# Molecular Analysis of the INCENPs (Inner Centromere Proteins): Separate Domains Are Required for Association with Microtubules During Interphase and with the Central Spindle During Anaphase

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*Abstract.* It has recently been proposed that mitotic chromosomes transport certain cytoskeletal proteins to the metaphase plate so that these proteins are able to subsequently participate in the assembly of the anaphase spindle and the cleavage furrow. To understand how such proteins accomplish their dual chromosomal:cytoskeletal role, we have begun a molecular and functional analysis of the inner centromere proteins (INCENPs), founder members of the class of "chromosome passenger proteins." cDNA clones encoding the open reading frames of the two chicken INCENPs were recovered. The predicted proteins, class I INCENP (96,357 D) and class II INCENP (100,931 D) are novel, and differ from each other by the inclusion of a 38-codon insert within the class II coding region. Transient expression of the chicken INCENPs in mammalian cells confirms that the signals and structures required for the transfer of these proteins from chromosomes to cytoskeleton are evolutionarily conserved. Furthermore, these studies reveal that INCENP association with the cytoskeleton is complex. The amino-terminal 42-amino acid residues are required for transfer of the INCENPs from the chromosomes to the mitotic spindle at anaphase, but not for binding of INCENPs to cytoplasmic microtubules. In contrast, an internal 200 amino acid coiled-coil domain was required for association with microtubules, but dispensable for spindle association. These experiments suggest that proteins required for assembly of specialized cytoskeletal structures during mitosis from anaphase onwards might be sequestered in the nucleus throughout interphase to keep them from disrupting the interphase cytoskeleton, and to ensure their correct positioning during mitosis.

THE onset of anaphase is a crucial time both for the distribution of chromosomes to the daughter cells and for the partitioning of the cytoplasm. Recent experiments have revealed the existence of a class of chromosomal proteins that may play important roles in both chromosomal and cytoplasmic events at this time. We have termed these components "chromosome passenger proteins," and suggested that they may be transiently associated with chromosomes as a means of transport to the spindle midzone, where they may perform essential roles in the later stages of mitosis (8).

The first chromosome passenger proteins to be described, the INCENPs[1] (inner centromere proteins), were originally identified as components of the mitotic chromosome scaffold (6). The INCENPs are restricted to the nucleus in interphase, and tightly bound to the chromosomes until early metaphase. However, during late metaphase, they leave the chromosomes to become concentrated in linear arrays that transect the metaphase plate between the chromosomes (9). As anaphase begins and the sister chromatids move towards the spindle poles, the INCENPs remain behind at the spindle midzone, where they are intimately associated with the bundled microtubules at the region of overlap between the two half spindles. Later in anaphase, a subpopulation of the INCENPs becomes closely associated with the cell cortex at the point where the cleavage furrow will subsequently form. By telophase, the INCENPs are concentrated at each side of the midbody in the intercellular bridge, with which they are discarded after cytokinesis.

Since the identification of the INCENPs (6), five other chromosomal passenger proteins have been identified by the use of experimental or autoimmune antibodies. The JB autoantigen (38 kD) (20) and antigen TD-60 (60 kD) (2) both have distributions through mitosis that are strikingly similar to that of the INCENPs. Two other proteins, CENP-E (312 kD) (37) and the 37A5 antigens (140 and 155 kD) (29) resemble the INCENPs in that these proteins associate with the centromeric region of chromosomes in early mitosis, and the spindle midzone and stem body from anaphase onwards. However, neither is associated with the cell cortex at the cleavage furrow, and neither is detected in interphase cells. Finally, MSA-36 (36 kD) is detected at centromeres in unblocked metaphase chromosomes, then migrates to the spindle at anaphase (32).

1. *Abbreviations used in this paper*: AMV, avian myoblastosis virus; INCENP, inner centromere protein; RT, reverse transcription.

Morphological studies have served to make the biological function of these proteins a tantalizing puzzle. The distributions of the chromosome passenger proteins during mitosis has led to suggestions that they could be important for such disparate events as regulation of sister chromatid pairing, interactions between the two half spindles, and assembly or function of the cleavage furrow (6). Nothing is known about the function in vivo of any of the chromosome passenger proteins. However, CENP-E has recently been proposed to be a member of the kinesin superfamily, and might function as a motor protein either at the kinetochore during prometaphase, or on the spindle during anaphase (36, 37).

The studies described here represent our initial steps towards determining the biological roles of the INCENPs. We describe the cloning of cDNAs encoding both INCENP polypeptides. Structural and functional analyses of these clones and of the polypeptides that they encode reveal the INCENPs to be multidomain proteins whose interaction with the spindle microtubules is likely to be complex and involve novel mechanisms.

## Materials and Methods

### Cloning

***Library Screening.*** mAb 3D3 (6) was used to screen a λgt11 chicken embryo fibroblast cDNA library (gift of B. Vennstrom, Karolinska Institute, Stockholm, Sweden) by standard techniques (3). The insert from one positive plaque was recloned in the pUC18 vector to generate clone I-14/pUC18. This sequence was used for further screening by nucleic acid hybridization as well as for antibody production. As 5′ extensions of I-14/pUC18 were not recovered from the λgt11 library, we constructed a λZAP II library. Poly(A)$^+$ RNA was recovered from log-phase MSB-1 cells by oligo-dT batch chromatography (4). Reverse Transcription with avian myoblastosis virus (AMV) reverse transcriptase and library construction were according to the manufacturer's instructions (Stratagene, La Jolla, CA). Multiple rounds of screening yielded overlapping INCENP clones (Fig. 1). However, no candidate translational start site was identified.

***Reverse Transcription–PCR.*** Reverse transcription (RT)–PCR primers were chosen from the 5′-most sequence of INCENP clones. RNA was denatured by exposure to 10 mM methylmercuric hydroxide (Aldrich Chemical Co., Milwaukee, WI) followed by 10 mM β-mercaptoethanol, then annealed to primer Anti-3 (5′TGTAGTGGCTGCCGAACATCC3′) (3). *TTh* DNA polymerase (Pharmacia Fine Chemicals, Piscataway, NJ) in Retro-Therm buffer adjusted to 90 mM KCl (Epicentre Technologies Corp., Madison, WI) directed first-strand cDNA synthesis (26).

15–30 dA residues were added to the 5′ end of the antisense strand by terminal deoxynucleotidyl transferase (GIBCO-BRL, Gaithersburg, MD). Second-strand synthesis was primed with RACE-1 (5′ GATGGATCCTGC AGAAGCTT$T_{13}$ 3′; Hind III site underlined) and accomplished with *Taq* polymerase (Perkin-Elmer Corp., Norwalk, CT) under standard conditions (13), but with 1 μM tetramethyl ammonium hydroxide added to suppress mispriming. Between steps, Sephadex S-300 (Pharmacia) spin-columns were used to separate unincorporated primers from reaction products.

5′ extensions were amplified by PCR (30 cycles: 30 s at 94°C, 30 s at 50°C, 30 s at 72°C; final incubation at 72°C for 7 min) with RACE-2 (5′ GATGGATCCTGCAGAAGC 3′) and nested antisense primer Anti-1 (5′ ATCTCTTCCAGCCACAACAG 3′). Products were digested with HindIII and SalI, size selected for 100–800-bp products, then ligated to HindIII–SalI digested and phosphatased Bluescript II KS+ vector (Stratagene). Clone PCR-1/BS was recovered in this fashion.

To verify the start site and obtain upstream sequence, some products were subjected to a second of round of PCR. Conditions were as described, except nested primer Anti-2 (5′ CTCAGGAGCTCCGCCAGCCGCTG 3′) was substituted for Anti-3, and annealing temperature was decreased to 48°C. Products were digested with HindIII and SacI, and cloned into Bluescript.

### Sequencing and Sequence Analysis

***Sequencing.*** λZAP II clones were transformed into double-stranded Bluescript inserts by in vivo excision, as recommended by the manufacturer. Exonuclease III–nested deletions were generated (18), and the resulting double-stranded templates were sequenced with Sequenase 2.0 (United States Biochemical, Cleveland, OH). Compressions and pauses were resolved by substituting deoxyinosine for deoxyguanosine, and by a terminal deoxynucleotidyl transferase chase, respectively.

***Data Analysis.*** MacVector 3.0 (IBI, New Haven, CT) was used for data entry and preliminary sequence analysis. The deduced open reading frames were compared to GenBank and SwissProt databases by the TFASTA search strategy (31), and by the BLASTP application maintained at the National Center for Biotechnology Information (Bethesda, MD) (1).

### Northern and Southern Blotting

Nytran nylon membranes were used for Northern and Southern blotting according to the manufacturer's recommendations (Schleicher & Schuell, Keene, NH). Agarose gels (FMC Corp. BioProducts, Rockland, ME) for RNA were run with 0.2 M formaldehyde (3). Nucleic acid probes were random-prime-labeled with $^{32}$P-dCTP (Amersham Corp., Arlington Heights, IL) by random-priming or PCR (33).

### Cloning Expression Constructs

All but the 5′-most end of the class I open reading frame was assembled in Bluescript from partial INCENP clones by combining BstXI fragments of I-75/BS and X24/BS to form clone 7/BS. Sequences near the translational start site were added by PCR. PCR-1/BS was used as a template for primers Start-sen (5′ CGGGATCCTCATATGGCGGTGGCAACG 3′; BamHI and start sites underlined) and Anti-1. The 89-bp product of BamHI and SalI digestion was directionally cloned into the 5′ end of clone 7/BS cut with the same enzymes to generate clone CI/BS. PCR-generated DNA was verified by sequencing.

The INCENP open reading frame was excised from CI/BS by digestion with BamHI and BglII and cloned into the BglII site of expression vector pECE (11) to generate clone INCENP$_I$/pECE. A similar full-length expression construct for class II (INCENP$_{II}$/pECE) was derived by digesting the class I construct with the unique-site enzymes BclI and BglII. The 3′ portion of the class I coding sequence was replaced by the corresponding BclI–BglII fragment from class II clone II-73/BS. DNA was purified by CsCl density gradient centrifugation (3).

### Construction of Deletion Clones

Two deletion mutants of INCENP$_I$/pECE were created to dissect INCENP domain structure. The sequence upstream from the unique SalI site at position 82 was eliminated by cutting INCENP$_I$/pECE with BamHI and SalI, then religating the blunted ends of the resulting large fragment. Translation was initiated at the first start codon encountered after this point, methionine 43. Accordingly, this construct was identified as INCENP$_I$-Δ1-42/pECE. In the second deletion construct, most of the putative coiled-coil region was removed by partial digestion of CI/BS with PstI. The fragment that was cut at two in-frame PstI sites (1532 and 2090) was isolated and self-ligated to generate INCENP$_I$-Δ511-696/BS. The BamHI–BglII fragment of this construct was recloned into pECE to generate INCENP$_I$-Δ511-696/pECE.

### Production of Antibodies to INCENPs

Clone I-14/pUC18 encodes the COOH-terminal 22-kD of the Class I INCENP open reading frame. It was digested with EcoRI and placed into the pATH11 vector (21), and a *trpE*-INCENP fusion protein was produced in *E. coli*. Fusion protein was immunoprecipitated with antibody 3D3 and run on a preparative SDS-polyacrylamide gel. The band migrating at 59 kD (37-kD *trpE* + 22-kD INCENP) was excised, ground into a powder under liquid nitrogen, and injected into the footpads of two New Zealand White rabbits, using acrylamide as adjuvant (17). Rabbits were boosted three times at 5-wk intervals with the same antigen. The third bleed from each rabbit (ra1-INCENP and ra2-INCENP) were used as immune sera, with preimmune sera serving as controls.

### Tissue Culture and Electroporation

DU-249 chicken cells (23), primary chick embryo fibroblasts, human HeLa cells, and porcine LLCPK cells were grown as monolayers in plastic flasks (Corning Inc., Corning, NY) in DME (GIBCO-BRL) with 10% FCS (HyClone Laboratories, Logan, UT). MSB-1 chicken lymphoblasts were grown in spinner culture as previously described (10).

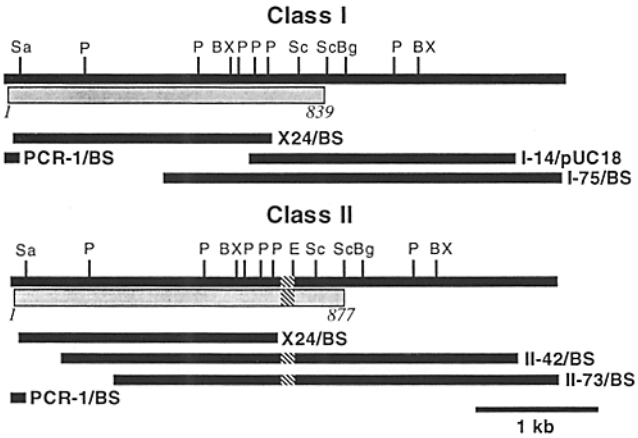


*Figure 1.* INCENP clones for class I and class II along with 5′ clones common to both classes. Clone PCR-1/BS was generated by RT-PCR, others were recovered from library screens. Class I and class II deduced open reading frames are shown (*shaded area*) with peptide residues italicized. The class II insert is striped. Restriction enzymes: BglII (Bg), BstXI (BX), EcoRI (E), PstI (P), SalI (Sa), ScaI (Sc). λZAPII library clones had 5′ EcoRI, and 3′ XhoI sites. In assembling contigs, a BamHI site was placed immediately upstream of codon 1 (see Materials and Methods).

HeLa and LLCPK cells were transiently transfected by electroporation (3). Adherent cells were grown to early log phase, trypsinized, rinsed, and resuspended in Opti-MEM I at 5 × $10^6$ cells/ml. 20 μg DNA was added to 0.4 ml of suspended cells in a 0.4-cm cuvette, then subjected to a pulse of 960 μF at 300 V by a Gene-Pulser (Bio Rad Laboratories, Richmond, CA). Cells were plated on dishes or glass cover slips in Opti-MEM-I, 10% FBS, and antibiotics, and incubated for 24 h.

### Electrophoresis and Immunoblotting

Chicken chromosomes were isolated from colcemid blocked cells as described (6). Transfected cells were rinsed in cold PBS in the presence of protease inhibitors (2 mM EDTA, 350 μg/ml TAME, 1 mM PMSF, 50 KIU aprotinin, 1 μg/ml leupeptin), and lysed in boiling SDS sample buffer. Methods for electrophoresis, immunoblotting, and affinity purification of antibodies from immunoblots have been described (10).

### Fluorescence Microscopy

Immunofluorescence was as previously described (9) with slight modifications. Cells grown on cover slips were fixed with cold methanol (5 min) to preserve mitotic spindle structure. Cover slips were placed in KB buffer (10 mM Tris-HCl, pH 7.7, 150 mM NaCl, 0.1% BSA) with 1:100 normal goat serum, followed by the same buffer with immunoadsorbed anti-INCENP antibody (1:500) and mouse mAbs to α-tubulin (1:100; Accurate Chem. and Sci. Corp., Westbury, NY) and β-tubulin (1:100; Amersham N-357). After rinsing, cover slips were incubated with biotinylated goat anti-rabbit IgG (1:500; Vector Laboratories, Inc., Burlingame, CA) and FITC-goat anti-mouse IgG (1:100; Cappell Laboratories, Durham, NC). Rinses were followed by application of streptavidin-Texas red (1:800; GIBCO-BRL) and 1 μg/ml 4′,6′-diamidine-2-phenylindole dihydrochloride (DAPI; Calbiochem Corp., San Diego, CA). All incubations were for 45 min at 22°C. Coverslips were mounted with Mowiol (Calbiochem Corp.).

Digital images were acquired directly from a Vanox microscope (Olympus) with a 100× apochromatic objective lens. A microscope-mounted SIT-66 camera (Dage-MTI, Inc., Michigan City, IN) delivered an analog signal to a PixelPipeline image analysis card (Perceptics, Knoxville, TN) in a Macintosh IIfx (Apple Computers, Cupertino, CA). Three 8-bit 480 × 640 pixel digitized images, one for each filter set (Texas red, fluorescein, and DAPI) were exported into Photoshop 2.5 (Adobe Systems, Mountain View, CA). Where filamentous cytoplasmic staining is shown, two images taken at different focal planes were merged into a single grayscale image. Photoshop was also used to adjust and sharpen images before printing onto T-MAX 100 film (Eastman Kodak Co., Rochester, NY) with a ColorFast film printer (GCC, Boston, MA).

## Results

### The INCENPs Are Novel Multidomain Proteins with a Predicted Central Coiled-Coil Motif

We have recovered cDNA clones that encode the bulk of the INCENP transcript. Restriction digests of 26 independent isolates revealed the presence of two classes of clones that could be distinguished by the absence (class I) or presence (class II) of an internal EcoRI site. Clone X24/BS extended farthest in the 5′ direction, but did not include an initiation codon. The 5′ portion of the cDNA was ultimately recovered by RT-PCR (see Materials and Methods). Using this method, we recovered an additional 49 base pairs of 5′ cDNA sequence. This contains a single candidate methionine codon in a context consistent with the consensus proposed for translational initiation (22). Four codons upstream is a TAA stop codon in the same reading frame (Fig. 2). Clone PCR-1/BS and six other clones containing both the ATG and upstream TAA were recovered from independent RT-PCR reactions.

The primary sequences of both classes of INCENP cDNAs were determined by the dideoxy method. Both contained single, long open reading frames that were virtually identical to one another, with the notable exception of an insert of 114 bp in the class II clones (see large box in Fig. 2). In addition, two single-base polymorphisms were observed: ACT to GCT led to a thr to ala substitution at residue 257, while an A to G change in the wobble position left residue 281 unaltered (Fig. 2).

The predicted INCENP polypeptides have molecular weights of 96,357 D (class I) and 100,931 D (class II). They are hydrophilic along their lengths and highly basic (pI ~9.5; ~22% lys + arg + his). Searches of the GenBank database with the TFASTA (31) and BLAST (1) search strategies indicated that the INCENPs do not closely resemble other known proteins.

The initial analysis of the predicted open reading frames of the INCENP cDNAs indicated that residues 503–717 (class I) and 503–752 (class II) contain heptad repeats that might permit the formation of an α-helical secondary structure (Fig. 3 *A*). Further analysis of the INCENP deduced amino acid sequences with the "coilcoil" computer program showed that the INCENP sequences in these regions have primary sequence similarities to the coiled-coil domains of myosins, tropomyosins, and keratins (24). Based on this comparison, the coilcoil program estimates that there is a high probability that these INCENP residues can form a coiled-coil structure. Interestingly, a single frame for the predicted heptad repeat is not maintained for the length of the putative coiled coil. Instead, "stutters" appear to break up the domain into eight (class I) or nine (class II) blocks (Fig. 3 *A*).

One possible mechanism for bringing about the changes in INCENP intracellular localization during mitosis would be posttranslational modification, as is the case for the nuclear lamins (16) and numerous other proteins. Examination of the deduced sequences for consensus phosphorylation motifs revealed many potential sites for modification shared by the two classes. These include possible sites for $p34^{cdc2}$ at residues 57 (KTPS), 193 (KSPK), and 448 (KTPS), and a tyrosine kinase site at residue 425 (KRAVDQRY) (Fig. 2). In addition, INCENPs may be substrates of casein kinase (12

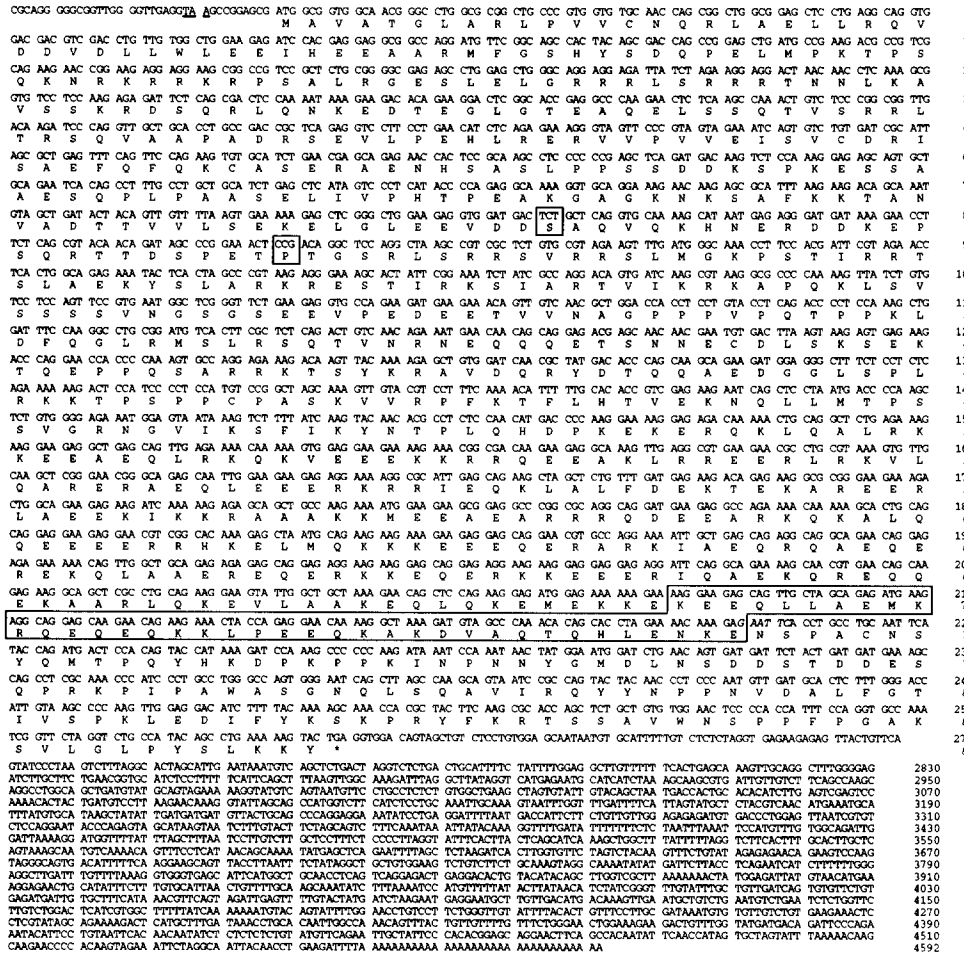

*Figure 2.* DNA and deduced amino acid sequence of the class II INCENP cDNA. This sequence was compiled from clones PCR-1/BS, X24/BS, II-73/BS, and I-75/BS. The in-frame stop codon at position -12 is underlined. Codons including polymorphisms (positions 769 and 843) are boxed; amino acid residue 257 is altered to thr in class I. The 114-bp (38 residues) that are omitted from class I (bases 2146 to 2259) are also boxed, and the class II internal EcoRI site (2259) is italicized. These sequences are available from EMBL and GenBank under accession numbers Z25419 (class I), and Z25420 (class II).

sites), cAMP-dependent protein kinase (27 sites), and protein kinase C (21 sites).

Because the INCENPs are nuclear proteins during interphase, we searched the deduced sequences for possible nuclear localization signals. Five widely spaced partial matches to the nucleoplasmin consensus signal (7) were identified beginning at residues 68, 303, 532, 592, and 614.

## *Polyclonal Antibodies Validate the cDNA Clones and Confirm That the INCENPs Are Chromosome Scaffold Components*

To generate polyclonal antisera against the INCENPs, cDNA clone I-14/pATH11 (encoding the COOH terminus of class I INCENPs fused to the *trpE* gene) was expressed in *E. coli*. The 59-kD expressed product was immunoprecipitated with mAb 3D3 (6) and used to immunize two rabbits. Ral-INCENP and ra2-INCENP each produced sera that recognized polypeptides of $M_r$ 133 and 145 kD in immunoblots of chicken chromosomes (Fig. 4 *B*). Both polypeptides are quantitatively retained in the chromosome scaffold fraction, confirming the tight association of the INCENPs with chromosomes from colcemid-arrested cells (Fig. 4 *B*). In indirect immunofluorescence, both rabbit sera recognized a set of antigens that distributed identically with the INCENPs across the cell cycle (9). Antibodies from both rabbit sera were affinity purified from the upper and lower INCENP bands on immunoblots. These affinity-purified antibodies recognized both bands on fresh immunoblots, and gave a pattern indistinguishable from whole serum by immunofluorescence of chicken cells (data not shown).

Comparison of the reactivities of mAb 3D3, ral-INCENP, and ra2-INCENP reveals at least three epitopes shared by the cloned and bona fide INCENPs. First, mAb 3D3 cross reacts in immunoblots with an 80-kD nuclear antigen that is not associated with chromosomes during mitosis (6). As neither rabbit antiserum recognizes this antigen in immunoblots, both rabbit antisera must recognize determinants distinct from that bound by antibody 3D3. Second, ra2-INCENP recognizes a human homologue of the INCENPs by indirect immunofluorescence and in immunoblots, where it binds to a polypeptide of 140-kD (Fig. 4). Ral-INCENP fails to recognize any human proteins by either method. Third, we have shown that antibody 3D3 and ral-INCENP, but not ra2-INCENP, fail to recognize the polypeptide encoded by $INCENP_I$-Δ511-696/pECE (see below).

To express the cloned chicken INCENPs in human (HeLa) and pig (LLCPK) cells, cDNA constructs encoding the entire class I and II open reading frames were assembled from RT-PCR clone PCR-1/BS, clone X24/BS, and either clone I-75/BS (class I) or II-73/BS (class II). These class I and II constructs were then cloned into the pECE expression vector, in which cloned genes are expressed under the control of the SV-40 early promoter. The constructs were termed $INCENP_I$/pECE and $INCENP_{II}$/pECE, and we will refer to

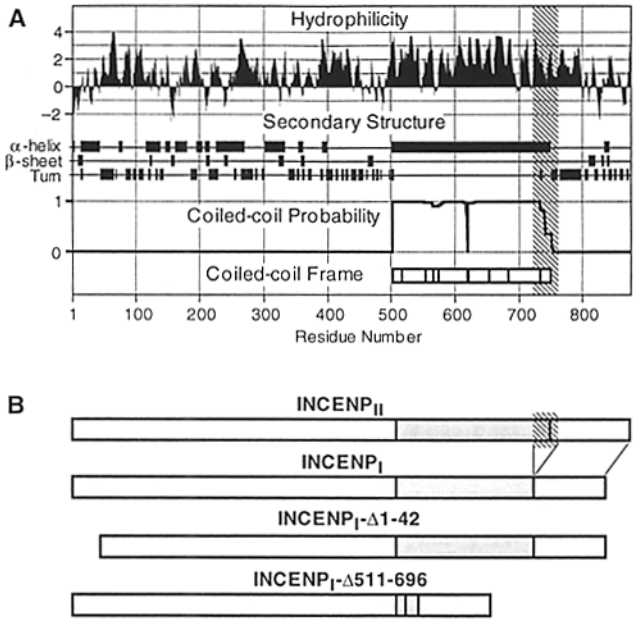


*Figure 3.* (*A*) Predicted structural organization of the class II chicken INCENP cDNA open reading frame. Kyle-Doolittle hydrophilicity plot (window = 7), Chou-Fasman secondary structure prediction, "coilcoil" (window = 21) (24) estimate of probability of coiled coil secondary structure for class II INCENP deduced open reading frame, and coilcoil prediction of the frames of the putative heptad repeats. Shaded area represents class II 114-bp insert. Hydrophilicity and secondary structure predictions were generated by MacVector software (IBI). (*B*) Schematic of polypeptides produced by full-length and deletion INCENP expression constructs in pECE. The region unique to class II is striped; the putative coiled-coil domain is shaded gray.

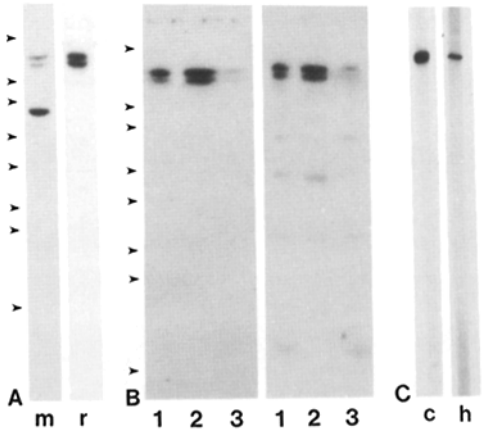


*Figure 4.* Antibodies to cloned INCENPs confirm that INCENPs are tightly associated with the chromosomal scaffold. Immunoblots of chromosomal proteins of MSB cells and HeLa cells were probed with antibody 3D3, ral-INCENP, and ra2-INCENP. (*A*) Mouse mAb 3D3 recognizes the INCENPs (133 and 145 kD) and nuclear protein 80 in whole cell lysates of chicken MSB cells (*m*), while ral-INCENP reacts only with the INCENPs (*r*). (*B*) Ral-INCENP (*left*) and ra2-INCENP (*right*) confirm that the INCENP doublet in isolated MSB chromosomes (lane *1*) remains in the chromosomal scaffold fraction (lane 2), and is not released by DNAse, and high salt treatments (lane *3*). (*C*) ra2-INCENP recognizes the INCENPs in MSB chicken cell chromosomal scaffold proteins (*c*), and a human homolog (140 kD) in chromosomal scaffold proteins from HeLa cells (*h*). Arrowheads denote molecular mass standards of 200, 116, 96, 68, 60, 43, 40, and 29 kD.

the expressed proteins as INCENP$_I$ and INCENP$_{II}$, respectively.

The molecular weights deduced from the sequences of INCENP$_I$ and INCENP$_{II}$ are 96,357 and 100,931 D, respectively. When expressed in mammalian cells, subjected to SDS-PAGE and immunoblotted, INCENP$_I$ was detected as a band with an apparent mobility of 125 kD, while INCENP$_{II}$ appeared to have a molecular mass of 140 kD. The INCENP$_{II}$ band migrated near the upper INCENP band of chicken cells, while the INCENP$_I$ band migrated close to the lower band (Fig. 5). The migration of the proteins encoded by the two INCENP cDNA is consistent with their identification as the bona fide, complete INCENPs. Post-translational modifications of both polypeptides apparently contribute to the slight differences between the INCENPs encoded by our cDNAs in mammalian cells and the two wild-type INCENPs. Taken together, these results strongly suggest that the cDNA clones described above are valid INCENP clones.

### *The Two INCENP Classes Appear to Arise by Alternative Splicing of a Single Transcript*

Northern (RNA) blotting analysis revealed a major poly(A)$^+$ INCENP mRNA of 4.5 kb in both normal and transformed chicken cells. A rapidly dividing ($t_d$ ~18 h) line of transformed lymphoblastoid cells (MSB-1) contained substantially higher amounts of this mRNA than did chicken embryo fibroblasts ($t_d$ ~30 h; data not shown). Surprisingly, two additional RNA species of 2.9 kb and 3.5 kb were detected when blots of MSB-1 poly(A)$^+$ RNA were probed with sequences contained within the coding region. Probes shared by both classes of message gave a distinct three-band pattern (Fig. 6 *A*), and a probe unique to the 114-bp Class II insert gave an identical result (data not shown). Although 2.9- and 3.5-kb mRNAs would be long enough to encode the 2.5 (class I)- and 2.6 (class II)-kb open reading frames of the INCENPs, these species were not observed when blots were probed with a 1.8-kb BglII–XhoI fragment immediately 3′ of the open reading frame of clone I-75/BS (Fig. 6 *A*). We conclude that INCENP mRNA processing may be complex. Chicken cells may contain poly(A)$^+$ INCENP RNA species other than those we have cloned and described here, and multiple forms of class I and class II messages may also exist. The existence of multiple splice variants has recently been shown for NuMa, another nuclear coiled-coil protein (34).

The INCENP mRNAs appear to be encoded by a single copy gene. This was seen when we probed Southern blots of chicken fibroblast genomic DNA with probes from the region unique to class II INCENPs and from the shared region immediately 5′ to it. In both cases, one or a few bands were present in each lane of restriction-digested genomic DNA (Fig. 6, *B* and *C*).

The most likely explanation of these results is that the two classes of INCENP mRNA are derived by alternative splicing of a primary transcript from a single gene.

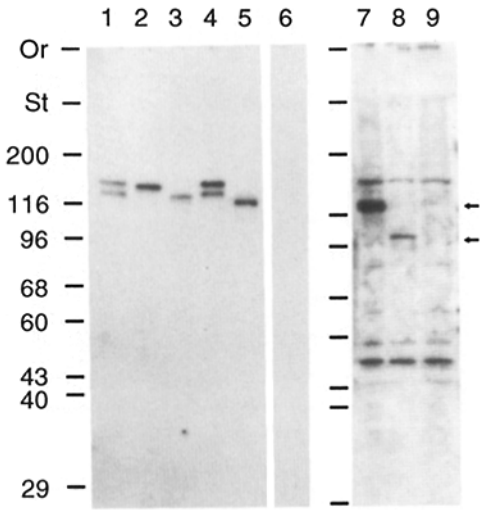


*Figure 5.* Recombinant INCENPs produced in transiently transfected mammalian cells migrate close to native chicken INCENPs from metaphase chromosomes in SDS-PAGE. Whole cell lysates of LLCPK cells were prepared 24 h after electroporation with INCENP expression constructs. Left panel (lanes 1–6): Immunoblot was probed with a mixture of ral-INCENP (1:1,000) and adsorbed ra2-INCENP (1:1,000). LLCPK cells were transfected with INCENP$_{II}$/pECE (lane *2*), INCENP$_{I}$/pECE (lane *3*), INCENP$_{I}$-Δ1-42/pECE (lane *5*), or pECE (lane *6*). Chromosomes from colcemid-blocked chicken cells (lanes *1* and *4*) served as controls. 18-h exposure. Right panel (lanes *7–9*): Immunoblot was probed with unadsorbed ra2-INCENP (1:500) to maximize detection of INCENP$_{I}$-Δ511–696. LLCPK cells were transfected with INCENP$_{I}$/pECE (lane *7*), INCENP$_{I}$-Δ511–696/pECE (lane *8*), or pECE (lane *9*). Arrows at 125 and 100 kD denote the positions INCENP$_{I}$, and INCENP$_{I}$-Δ511–696, respectively. Bands visible at 50, and 160 kD result from the recognition by ra2-INCENP of unidentified, non-INCENP cellular proteins. 36-h exposure. *Or*, origin; *St*, stacker.

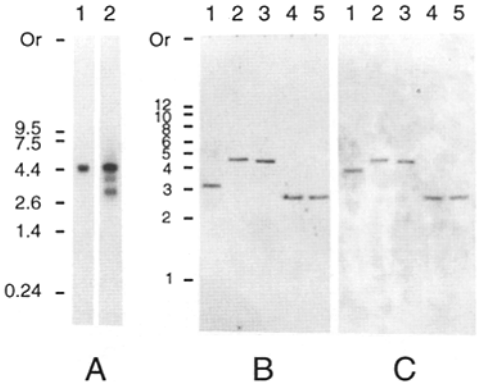


*Figure 6.* A single chromosomal locus gives rise to several INCENP mRNAs. (*A*) Northern blots of poly(A)$^+$ mRNA isolated from log-phase chicken MSB cells. Blots were probed with a 1.7-kb BstXI–XhoI fragment from the 3′ untranslated region of clone I-75 (lane *1*) or a 193-bp EcoRI–ScaI fragment (bases 2260–2452) common to the coding regions of both classes of cDNA (lane *2*). (*B* and *C*) Genomic Southern blot of DNA extracted from chick embryo fibroblasts and digested with XbaI (lane *1*), BglII (lane *2*), BstXI (lane *3*), AvaII (lane *4*), BstXI and AvaII (lane *5*). The blot was probed with a 193-bp EcoRI–ScaI fragment (bases 2260–2452) common to both classes of cDNA, and immediately 3′ to the 114-bp class II insert (*B*). After stripping, the blot was reprobed with a labeled 123-bp PCR product covering the adjacent region unique to class II (*C*). *Or*, origin.

### *Cloned Chicken INCENPs Localize Correctly in Transfected Mammalian Cells*

We examined the ability of class I and class II INCENPs to localize in mammalian cells when cells were transiently transfected with INCENP cDNA expression constructs. With each of the full-length and deletion constructs that we have expressed using the pECE vector, we could find many cells in which INCENP products accumulated to near physiological levels, as judged by indirect immunofluorescence. This is a significant advantage when examining localization of the protein to intracellular compartments.

Within 24 h of electroporation of the constructs encoding INCENP$_{I}$ or INCENP$_{II}$ into HeLa or LLCPK cells, 30 to 60% of the cells expressed recombinant INCENP antigen that was readily detectable by immunoblotting and by indirect immunofluorescence. We routinely detected the expressed proteins with ral-INCENP (Fig. 7) or mAb 3D3 (data not shown), since these antibodies do not recognize the endogenous INCENPs of mammalian cells. Thus, the transfected proteins could be detected selectively in the presence of a background of endogenous proteins without the necessity of attaching epitope tags.

As mammalian cells expressing chicken class I INCENPs traversed the mitotic cycle, the expressed proteins underwent the characteristic distribution pattern originally observed in chicken cells (Fig. 7). In interphase, INCENP$_{I}$ staining was typically confined to the nucleus. In mitosis, the expressed proteins were concentrated on the chromosomes from prophase through metaphase. The expressed proteins separated from the chromosomes by earliest anaphase, becoming localized to streaks in the spindle midzone. These streaks were parallel to the long axis of the spindle. The cell cortex in the neighborhood of the nascent cleavage furrow was also stained. As anaphase proceeded, the INCENP$_{I}$ polypeptide became progressively more concentrated at the spindle midzone. At the conclusion of telophase, the intercellular bridge stained intensely for INCENP$_{I}$ on each side of the midbody. In some cells with very high expression, additional staining could be seen in the reforming nucleus (data not shown). Expression of moderate levels of INCENP$_{I}$ polypeptide did not appear to have deleterious effects on transiently transfected LLCPK cells. Most mitotic figures were normal in appearance (Fig. 7), and daughter cells separated by chicken INCENP$_{I}$-containing intercellular bridges were commonly seen.

These results provide conclusive proof that the cDNA clones described above encode INCENP polypeptides that contain all the signals needed for proper INCENP$_{I}$ localization in the cell (Fig. 7). In addition, the results demonstrate that the cellular factors responsible for the various alterations in targeting of the INCENPs across the mitotic cycle are conserved between chicken and mammalian cells.

A small proportion of cells transfected with class I INCENP cDNA greatly overexpressed expressed the protein ("jackpot" cells). As expected, the nuclei of such cells stained intensely for chicken INCENP. Unexpectedly, however, significant levels of INCENP$_{I}$ staining were also observed in the cytoplasm as a bright, fibrillar network. This cytoplasmic network was completely coincident with that

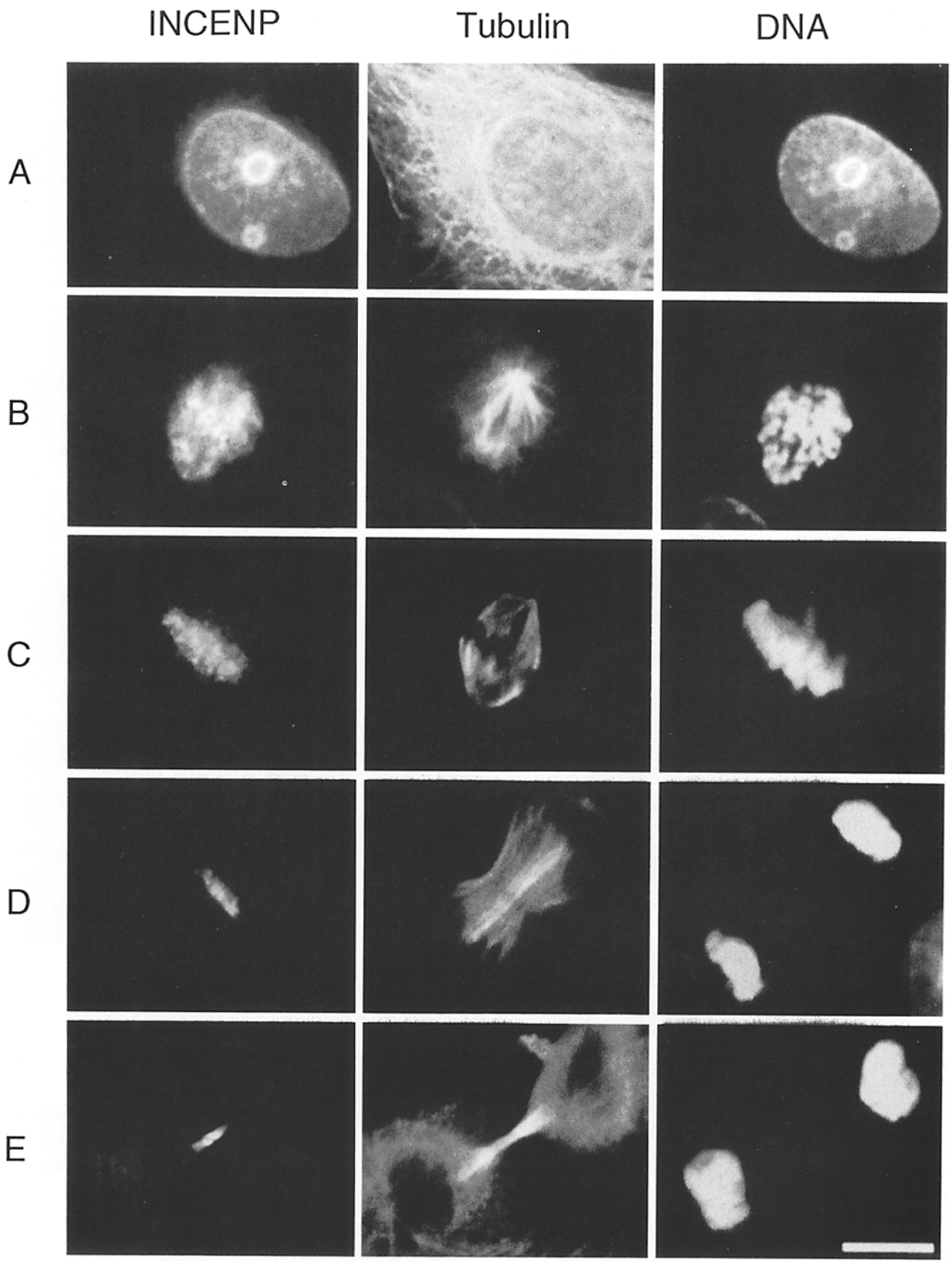


*Figure 7.* Class I chicken INCENP distribution in transiently transfected mitotic LLCPK cells mimics the INCENP distribution in chicken cells. Cells were fixed 24 h after electroporation with INCENP$_1$/pECE. Interphase (*A*), prophase (*B*), metaphase (*C*), anaphase (*D*), and late telophase/early G1 (*E*). Cells were stained with ral-INCENP (*left column*), mouse monoclonal anti-tubulins (*center column*), and DAPI (*right column*). Bar, 10 μm.

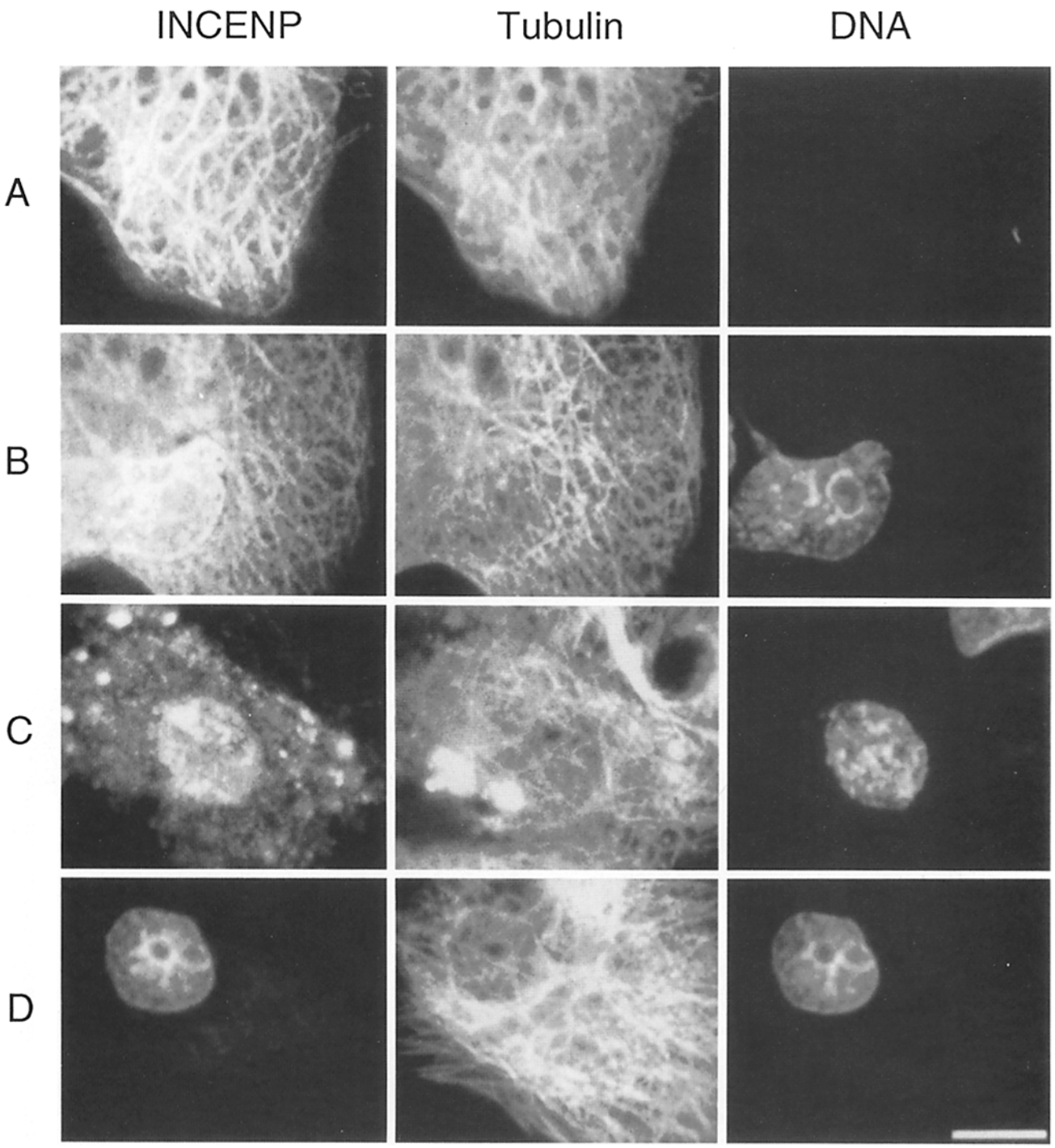


*Figure 8.* INCENP$_I$ and INCENP$_I$-Δ1–42 decorate cytoplasmic microtubules in interphase "jackpot" LLCPK cells, while INCENP$_I$-Δ511–696 does not. Cells were fixed 24 h after electroporation with INCENP$_I$/pECE (*D*), INCENP$_I$-Δ1-42/pECE (*B*), or INCENP$_I$-Δ511–696/pECE (*C*). Cells in *A–C* were "jackpot" cells, while the cell shown in *D* was expressing INCENP$_I$ at a lower level. Cells were stained with ral-INCENP (*left column*, *A*, *B*, *D*) or adsorbed ra2-INCENP (*left column*, *C*), mouse monoclonal anti-tubulins (*center column*), and DAPI (*right column*). Bar, 10 μm.

visualized by staining with antibodies to tubulin (Fig. 8 *A*). The INCENP-decorated fibrils did not colocalize with either intermediate filaments (visualized with antibodies to keratins and vimentin), or with actin (visualized with phalloidin staining—data not shown). We do not yet know if the INCENP$_I$ polypeptide binds directly to microtubules, or interacts indirectly via microtubule-associated proteins.

The appearance of INCENP$_I$ in the cytoplasm seemed to alter the structure of cytoplasmic microtubules. The microtubules appeared to be bundled into coarser fibrils than those seen in control cells. While many microtubules in control cells ran in more or less straight lines from near the nucleus to the periphery of the cell, those in "jackpot" cells were usually curly. Rather than terminating at the periphery of the cell, fibrils seemed to curve back into the cell (Fig. 8 *A*). In addition, cytoplasmic INCENPs appeared to enhance mi-

crotubule stability. Even when transfected cells were fixed under conditions that were not favorable for microtubule preservation, the microtubule network of "jackpot" cells could be visualized. No "jackpot" cells were ever observed in any stage of mitosis.

### *Identification of an Amino Acid Motif Required for Targeting of the INCENPs to the Anaphase Spindle and Midbody*

The deduced amino acid sequence of the INCENPs suggested an obvious multidomain organization for these proteins, but gave no clues as to the biological function of any given domain. We therefore began a functional dissection of the INCENP polypeptide by creating chimeric forms of the protein to determine how these localized during the cell cycle. The properties of two of these chimeric INCENP polypeptides will be described here.

The first of these constructs identified a region of the INCENPs that is required for the transfer of class I INCENPs from the chromosomes to the spindle at the metaphase–anaphase transition. This region includes the amino-terminal 42 amino acids of class I INCENPs. These residues were deleted in the amino-terminal deletion mutant $INCENP_I$-Δ1-42/pECE. The resulting truncated INCENP was detected in transiently transfected mammalian cells. We will refer to this chimeric protein as $INCENP_I$-Δ1-42.

While $INCENP_I$-Δ1-42 localized normally to interphase cells and in mitosis through metaphase, a dramatic difference in localization occurred at the metaphase–anaphase transition. At this time, $INCENP_I$-Δ1-42 completely failed to transfer to the spindle midzone. Instead, it remained attached to the chromosomes as they moved towards the poles in anaphase (Fig. 9 *D*).

Interestingly, the inability of the $INCENP_I$-Δ1-42 polypeptide to transfer from the chromosomes to the spindle does not appear to reflect an inability of the protein to bind to and bundle microtubules. As with full length $INCENP_I$, cytoplasmic $INCENP_I$-Δ1-42 polypeptide apparently attaches to microtubules and bundles them in "jackpot" cells (Fig. 8 *B*).

### *The INCENP Coiled-Coil Domain Is Apparently Required for Microtubule Colocalization and Bundling*

Perhaps the most obvious feature of the deduced INCENP polypeptide sequence is the predicted α-helical coiled coil that spans residues 503–717 of the class I polypeptide. In addition to serving as rod-like "spacers," such coiled-coil domains are essential for the oligomerization of many structural proteins, including intermediate filament proteins (30) and myosin (14). We would thus predict that disruption of the coiled coil might have a profound effect on the INCENP structure and function.

To investigate the role of the predicted coiled-coil domain in INCENP function, we generated a construct in which most of this domain was deleted, reducing the predicted coiled-coil from 215- to 29-amino acid residues. We refer to this expressed protein as $INCENP_I$-Δ511–696 (Fig. 3 *B*). $INCENP_I$-Δ511–696 was not recognized by antibodies 3D3 or ral-INCENP. It was, however, recognized by ra2-INCENP which had been pre-adsorbed against fixed LLCPK cells to remove its weak cross-reactivity to porcine INCENP homologues.

Most surprisingly, removal of the coiled-coil domain had no detectable effect on the ability of $INCENP_I$-Δ511–696 to undergo the normal pattern of INCENP rearrangements as transfected cells traversed the mitotic cycle. Thus, the protein entered the nucleus normally during interphase, was associated with the chromosomes through metaphase, and transferred to the central spindle, cleavage furrow and stem body during anaphase and telophase (data not shown).

Since $INCENP_I$-Δ511–696 apparently assembled and targeted correctly throughout mitosis, the behavior of this protein in "jackpot" cells was surprising. As was the case for all INCENP constructs, when grossly overexpressed, a substantial portion of $INCENP_I$-Δ511–696 was observed to accumulate in the cytoplasm. Unlike all other constructs examined to date, however, this altered protein did not associate with the cytoplasmic microtubules, which remained unbundled (Fig. 8*C*). However, some "jackpot" cells displayed disordered microtubule networks, and were rounded instead of flattened on the growth substrate (data not shown). It appears that the coiled-coil domain of $INCENP_I$ is required for this protein to associate with and bundle cytoplasmic microtubules.

## *Discussion*

### *Cloning of Two Classes of INCENP cDNAs*

The INCENPs were first identified with a mAb elicited by immunization with chromosome scaffolds. The antibody recognized two polypeptide antigens (135 and 150 kD) that are chromosomal through metaphase, and associated with the cytoskeleton during the final stages of mitosis (6). The deduced amino acid sequences obtained from two classes of cDNA clones that span the entire open reading frames of the two INCENPs yield molecular masses of 96,357 D for class I INCENP (referred to here as $INCENP_I$) and 100,931 D for class II INCENP ($INCENP_{II}$). This size difference arises from the insertion of an additional 38 residues in the $INCENP_{II}$ polypeptide, apparently as the result of alternative splicing of a single primary transcript. The deduced INCENP polypeptides are very basic (pI ~9.5) polypeptides that contain long predicted coiled-coil domains. They do not share significant sequence relationships with other known proteins.

The cDNA clones described here seem to cover the entire INCENP coding region. All 5′ cDNA clones contain an in-frame stop codon just upstream from the presumptive initiation codon. In addition, when the cloned cDNAs were transiently expressed in mammalian cells, they produced proteins that migrated close to the endogenous forms of these proteins in SDS polyacrylamide gels.

### *The INCENPs and Their Intracellular Ligands Are Functionally Conserved between Birds and Mammals*

The characteristic pattern of movements undertaken by the INCENPs during the mitotic cycle suggests that these proteins interact specifically with at least four different cellular ligands. These include: (*a*) the nuclear import machinery (7, 15, 27); (*b*) chromatin, particularly in the region of the centromere; (*c*) the anti-parallel microtubules of the spindle overlap zone; and (*d*) the inner surface of the plasma membrane at the cleavage furrow. In addition, examination of

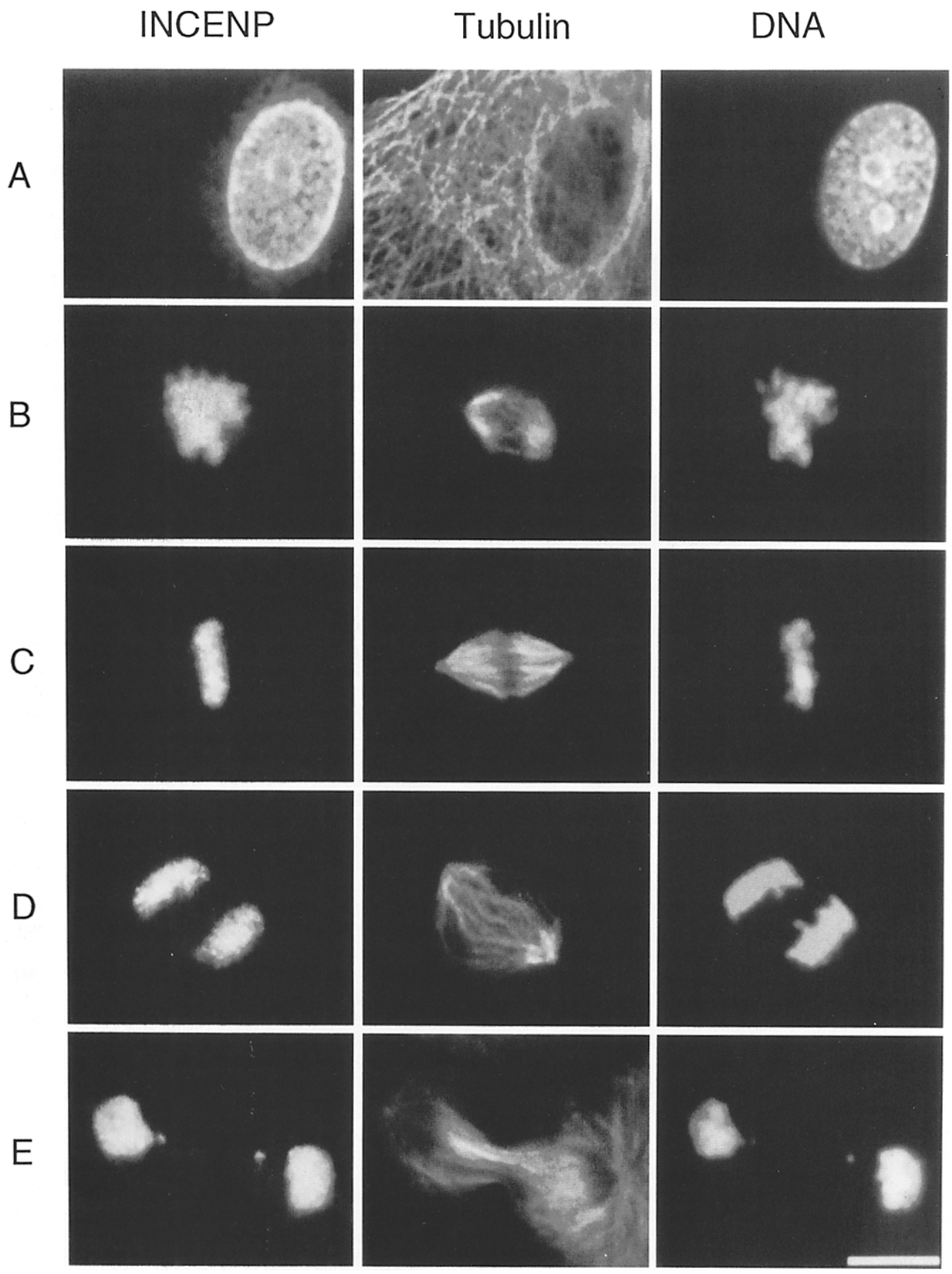


*Figure 9.* The first 42 amino acids of INCENP$_I$ are required for INCENP$_I$ transfer from chromosomes to the mitotic spindle. LLCPK cells were electroporated with INCENP$_I$-Δ1–42/pECE, fixed after 24 h, and stained with ral-INCENP (*left column*), mouse monoclonal anti-tubulins (*center column*), and DAPI (*right column*). Interphase (*A*), prophase (*B*), metaphase (*C*), anaphase (*D*), and telophase (*E*). Bar, 10 μm.

cells that grossly overexpress the INCENP polypeptide in transient transfection experiments ("jackpot" cells) reveals that the INCENPs are capable of associating with and bundling parallel microtubules.

The present studies indicate that the intracellular ligands with which the INCENPs interact and the regulatory signals that govern these interactions during mitosis are functionally conserved between birds and mammals. When chicken INCENP cDNAs were expressed in human (HeLa) and pig (LLCPK) cells, both $INCENP_I$ and $INCENP_{II}$ polypeptides showed a normal pattern of distribution across the cell cycle. In addition, antibody ra2-INCENP clearly recognizes proteins homologous to the chicken INCENPs in human and Indian muntjac cells. Antibody ra2-INCENP reactivity against porcine homologs in LLCPK cells is weaker, but still detectable.

At present, nothing is known about how INCENP affinities for their ligands change across the mitotic cycle.

### *The INCENPs Are Cytoskeletal Proteins That Can Alter Microtubule Morphology*

Throughout most of the cell cycle, the INCENPs are located in the nucleus and on the chromosomes. In fact, when chromosomes are isolated from colcemid-blocked cells, the INCENPs are among the most tightly associated proteins known, being nearly quantitatively retained in the chromosome scaffold fraction.

The data obtained here demonstrate that the INCENPs may act as cytoskeletal proteins that are potentially capable of altering the morphology of the cellular microtubule network during interphase. We have shown here that when the INCENPs are sufficiently overexpressed, as in jackpot cells, the proteins appear to exceed the ability of the nucleus to sequester them, and accumulate in the cytoplasm. There, the cytoplasmic INCENPs associate with the microtubules and cause them to assemble into bundles, which curl around and terminate in the cellular interior rather than at the edge of the cell.

The apparent exclusion of INCENPs and INCENP homologs from the cytoplasm of wild-type, untransfected cells complicates the interpretation of jackpot cells. The association of $INCENP_I$ with microtubules could result from relatively unspecific, low-affinity interactions that are not reflective of any physiological role of the INCENPs. However, a number of lines of evidence suggest that $INCENP_I$-microtubule interactions may be relevant to INCENP function. First, INCENPs appear to be closely associated with microtubules during the later stages of mitosis (8). Second, while few proteins that have been overexpressed by transient transfection of mammalian cells have been demonstrated to associate with fibrillar cytoplasmic networks, binding to and bundling of microtubules has been shown for the microtubule-associated proteins tau (19) and MAP2C (35). Finally, the failure of $INCENP_I$ to bind to nonfilamentous cytoplasmic structures, microfilaments, or intermediate filaments of jackpot cells suggests that the affinity of $INCENP_I$ for microtubules must be significantly higher than its affinity for other cytoplasmic components.

One role of the INCENPs may be to promote bundling of microtubules in the midzone of the mitotic spindle during anaphase. It appears that the inappropriate expression of this activity during interphase is deleterious to the cell. We have never observed any cell displaying gross overexpression of the INCENPs in any phase of mitosis. This observation is consistent with the notion that sequestration of mitosis-specific cytoskeletal proteins in the nucleus during interphase may be an important functional requirement.

### *Identification of a Function Domain Required for INCENP Localization to the Spindle Midzone*

Deletion of the amino-terminal 42 amino acid residues in $INCENP_I$-Δ1–42 prevented the polypeptide from transferring from chromosomes to the metaphase spindle at the metaphase:anaphase transition in mammalian cells (Fig. 9 *D*). This is the first identification of a peptide motif required for localization of a protein to the spindle midzone and midbody during anaphase.

This result could be explained in two ways. First, residues 1–42 could constitute all or part of an autonomous targeting sequence that directs the localization of the INCENPs to the spindle midzone. Observation of this region by thin-section EM has previously revealed the presence of an amorphous electron-dense substance that apparently coats the antiparallel microtubules where they are in the most intimate contact during anaphase and telophase (5, 25). This "stem body" material later attains prominence as the midbody in the intercellular bridge during cytokinesis. The composition of this material is presently unknown, but might include the INCENPs. Thus, residues 1–42 of the INCENPs could bind to a structural component of the stem body material. It is also possible that the INCENPs bind directly to the adjacent anti-parallel microtubules of the midzone. No significant similarities were identified between the amino-terminal 80 residues of the INCENPs and the sequences of two other polypeptides that bind to the central spindle during anaphase, CENP-E (37) and MKLP-1 (28). Experiments are presently underway to determine whether INCENP residues 1–42 can direct the targeting of an exogenous polypeptide to the spindle midzone and stem body.

Alternatively, transfer of the INCENPs to the spindle in late metaphase might be governed by their release from the chromosomes, rather than by binding to the spindle. Residues 1–42 could comprise a release "switch" that is activated by some stage-specific modification such as proteolysis, phosphorylation, or dephosphorylation. It appears unlikely that this region of the INCENP polypeptide is a substrate for phosphorylation, as it lacks serine residues, and the sole threonine is not in a consensus recognition motif for any described kinase. However, one of the three INCENP consensus sites for phosphorylation by $p34^{cdc2}$ is nearby, at residue 60.

### *Most of the Coiled-Coil Domain Is Dispensable for Localization of the INCENPs during Mitosis*

Computer analysis of the deduced INCENP polypeptide sequence predicts the existence of an internal coiled-coil domain (residues 503 to 717 in class I, residues 503 to 733 in class II). Studies of proteins such as myosin have shown that such coiled-coil domains can be crucial for dimerization and higher-order assembly of structural proteins (14). Thus, the INCENPs are likely to form homodimers or heterodimers in vivo.

Given this prediction, together with the importance of coiled-coil interactions for intermediate filament structure

and organization (12) we anticipated that truncation of the coiled-coil domain should severely impair INCENP function. We were thus surprised to note that the distribution of INCENP$_I$-Δ511–696 was apparently normal across the mitotic cycle. In this polypeptide, the coiled-coil has been reduced from 215 amino acid residues to 29.

This unexpected result could be explained if the remaining vestige of the coiled coil domain were sufficient to permit INCENP$_I$-Δ511–696 to oligomerize with its normal binding partners. Alternatively, the coiled-coil might not be essential for the proper placement of INCENPs in mitotic cells. Thus, the subcellular localization of the INCENPs during the mitotic cycle could be primarily dictated by interactions of the amino and carboxy-terminal domains with ligands in the chromosome and spindle. If this is the case, then our results demonstrate that the spacing between these domains is not critical.

### *The Ability to Associate with Microtubules Is Insufficient to Target the INCENPs to the Mitotic Spindle*

Comparison of the behavior of the mutant INCENP lacking the amino terminus with the one lacking the coiled-coil domain suggested an unexpected level of complexity in the associations of the INCENPs with the spindle midzone. On the one hand, the mutant that could not asssociate with the spindle midzone (INCENP$_I$-Δ1-42) retained its ability to associate with and bundle cytoplasmic microtubules in jackpot cells. On the other hand, the mutant polypeptide lacking the coiled coil (INCENP$_I$-Δ511–696) remained able to associate with the spindle and midbody throughout anaphase and telophase, but failed to associate with cytoplasmic microtubules in interphase jackpot cells. To our knowledge, this is the first instance where a putative coiled-coil domain has been implicated in association of a protein with microtubules.

These seemingly paradoxical observations could be reconciled if the coiled-coil regions of the INCENPs were required for interactions with parallel microtubules, while the amino-terminal 42 amino acids were required only for association with anti-parallel microtubules in the central spindle. Alternatively, INCENP localization in the central spindle might be due to interactions with spindle components other than microtubules, such as components of the stem body material. It will be important in future experiments to examine the binding of purified INCENPs to microtubules and isolated mitotic spindles in vitro.

### *Conclusions*

The functional analysis of the INCENPs presented here provides support for the notion that interphase cells regulate the activity of cytoskeletal proteins that play an important role during the latter stages of mitosis. For example, the INCENPs are sequestered in the nucleus so that they cannot make contact with the interphase cytoskeleton. Thus, when chromosomes move to the metaphase plate during mitosis, they apparently bring with them not only the DNA in a form suitable for transmission to the daughter cells, but in addition, a class of cytoskeletal "passenger" proteins whose role is to participate in the construction of the mitotic spindle and cleavage apparatus. Further analysis of proteins such as the INCENPs should reveal the role this chromosomal:cytoskeletal "collaboration" plays in shaping events during the closing phases of mitosis.

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