

# The Inner Centromere Protein (INCENP) Antigens: Movement from Inner Centromere to Midbody during Mitosis

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**Abstract.** We describe a novel set of polypeptide antigens that shows a dramatic change in structural localization during mitosis. Through metaphase these antigens define a new chromosomal substructure that is located between the sister chromatids. Because the antigens are concentrated in the pericentromeric region, we have provisionally termed them the INCENPs (inner centromere proteins). The INCENPs (two polypeptides of 155 and 135 kD) were identified with a monoclonal antibody that was raised against the bulk proteins of the mitotic chromosome scaffold fraction. These two polypeptides are the most tightly bound chromosomal proteins known. When scaffolds are prepared, 100% of the detectable INCENPs remain scaffold associated. We were therefore unprepared for

the fate of the INCENPs at anaphase. As the sister chromatids separate, the INCENPs dissociate fully from them, remaining behind at the metaphase plate as the chromatids migrate to the spindle poles. During anaphase the INCENPs are found on coarse fibers in the central spindle, and also in close apposition to the cell membrane in the region of the forming contractile ring. During telophase, the INCENPs gradually become focused onto the forming midbody, together with which they are ultimately discarded. Several possible *in vivo* roles for the INCENPs are suggested by these data: regulation of sister chromatid pairing, stabilization of the plane of cleavage, and separation of spindle poles at anaphase.

**T**HE centromere has long been a subject of curiosity due to its prominent role in the attachment of sister chromatids to each other and to the mitotic spindle. However, with the exception of scattered ultrastructural and cytochemical analyses (reviewed in 39), the centromere has remained an elusive subject, a consequence of the difficulty of purifying it free from bulk chromatin.

In recent years experimental breakthroughs have begun to permit the identification and functional analysis of kinetochore components. (The kinetochore is the centromeric substructure to which microtubules attach). Genetic manipulations in *Saccharomyces* led first to the isolation of functional kinetochore DNA sequences (reviewed in 2, 5) and ultimately to the construction of stable artificial chromosomes (6, 32). At the same time, substantial progress has been achieved in the identification of human centromere proteins (13, 18, 33, 42, 48, 49) subsequent to the discovery of centromere-specific autoantibodies in the sera of certain patients with rheumatic diseases (31). At least some of these autoantigens are thought to be located in the kinetochore (3).

Dramatic improvements in our understanding of the dynamics of microtubule assembly and disassembly (28) and the organization of microtubules in the spindle (16, 20, 45) have yielded new insights into the way in which kinetochores and microtubules interact *in vivo* (17, 29). The convergence of this diverse array of knowledge has permitted elaboration of detailed hypotheses to explain the function of the kineto-

chore in mitosis. More importantly, the reagents required to test these hypotheses are now available.

The kinetochore provides only one aspect of centromere function, however. It would be pointless for chromosomes to be subjected to the poleward forces generated by the mitotic apparatus were there not a mechanism for the controlled separation of sister chromatids at the metaphase/anaphase transition. Cytologists have long been interested in the mechanism of mitotic sister chromatid disjunction, although studies of the process have progressed little past the descriptive stage.

It is known that traction forces applied by the spindle are not required for mitotic chromosome disjunction since both acentric fragments (4) and chromosomes in colcemid (or colchicine)-treated cells (30, 40) will separate in a synchronous (though in the latter case, delayed) fashion. This implies that a regulated chromatid-separation activity must exist in the cell. This activity has been postulated to involve either delayed replication of centromeric DNA (5; for which little direct evidence exists), or a regulated action of centromere-specific type II topoisomerases (43). The latter do appear to be involved in mitotic disjunction, since cell death of conditional lethal topoisomerase II mutants of both *Saccharomyces* and *Schizosaccharomyces* appears to arise from an abortive attempt to disjoin sister chromatids (22, 47). Type II topoisomerases are located all along the mitotic chromosome, however (10), and it is not known whether a centro-

mere-specific regulator of topoisomerase II function operates *in vivo*. Certainly, the observation that acentric fragments disjoin at the normal time (4), together with the fact that in many cell types the sister chromatids remain tightly apposed along their entire lengths throughout mitosis up to anaphase, argues against a special sequestration of disjoining activities in the centromere.

Here we describe a class of polypeptides that we provisionally term INCENPs (inner centromere proteins).<sup>1</sup> INCENPs are localized between the sister chromatids of mitotic chromosomes through metaphase. At anaphase the antigens remain behind at the metaphase plate as the chromosomes begin their poleward migration. This exit from the chromosome is particularly striking in view of the observation that in chromosomes isolated from colcemid-blocked cells the INCENPs show an extremely tight association with the chromosome scaffold fraction. As the separated chromatids migrate to the spindle poles the INCENPs gradually become concentrated onto the tightly bundled microtubules of the midbody. Several possible roles of INCENPs *in vivo* are discussed.

## Materials and Methods

### Isolation of Mitotic Chromosomes

Mitotic chromosomes were isolated from MSB-1 cells grown in RPMI 1640 with 5% iron-supplemented calf serum and blocked overnight with 0.1  $\mu$ g/ml colcemid as previously described (14). Scaffolds were prepared from these chromosomes using 2 M NaCl lysis mix (1). The final scaffold pellet was resuspended in D-PBS (8.06 mM Na<sub>2</sub>HPO<sub>4</sub>·7H<sub>2</sub>O, 1.47 mM KH<sub>2</sub>PO<sub>4</sub>, 137 mM NaCl, 2.7 mM KCl, 0.68 mM CaCl<sub>2</sub>, 0.492 mM MgCl<sub>2</sub>) for immunizing mice.

### Immunization of Mice and Isolation of Monoclonal Antibodies

6-wk-old BALB/c mice were injected intraperitoneally with an emulsion consisting of Freund's complete adjuvant and chromosome scaffolds isolated from  $\sim 1 \times 10^9$  MSB-1 cells. After 4 wk the mice were boosted intraperitoneally with the same amount of antigen in incomplete adjuvant. 10 d after the first boost the mice were bled and the serum tested for anti-scaffold antibodies on immunoblots of MSB-1 chromosomes. Mice producing anti-scaffold antibodies were given a final boost of antigen in D-PBS alone 10 d after the first bleed. 3 d after the final boost the spleen cells were fused with P3-X63-Ag8.653 mouse myeloma cells (24) according to published procedures (25). After the fusion, the cells were resuspended in DME containing 20% (vol/vol) FCS, 10% (vol/vol) NCTC 135 and HAT (100  $\mu$ M hypoxanthine, 0.4  $\mu$ M aminopterin, and 20  $\mu$ M thymidine) and plated in 24-well dishes (Flow Laboratories, Inc., McLean, VA) at a density of  $1-10 \times 10^6$  spleen cells/well in 1 ml of growth medium. Culture supernatant from wells with cell growth was screened on immunoblots of MSB-1 chromosomes. Wells positive for chromosomal proteins were cloned twice in agar (25) and later grown in flasks.

### Bookkeeping Experiments

To assess the distribution of the INCENP antigens between mitotic chromosomes, interphase nuclei and cytoplasm we first pelleted 50 ml of cells taken from a 500-ml MSB-1 spinner culture ( $\pm$  a 15-h block with 0.1  $\mu$ g/ml colcemid). The cells were gently resuspended in RSB (10 mM Tris: HCl pH 7.4, 10 mM NaCl, 5 mM MgCl<sub>2</sub>) and swollen for 5 min at room temperature. After centrifugation, swollen cells were disrupted in 3 ml of lysis buffer (26) by Dounce homogenization, and the lysates centrifuged at 3,200 *g* for 20 min. The supernatants were removed and placed on ice (cytoplasmic

1. *Abbreviations used in this paper:* DAPI, diaminoindophenylindole; D-PBS, 8.06 mM Na<sub>2</sub>HPO<sub>4</sub>·7H<sub>2</sub>O, 1.47 mM KH<sub>2</sub>PO<sub>4</sub>, 137 mM NaCl, 2.7 mM KCl, 0.68 mM CaCl<sub>2</sub>, 0.492 mM MgCl<sub>2</sub>; INCENP, inner centromere protein.

fraction). The pellets were then resuspended in a volume of final chromosome buffer (buffer 4-26) equivalent to the volume of the starting lysate (chromosomal and nuclear fraction). Aliquots (0.2 ml) of the nuclear/chromosomal and cytoplasmic fractions were resuspended in 0.1 ml of 3 $\times$  SDS-PAGE sample buffer (14), sonicated, and boiled before SDS-PAGE.

Percoll-purified chromosomes and chromosome scaffolds were then isolated from the remaining 450 ml of colcemid blocked cells (14). The chromosome scaffolds were pelleted at 16,000 *g* for 15 min and resuspended directly in 1 $\times$  SDS-PAGE sample buffer. The supernatant of proteins solubilized during the scaffold extraction was precipitated in 15% TCA at 4° for 30 min and later resuspended in 1 $\times$  SDS-PAGE sample buffer.

### Immunofluorescence

Procedures for indirect immunofluorescence have been modified from those described previously (12). For Figs. 1, 5, 6, 7, 9, and 10, cells (chicken hepatoma-derived line 249) were either grown on coverslips, or obtained by mitotic shakeoff, concentrated in warm medium, and centrifuged onto glow-discharged coverslips (12).

**Protocol 1.** (All operations performed at room temperature.) For localization of INCENPs (Figs. 1, 5, 9, and 10), the samples were rinsed in D-PBS, fixed in 3% HCHO in D-PBS, and subsequently all operations were performed in StB (10 mM Tris:HCl pH 7.4, 150 mM NaCl, 0.1% BSA [pentex grade; Miles Laboratories, Naperville, IL]) plus 0.02% Triton X-100. Similar distributions of the INCENP antigens were observed if HCHO fixation was replaced by immersion in acetone at -20°C (5 s), followed by rehydration in D-PBS.

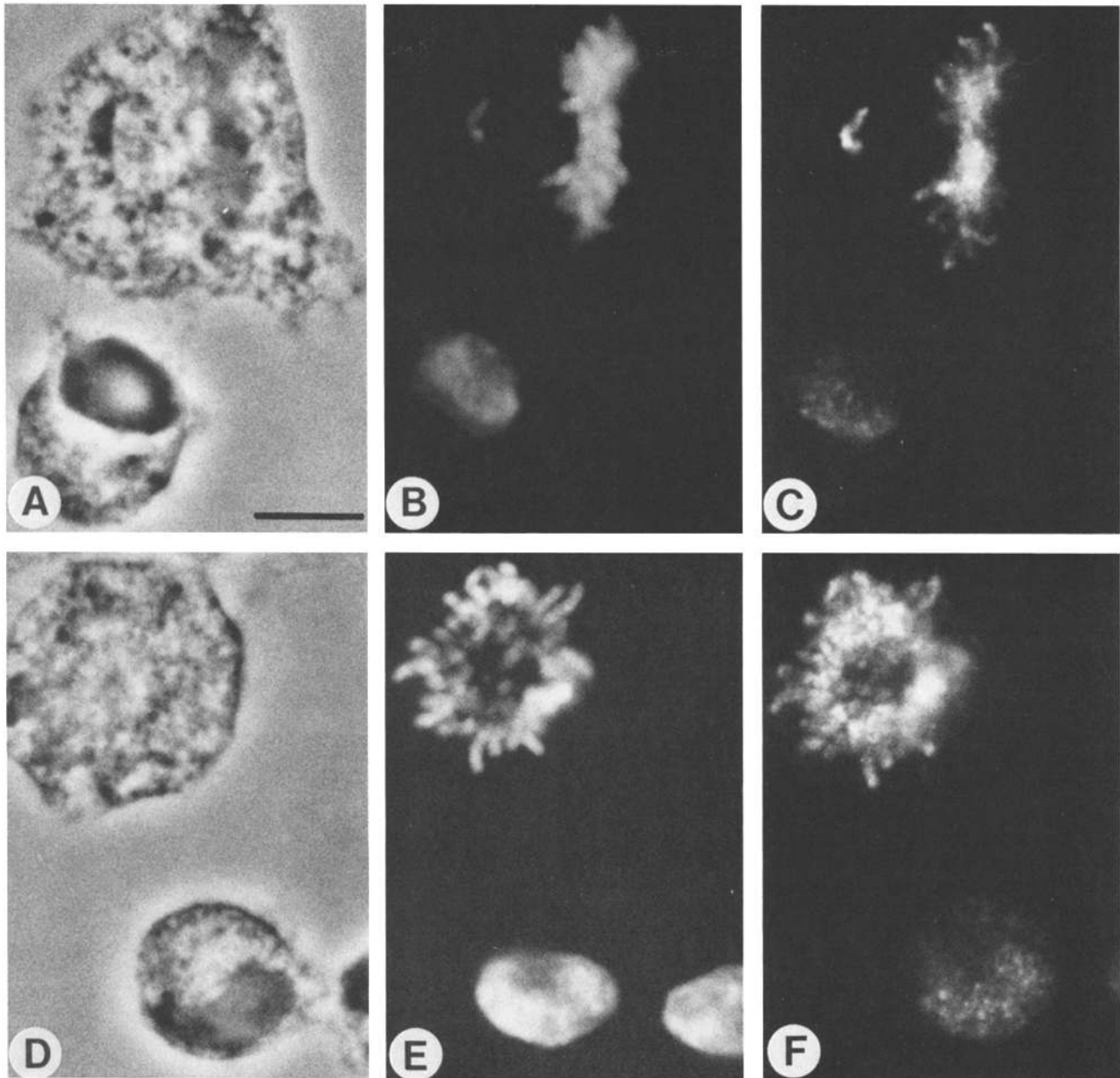
**Protocol 2 (modified from reference 34).** For simultaneous localization of INCENPs and tubulin, the coverslips were incubated in StB (stabilization buffer = 0.1 M Pipes/HCl pH 6.8, 1 mM EGTA, 1 mM GTP, 4% polyethylene glycol [6,000-8,000; Sigma Chemical Co., St. Louis, MO]) at 37°C for 30 s, incubated in StB + 0.2% Triton X-100 for 4 min at 37°C, washed twice in StB for 30 s at 37°C, rinsed twice in D-PBS at 25°C, fixed in 3% HCHO for 30 s at 25°C, rinsed three times (2, 5, 3 min) in KB<sub>light</sub> (KB with 0.01% Triton X-100), and incubated for 30 min at 37°C with a 1:1 mixture of mAb-3D3 and affinity-purified rabbit anti- $\beta$ -tubulin (gift of D. Murphy, Johns Hopkins School of Medicine). The coverslips were rinsed as above (three times with KB<sub>light</sub>) and then incubated with a mixture of fluoresceinated goat anti-rabbit Ig (1:200; Cappel Laboratories, Inc., Cochranville, PA), and biotinylated goat anti-mouse Ig (1:100; Vector Laboratories, Inc., Burlingame, CA) for 30 min at 37°C. After washing (as above), the coverslips were incubated with a 1:800 dilution of streptavidin/Texas red (Bethesda Research Laboratories, Gaithersburg, MD) in KB<sub>light</sub> for 30 min at 37°C, washed as above, and mounted in Mowiol (34).

**Protocol 3.** For higher resolution localization of INCENPs in mitotic chromosomes, cells that had been incubated with colcemid (0.1  $\mu$ g/ml) for 3 h were collected by shakeoff, centrifuged, resuspended in hypotonic medium for 5 min (RSB buffer), and centrifuged onto glow discharged coverslips. These were then processed as above (protocol 1), with the exception that the KB buffer used contained 0.1% Triton X-100 throughout.

**Protocol 4.** For localization of INCENPs in extracted chromosomes and chromosome scaffolds, highly purified mitotic chromosomes were centrifuged onto glow-discharged coverslips, which were then immersed in D-PBS. Where production of scaffolds was desired, the coverslips were first incubated with micrococcal nuclease at 40  $\mu$ g/ml for 20 min at 4°C. (This incubation was omitted for the experiment of Fig. 3, A and B.) Coverslips were then incubated with polyanion lysis mix (10 mM Tris:HCl pH 9, 10 mM EDTA pH 9, 0.4 mg/ml dextran sulphate [Pharmacia Fine Chemicals, Piscataway, NJ], 0.04 mg/ml heparin [Sigma Chemical Co.], and 0.1% Ammonyx Lo [gift of the Onyx Chemical Co., Jersey City, NJ]) three times (2, 5, 3 min) at 4°C, and processed for indirect immunofluorescence as above for protocol 1, with the exception that KB<sub>light</sub> was used throughout. Similar results were obtained if coverslips were extracted with lysis mix containing 2 M NaCl in place of the polyanion mixture (data not shown).

### Other Immunological Techniques

Immunoblotting procedures have been described previously (15), as have been procedures for affinity purification of antibodies from nitrocellulose blots (13), and methods for immunoadsorption of antigens from cells disrupted by boiling in SDS solubilization buffer (19, 35). For the experiment of Fig. 11, and in all of our more recent immunoblotting work, the blots were washed in Triton/SDS buffer (50 mM triethanolamine/HCl pH 7.4, 100 mM NaCl, 2 mM K-EDTA, 0.5% Triton X-100, 0.1% SDS; reference 35), which significantly decreases background nonspecific binding.



**Figure 1.** Use of mAb-3D3 to stain mitotic cells. (A-C) Side view of metaphase plate. (D-F) End view of plate. The images shown are: (A and D) phase contrast; (B and E) DAPI stain of DNA; (C and F) mAb-3D3 staining. Note that one chromosome (A-C) has not yet congressed to the plate. Note also that the interphase nuclei are also weakly immunopositive. Bar, 10  $\mu$ m.

### Centrifugal Elutriation

MSB-1 cells were grown in RPMI 1640 medium (Gibco, Grand Island, NY) supplemented with 5% FCS (HyClone Laboratories, Logan, UT).  $2-4 \times 10^8$  cells in log phase were pelleted at 2,000 *g* and resuspended in 20 ml of elutriation buffer (Hanks balanced salt solution [Gibco] containing 5% FCS). EDTA was added to a final concentration of 0.02% and the cell suspension passed through a 20-cc syringe fitted with a 22-gauge needle. (This step decreased the percentage of G<sub>1</sub> cells contaminating the sorted G<sub>2</sub> + M populations as a result of the tendency of daughter cells to remain associated after cytokinesis.) The cell suspension was then separated according to size (approximate position in the cell cycle) by centrifugal elutriation using a JE-6B elutriator rotor mounted in a J2-21 centrifuge (Beckman Instruments, Inc., Palo Alto, CA). Rotor speed was maintained constant at 1,800 rpm throughout the separation. The starting flow rate of 6 ml/min was increased by  $\sim 1$  ml/min increments. 50 ml of cell suspension was collected on ice af-

ter each increase in flow rate. The elutriation finished by the time the flow rate reached  $\sim 20$  ml/min. Fractions were analyzed for size and number of cells with a Coulter counter, and cell cycle position determined by flow cytometry. After elutriation, cells were washed in cold D-PBS and solubilized in SDS-PAGE sample buffer at a concentration of 10,000 cells/ $\mu$ l. An equal number of cells/lane ( $2.5 \times 10^5$ ) from each fraction (including one loading from the initial unsorted culture) was subjected to electrophoresis through replicate 10% polyacrylamide gels (26). One gel was stained for protein content and the others transferred electrophoretically to nitrocellulose for immunoblotting analysis with various antisera (15). In these experiments the protein content of fractions 1 and 15 is often underrepresented. This is due to the presence of some cell debris in fraction 1 that the Coulter counter detects as intact cells, and to mitotic cells in fraction 15 that elutriate joined by a midbody, but subsequently separate and are counted by the Coulter counter as two separate cells.

## Results

### Isolation of a Monoclonal Antibody Recognizing Two Chromosome Scaffold Proteins

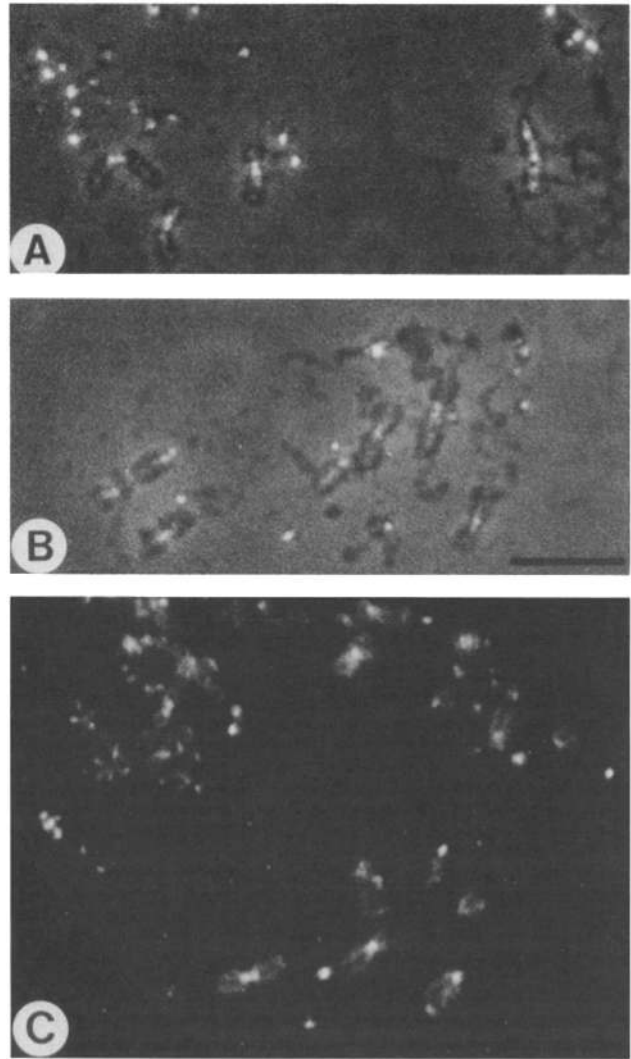
In this study we set out to obtain monoclonal antibodies that would recognize previously unidentified components of the mitotic chromosome scaffold fraction. We prepared scaffolds from isolated chicken mitotic chromosomes and injected them into mice without further subfractionation, eventually obtaining a single hybridoma line that was cloned twice in soft agar. This cell line secretes a monoclonal antibody, mAb-3D3 (an IgG-2b; data not shown) that recognizes three antigens of  $M_r$  80,000, 135,000 and 155,000 in immunoblots of whole cells (see Fig. 4, below). For ease in description, we initially termed these antigens ag80kD, ag135kD, and ag155kD.

### Localization in Mitotic Chromosomes

mAb-3D3 stains the condensed chromosomes in metaphase cells (Fig. 1). In these cells, the chromosomes are organized in a ring on the metaphase plate (Fig. 1, *D-F*). The inner part of this ring appears more intensely stained with antibody than is the periphery. This is not due simply to the overall packing of chromosomes tightly into the plate. The diaminophenylindole (DAPI) image of total DNA in these structures fails to show evidence for a dense inner ring of chromatin (Fig. 1, *B* and *E*).

In an isolated chromosome that had (presumably) not yet congressed to the plate (Fig. 1 *C*) the centromere was more intensely stained than were the distal regions of the arms. However it is important to note that mAb-3D3 did significantly stain the entirety of the two sister chromatids, which were tightly paired along their entire length.

To analyze the chromosomal location of the 3D3 antigens at higher resolution, chicken cells were blocked in mitosis with colcemid, swollen in hypotonic medium, disrupted by centrifugation onto coverslips, and subsequently processed for immunofluorescence analysis (Fig. 2). Colcemid treatment causes the association between sister chromatids to loosen so that they adopt the classical "X" conformation (27). This is accompanied by a change in the distribution of the 3D3 antigen, which is now localized almost exclusively to the centromere. Furthermore, this localization has two unusual features. (*a*) In many chromosomes the centromeric staining is resolved into two dots flanking the centromere. These dots are reminiscent of the double dots seen upon staining chromosomes with anti-centromere antibodies (15), except that the axis connecting the dots is parallel to the long axis of the chromosome for the 3D3 antigen and perpendicular to it for centromeric autoantigens. Thus the INCENPs are apparently present in a chromosomal substructure, distinct from that containing the CENP family of centromeric autoantigens (13). (*b*) The 3D3 antigens are localized between the sister chromatids. Thus the two dots appear to correspond to the two regions of the proximal arms previously described by Lima de Faria (27) as the last points of contact between the sister chromatids just before separation at anaphase. This suggests that the 3D3 antigens might have something to do with sister chromatid adhesion, a hypothesis supported by the observation that the antigens are apparently

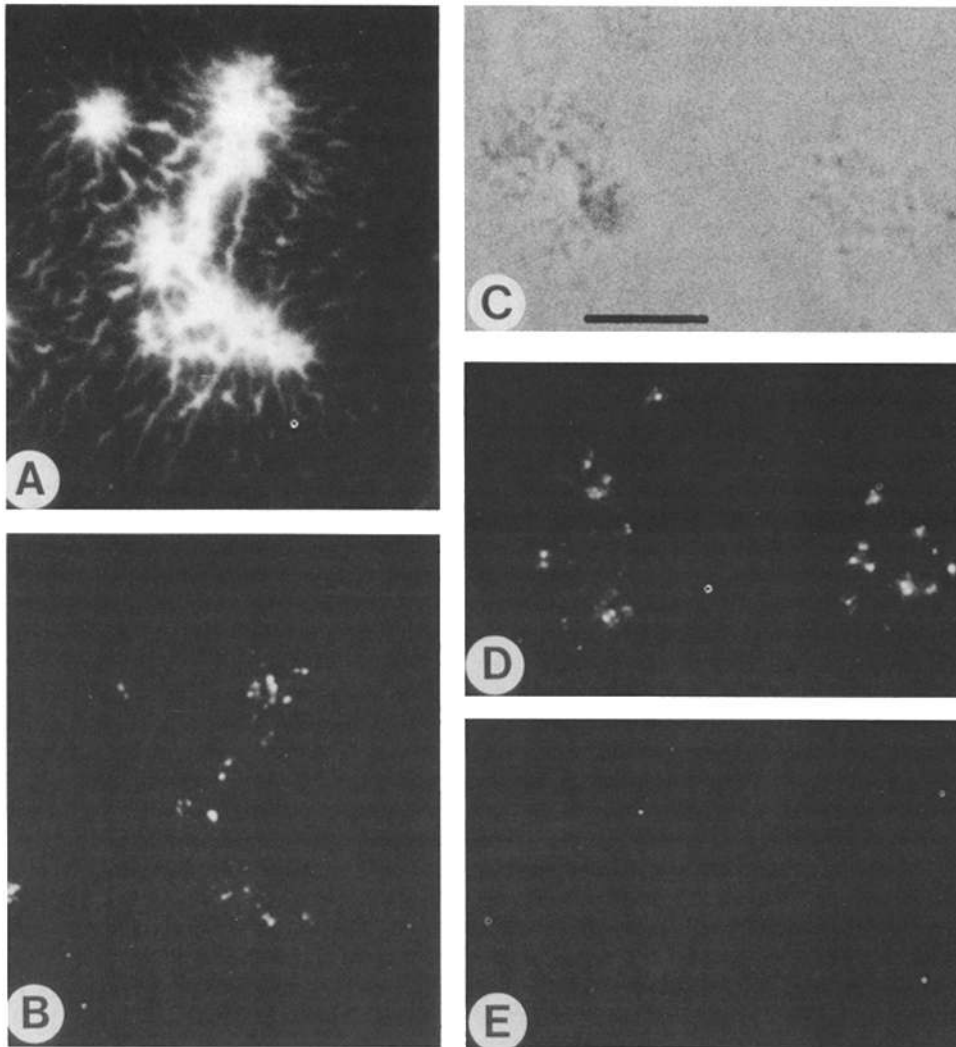


**Figure 2.** Localization of the INCENP antigens in chromosomes from colcemid-blocked cells. Blocked cells were spread by centrifugation on glow-discharged coverslips (12) and stained with mAb-3D3. (*A* and *B*) Simultaneous phase contrast/immunofluorescence double exposures. (*C*) The fluorescence image alone. Note that the staining is predominantly just above and below the centromere, and is concentrated between the sister chromatids. Bar, 10  $\mu$ m.

found wherever the sister chromatid arms remain in contact (this is particularly noticeable in Fig. 2 *A*).

Because of this striking localization of the 3D3 antigens between the sister chromatids and especially in the centromere region, we have given the antigens the provisional designation of INCENPs (inner centromere proteins).

We have performed two extraction experiments to further map the location of the INCENPs in mitotic chromosomes. In the first, purified mitotic chromosomes on coverslips were extracted with a dextran sulphate/heparin lysis mix shown previously to efficiently remove the bulk of chromosomal proteins, while leaving behind the insoluble proteins of the chromosome scaffold fraction (1, 11). When these extracted chromosomes were examined under fluorescence after staining with the DNA-binding dye DAPI, the DNA was seen to



**Figure 3.** Localization of INCENPs in extracted mitotic chromosomes and chromosome scaffolds. Highly purified mitotic chromosomes were centrifuged onto coverslips and treated as follows. (A and B) Coverslips were treated with dextran sulphate/heparin lysis mix to remove histones. (A) DAPI image of DNA; (B) immunolocalization of INCENP antigens. (C–E) Coverslips were immersed in a solution with 1  $\mu\text{g}/\text{ml}$  micrococcal nuclease and subsequently extracted with the polyanion lysis mix. (C) Phase contrast; (D) immunolocalization of INCENP antigens; (E) DAPI staining of DNA. In both cases the centromere-linked dots of INCENP antigens resist the extraction. Bar, 10  $\mu\text{m}$ .

be widely dispersed (Fig. 3 A), as shown previously in the electron microscope (36). In contrast, the INCENPs remained localized in discrete dots, many of which appear double (Fig. 3 B). If both DNA and histones were removed by nuclease digestion followed by polyanion extraction, the dot-like localization of the INCENPs remained unaltered (Fig. 3 D). Staining with DAPI confirms that the vast majority of the DNA is removed by this procedure (Fig. 3 E). Thus, in chromosome scaffolds the INCENPs are localized in discrete foci.

#### **Mapping the Intracellular Distribution of INCENPs by Subcellular Fractionation**

The immunofluorescence data suggest strongly that the INCENP antigens are localized in mitotic chromosomes and in chromosome scaffolds. However, to control for possible losses of antigen during processing for immunofluorescence and for possible masking of epitopes in situ, we have also examined the cellular localization of the INCENP antigens by subcellular fractionation, SDS-PAGE, and immunoblotting.

Fig. 4 presents results of a bookkeeping experiment designed to examine the association of the INCENP antigens

with interphase nuclei and mitotic chromosomes. Interphase or mitotic (colcemid-blocked) cells were gently disrupted by Dounce homogenization, and centrifuged at low speed (10, 11) to give a pellet of crude chromosomes and nuclei (Fig. 4, lanes 1 and 3, respectively) and a supernatant of mitotic or interphase cytoplasm (lanes 2 and 4). The protein composition of these samples, stained with Coomassie Blue, is shown in Fig. 4 A. The immunoblot (Fig. 4 B) shows that mAb-3D3 recognizes the three antigens (ag155kD, ag135kD, ag80kD) in both interphase nuclei and mitotic chromosomes. The two larger antigens appear exclusively nuclear/chromosomal, while a trace of ag80kD is found in the cytoplasm. We demonstrate below that the two larger antigens are responsible for the INCENP staining pattern of mAb-3D3. We therefore designate them INCENP A (155 kD) and INCENP B (135 kD).

When the crude chromosomes were further purified by centrifugation through sucrose and Percoll gradients (Fig. 4, lanes 5), ag80kD was removed. Thus this component, while weakly associated with mitotic chromosomes (or possibly other rapidly sedimenting cellular debris), is apparently not an intrinsic protein of mitotic chromosomes. The

cellular localization of this antigen has been examined further using specific polyclonal antibodies (see below).

In contrast, the two high molecular mass antigens are intrinsic chromosomal proteins. Both cofractionate with mitotic chromosomes and are quantitatively retained in chromosome scaffolds (Fig. 4, lanes 5 and 6). They are undetectable among the proteins released from chromosomes by nuclease digestion and 2 M NaCl extraction (Fig. 4, lanes 7). Thus INCENP A and INCENP B are among the most tightly associated mitotic chromosome scaffold components described to date.

### *Dynamic Redistributions of the INCENPs during Mitosis*

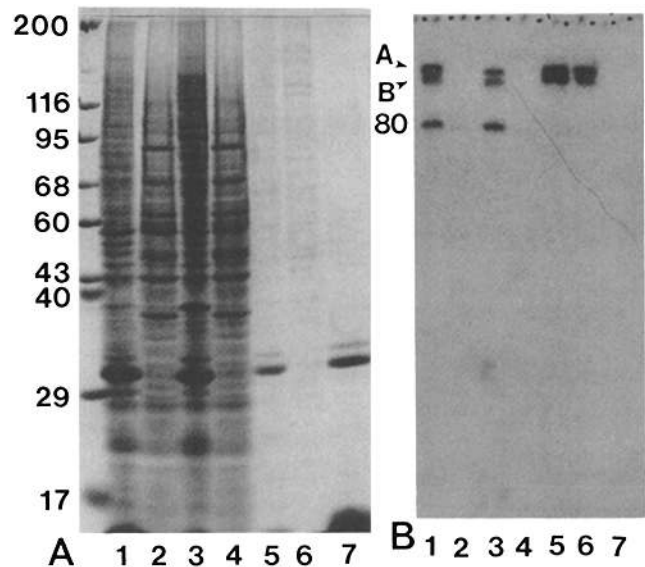
The biochemical and immunocytochemical experiments described above indicate clearly that the INCENPs are an integral part of the mitotic chromosome infrastructure. In fact, we know of only one other mitotic chromosome scaffold component that is recovered quantitatively in the chromosome scaffold fraction. (This is CENP C, the 140-kD centromeric autoantigen [Earnshaw, W. C., unpublished observations].)

We were therefore unprepared for the fate of the INCENPs during mitosis. As is shown in Fig. 5, they appear to dissociate completely from the chromosomes at the metaphase-anaphase transition. Fig. 5 (G-I) shows a metaphase plate (end view) and midanaphase side by side. The former shows the typical chromosomal staining seen above (Fig. 1), while in the latter cell the INCENPs appear distributed in a belt across the central region. Other examples of early, mid, and late anaphases are also shown in Fig. 5 (A-F). In all cases the antigen appears to be localized on coarse fibers in the zone of overlap of the two half spindles. In through-focus series, the antigen is also seen to be closely apposed to the cell membrane in the region of the cleavage furrow (see *arrowheads* in Fig. 5, D and G).

As mitosis continues, the INCENPs ultimately end up in the midbody (Fig. 5, D-F; see also Fig. 6, D-F, and Fig. 9, D-F). We have never detected staining of the nucleus of a cell to which a prominent midbody was attached.

The experiments shown in Figs. 1 and 5 used gentle fixation conditions that were chosen for optimal observation of chromosomal antigens. However, such conditions give poor preservation of microtubules. In view of the apparent localization of the INCENP antigens in the central spindle after sister chromatid separation, we therefore decided to examine cells that were fixed under conditions that better preserve microtubule structures. Fig. 6 shows the simultaneous colocalization of tubulin and INCENPs in metaphase, late anaphase, and telophase cells. Fig. 7 presents similar data for two cells in early and midanaphase. These images confirm that the INCENPs are present on the metaphase chromosomes and are not found on the spindle microtubules. At anaphase, the antigen is distributed across the center of the cell in a zone about the thickness of the original metaphase plate. While exact comparison of the fluorescence images is difficult, it appears that the INCENP antigen may show some association with the microtubules of the central spindle. At telophase (Fig. 6, D-F, an abnormal three-way division is shown) the INCENPs are restricted to the midbody, while microtubule bundles project out from this structure.

These data indicate that subsequent to the onset of ana-



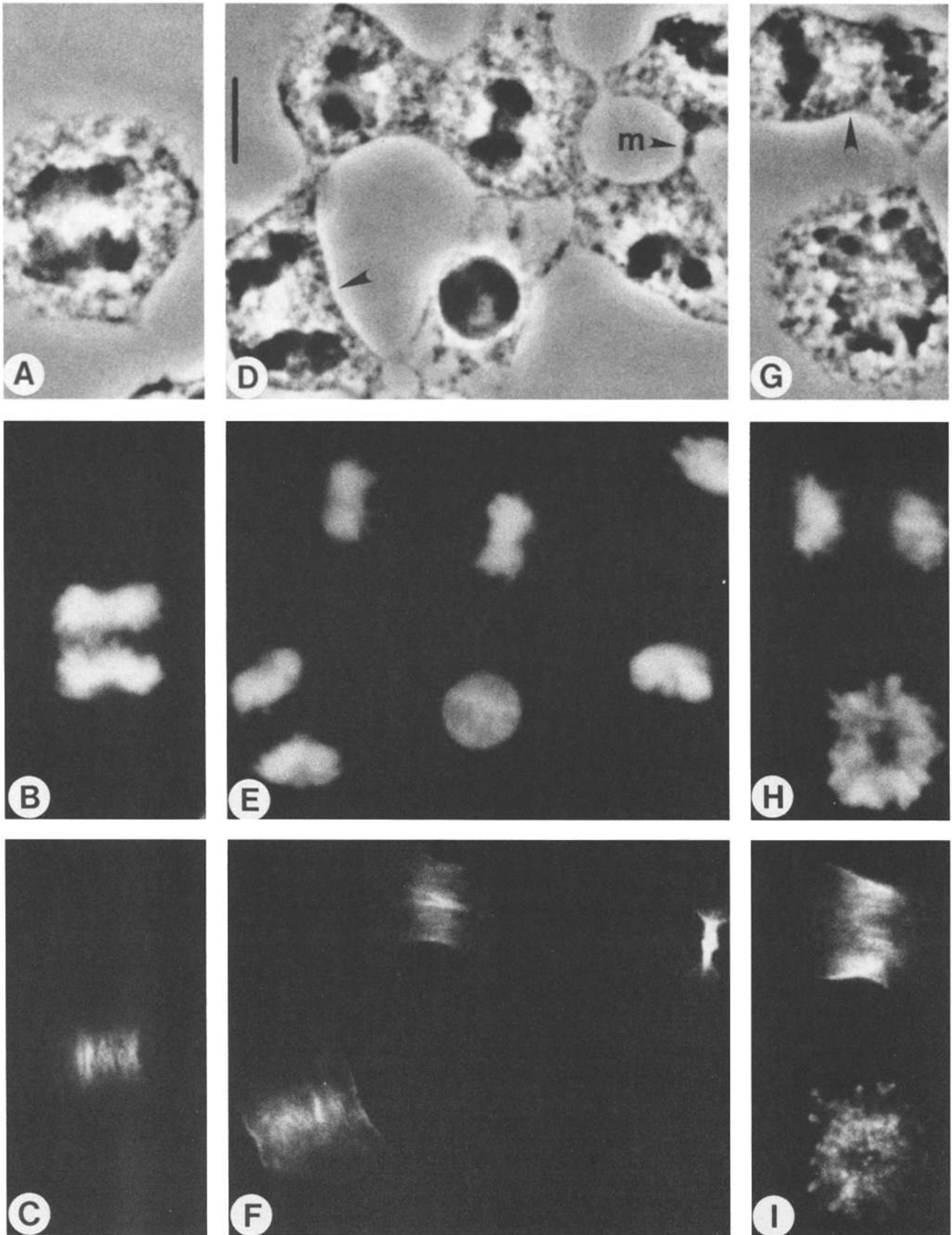
**Figure 4.** Distribution of the INCENP antigens in subcellular fractions. (A) Coomassie Blue-stained gel with molecular mass markers (in kilodaltons) at left. (B) Immunoblot of parallel samples showing species recognized by mAb-3D3. The lanes show: (1) crude interphase nuclei; (2) interphase cytoplasm; (3) crude mitotic chromosomes; (4) mitotic cytoplasm; (5) highly purified mitotic chromosomes; (6) the chromosome scaffold fraction prepared from these chromosomes by the 2 M NaCl extraction; (7) supernatant fraction of proteins solubilized by the 2 M NaCl extraction. In all cases comparable numbers of cell equivalents were loaded per lane. Binding of mAb was detected with  $^{125}\text{I}$ -goat anti-mouse Ig.

phase the INCENPs may associate with specific regions of the microtubules. Alternatively, the primary association may be with other structures in the region of the forming cleavage furrow.

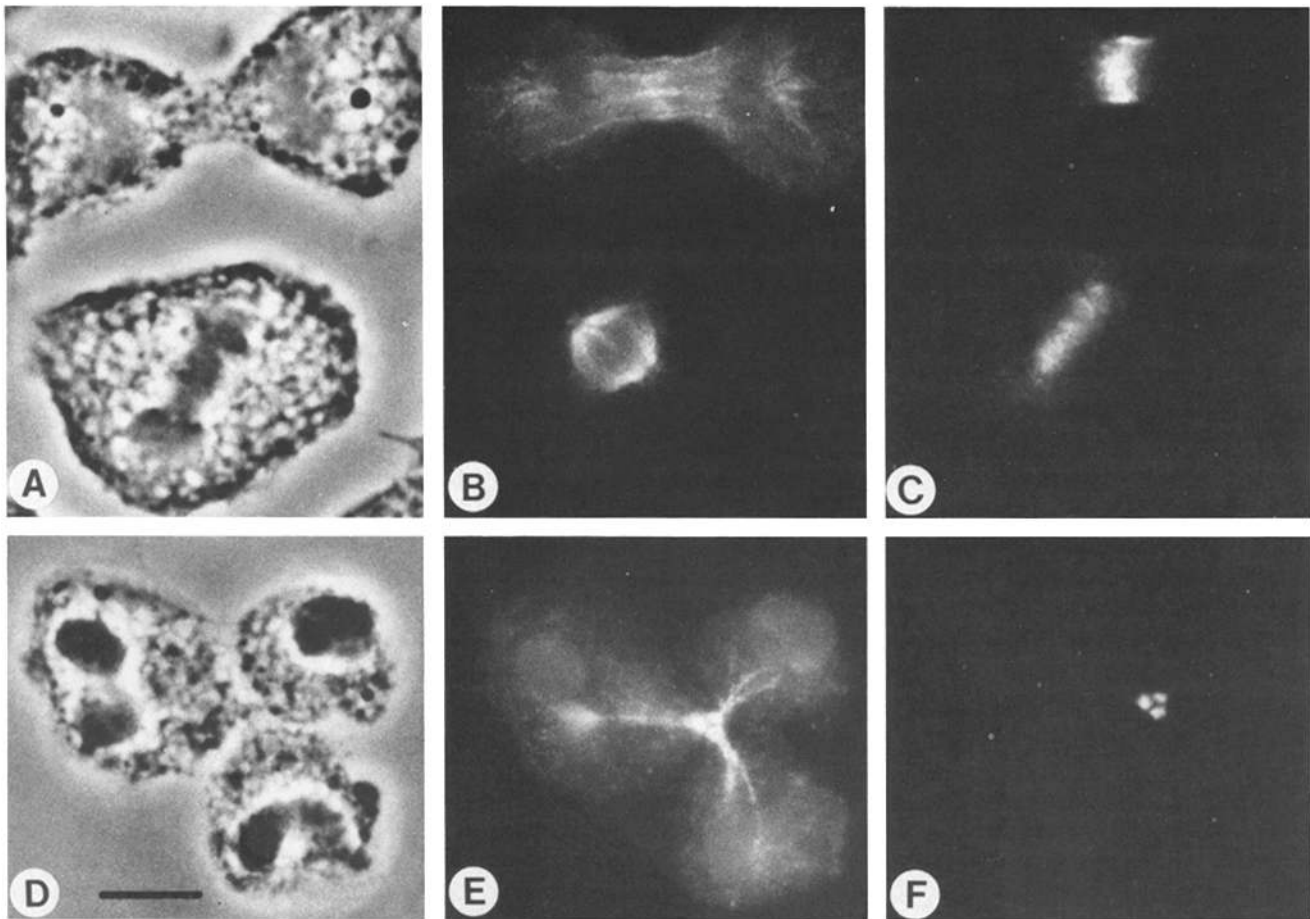
### *Demonstration that the Antigens Identified in Immunoblotting Give Rise to the INCENP Immunofluorescence Pattern*

It is very difficult to prove that an immunofluorescence staining pattern obtained with a single mAb is due to specific polypeptides recognized by that mAb in immunoblots. The possibility that different species are recognized in immunofluorescence and immunoblots must always be considered, particularly in view of the well known tendency of mAbs for unexpected cross-reaction. However three lines of evidence suggest that the striking immunofluorescence patterns obtained with mAb-3D3 are due to recognition of INCENPs A and B. (a) Immunofluorescence of highly purified mitotic chromosomes gives the characteristic inner centromere staining. Immunoblotting analysis of the same material demonstrates the presence only of immunoreactive INCENPs A and B (not shown). (b) The nuclei of early G<sub>1</sub> cells (still connected by a prominent cytoplasmic bridge) do not bind mAb-3D3 in indirect immunofluorescence. Immunoblotting of cells separated according to cell cycle position by centrifugal elutriation confirms that INCENPs A and B are much reduced in early G<sub>1</sub> cells (see below).

A more definitive experiment would be to affinity purify antibody from INCENPs A and B on nitrocellulose blots and show that this antibody gives the characteristic immunofluo-



**Figure 5.** Redistribuition of the INCENPs at the metaphase-anaphase transition. The images are as follows: (A, D, and G) phase contrast; (B, E, and H) DAPI staining of DNA (C, F, and I) immunolocalization of INCENP antigens. (A-C) Early anaphase. (D-F) Midanaphase, early telophase, late telophase (with forming midbody indicated by *m*). (G-I) Side by side comparison of a metaphase plate (end view) and midanaphase. At anaphase the antigens are localized in coarse fibers covering the central zone of overlapping microtubules in the spindle. In D and G, arrowheads indicate two cells in which an apparent association of the antigens with the membrane at the cleavage furrow can be seen. Bar, 10  $\mu$ m.



**Figure 6.** Simultaneous localization of tubulin and INCENPs in mitotic cells. Images shown are (A and D) phase contrast, (B and E) immunolocalization of rabbit tubulin with polyclonal antibody, (C and F) immunolocalization of INCENPs. The stages shown are (A–C) metaphase and late anaphase; and (D–F) telophase of an abnormal division. The INCENPs appear to colocalize with very limited regions of microtubules in the postmetaphase cells. Bar, 10  $\mu$ m.

rescence pattern. Unfortunately it is meaningless to do this experiment with a mAb, since only a single-binding specificity is present to begin with. We have, however, performed the experiment using polyclonal serum from a mouse immunized with chicken chromosome scaffolds. This mouse was immunized in an earlier attempt to obtain anti-scaffold mAbs. (This was not the mouse that eventually gave rise to mAb-3D3.) When serum from this mouse was used to probe an immunoblot of the proteins of isolated chicken chromosomes, >15 polypeptides were recognized, including four prominent species in the 130–170-kD molecular mass range (Fig. 8 A).

Antibodies were affinity purified from each of these four polypeptides as described previously (13), and the specificity of the eluted antibodies confirmed by reblotting against the proteins of isolated chromosomes (Fig. 8 B). It is apparent from Fig. 8 B that bands 1 and 2 contain distinct antigens, while the antibodies that bind to bands 3 and 4 cross react strongly (Fig. 8 B, lanes 1–4). The immunoblotting pattern obtained with the latter two affinity-purified antibodies is indistinguishable from that obtained with mAb-3D3 (Fig. 8 B, lanes 3–5). Thus both polyclonal and monoclonal antibodies to INCENPs A and B cross react strongly.

In indirect immunofluorescence, anti-band 1 and anti-

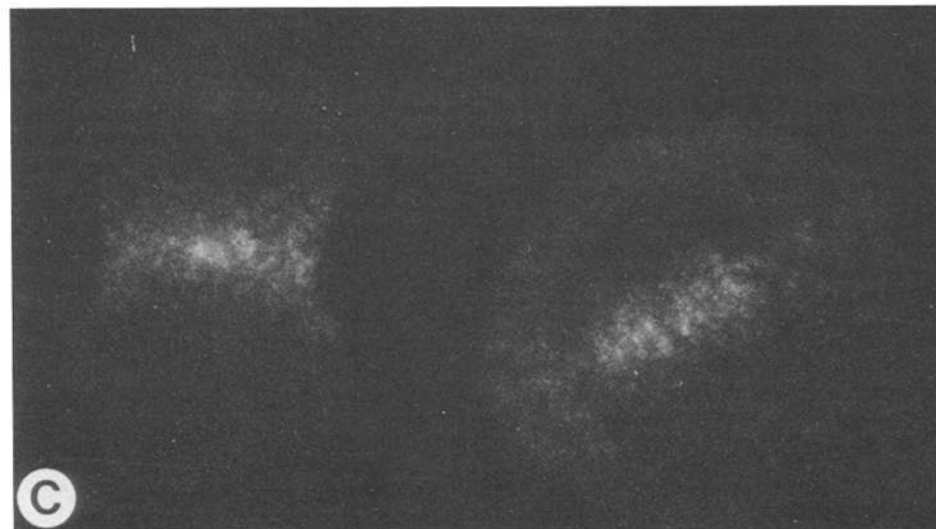
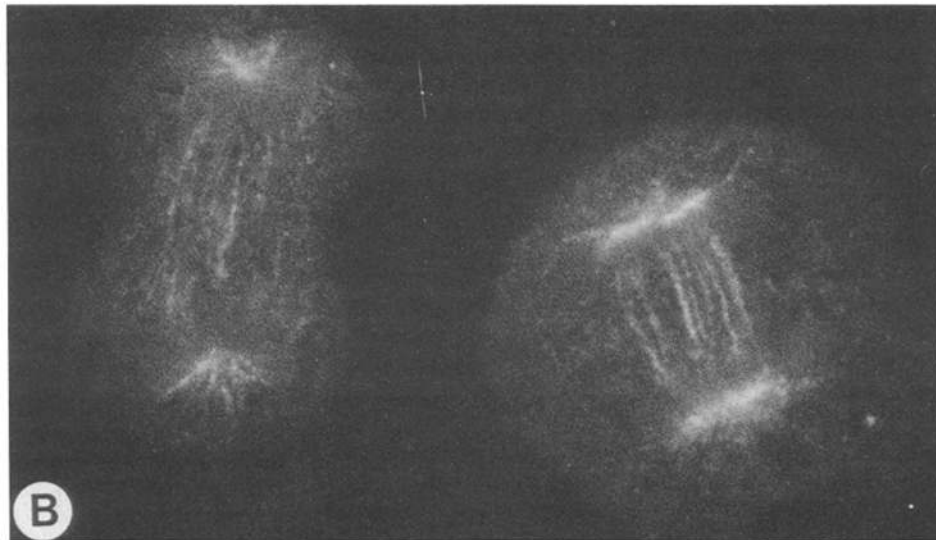
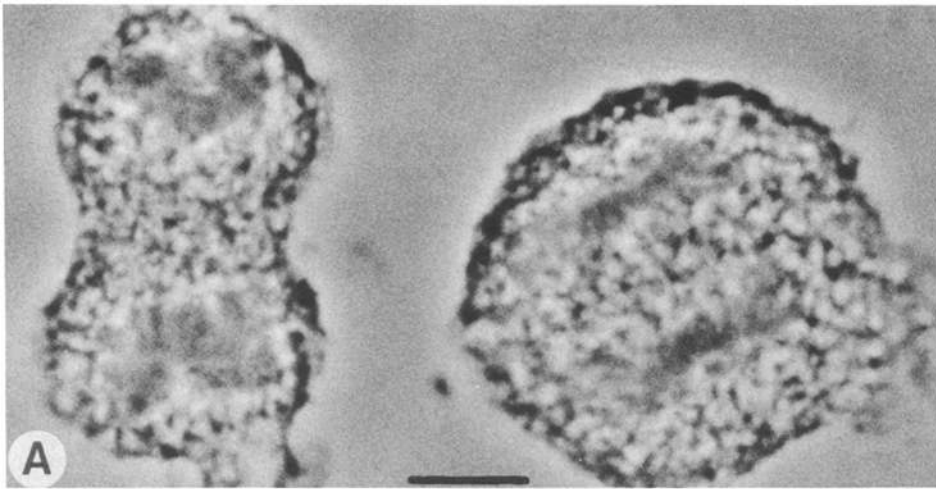
band 2 gave patterns distinct from that obtained with mAb-3D3. Anti-band 1 recognizes the condensed chromosomes in metaphase, anaphase, and telophase cells. In the latter an extremely faint staining of midbodies may be seen (perhaps due to contaminating anti-band 3 and anti-band 4, which may be faintly seen in the control immunoblot; Fig. 8 B, lane 1). Anti-band 2 yields a similar fluorescence pattern, with stronger recognition of midbodies (again possibly due to presence of contaminating anti-band 3 and anti-band 4).

Indirect immunofluorescence of dividing cells using affinity-purified anti-band 3 is shown in Fig. 9. This fluorescence is essentially identical to that obtained using mAb-3D3, showing strong binding to metaphase chromosomes (Fig. 9, A–C), the central zone of overlap of the anaphase spindle (Fig. 9, A–C), and to the midbody (Fig. 9, D–F). Similar fluorescence images were obtained with anti-band 4 (not shown). Thus the unusual pattern of immunostaining of dividing cells with mAb-3D3 appears to derive from recognition of INCENPs A and B.

#### *The 80-kD Antigen*

We have further investigated the relationship between ag80kD and INCENPs A and B by preparing a polyclonal antibody specific for ag80kD. The ag80kD band was excised

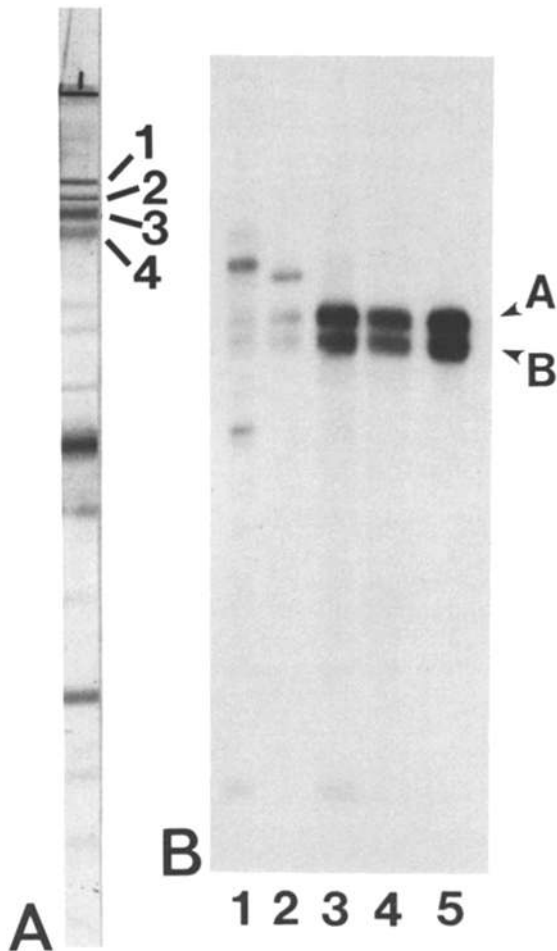




*Figure 7.* Simultaneous localization of tubulin and INCENPs in anaphase cells. Images shown are (A) phase contrast, (B) immunolocalization of tubulin, and (C) immunolocalization of INCENPs. Late (*left*) and mid-anaphase are shown. The INCENPs are concentrated at the original location of the metaphase plate. Bar, 10  $\mu$ m.

from a gel of total cellular proteins immunoprecipitated with mAb-3D3, macerated, and injected into a guinea pig. The resultant serum recognized only ag80kD on immunoblots of whole cell lysates (not shown). This polyclonal serum and mAb-3D3 recognize the same polypeptide. When immuno-

reactive species were precipitated from cells solubilized in SDS using anti-ag80kD (19, 35), subjected to SDS-PAGE, and analyzed by immunoblotting with mAb-3D3, the monoclonal antibody reacted strongly with the immunoprecipitated ag80kD (data not shown).



**Figure 8.** Affinity purification of polyclonal antibodies recognizing INCENPs A and B. (A) Serum from a mouse immunized with chromosome scaffolds was used to stain an immunoblot of total chromosomal proteins (immunoperoxidase detection). (B) Antibodies eluted from the four bands indicated in (A) were used to probe parallel strips of chromosomal proteins (lane number corresponds to band number in A). The immunoblots obtained with polyclonal anti-bands 3 and 4 were indistinguishable from that obtained with mAb-3D3 (lane 5).

The pattern of immunofluorescence observed upon staining cells with anti-ag80kD (Fig. 10) differs from that obtained with mAb-3D3 in two significant ways. First, neither prophase or metaphase chromosomes are stained (Fig. 10, A-C). Anti-ag80kD stains the entire cytoplasm diffusely in mitotic cells, being excluded from the condensed chromosomes. Second, the antibody does not stain midbodies (*m* in Fig. 10 D). The latter is the most consistent and unmistakable feature of staining exhibited with mAb-3D3.

Given the failure of anti-ag80kD to decorate mitotic chromosomes *in situ*, we wished to know the reason for the biochemical cofractionation of this antigen with crude mitotic chromosomes (Fig. 4). Indirect immunofluorescence analysis of crude chromosome preparations indicated that anti-ag80kD was present solely on contaminating interphase nuclei and debris (data not shown). This material is efficiently removed by the Percoll gradient during the standard chromosome isolation procedure.

We conclude that ag80kD exhibits a different cellular distribution from INCENPs A and B. Thus ag80kD is not an INCENP, and the basis of its reaction with mAb-3D3 is not known.

### Cell Cycle Analyses

The nuclei of early G<sub>1</sub> cells (recognized as small cells that remain joined by a conspicuous intercellular bridge with midbody) were consistently negative in immunofluorescence with mAb-3D3. This suggested that the cellular levels of INCENPs might vary widely across the cell cycle.

To test such a hypothesis, cells were separated according to size (a function of cell cycle position) by centrifugal elutriation. An equal number of cells from each fraction was then boiled in SDS sample buffer and analyzed by SDS-PAGE and immunoblotting (Fig. 11). The total protein in each fraction is shown in Fig. 11 A. The intensity of Coomassie Blue staining gradually increases in subsequent fractions, reflecting the increase in cell size.

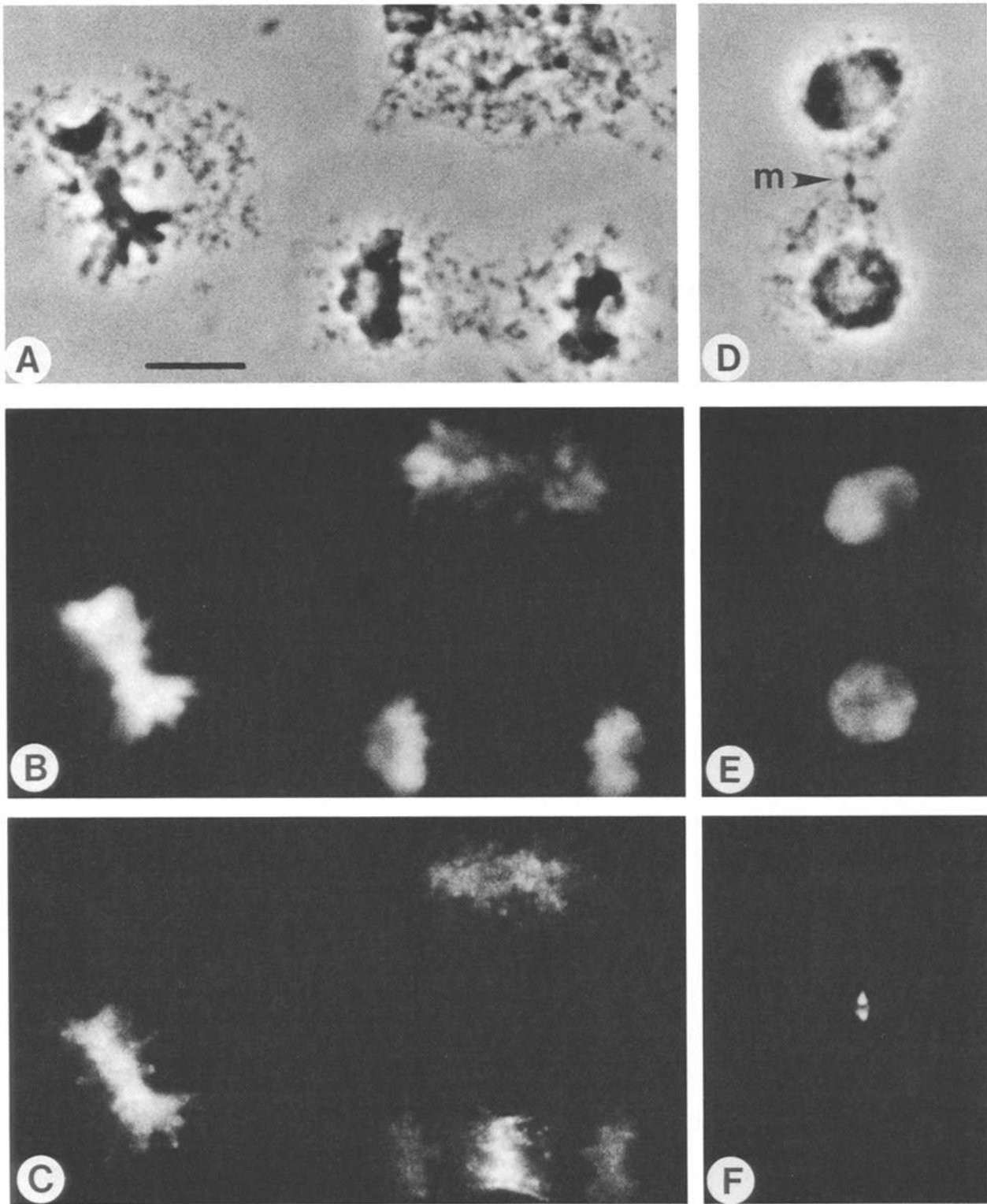
In the immunoblot with mAb-3D3 (Fig. 11 B) INCENPs A and B were observed to vary dramatically across the cell cycle. They are barely detectable in early G<sub>1</sub> cells (fractions 1 and 2), and increase to a maximum value in G<sub>2</sub>. This variation in staining is consistent with the INCENP staining pattern seen in immunofluorescence experiments. In contrast ag80kD shows much less variation across the cell cycle.

### Discussion

#### Protein Antigens that React with mAb-3D3

One major concern when using monoclonal antibodies to analyze hitherto unknown structures is the possibility that different aspects of the staining may arise from cross-reactions with different antigens. We have therefore examined the interactions of mAb-3D3 with cellular antigens in a number of ways. mAb-3D3 interacts with three polypeptides in immunoblots: INCENP A (155 kD), INCENP B (135 kD), and ag80kD. Only INCENPs A and B can account for the observed immunofluorescence patterns, however. This is deduced from the following observations. (a) Polyclonal serum from an independently injected mouse was affinity purified from INCENPs A and B. Both purified antibodies reacted with both polypeptides (as does mAb-3D3), and both gave immunofluorescence staining patterns similar to that seen with mAb-3D3. (b) Purified mitotic chromosomes contain only INCENPs A and B (detectable by immunoblotting). These chromosomes show the characteristic INCENP staining by indirect immunofluorescence. (c) Measurement of the levels of total INCENPs A and B across the cell cycle reveals that early G<sub>1</sub> cells, which show no nuclear fluorescence with mAb-3D3, have only minute amounts of the two antigens detectable by immunoblotting. (d) Polyclonal anti-ag80kD has been prepared, and precipitates the same 80-kD polypeptide recognized by mAb-3D3. This polyclonal serum gives an immunofluorescent staining pattern distinct from that seen with mAb-3D3. ag80kD shows no association with either mitotic or prophase chromosomes (at which stage it is still nuclear). Thus ag80kD is not an INCENP.

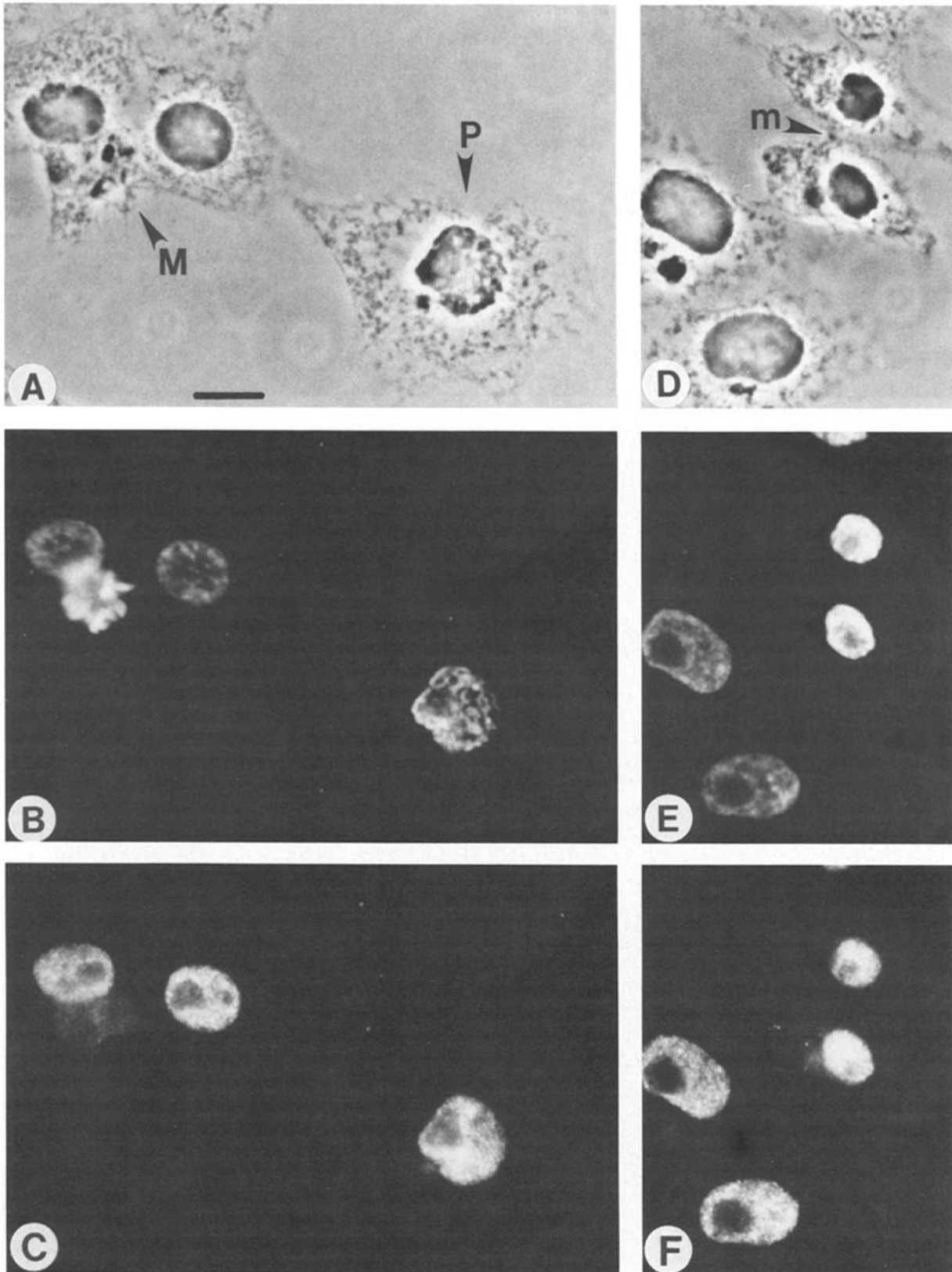
The basis of the crossreaction of mAb-3D3 with ag80kD remains obscure. An obvious possibility is that the mAb recognizes a posttranslational modification, as previously



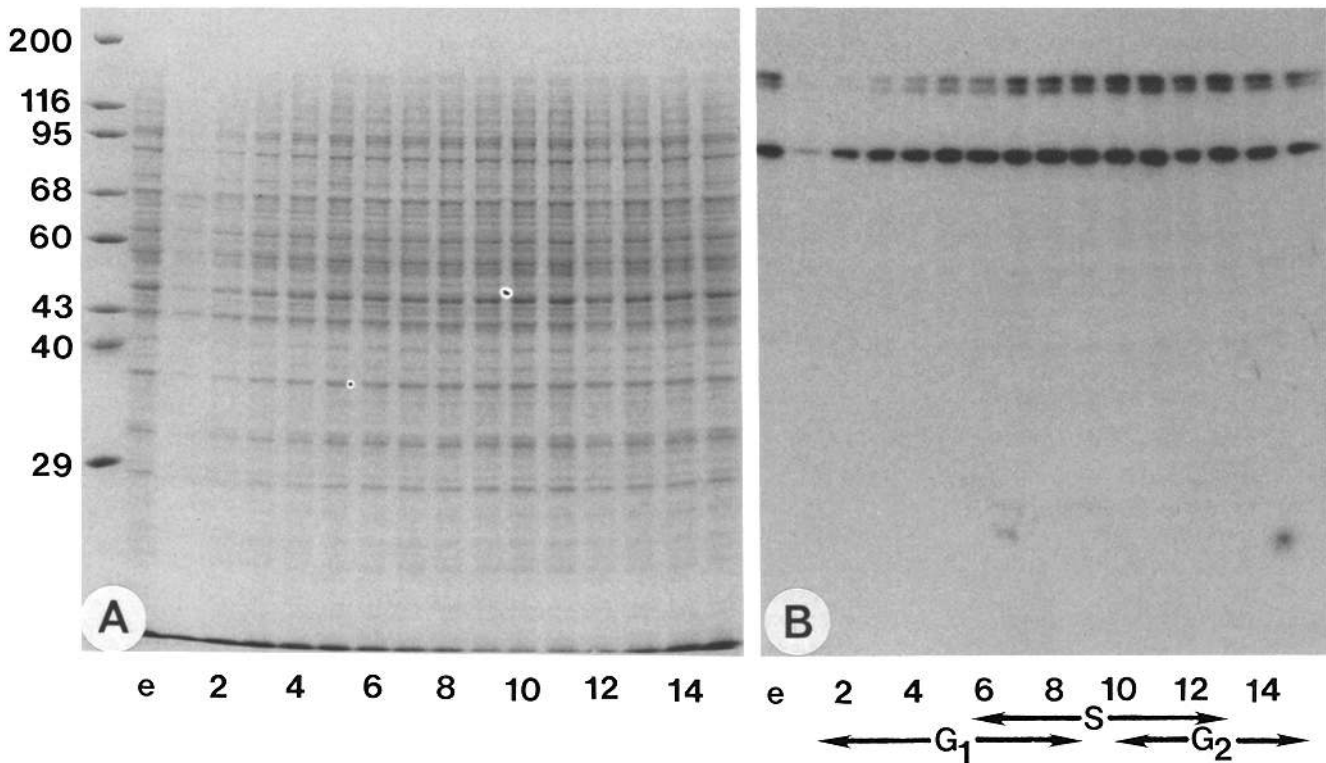
**Figure 9.** Indirect immunofluorescence with polyclonal anti-band 3 (see Fig. 8). Images are: (A and D) phase contrast; (B and E) DAPI binding to DNA; (C and F) immunostaining with affinity-purified polyclonal anti-band 3. The antibody binds to mitotic chromosomes (A–C), the midzone of anaphase spindles (A–C), and midbodies (D–F, *m* in D). Bar, 10  $\mu$ m.

shown for certain other mitosis (7) and nuclear envelope (8, 42)-specific mAbs. The former has been shown to recognize specific mitotic phosphoproteins (7), while the latter recognize an unusual O-linked glycosylation (23, 41). We have attempted to exclude these obvious possibilities by showing

that the interaction of INCENPs A and B with mAb-3D3 is not inhibited by preincubation of the antigens with alkaline phosphatase, hexoseaminidase, or galactosyl transferase (data not shown). Recent experiments suggest strongly that mAb-3D3 recognizes a peptide epitope, since the antibody has



**Figure 10.** Immunolocalization of ag80kD in interphase and mitotic cells. Images are (A and D) phase contrast; (B and E) DAPI staining of DNA; (C and F) immunolocalization of ag80kD. In A a metaphase (M) and prophase (P) are indicated. In D a midbody (m) is indicated. This polyclonal antibody does not bind to condensed chromosomes of either prophase or metaphase cells, and shows no binding to midbodies. Bar, 10  $\mu$ m.



**Figure 11.** Levels of INCENP antigens across the cell cycle. Cells were sorted according to size by centrifugal elutriation, counted with a Coulter counter, and solubilized in boiling SDS-PAGE sample buffer. Equal numbers of cells were loaded per lane. (A) Coomassie Blue stain. (B) Parallel gel analyzed by immunoblotting with mAb-3D3. Elutriation fraction numbers are indicated below each panel (e, unfractionated population). The cell cycle phases of the various fractions (determined by flow cytometry) are displayed at the bottom of B.

been used to obtain immunopositive clones from a  $\lambda$ -gt11 expression library (Earnshaw, W. C., unpublished data). Bacteria are not thought to perform the same spectrum of post-translational modifications observed in mammalian cells. (Significantly, the immunopositive clones are not recognized by anti-ag80kD.)

We conclude that the immunofluorescence patterns presented above are due to the specific recognition of peptide epitopes on INCENPs A and B by mAb-3D3.

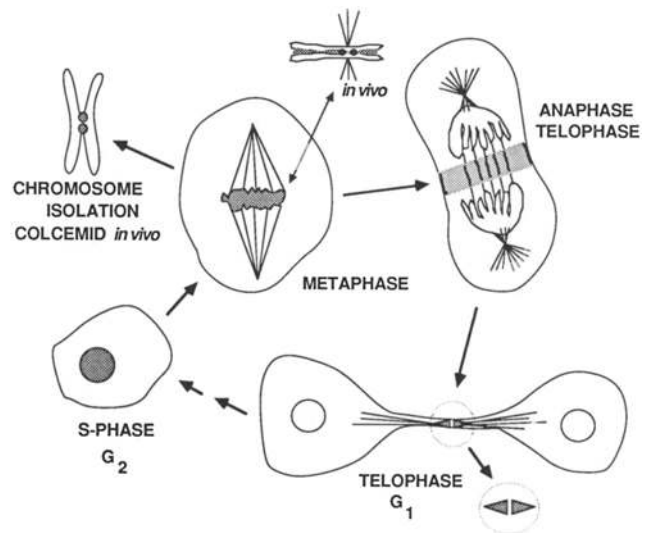
The relationship between INCENPs A and B is unknown. It is possible that they represent the products of a single gene and differ as a result of posttranslational modification. If this is so, then the modification is unlikely to be either phosphorylation or O-linked glycosylation for the reasons mentioned above. However, other modifications (such as poly-ADP ribosylation) might also cause alterations in mobility of the magnitude observed. It is also possible, however, that INCENPs A and B are the products of distinct genes.

#### **Localization of the INCENP Antigens in Mitotic Chromosomes and in Cells**

Separation of cells by centrifugal elutriation indicates that levels of INCENPs A and B vary widely across the cell cycle. The antigens are present in much reduced amounts in early G<sub>1</sub> cells, begin to increase dramatically during S phase, and attain their maximum levels during G<sub>2</sub>.

Fig. 12 provides a recapitulation of the changes in localization and amounts of the INCENPs that occur during the cell cycle. In early G<sub>1</sub> the INCENPs are detectable only in the

midbody, which remains from the previous mitosis. Immunofluorescent staining of interphase cells shows a speckled nuclear distribution of antigen (Fig. 1). The INCENPs undergo a dramatic series of changes in their cellular localization during mitosis. Through metaphase, the antigens are intimately associated with the mitotic chromosomes, where they are concentrated between the sister chromatids at points



**Figure 12.** Summary diagram showing the location of the INCENPs at various times during the cell cycle.

of contact. At the onset of anaphase the antigens separate from the chromosomes, remaining behind at the site of the metaphase plate. As the chromosomes migrate to the poles, the INCENP antigens are localized in a belt across the central spindle, with local regions of concentration proximal to the cell membrane in the region of the contractile ring. As cytokinesis proceeds, the INCENP antigens gradually become concentrated in the midbody.

### ***Speculations Concerning the Possible Role of the INCENP Proteins In Vivo***

The localization of the INCENP antigens in mitotic chromosomes and the dramatic alterations in this localization that occur during mitosis suggest several possible roles for these polypeptides during mitosis.

***The INCENPs May Regulate the Pairing of Sister Chromatids.*** That sister chromatid separation is a regulated process is suggested by the classical observation that the onset of anaphase is abrupt, with the vast majority of chromosomes separating at nearly the same time. Further, the spindle is not required for this separation, which also occurs in certain cells in the presence of levels of colchicine or colcemid sufficient to prevent the assembly of spindle microtubules (30, 40). This regulation might be achieved via direct structural interactions (i.e., the antigens might form a "zipper" between sister chromatids that dissociates in response to some signal). Alternatively, the INCENPs might act as modulators of the enzymatic activity of topoisomerase II (a major structural component of mitotic chromosome scaffolds [14], that is known to be required for mitotic disjunction in lower eukaryotes [9, 46]).

Two observations support such a role for the INCENPs. First, through metaphase, the INCENPs are the two most tightly bound chromosome scaffold components identified to date. This association is strikingly reversed at the onset of anaphase, at which point the antigens appear to leave the chromosomes entirely. It is tempting to speculate that some irreversible modification of the INCENPs (proteolysis?) serves as the trigger for anaphase chromatid separation, with the modified antigen being subsequently removed from the cell by sequestration in the midbody.

The distribution of the INCENPs within metaphase chromosomes from both normal and colcemid-blocked cells is also consistent with their potential involvement in sister chromatid pairing. In normal metaphase, the sister chromatids are tightly apposed along their entire lengths (Fig. 1). Furthermore, the ability of sister chromatids to undergo a timed anaphase separation is not limited to the centromere; acentric fragments derived from the distal arms also separate coordinately with their centric counterparts (4). This implies that the components that regulate pairing are distributed along the entire length of the chromosome, as are the INCENP antigens.

In colcemid-blocked cells, the distal chromatid arms separate and the sister chromatids remain joined by two bridges between the proximal arms just above and below the centromere (which itself may separate; 27). The localization of the INCENP antigens in colcemid-blocked chromosomes shows a remarkable correlation with this pattern of sister chromatid separation. As the distal arms separate, the distal INCENPs disappear. The INCENPs are ultimately found in two small dots just above and below the centromere (Fig. 2). The coincidence between the classical observation of sister chromatid

attachment and the localization of the INCENP antigens is striking.

***The INCENPs Might Be Involved in Stabilization of the Cleavage Furrow at Cytokinesis.*** Examination of through-focus series of images from anaphase cells shows that the INCENP antigens permeate the central spindle, extending outward to form a concentrated zone subjacent to the cell membrane in the region of the contractile ring (Fig. 5). It is thus tempting to speculate that INCENP antigens, which define the center of the metaphase plate (as the point midway between oppositely oriented sister kinetochores), might, upon their release from the chromosomes, interact with the components of the contractile ring.

Classical experiments suggested that the location of the metaphase plate specifies the location of the cleavage furrow at cytokinesis (reviewed in 37), but more recent observations cast doubt on this. For example, disruption (44) or removal (21) of the spindle does not prevent formation of the cleavage furrow, provided that late metaphase or early anaphase has first been reached. These results suggest that spindle components are not directly involved in stimulating formation of the cleavage furrow. However they do not rule out a mechanism whereby components essential for furrow formation are moved to the metaphase plate as part of the spindle, and then are released from the spindle at anaphase onset.

The micromanipulation studies of Rappaport, which have been interpreted as showing that cleavage furrows always appear midway between asters regardless of whether or not a spindle is present in this region (37, 38), suggest that cleavage furrow initiation is independent of the structure of the central spindle. However a factor liberated from the spindle at anaphase onset might be required in order to stabilize the forming furrow so that cytokinesis may proceed to completion. In future experiments it will be informative to determine the distribution of INCENPs in echinoderm eggs, both during normal cytokinesis and subsequent to micromanipulations that induce the formation of extra furrows. This will be particularly important, since the presence of maternal stores of INCENPs in eggs and oocytes (if these exist) could conceivably lead to a "deregulation" of normal pathways of furrow formation.

***The INCENPs Might Promote the Microtubule-based Movements of Mitosis.*** The anaphase movements of mitosis have two components: migration of the chromosomes to the spindle poles, and the separation of the poles themselves. The localization of the INCENPs in anaphase is consistent with their participation in the latter. The distribution of INCENPs on anaphase spindles coincides with the zone of overlapping microtubules that project inward from the two poles, suggesting that they could somehow be involved in microtubule sliding. Alternatively, the INCENPs could be part of a stable anchoring complex against which the separating spindle poles exert force.

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