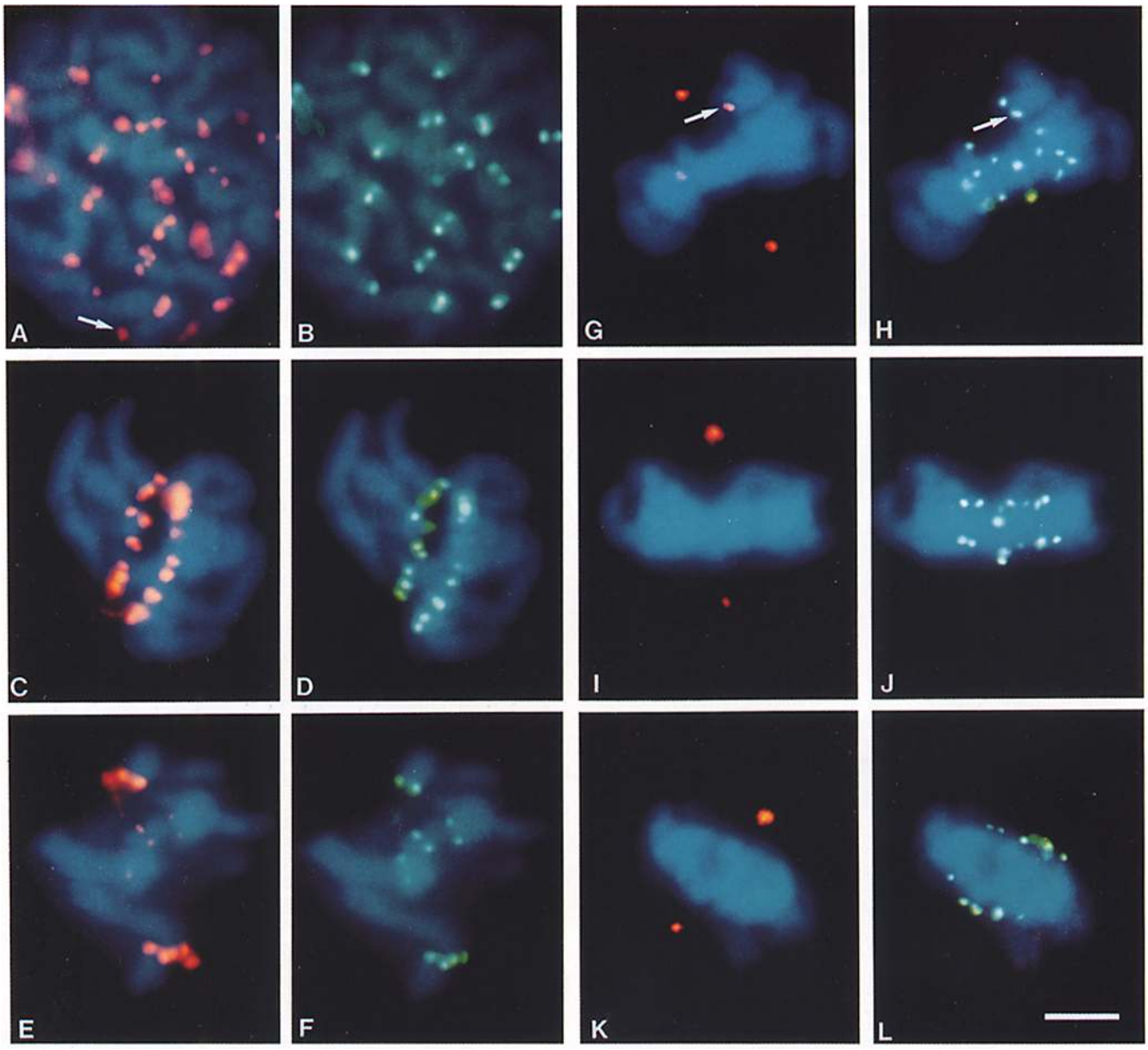

Figure 3. The 3F3/2 antibody epitope is expressed differentially at kinetochores during the mitotic cycle. Paired images of cells labeled with 3F3/2 antibody (*A*, *C*, *E*, *G*, *I*, and *K*), CREST antikinetochore antibody (*B*, *D*, *F*, *H*, *J*, and *L*), dapi dye for DNA (*blue label* in all images). During prophase (*A* and *B*), the 3F3/2 antibody epitope becomes strongly expressed at the kinetochores and at the mitotic poles (*arrow*). At early prometaphase (*C* and *D*), all kinetochores express the 3F3/2 antibody epitope intensely. At mid prometaphase (*E* and *F*), kinetochores of chromosomes near the metaphase plate express the epitope weakly while those of chromosomes near the poles express it strongly. At late prometaphase (*G* and *H*), only a few kinetochores are labeled by the 3F3/2 antibody. The epitope is often expressed more brightly on one of the two partner kinetochores of a single chromosome (*arrows*). At metaphase (*I* and *J*) and anaphase (*K* and *L*), the mitotic poles but not the kinetochores express the 3F3/2 antibody epitope. Bar, 5 μm.

injected antibody. Simultaneously they were colabeled with CREST serum. Only the injected cells showed label with the mouse secondary antibody. Injected cells show identical patterns to those seen through our conventional immunolabeling (Fig. 7). Thus in the living cells, antibody binds to kinetochores in prometaphase cells and binds differentially at mid and late prometaphase. This result supports the notion that the epitope is found in living cells. We have not yet examined the detailed behavior of chromosomes in injected cells. In a few cells that were incubated at 37°C for 30 min after microinjection, there was no indication that the antibody, at the concentration used, impaired progression through mitosis (data not shown).

The 3F3/2 Antibody Recognizes a Limited Number of Polypeptide Bands in Immunoblots of Mitotic Chromosomes

The fact that cells incubated with microtubule inhibitors ex-

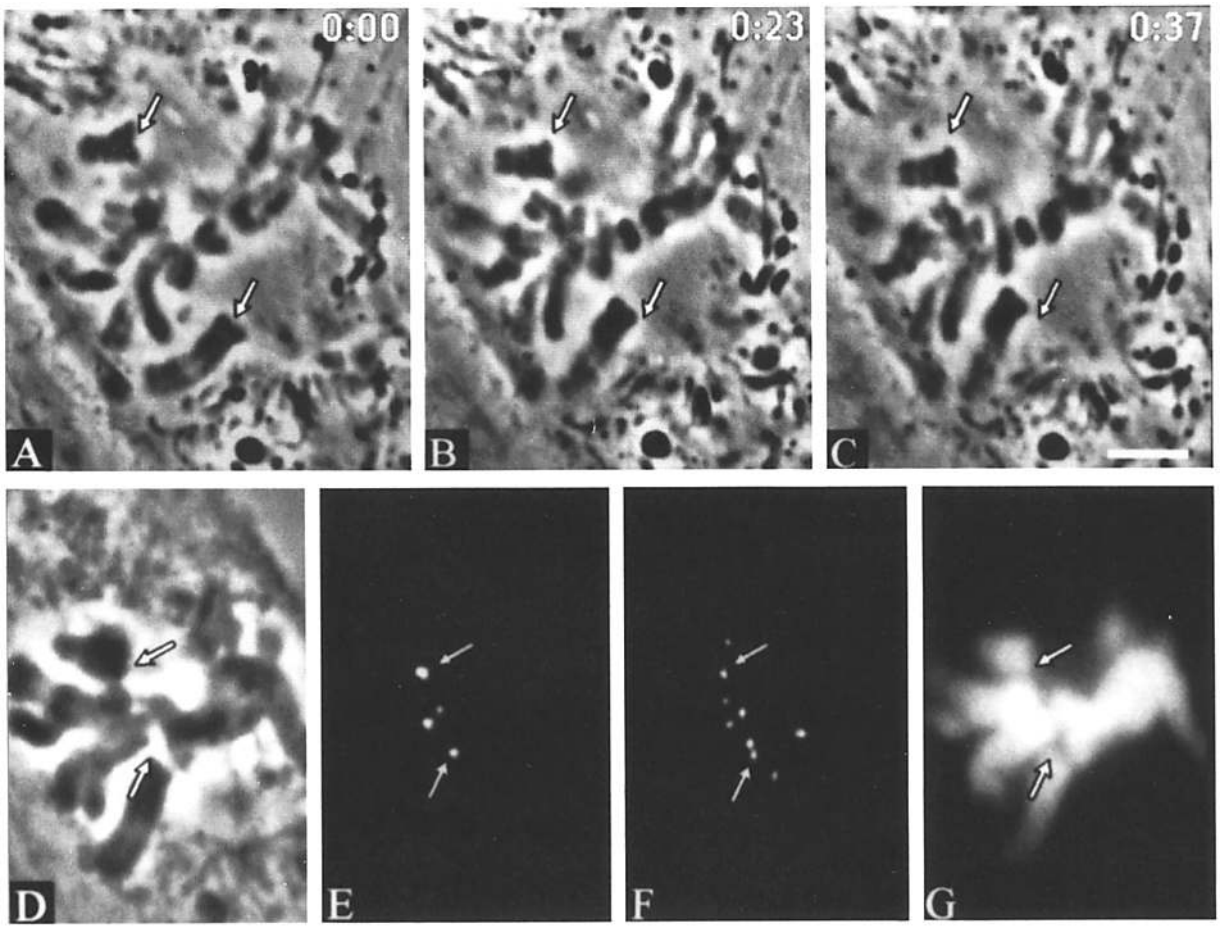

Figure 4. Chromosomes moving toward the metaphase plate express the 3F3/2 antibody epitope more strongly on the leading kinetochore. Cells were imaged by phase contrast microscopy while alive (*A*, *B*, and *C*). Arrows in live images are stationary references. Time in min:s indicated in upper right. At 50 s the cell was detergent-extracted, fixed, and prepared for double label immunofluorescence. Comparison of the phase contrast image of the lysed cell (*D*), the 3F3/2 antibody (*E*), the CREST antikinetochore antibody (*F*), and dapi label (*G*) indicates that the two moving chromosomes express the 3F3/2 antibody epitope more intensely on the kinetochore leading the movement toward the metaphase plate (*arrows*). Bar, 5 μm.

hibit strong labeling of all their kinetochores with the 3F3/2 antibody (not shown) allowed us to prepare chromosome fractions to test by immunoblotting. Fig. 8 shows that chromosomes prepared by a simple glycerol step gradient exhibit four major immunoreactive bands as well as several minor ones.

Discussion

The intriguing changes in the distribution of 3F3/2 epitope at kinetochores of mitotic chromosomes suggests that the epitope may play important roles in microtubule-kinetochore interactions. The epitope may signal a regulatory phosphorylation involved in turning on or off of the activity of dynein and kinesin-like microtubule motors resident at the kinetochore. Alternatively the epitope might be involved in controlling the binding or assembly and disassembly of kinetochore microtubules. Until more is understood about the biochemistry of the epitope, detailed discussion of how it functions remains the realm of conjecture. Below we provide a few simple ideas of the many that are possible to explain how the epitope might operate in regulating chromosome motion in prometaphase.

We hypothesize that expression of the epitope signals the activation of minus-end-directed motility at kinetochore, either by activating a dynein motor or inactivating an opposing, kinesin-like motor. In prophase, the kinetochores begin to express the epitope and thus become activated for minus-end-directed microtubule motility. Each kinetochore attaches to the first microtubules that penetrate nearby after the breakdown of the nuclear envelope. This widescale activation of minus-end-directed motility at the kinetochores would serve to efficiently bring each chromosome into the mitotic spindle oriented near one of the mitotic poles. For a chromosome to then move toward the metaphase plate, it would be necessary to downregulate the minus-end-directed motility of the kinetochore proximal to the nearby pole. We hypothesize that this downregulation occurs through dephosphorylation which is recognized as a loss of 3F3/2 antibody immunoreactivity. At this point the dynein-like motors of the distal kinetochore can move along microtubules from the op-

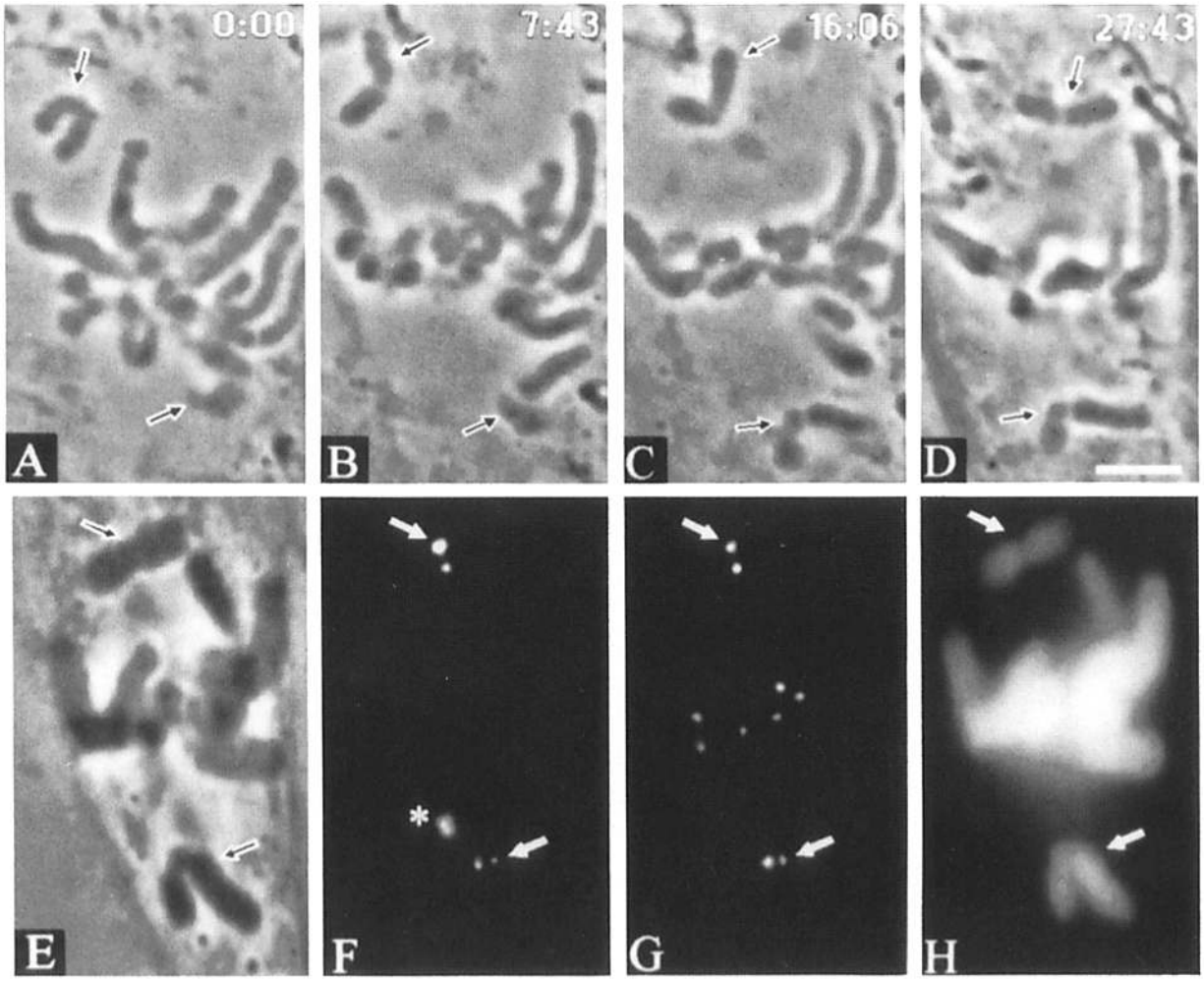

Figure 5. Chromosomes remaining near the mitotic poles continue to express the 3F3/2 antibody epitope for long periods in prometaphase. This cell was video recorded by phase contrast microscopy. At time 0:00, when recording was begun, the cell contained a well developed bipolar spindle with most chromosomes near the metaphase plate (*A*). Two chromosomes (*arrows*), one in each half spindle, remained near the mitotic poles. The two noncongressed chromosomes moved slowly near the poles for over 20 min (*B*, *C*, and *D*) after which the cell was extracted and fixed (*E*). Labeling of the cell with the 3F3/2 antibody (*F*), CREST antikinetochore antibody (*G*), and dapi (*H*) shows that both kinetochores of the noncongressed chromosomes express the 3F3/2 antibody epitope while those of the chromosomes at the metaphase plate do not. The 3F3/2 antibody epitope is also expressed at the mitotic poles one of which (*asterisk*) is in focus. Bar, 5 μm.

posite pole and thus move the chromosome toward the metaphase plate.

At a molecular level we suggest that expression of the epitope may signal a regulatory phosphorylation of a microtubule motor protein resident at the kinetochore. The experiments of Hyman and Mitchison (25, 26) indicate that kinetochores in isolated chromosomes contain minus-end-directed and plus-end-directed microtubule motors that are regulated by phosphorylation. Their interpretation, based on the use of ATP-γ-S to hyperphosphorylate chromosomes, was that phosphorylation would activate plus-end-directed microtubule motors. However, this effect of phosphorylation would contradict our model presented above where phosphorylation would activate minus-end-directed motors and/or inactivate plus-end-directed motors.

Based on the differences in the structure of the kinetochore fibers in prometaphase and anaphase cells, Hyman and Mitchison (25) speculated that minus-end-directed motor molecules at the kinetochore may be completely turned off after prometaphase and that anaphase chromatid motion may be driven by microtubule disassembly. The notion of different mechanisms of force production acting in prometaphase versus anaphase is consistent with the fact that expression of the 3F3/2 antibody epitope correlates only with chromosome motion in prometaphase cells and is not detected in anaphase.

During mid-prometaphase chromosomes also undergo transient reversals of direction and again move toward the poles. It is of interest to determine whether the pattern of 3F3/2 antibody binding is altered when chromosomes change direction and move toward the poles. Unfortunately, while movements toward the metaphase plate are often relatively long in duration, movements in the opposite direction, toward the pole, tend to be very short and of unpredictable persistence. Since our protocol, by necessity, involves a delay of ~12–15 s in order to rinse off the medium, we lack the time resolution to accurately test whether chromosomes that change direction and move toward the pole show an al-

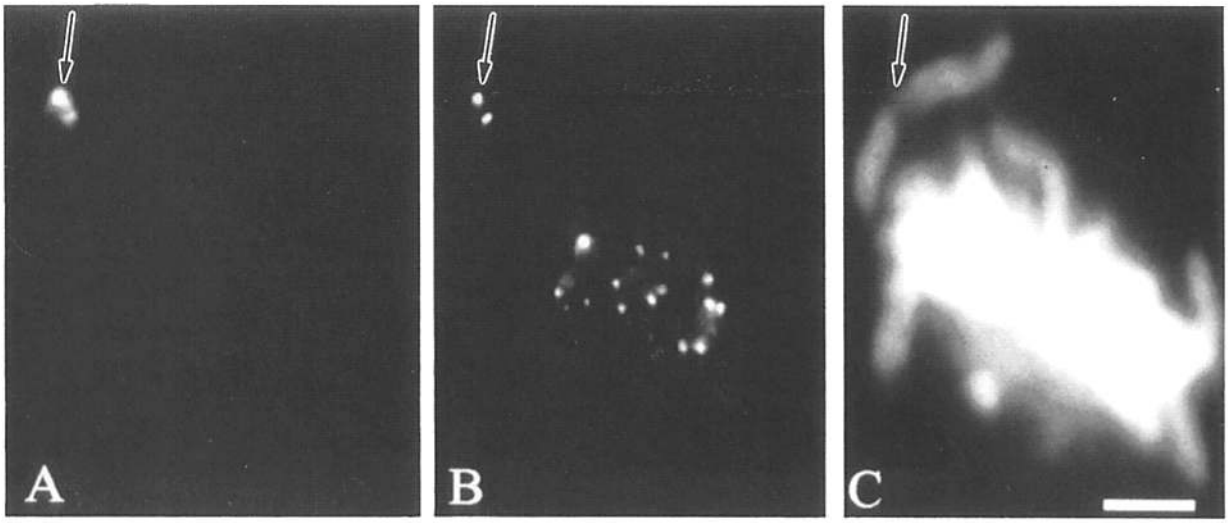

Figure 6. The 3F3/2 antibody epitope is expressed strongly on "lost" chromosomes that are unattached to the mitotic spindle. Cells were detergent extracted, fixed, and labeled with the 3F3/2 antibody (*A*), CREST antikinetochore antibody (*B*), and dapi (*C*). The cell shown contains a lost chromosome whose kinetochores express the 3F3/2 antibody epitope strongly (*arrows*). The other kinetochores of the other chromosomes gathered at the metaphase plate exhibit no expression of the 3F3/2 antibody epitope. Bar, 5 μm.

tered pattern of expression of the 3F3/2 antibody at their kinetochores. In the few chromosomes of mid-prometaphase cells that we have observed to show extended movement toward a mitotic pole, nearly all, when later examined, had both kinetochores oriented toward that pole (data not shown). We are currently developing methods by which to improve our temporal resolution to examine the more transient reversals of direction.

Tissue culture cells at metaphase which contain misaligned chromosomes are delayed in the onset of anaphase (54). The biochemical basis for this checkpoint regulation of the mitotic cycle is not understood. We observed that misaligned chromosomes express the 3F3/2 antibody epitope at high levels even in cells in which all the other chromosomes have congressed to the metaphase plate and show no expression. We speculate that the expression of this epitope may be a component of an inhibitory signal that blocks cell cycle progression.

The protection of the epitope rendered by the phosphatase inhibitor microcystin in the lysis protocol and the sensitivity of the epitope in fixed cells to exogenously applied phosphatases indicate that the 3F3/2 antibody recognizes a phosphoepitope. The protection afforded by microcystin indicates that the phosphorylation is of the serine/threonine class since microcystin is reported to be without effect on tyrosine phosphatases (22, 33). The differential expression of the epitope on kinetochores may be due to differential phosphorylation of a target protein present throughout mitosis. Alternatively,

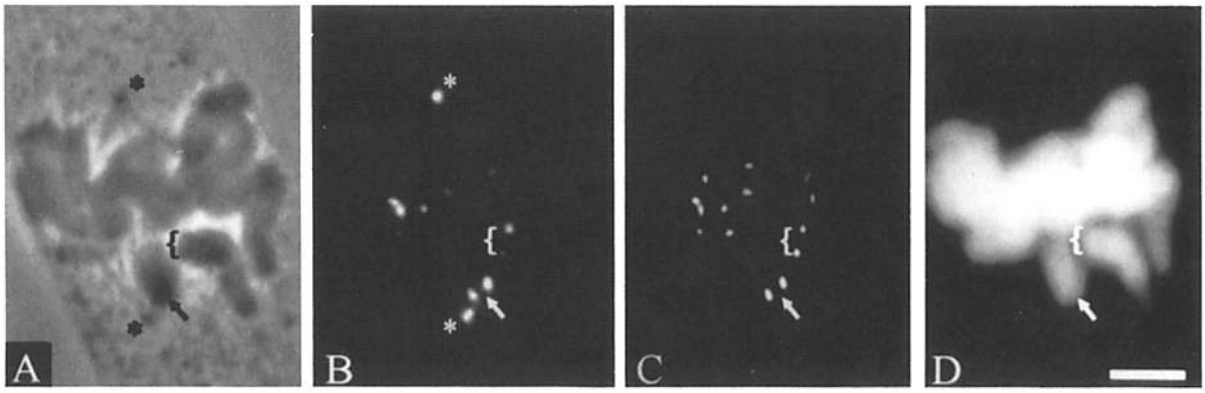

Figure 7. Microinjected 3F3/2 antibody binds to kinetochores differentially in prometaphase cells. This cell was injected in prophase with 3F3/2 antibody. After incubation for 15 min at 37°C, the cell was detergent-extracted and fixed. The cell was labeled with CREST antikinetochore antibodies and subsequently with anti-mouse and anti-human secondary antibodies containing different fluorophores. Only the injected cells exhibited labeling with the antimouse secondary antibodies. A cell injected in prophase and fixed in late prometaphase is shown by phase contrast microscopy in *A*. The 3F3/2 antibody (*B*) labels the kinetochores to different degrees. All the kinetochores are labeled brightly by the CREST antikinetochore serum (*C*). An image of the dapi-labeled chromosomes is shown in *D*. Note that the 3F3/2 antibody label on the chromosome midway between the pole and the metaphase plate is more intense on the kinetochore toward the metaphase plate (*brackets*). On a chromosome still oriented near the lower mitotic pole, the 3F3/2 antibody labels both kinetochores strongly (*arrows*). The 3F3/2 antibody also binds at the mitotic poles (*asterisks*). Bar, 5 μm.

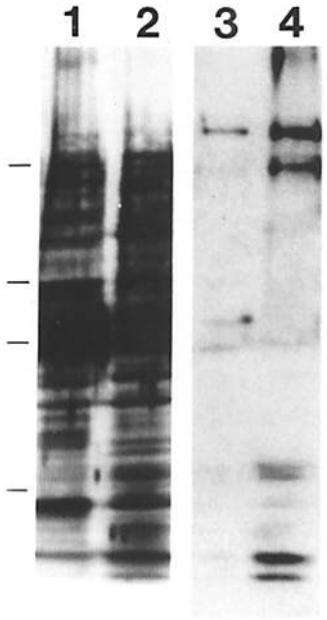

Figure 8. The 3F3/2 antibody recognizes chromosomal proteins by immunoblotting. Interphase extracts (lanes *1* and *3*) or mitotic chromosomes (lanes *2* and *4*) were electrophoresed on 5–20% polyacrylamide SDS gels and transferred to nitrocellulose. Strips were stained with India ink to reveal total protein (lanes *1* and *2*) or probed with 3F3/2 antibody. Weak immunoreactivity, principally in a high molecular weight protein, is detected in the interphase sample. The chromosome fraction reveals four major labeled bands, two of high molecular weight and two of low molecular as well as several less prominent immunoreactive bands. The location of molecular weight markers of 200, 97, 69, and 32 kD are indicated by the dashes on the left.

the protein containing the epitope may be physically released from some kinetochores during prometaphase or the epitope may be differentially masked by the formation of complexes with other proteins.

The identity of the protein or proteins that contain the 3F3/2 epitope remains unknown. The epitope is widespread. We have tested cell lines from seven mammalian species and one from *Drosophila*. All showed label at the kinetochores of early prometaphase cells (data not shown). In relatively crude chromosome preparations, immunoblotting with the 3F3/2 antibody identified a limited subset of protein bands, the most prominent being two bands of high molecular weight and two of low molecular weight. We are uncertain of the identity of the two high molecular weight bands. The two low molecular weight bands comigrate with major protein bands that we assume to be histones. Since the immunofluorescence data indicate that the epitope is restricted to the kinetochore region and not present in the chromosome arms, we feel that it is unlikely that the antibody binds to all histones. However, it is possible that histones at the centromere/kinetochore region are specifically phosphorylated and become immunoreactive. Alternatively there may be certain classes of minor histones that comigrate with the major histones and are centromere-specific. Cenp-A is a histone-like protein of the centromere that reacts with certain CREST autoimmune sera (41). We are yet uncertain which of the polypeptides labeled by immunoblotting of chromosome fractions represent the protein(s) of the kinetochore identified by immunofluorescence. Heretofore, attempts to further purify or subfractionate the chromosome preparations have resulted in a general loss of immunoreactivity. We are currently developing methods to overcome this extreme lability of the epitope.

In the course of our work we developed an alternative hypothesis concerning the molecular nature of the 3F3/2 epitope. We believe that it is possible that the endogenous target of the 3F3/2 antibody in living cells is itself a phosphatase. The phosphortyrosine phosphatases and presumably the cdc25 phosphatases function through a relatively long-lived phosphocysteine intermediate that can be trapped by rapid denaturation (18). While phosphocysteine intermediates have not been demonstrated for serine/theonine phosphatases, these phosphatases do contain highly conserved, essential cysteine residues and are strongly inhibited by sulfhydryl reagents (46). We speculate that the 3F3/2 epitope could be a phosphocysteine generated when a phosphatase is trapped as the enzyme intermediate by fixation or by treatment with microcystin. If this hypothesis is accurate, kinetochores with high expression of the 3F3/2 antibody epitope would be those with high phosphatase activity.

The regulation of chromosome alignment and the onset of anaphase are among the oldest questions in cell biology. However, because of the fragility and transitory nature of the mitotic spindle, they have been difficult to address. While we have not yet established a causal relationship between expression of the 3F3/2 antibody epitope and mitotic events, we believe our observations are highly significant. For the first time we demonstrate a biochemical difference among the kinetochores of a single cell and a difference between the sister kinetochores single chromosome in a manner that suggests a close involvement in the regulation of chromosome movement and the progression of the cell cycle.

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