Human CENP-A Contains a Histone H3 Related Histone Fold Domain That Is Required for Targeting to the Centromere

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Abstract. Centromeres are the differentiated chromosomal domains that specify the mitotic behavior of chromosomes. To examine the molecular basis for the specification of centromeric chromatin, we have cloned a human cDNA that encodes the 17-kD histone-like centromere antigen, CENP-A. Two domains are evident in the 140 aa CENP-A polypeptide: a unique NH_2-terminal domain and a 93-amino acid COOH-terminal domain that shares 62% identity with nucleosomal core protein, histone H3. An epitope tagged derivative of CENP-A was faithfully targeted to centromeres when expressed in a variety of animal cells and this targeting activity was shown to reside in the histone-like COOH-terminal domain of CENP-A. These data clearly indicate that the assembly of centromeres is driven, at least in part, by the incorporation of a novel core histone into centromeric chromatin.

CENTROMERES are the chromosomal loci that specify the segregation behavior of chromosomes during mitosis and meiosis (Schulman and Bloom, 1991). In mammals, centromeres are visible as the primary constriction of mitotic chromosomes and contain densely packed heterochromatic satellite DNA. The centromere appears to direct the assembly of the kinetochore, a differentiated trilaminar plaque present at the surface of the centromere that interacts directly with microtubules of the mitotic spindle apparatus (Brinkley and Stubblefield, 1966; Ris and Witt, 1981). Analysis of centromeres of yeast chromosomes reveal that centromere function is specified by *cis*-acting DNA sequences that interact with sequence-specific DNA binding proteins to assemble a microtubule-dependent motor complex on the chromosome (Middleton and Carbon, 1994; Hyman et al., 1992; Lechner and Carbon, 1991; Clarke and Carbon, 1980). In addition to acting as a site for microtubule binding and force generation, the centromere mediates attachment of the sister chromatids throughout mitosis until their separation at the metaphase–anaphase transition.

The functions of the centromere are associated with a chromatin structure that is differentiated from that of the chromosome arms. In animal cells, this is observed as the densely packed constitutive heterochromatin of the primary constriction. In budding yeast it has been possible to examine centromeric chromatin at the molecular level, where the ca 125-bp centromere DNA sequences are folded within a 150–200-bp nuclease-resistant "particle" flanked on either side by precisely phased nucleosomes (Bloom and Carbon, 1992; Funk et al., 1989). Experiments using transcription to disrupt the unique chromatin configuration of the centromere domain have shown that this structure is essential for proper segregation of chromosomes during mitosis (Hill and Bloom, 1989). Similarly, in the fission yeast *Schizosaccharomyces pombe*, the essential central core DNA sequences of the centromere are packaged in a region with a distinctive nonuniform and highly varied nucleosome distribution, as assayed by nuclease digestion techniques (Polizzi and Clarke, 1991). This modified chromatin is only observed in the context of functional centromeres: the same sequences are assembled into normal chromatin when present on a minichromosome in *Saccharomyces cerevisiae*. These data indicate that altered chromatin structure is intimately related to the function of centromeres.

The discovery of centromere-specific autoantibodies in patients with limited systemic sclerosis (CREST syndrome) and characterization of the antigens recognized by these sera have provided tools for analysis of centromere structure and function in mammalian cells (Bernat et al., 1990; Simerly et al., 1990; Earnshaw and Rothfield, 1985; Brenner et al., 1981; Moroi et al., 1980). Three major antigens, centromere proteins (CENP)[1]-A, -B, and -C have been identified using these sera. CENP-C is a 140-kD polypeptide that is a component of the inner plate of the kinetochore and thus lies at the interface between the chromosome and the kinetochore (Saitoh et al., 1992). While the function of CENP-C is not clear, antibody microinjection experiments have shown that it is required for normal kinetochore assembly (Tomkiel et al., 1994). CENP-B was the first centromere protein to be cloned (Earnshaw et al., 1987) and has been shown to be a sequence-specific DNA binding protein that is localized to

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1. *Abbreviations used in this paper*: CENP, centromere protein; RT-PCR, reverse transcript PCR; SF, serum-free; TX, Triton X-100.

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the heterochromatic interior of the centromere, underlying the kinetochore (Masumoto et al., 1989; Cooke et al., 1990). CENP-B binds to a subset of the alphoid satellite DNA that comprises the major DNA component of human centromeres and may play a role in the higher order folding of alphoid chromatin through self-assembly (Yoda et al., 1992).

The biochemical studies of Palmer and colleagues point to a more direct role for CENP-A in establishing a differentiated chromatin structure at the centromere (Palmer and Margolis, 1985; Palmer et al., 1987, 1991). These workers demonstrated that CENP-A is present on nucleosome-like structures following micrococcal nuclease digestion of nuclei and that it co-purifies with nucleosome core particles under stringent isolation conditions (Palmer et al., 1987; Palmer and Margolis, 1985). Direct purification of CENP-A from bovine sperm nuclei led to partial amino acid sequence analysis which showed that CENP-A shared homology with the core nucleosomal protein histone H3 (Palmer et al., 1991). On the basis of these biochemical properties and amino acid sequence, these authors proposed that CENP-A is a centromere-specific histone.

In this work, we isolated a partial cDNA for bovine CENP-A using reverse transcript PCR (RT-PCR) and have used this as a probe to isolate a full-length cDNA for human CENP-A. Sequence analysis reveals that CENP-A is indeed a divergent histone H3-related protein. It possesses two domains defined by homology with histone H3: a divergent NH_2-terminal domain and a homologous COOH-terminal domain that shares 60% amino acid identity with histone H3. We constructed an epitope-tagged derivative of human CENP-A and showed that it is faithfully targeted to centromeres when expressed in mammalian cells. This expression system was used to examine centromere-targeting signals within CENP-A. Surprisingly, the histone-like domain specified the centromeric localization of CENP-A rather than its unique NH_2-terminus. These experiments clearly establish that the centromere is differentiated from the chromosome arms at the most fundamental level of chromatin structure and, further, that key molecular recognition events required for centromere assembly occur at the level of the nucleosome.

Materials and Methods

Cell Culture

HeLa cells were maintained in DME supplemented with 10% fetal bovine serum. Indian muntjac fibroblasts (CCL-157; American Type Culture Collection, Rockville, MD) were grown in Ham's F12 medium with 10% fetal bovine serum. Both cell lines were grown at 37°C in a 5% CO_2 atmosphere. For immunofluorescence cells were grown directly on sterile acid-washed 12-mm diam cover slips in a 24-well dish. Materials for cell culture were obtained from GIBCO BRL (Gaithersburg, MD).

Cloning

Routine nucleic acid techniques were performed essentially as described in Ausubel et al. (1990). For isolation of bovine CENP-A, cDNA degenerate oligonucleotides were synthesized based on reverse translation of bovine CENP-A peptide sequences reported by Palmer et al. (1991). The 5' oligonucleotide, GGAATTCCCARAARACNACNCA, was derived from peptide C1 corresponding to amino acid sequence QKTTH, and the 3' oligonucleotide, TTYCCNAARGAYGTNCAGAATTCC (R = A or G, Y = C or T, N = A, G, C, or T), derived from the sequence FPKDVQ of peptide C10/D5/C5. Total RNA from MDBK cells (a generous gift of Dr. Ed Chan, The Scripps Research Institute) was reverse transcribed with random primers using a commercial cDNA synthesis kit (Red Module, Invitrogen, San Diego, CA). PCR reactions contained cDNA from 1 μg of RNA and oligonucleotide primers at a concentration of ~0.5 μM times the degeneracy of each primer (64-fold for C1 primer and 128-fold for C10/D5/C5 primer) in a total volume of 100 μl with 2.5 U of Taq polymerase (Promega Biotec, Madison, WI). Samples were amplified in a thermal cycler (Ericomp, San Diego, CA) according to the following program: 2 min at 99°C (2 min × 99°C) for initial denaturation; two cycles with 30 s × 95°C denaturation, 60 s × 37°C annealing, 90 s × 72°C extension; 28 cycles with 30 s × 95°C, 60 s × 55°C, 90 s × 72°C; 10 min at 72°C. Products were analyzed by electrophoresis on 2% agarose-TEA and the predominant product at the expected size was isolated, purified using Qiaex resin (Qiagen, Chatsworth, CA), digested with EcoRI (New England Biolabs, Beverly, MA) and cloned into pBluescript (Stratagene Corp., La Jolla, CA). Sequence analysis of the bovine cDNA clone (bCNPA) was performed by the dideoxy method using Sequenase (United States Biochemical Corp., Cleveland, OH) with ^{35}S-labeled thio-dATP from Amersham Corp. (Arlington Heights, IL). DNA sequences were analyzed using the GCG software suite (Devereaux et al., 1984).

bCNPA was labeled with α-[^{32}P]dCTP or dATP (Amersham Corp.) using the random primer method (Feinberg and Vogelstein, 1983) and used to screen a human endothelial cell cDNA library constructed in λ gt11 (a gift of Dr. Evan Sadler, Washington University, St. Louis, MO) resulting in the isolation of three cDNA clones. One of these was used to screen a human T-lymphoblastoma (MOLT-4) cDNA library in λ gt11 (a gift of Dr. Ed Chan), resulting in the isolation of the full length clone, cDNA 211, described here. cDNA 211 was subcloned into the EcoRI site of pBluescript and DNA sequence analysis was performed as described above.

Expression Constructs

For expression, isolated cDNA 211 was made flush by treatment with Klenow fragment and ligated with pcDL SRα-296 (Takabe et al., 1987) that had been digested with XhoI and filled with Klenow. To construct an epitope-tagged derivative of CENP-A, a bottom strand primer was prepared that contained the sequence of 10 codons encoding the influenza hemagglutinin epitope HA1 (Niman et al., 1983) following the terminal Gly_{140} codon of CENP-A (5' CACTGGGTGCAGGAGCTCGTTAAGTCAGCTAGCGTAGTCCGGCACGTCGTACGGGTACCCGAGTCCCTCCTCAAGGC). A top strand primer, 5' GGCCCCTCCTTAGG, was also prepared and the two primers were used to amplify a fragment using cDNA 211 as a template. The PCR product was digested with SfiI and SacI and subcloned into cDNA 211, replacing the wild type SfiI-SacI segment of the cDNA, to form plasmid pCA-HA1. The EcoRI insert of pCA-HA1 was subcloned into pcDL SRα 296 as described above for the wild-type cDNA to generate plasmid pcDL CA-HA1. An epitope-tagged version of mouse histone H3.2 was constructed in an analogous manner in plasmid pMH3.2, a cloned mouse histone H3 gene kindly provided by Dr. William Marzluff (University of North Carolina, Chapel Hill, NC) (Taylor et al., 1986). To construct pcDL H3-HA1, the histone H3-HA1 coding region was excised by digestion with NcoI, which cut at the initiator ATG codon, and KpnI, the latter site comprising part of the epitope sequence. A second fragment was prepared from cDNA 211 by PCR, spanning the 5'-untranslated region of the cDNA and introducing a NcoI site at the ATG initiator codon compatible with the NcoI site of MH3.2. This fragment was digested with SacII and NcoI. A vector was prepared from pcDL CA-HA1 by digestion with SacII and KpnI and the three fragments were ligated together to form pcDL H3HA1. Chimeric construct pcDL CA/H3-HA1 was constructed using a combination of PCR and restriction fragments to join CENP-A residues 1-51 with histone H3 residues 52-135, using a BstYI site in histone H3. Similarly, pcDL H3/CA-HA1 joined histone H3 residues 1-52 with CENP-A residues 53-140, using a HindIII site in CENP-A. All of these constructs maintained the 5'- and 3'-untranslated regions of human CENP-A. A CENP-B expression plasmid was prepared by cloning the 1.95-kb human genomic SmaI fragment of CENP-B into the XhoI site of pcDL-SRα296 after treatment with T7 DNA polymerase to fill in the ends.

Transfection

For analysis by immunofluorescence 2–5 × 10^4 cells were plated onto 12-mm coverslips in 24-well dishes the night prior to transfection. DNA was introduced using cationic-lipid mediated transfection (Felgner et al., 1987) with Lipofectamine reagent (GIBCO BRL). DNA (250 ng) was diluted in 25 μl of serum-free DME (DME-SF) and combined with 25 μl of DME-SF

containing 2.5 μl (5 μg) of Lipofectamine. DNA–lipid complexes were allowed to form at room temperature for 30–60 min and then diluted with 200 μl of DME-SF. Cells were washed once with DME-SF and DNA–lipid complexes were applied in a total volume of 250 μl. Plates were returned to the incubator for 5–6 h and then supplemented with 500 μl of complete DME. The following morning the medium was replaced with fresh DME and cover slips were processed 24 h later. For analysis of transfected cells by immunoblot, transfections were performed in 10-cm dishes using 5 μg of DNA and 100 μg of Lipofectamine in 5 ml of DME-SF. Cells were harvested 48 h after application of DNA by lysis in 250 μl of SDS-PAGE sample buffer.

Immunofluorescence Microscopy

Transfected cells on 12-mm cover slips were washed in 37°C PBS and fixed for 10 min with 4% formaldehyde in PBS prepared from paraformaldehyde. Cover slips were washed once in PBS and twice in PBS with 0.1% Triton-X 100 (PBS-TX) for 3 min and then blocked with 1% BSA (Boehringer-Mannheim Biochemicals, Indianapolis, IN) in PBS-TX for 15 min, all at room temperature. Primary antibodies were diluted in PBS-TX/1%BSA. Antibodies used were: monoclonal antibody 12CA5 against the HA-1 epitope (a gift of Dr. Ian Wilson, The Scripps Research Institute, La Jolla, CA) at a concentration of 10 μg/ml, monoclonal antibody mACA-1 against human CENP-B (Earnshaw et al., 1987) at a concentration of 20 μg/ml and human CREST autoantiserum hACA-M (Sullivan and Glass, 1991) at a dilution of 1:2,000. In control experiments, specificity of antibodies was verified individually; for analysis of transfected cells primary antibodies were combined in a single incubation for 30 min at 37°C. Following incubation, primary antibody solution was removed and cover slips were washed four times with PBS-TX for 4-min each. Secondary antibodies (Southern Biotechnologies, Inc., Birmingham, AL) were fluorescein-conjugated anti-mouse IgG-2b at 1:25 dilution to detect antibody 12CA5 and either rhodamine-conjugated sheep anti-human IgG at 1:200 dilution (for autoantiserum hACA-M) or anti-mouse IgG-1 (for mACA-1). Secondary antibodies were diluted in PBS-TX/1% BSA and incubated at 37°C for 30 min. Cover slips were then washed once in PBS-TX and three times with PBS for 4-min each and then rinsed briefly in distilled water and air dried. Slips were then mounted on slides with an anti-fade mounting medium (Molecular Probes, Eugene, OR) and sealed with a solution of 50% clear nail polish in acetone. Microscopy was performed using a MRC-600 confocal laser scanning instrument (Bio Rad Laboratories, Cambridge, MA) fitted to a Zeiss Axiovert epifluorescence microscope using the 63× (planapo) objective. Images were collected from a single focal plane (ca. 0.4 μm) using 30 scans averaged by the Kalman method with the Bio Rad COMOS software package, using the minimal slit opening and maximum photomultiplier gain settings for the laser. Differential phase contrast images were collected after fluorescence images. Images were recorded onto Ektar ASA 25 film using a film recorder. For standard epifluorescence microscopy, a Zeiss Axiophot microscope with 100× planapo objective lens was used and images were recorded using Ektachrome ASA 400 or T-MAX ASA 400 film.

Immunoblots

Proteins were resolved by electrophoresis on 15% SDS-PAGE gels (Laemmli, 1970) and transferred to nitrocellulose (BA83; Schleicher & Schuell, Keene, NH) in an electroblotting apparatus (Bio Rad Laboratories) at 70 V for 2.5 h. Filters were blocked in 10 mM Tris-HCl, pH 8, 150 mM NaCl, 0.1% Tween-20 (TBST) with 5% nonfat dried milk for 1 h at room temperature or overnight at 4°C. Filters were washed once in TBST and then incubated with primary antibodies diluted in 1% BSA in TBST for 1 h at room temperature. Antibodies were 12CA5 used at 1 μg/ml or hACA-M used at 1:5,000. Filters were washed five times for 5-min each in TBST and then incubated with horseradish peroxidase–coupled secondary antibodies at a dilution of 1:5,000–1:20,000 in 1% BSA-TBST for 1 h at room temperature. Filters were washed five times in TBST as for primary antibody incubations and processed for chemiluminescent detection using an ECL kit (Amersham Corp.) according to the manufacturer's instructions.

Results

Isolation of cDNA Clones Encoding CENP-A

Degenerate oligonucleotide primers for amplification of

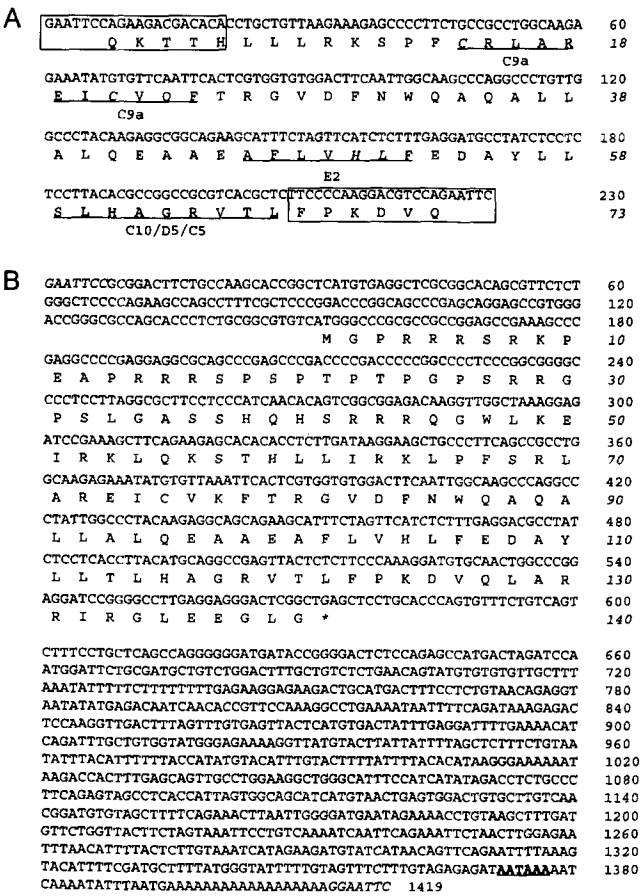

Figure 1. Nucleotide and encoded amino acid sequence of mammalian CENP-A clones. (*A*) The sequence of the bovine CENP-A RT-PCR product is shown with the corresponding amino acid sequence below. Primer derived sequences at the 5′ and 3′ ends of the cDNA fragment are boxed. Amino acid sequences corresponding to peptide sequences of Palmer et al. (1991) are underlined, with the identity of each peptide indicated below. (*B*) Sequence of human CENP-A cDNA 211. cDNA 211 was the longest of eight human cDNAs isolated by screening libraries from MOLT4 and human endothelial cells as described in Materials and Methods. The complete nucleotide sequence is shown with the amino acid sequence of the open reading frame shown below. The translational start indicated is the sole in-frame ATG codon in the reading frame and initiates an open reading frame of 140 codons. A consensus polyadenylation signal sequence found at nucleotide 1372 is underlined. Recognition sites for restriction enzyme EcoRI, introduced as linkers during cDNA synthesis are shown in italics. These sequence data are available from EMBL/GenBank/DDBJ under accession numbers U14518 (human) and U14519 (bovine).

CENP-A were designed from peptide sequences reported for bovine CENP-A (Palmer et al., 1991). cDNA was prepared from total bovine RNA, derived from cultured MDBK cells, and used as template for PCR, resulting in the amplification of a 230-bp cDNA fragment which was subcloned and subjected to DNA sequence analysis (Fig. 1 *A*). The cDNA spans 73 codons including five flanking codons at each end that are derived from the primers. Within the amplified region three segments match peptide sequences reported by Palmer et al. (1991), differing only in positions assigned ambiguously in that work (Fig. 1 *A*, *italics*). We conclude that the isolated PCR product corresponds to the gene encoding bovine CENP-A as identified by Palmer and co-workers

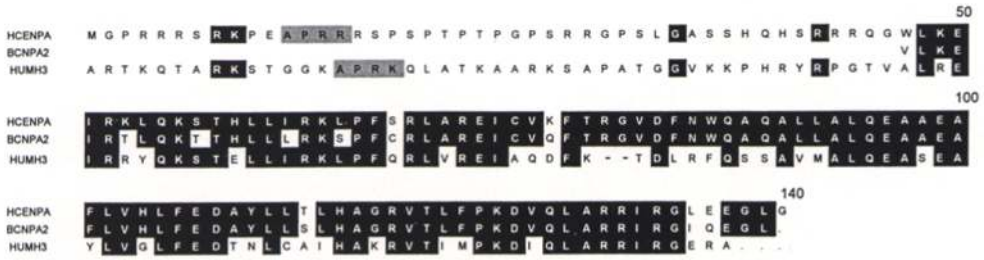

Figure 2. Alignment of CENP-A sequences with histone H3. The amino acid sequences encoded by human CENP-A was aligned with the contiguous sequence assembled for bovine CENP-A and with human histone H3 (SwissProt H31 HUMAN, P16106) using the GAP program of the University of Wisconsin Genetics Computer Group software (Milwaukee, WI). Residues identical to human CENP-A are highlighted (*black*); a short motif shared between CENP-A and histone H3 in the NH_2 terminus is highlighted (*grey*). Note the presence of a two amino acid insertion in CENP-A relative to histone H3 following residue 79.

(1991). Two additional segments of peptide sequence flank the region amplified by PCR, allowing a contiguous sequence of 93 amino acid residues to be assembled for bovine CENP-A (Fig. 2).

The bovine RT-PCR product was used to screen human cDNA libraries, resulting in the isolation of a 1,419-bp human cDNA, designated cDNA 211 (Fig. 1 *B*). The open reading frame of cDNA 211, identified by homology with bovine CENP-A, contains a single in-frame methionine and spans 140 codons, predicting a protein of 15,990 D. Comparison of the predicted human protein sequence with the contiguous 93-residue segment of bovine CENP-A compiled from PCR and peptide sequences reveals 90.1% identity (94.5% similarity) between the two proteins in this segment (Fig. 2).

CENP-A Is a Histone H3 Homologue

The mammalian CENP-A sequences share significant homology with histone H3 (Fig. 2) as predicted from the partial sequence data of Palmer et al. (1991). This homology is restricted to the COOH-terminal portion of histone H3, residues 48-135. In this region the CENP-A sequences share an average of 60% identity and about 75% similarity with human histone H3. This region corresponds to the element of histone H3 that is essential for viability in yeast (Mann and Grunstein, 1992; Morgan et al., 1991) and to the ordered "histone fold" domain revealed by x-ray crystallographic analysis of the histone octamer (Arents et al., 1991). Thus the structure of CENP-A is compatible with a role as a component of a modified nucleosome or nucleosome-like structure in which it replaces one or both copies of conventional histone H3 in the $(H3\text{-}H4)_2$ tetrameric core of the nucleosome particle, a hypothesis consistent with the known biochemical properties of CENP-A (Palmer et al., 1987).

In contrast to the homology observed in the histone fold domain, the NH_2 terminus of human CENP-A shares essentially no sequence identity with the corresponding region of histone H3. In addition, a search of the comprehensive amino acid sequence database using the NCBI BLAST server (Altschul et al., 1990) failed to reveal significant sequence similarity between the NH_2 terminus of CENP-A and any other known proteins. The NH_2 terminal domain of human CENP-A shares the basic character of the histone H3 NH_2 terminus, but is skewed toward arginine (12 Arg, 1 Lys) while histone H3 is roughly balanced between Arg (6) and Lys (8). Four copies of the dipeptide (Ser/Thr)-Pro, residues 17-24, are present in the NH_2 terminus and could represent substrates for phosphorylation (Nigg, 1993).

Taken together these data indicate that CENP-A, like histone H3, is a two domain protein that possesses a histone fold domain with significant similarity to histone H3 and a divergent basic NH_2 terminus.

Expression of cDNA 211 Demonstrates That It Encodes Bona Fide CENP-A

CENP-A was originally identified as a 17-kD autoantigen that reacts specifically with anticentromere autoantibodies present in patients with limited system sclerosis or CREST syndrome (Earnshaw and Rothfield, 1985). To verify that cDNA 211 encodes bona fide CENP-A, we directly analyzed the properties of the gene product by expression in mam-

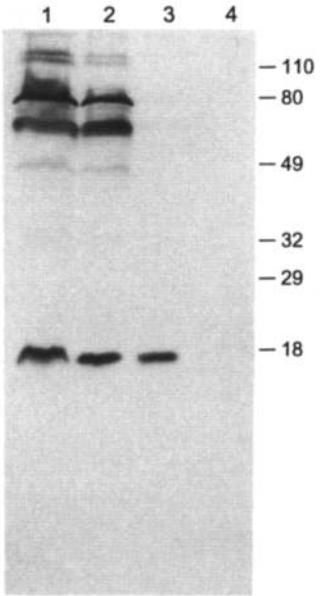

Figure 3. The polypeptide encoded by human CENP-A cDNA 211 reacts with human anti-centromere autoantibodies. A dish (10 cm) of Indian muntjac cells at 70% confluence were transfected with 5 μg of pcDL 211 using cationic liposomes and harvested by lysis in SDS-PAGE sample buffer 48 h after transfection. A control dish was mock transfected without DNA and harvested at the same time. Exponentially growing HeLa cells were harvested by trypsinization and dissolved in SDS-PAGE lysis buffer at a concentration of 5×10^7 cells/ml. Proteins were resolved by SDS-PAGE on 15% gels, transferred to nitrocellulose, and then immunoblotted with hACA-M at a dilution of 1:1,000. Lane *1* shows HeLa cell extract (10^6 cells); lane *2*, a mixture of HeLa cell extract (5×10^5 cells) and pcDL 211-transfected Indian muntjac cell extract ($\sim 2.5 \times 10^4$ cells); lane 3, pcDL 211-transfected Indian muntjac cell extract alone ($\sim 5 \times 10^4$ cells); lane *4*, mock-transfected Indian muntjac ($\sim 5 \times 10^4$ cells). The 17-kD hACA-M-reactive peptide is specifically observed in transfected Indian muntjac cell extracts and co-migrates with HeLa cell CENP-A antigen.

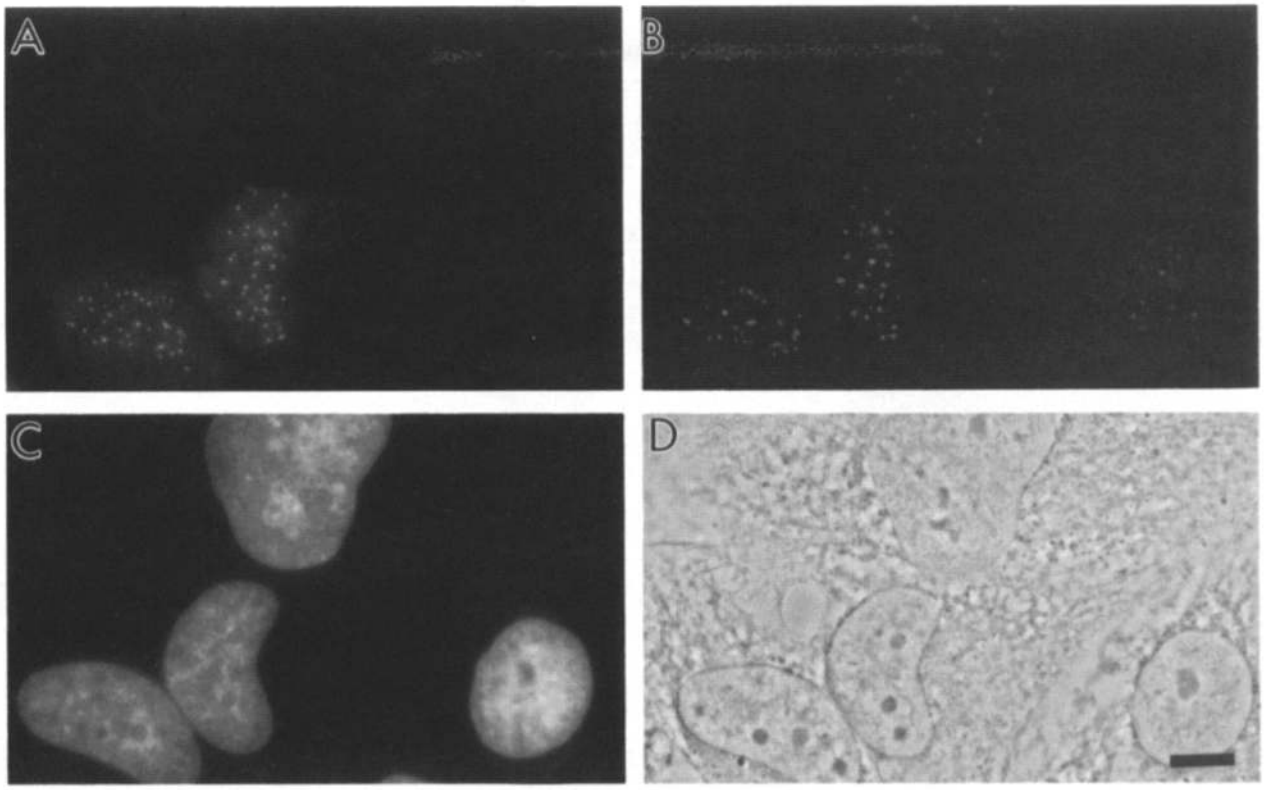

Figure 4. Centromeric localization of epitope tagged CENP-A. HeLa cells were transfected with plasmid pcDL CA-HA1 and processed for dual-label immunofluorescence 48 h after transfection. Each image includes one or more untransfected cells as a control for staining specificity with mAb 12CA5. (*A*) Detection of epitope-tagged transfected gene product with mAb 12CA5; (*B*) endogenous centromere antigens detected with hACA-M; (*C*) DAPI staining; and (*D*) phase contrast. Bar, 10 μm.

malian cells. cDNA 211 was cloned into pcDL SRα-296, a constitutive expression vector based on the SV40 early promoter (Takabe et al., 1987), to produce plasmid pcDL-211 and this was introduced into mammalian cells by cationic lipid-mediated transfection (Felgner et al., 1987). Transfected cells were harvested after 48 h by lysis in SDS-PAGE sample buffer and protein expression was assayed by western blot using hACA-M, a CREST autoantiserum containing antibodies against human CENP-A (Fig. 3). In preliminary experiments conditions allowing selective identification of human CENP-A expressed in Indian muntjac cells were identified (Fig. 3, lane *4*). Transfected cells expressed an immunoreactive 17-kD antigen (Fig. 3, lane *3*) that co-migrated with HeLa CENP-A in SDS-PAGE (Fig. 3, lanes *1* and *2*). Thus the cDNA 211 encoded protein co-migrates with and shares the antigenic properties of bona fide human CENP-A.

The most distinctive property of CENP-A is its centromeric localization. To examine the intracellular distribution of the transfected gene product in human cells, an epitope-tagged derivative of CENP-A was prepared. A decapeptide coding sequence corresponding to the influenza hemagglutinin epitope HA-1 (Niman et al., 1983) was introduced after the last codon of the cDNA 211 open reading frame using PCR. The epitope tagged cDNA was subcloned into pcDL to form plasmid pcDL CA-HA1 and was analyzed by transient expression in HeLa cells. Cells were transfected on cover slips and processed after 48 h for dual-label immunofluorescence microscopy. The transfected gene product was detected using HA-1 specific MAb 12CA5 (Fig. 4 *A*), while the location of endogenous centromere antigens was determined using hACA-M (Fig. 4 *B*). In 40–70% of cells that expressed transfected CENP-A, the protein was localized in the nucleus in a number of discrete foci that co-localized with centromeres defined by staining with human anti-centromere autoantibodies. Since the human serum recognizes the transfected gene product as well as endogenous centromere antigens, a parallel cover slip was processed for detection using anti-CENP-B monoclonal antibody mACA-1 to visualize centromeres. Transfected CENP-A co-localized with endogenous CENP-B confirming that the cDNA encoded polypeptide was faithfully directed to centromeres in human cells (data not shown). We conclude on the basis of sequence homology, molecular size, immunochemical reactivity and the centromeric localization of the gene product that cDNA 211 encodes a full-length copy of human CENP-A.

The heterogeneity of exogenous gene expression in individual cells in transient transfection assays is well documented. In these experiments, CENP-A was expressed at widely varying levels as judged by total fluorescence signal in nuclei of transfected cells. In cells that expressed CENP-A at high levels, it was distributed throughout the nucleus in interphase cells and was retained on condensed mitotic chromosomes, suggesting that it is associated with chromatin throughout the nucleus (Fig. 5). This was not an artifact of the epitope tag as it was also observed with an untagged construct that expressed native CENP-A (data not shown). It is not clear how nucleoplasmic CENP-A is associated with

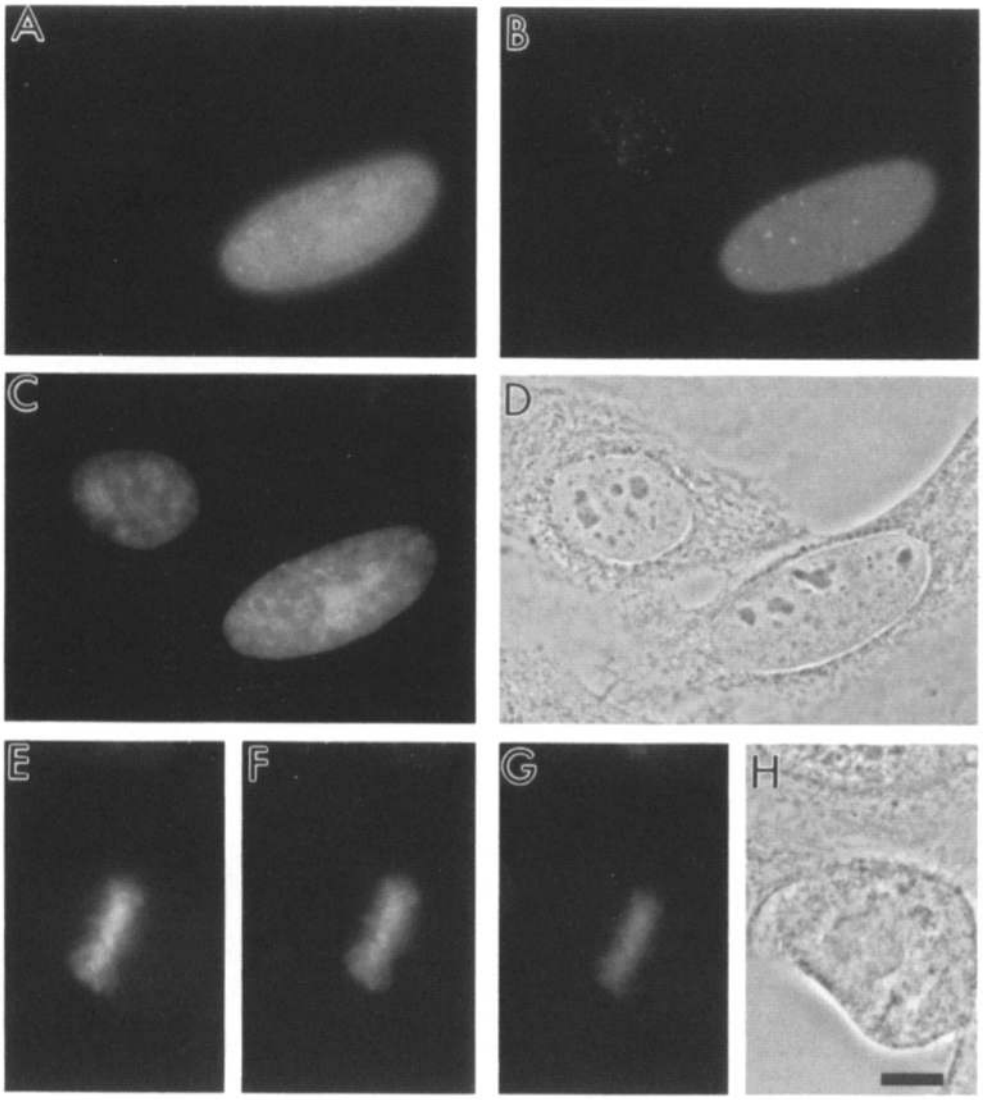

Figure 5. Overexpression of CENP-A results in promiscuous localization throughout the chromosomes. HeLa cells were transfected with plasmid pcDL CA-HA1 and processed for immunofluorescence as in Fig. 4. Cells that overexpressed the transfected CENP-A gene product, as judged by total fluorescence signal and distribution, were selected for analysis. *A–D* show interphase cells and *E–H* show a mitotic cell in metaphase. (*A* and *E*) mAb 12CA5; (*B* and *F*) hACA-M; (*C* and *G*) DAPI; and (*D* and *H*) phase contrast. Bar, 10 μm.

chromosomes or if it is assembled into nucleosomes. Surprisingly, cells containing chromosomes uniformly labeled with CENP-A appeared to proceed through mitosis normally as judged by observation of cells at all stages of mitosis, including metaphase (Fig. 5, *E–H*), late anaphase and telophase. Nevertheless, CENP-A is preferentially targeted to centromeres when expressed at or near physiological levels.

The Histone Fold Domain of CENP-A Is Required for Centromeric Localization

CENP-A in vivo is detected solely at centromeres using anti-centromere antisera. There remains a formal possibility that CENP-A is distributed outside centromeres below the limits of detectability of immunocytochemical assays. However, the possibility that human anti-centromere autoantibodies recognize a distinct centromeric form of CENP-A can be eliminated since we have shown that these antibodies detect non-centromeric CENP-A in transfected cells (Fig. 5). These observations underscore the idea that under normal conditions a mechanism exists to selectively target CENP-A for assembly into centromeres in the presence of a large pool of potentially competitive histone H3. These considerations prompted us to ask what features of the CENP-A polypeptide play a role in the selective assembly of CENP-A at centromeres.

The information that specifies the centromeric localization of CENP-A could reside in the unique NH_2 terminus, within the histone fold domain or could require the presence of both portions of the protein. To test the roles of these two regions, a pair of CENP-A/histone H3 chimeras were constructed to examine targeting by transfection in HeLa cells. Plasmid pcDL CA/H3-HA1 was constructed by fusion of CENP-A NH_2-terminal codons 1-51 with codons 52-135 of histone H3 (Fig. 6 *A*). The reciprocal construct, pcDL H3/CA-HA1 fused codons 1-52 of histone H3 with the histone fold domain of CENP-A, residues 53-140. As a control, the coding region of mouse histone H3 was cloned into pcDL-211 to generate pcDL H3-HA1. These constructs maintained the CENP-A 5′ and 3′ untranslated regions to eliminate or minimize effects of mRNA structure or regulation on the experimental results. Plasmids, including pcDL CA-HA1, were introduced into HeLa cells and after 48 h, ex-

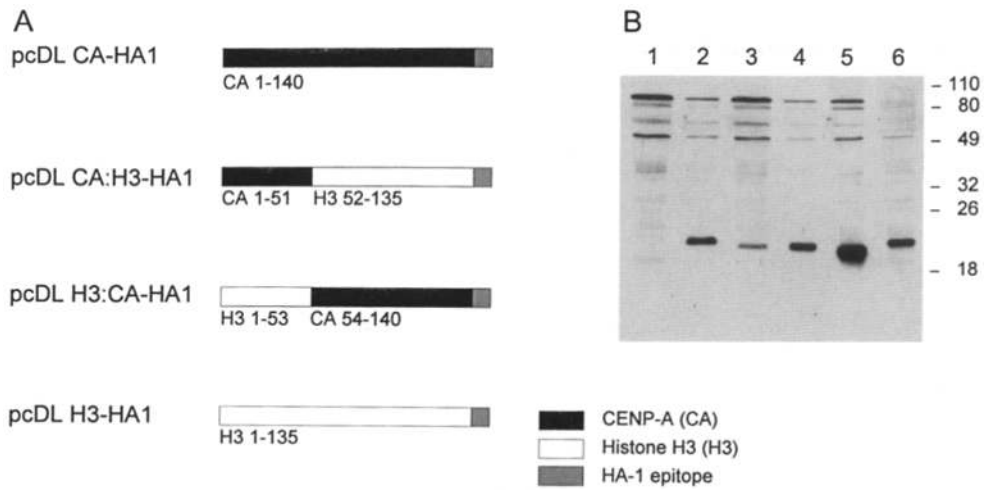

Figure 6. Chimeric constructs used for analysis of CENP-A localization requirements. (*A*) Diagram of chimeras constructed for analysis of localization signals within CENP-A. CENP-A sequences are indicated in black while histone H3 sequences are indicated in white. The COOH-terminally located HA-1 epitope is indicated in grey. Numbers beneath the diagrams show the amino acid residues of CENP-A (*CA*) or histone H3 (*H3*) present in each construct. (*B*) Expression of epitope-tagged proteins in HeLa cells. Each of the plasmids shown in part A was transfected into HeLa cells and cell lysates were prepared after 48 h for immunoblot analysis with mAb 12CA5. (Lane *1*) Mock-transfected cells. Note the presence of endogenous cross-reactive antigens, most notably of 50 and 85 kD. (Lane *2*) CA/HA1; (lane *3*) CA/H3-HA1; (lane *4*) H3/CA-HA1; (lane *5*) H3-HA1. Epitope-tagged gene products are observed as single bands in the range of 19–21 kD. (Lane *6*) pcDL CA-HA1 transfected into Indian muntjac cells. Positions of molecular weight standards are indicated at the right.

pression of epitope-tagged proteins was assayed by immunoblot analysis using mAb 12CA5 (Fig. 6 *B*). The intracellular distribution of the transfected gene products was also assayed at this time by immunofluorescence with 12CA5 and hACA-M as described, using a confocal laser scanning microscope. Epitope-tagged CENP-A-HA1 localized to centromeres as described above (Fig. 7 *A*) while tagged histone H3-HA1 localized throughout the nucleus as expected for histone H3 (Fig. 7 *D*; Kurth et al., 1978). The chimeric CA/H3-HA1, possessing the unique NH_2 terminus of CENP-A, failed to localize at centromeres but rather was distributed throughout the nucleus (Fig. 7 *B*). In contrast, H3/CA-HA1, which possesses the COOH-terminal histone-fold domain of CENP-A, was localized at centromeres in numerous cells (Fig. 7 *C*). This protein appeared to localize at centromeres with a somewhat reduced efficiency or selectivity, evidenced by greater levels of general nuclear staining in transfected cells than observed with wild type CENP-A-HA1. Nevertheless, it is clear that the signals required to target CENP-A to centromeres reside within the histone fold domain of CENP-A rather than in the unique sequences of the NH_2 terminus.

CENP-A Recognizes a Conserved Element of the Mammalian Centromere

The identification of a centromere localization signal in the histone fold domain of CENP-A, which is highly conserved in mammals (Figs. 1 and 2), raised the possibility that CENP-A recognizes an evolutionarily conserved element of the centromere. To test this hypothesis, pcDL CA-HA1 was transfected into Indian muntjac cells and assayed by immunofluorescence microscopy as described for HeLa cells above. It is important to note that, while not detected under the conditions used for immunoblotting in Fig. 3 (hACA-M serum at 1:5,000 dilution), we have detected a homologue of CENP-A in the Indian muntjac by Northern blot and by immunoblot analysis using hACA-M at a dilution of 1:100 (data not shown). Human CENP-A localized to centromeres of Indian muntjac chromosomes, identified on the basis of number, co-localization with endogenous centromere antigens and the characteristic G2/M phase morphology of muntjac centromeres (Fig. 8, *A* and *B*). Chimera H3/CA-HA1 also localized to muntjac centromeres, indicating that the conserved targeting element resides within the histone fold domain of CENP-A. Thus, the histone fold domain of CENP-A recognizes a conserved component of centromere structure or assembly in mammalian cells.

CENP-B, the Sequence-specific Satellite DNA Binding Protein of the Centromere, Does Not Specify Localization of CENP-A

One plausible mechanism for the centromeric localization of CENP-A is that it is mediated by protein-protein interactions with another centromere component. A candidate for such a targeting protein is CENP-B, which is localized to the centromere by virtue of a sequence-specific centromere DNA binding activity (Masumoto et al., 1987; Pluta et al., 1992). In preliminary experiments, we noticed that human CENP-B expressed in the Indian muntjac does not preferentially localize at centromeres (Fig. 9 *C*). This observation allowed us to test the hypothesis that CENP-B plays a role in targeting of CENP-A.

Human CENP-A and CENP-B were simultaneously introduced into Indian muntjac cells by co-transfection and their localizations were independently determined by dual-label immunofluorescence microscopy. In these experiments, epitope tagged CENP-A-HA1 was detected with antibody 12CA5 while human CENP-B was detected with monoclonal antibody mACA-1 (Earnshaw et al., 1987). This antibody is specific for human CENP-B, allowing unambiguous identification of human CENP-B expressed in transfected Indian muntjac cells. CENP-B localized to numerous discrete foci within the nucleus, in large excess of the number of centromeres, presumably by binding to cryptic recognition sites distributed throughout the muntjac genome (Fig. 9 *C*). In contrast, CENP-A-HA1 in these same cells was restricted to

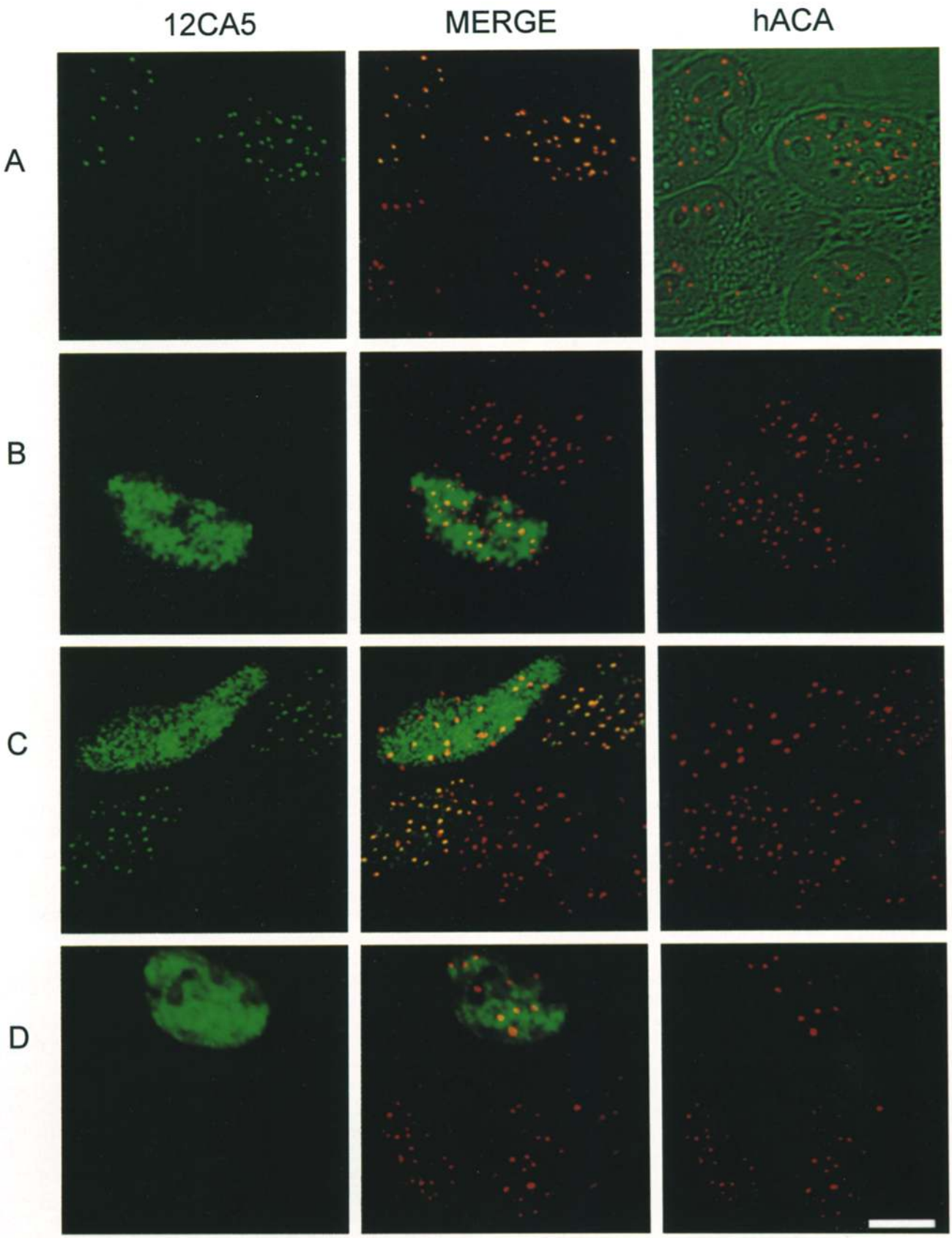

Figure 7. The histone fold domain of CENP-A is required for centromere localization. Plasmids were introduced into HeLa cells as described and processed for dual-label immunofluorescence with MAb 12CA5 (*left*) or hACA-M (*right*) using a Bio Rad MRC 600 confocal laser scanning microscope. To evaluate co-distribution of epitope-tagged gene products with endogenous centromere antigens, the fluorescein (*12CA5*) and rhodamine (*hACA-M*) signals were merged using the MERGE feature of the MRC 600 COMOS software package, shown

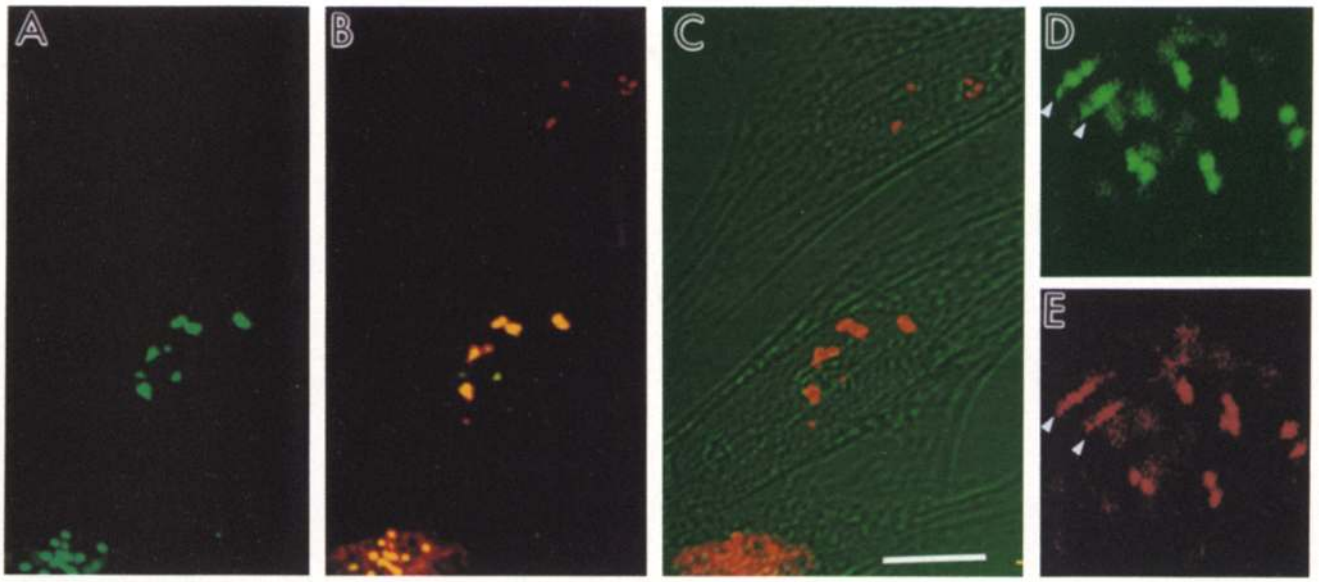

Figure 8. Human CENP-A is faithfully targeted to centromeres of the Indian muntjac. Indian muntjac cells were transfected with pcDL CA-HA1 and processed for immunofluorescence using the confocal microscope as described in Fig. 7. *A–C* show a field of interphase cells. (*A*) mAb 12CA5; (*B*) merged signals mAb12CA5 + hACA-M; and (*C*) hACA-M superimposed on differential interference contrast image. A G_2 or early prophase cell is shown in *D* and *E*, revealing the characteristic double dot staining of duplicated centromeres; arrowheads indicate the large centromere of the muntjac X chromosome. (*D*) mAb 12CA5; and (*E*) hACA-M. Bar, 10 μm.

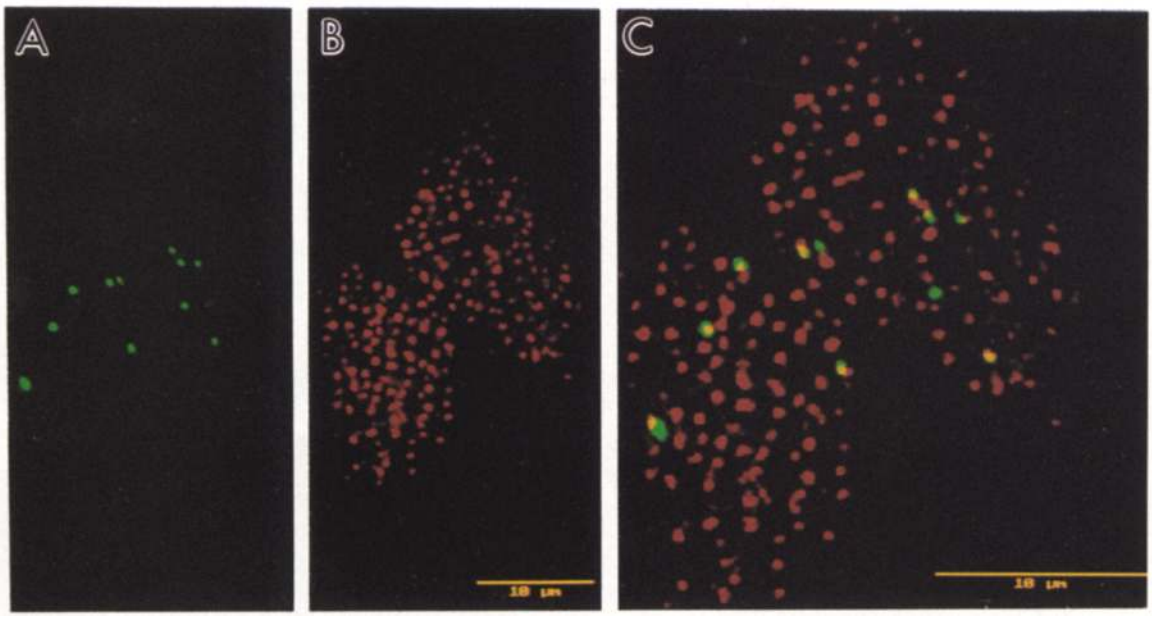

Figure 9. CENP-B alone is insufficient to specify the localization of CENP-A. Indian muntjac cells were cotransfected with pcDL CA-HA1 and pcDL CB, expressing human CENP-B. Distribution of antigens was assayed by dual label immunofluorescence microscopy using the confocal microscope as described in Fig. 7. (*A*) CENP-A-HA1 localization; (*B*) human CENP-B localization; (*C*) enlarged merge of *A* and *B*. Bars, 10 μm.

in the center panel. In this analysis, co-distributed antigens are visualized as yellow. A differential interference contrast image is shown superimposed on the hACA-M fluorescence signal in the upper right panel, showing that the antibody signals are restricted to nuclei. Each image includes one or more untransfected cells as a control for staining specificity with mAb 12CA5. (*A*) pcDL CA-HA1; (*B*) pcDL CA/H3-HA1; (*C*) pcDL H3/CA-HA1; and (*D*) pcDL H3-HA1. Bar, 10 μm.

centromeres as shown previously (Fig. 9 *A*). Some of the CENP-B–containing foci partially overlapped with centromeres as defined by CENP-A-HA1, but some centromeres were unlabeled with human CENP-B (Fig. 9 *B*). CENP-A was clearly absent from the majority of CENP-B–containing foci in co-transfected cells. Thus, the localization of CENP-A cannot be specified (solely) by an interaction with CENP-B, but rather must depend on other structural features within the centromere.

Discussion

CENP-A Is a Centromere-specific Core Histone

Human CENP-A was originally identified as a 17-kD centromere-associated CREST antigen present on human chromosomes (Earnshaw and Rothfield, 1985). The possibility that one of the CREST antigens might resemble histone proteins was first raised by Palmer and Margolis and their co-workers, who showed that CENP-A was present on nucleosome like particles and co-fractionated with histones during biochemical purification of histones from nucleosome core particles (Palmer and Margolis, 1985; Palmer et al., 1987). This idea was supported by direct peptide sequence analysis of bovine CENP-A, revealing amino acid sequence similarity between CENP-A and histone H3 (Palmer et al., 1991). In this work we have isolated a full-length cDNA for human CENP-A, allowing the first complete sequence analysis of the protein. These data, coupled with functional expression of CENP-A in mammalian cells demonstrate that, indeed, CENP-A is a centromere-specific homologue of the core nucleosomal protein histone H3.

Nucleosome structure and the core histone proteins have been generally thought to be highly conserved. The divergence of histone H3 over the entire phylogenetic spectrum does not exceed 10% and within a single species variation among different histone H3 genes is restricted to just a few amino acid positions (Wells and McBride, 1989). Until recently, the existence of divergent homologues of the core histone proteins was unexpected. The identification of macro-H2A (mH2A), a liver nucleosomal protein that possesses a histone H2A domain coupled to a large nonhistone region, provides the only other example of a divergent core nucleosomal protein identified at the biochemical level (Pehrson and Fried, 1992). However, the characterization of CENP-A and mH2A, coupled with the discovery of genes encoding histone H3-like proteins by large scale DNA sequence analysis in *Caenorhabditis elegans* (Wilson et al., 1994), clearly indicates that modification of chromatin through incorporation of divergent core histones may be an important theme for chromosome structure.

Structure of CENP-A and Histone Function

The organization of CENP-A parallels that of histone H3. Histone H3, like the other core histones, possesses two domains: a flexible and highly basic NH_2-terminal tail that is dispensable for nucleosome assembly (Allen et al., 1982) and viability in yeast (Mann and Grunstein, 1992), and a globular COOH-terminal domain that assembles with histone H4 to form the proteinaceous core of the nucleosome (Arents et al., 1991; Richmond et al., 1984). The NH_2 terminus of CENP-A, residues 1-47, shares the basic and flexible nature of the histone tail but no amino acid sequence similarity with histone H3.

The COOH-terminal portion of histone H3 is folded into an extended dumbbell-shaped structure termed the histone fold domain that typifies all four core histones (Arents et al., 1991). The homology between CENP-A and histone H3 begins abruptly at the border between the NH_2-terminal domain and this histone fold domain. The histone fold domain of human CENP-A is 62% identical to that of human histone H3. Since the available biochemical data demonstrate that CENP-A is found in association with histone H4 and the other core histones in particles that co-purify with nucleosome core particles, it is reasonable to assume that CENP-A acts as a histone H3 homologue replacing one or both copies of histone H3 in a certain set of centromeric nucleosomes.

The major function of the core histones is to bind to DNA, folding it across the nucleosome surface. In particular, nucleosomal DNA makes several contacts with histone H3 in its path across the surface of the histone octamer (Mirzabekov et al., 1978; Arents and Moudrianakis, 1993). How is the CENP-A histone H3 domain expected to impact the structure of the nucleosome? The high degree of sequence identity shared between CENP-A and histone H3 would suggest that CENP-A nucleosomes are very similar to normal nucleosome. CENP-A sequences that correspond to the positions where DNA enters and exits the nucleosome are highly conserved relative to histone H3. However, CENP-A is diverged from a conserved region of histone H3 that is found near the nucleosome twofold axis, around residue 110 (Camerini-Otero and Felsenfeld, 1979). The positioning of nucleosomes on DNA in vitro is facilitated by placing intrinsically bent or flexible DNA near the dyad axis of the nucleosome (Constanzo et al., 1990; Schrader and Crothers, 1990). Thus, CENP-A is differentiated from histone H3 in a region that may be involved in nucleosome/DNA recognition. Whether CENP-A can impart selectivity for centromeric DNA sequence or structure to nucleosomes remains to be experimentally determined, but the results presented here demonstrate a role for CENP-A in recognition of the centromere.

Targeting of CENP-A Is Dependent on the Histone Fold Domain

CENP-A is normally detected only at centromeres. To localize the structural determinants of CENP-A that are involved in centromeric targeting, we constructed chimeric molecules comprised of CENP-A and histone H3. The natural division of CENP-A and histone H3 into two domains allowed us to test the two segments of the protein while maintaining the overall structural organization of the resultant chimeras. Our initial hypothesis was that the unique NH_2 terminus of CENP-A would be required for centromere localization while the histone fold domain would provide the structure necessary for nucleosomal assembly. Surprisingly, the NH_2 terminus was incapable of selectively directing the histone fold domain of histone H3 to the centromeres. Rather, the COOH-terminal histone fold domain of CENP-A is itself sufficient for selective assembly at centromeres.

Recognition of the centromere must, at some level, be based on DNA sequence recognition. One possibility is that

CENP-A does not recognize centromeric DNA itself, but that it is guided to centromeres indirectly by interaction with a protein that directly recognizes and binds to centromeric DNA sequences. The possibility that CENP-B mediates the localization of CENP-A to centromeres was tested by co-transfection of the human genes into a heterologous cell type in which CENP-A is faithfully localized to centromeres while CENP-B is promiscuously localized to noncentromeric nuclear assembly sites. In this experiment, co-expression of CENP-B did not affect the centromeric localization of CENP-A. This result rules out the simple hypothesis that CENP-A is directed to centromeres by binding to CENP-B. CENP-A localization is either independent of CENP-B or requires additional molecular recognition events. The fact that the histone fold domain of CENP-A is sufficient for centromeric localization suggest that CENP-A may directly recognize centromeric DNA sequence or structure.

Why Does the Centromere Use a Different Core Histone?

The unique structure of CENP-A reported here coupled with previous biochemical studies (Palmer et al., 1987) indicate that the centromere is differentiated from the chromosome arms at the most fundamental level of chromatin structure, the nucleosome. Modified nucleosomes are unlikely to be necessary for the actual assembly of microtubule-dependent motor proteins onto the chromosome, since these interactions can be reconstituted in vitro using naked DNA (Middleton and Carbon, 1994; Hyman et al., 1992). Nevertheless, the differentiation of centromeric nucleosomes may be required for centromere function: recently a yeast gene required for chromosome segregation has been identified that, like CENP-A, is a highly diverged histone H3 homologue (Stoler, S., and M. Fitzgerald-Hayes, personal communication). The specific configuration of chromatin at the centromere could be important for insulating the centromere from transcriptional activity or provide a necessary component of the sister chromatid pairing mechanism. Alternatively, CENP-A could provide a mechanical function within the centromere. Mitotic motors can exert force in vast excess of that required for chromosome movement (Nicklas, 1988). One possible role of differentiated centromeric chromatin could be to provide the chromatin fiber with the mechanical stability necessary to integrate forces generated at the kinetochore with the chromosome scaffold, to effectively transduce force generated locally at the surface of the chromosome to the global chromosome movements of mitosis. The recent demonstration that tension at the kinetochore plays a signalling role in modulating microtubule stability and motor protein directionality further underscores the importance of the mechanical properties of the centromere at the level of regulation of chromosome movement and spindle function (Skibbens et al., 1993; Ault and Nicklas, 1989). The identification of CENP-A as a unique centromeric histone will facilitate experimental analysis of the role of chromatin structure in the assembly and function of the centromere.

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References

Allen, J., N. Harborne, D. C. Rau, and H. Gould. 1982. Participation of core histone 'tails' in the stabilization of the chromatin solenoid. *J. Cell Biol.* 93:285–297.

Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman. 1990. Basic local alignment search tool. *J. Mol. Biol.* 215:403–410.

Arents, G., R. W. Burlingame, B. C. Wang, W. E. Love, and E. N. Moudrianakis. 1991. The nucleosomal core histone octamer at 3.1Å resolution: a tripartite protein assembly and a left-handed superhelix. *Proc. Natl. Acad. Sci. USA.* 88:10148–10152.

Arents, G., and E. N. Moudrianakis. 1993. Topography of the histone octamer surface: repeating structural motifs utilized in the docking of nucleosomal DNA. *Proc. Nat. Acad. Sci. USA.* 90:10489–10493.

Ault, J. G., and R. B. Nicklas. 1989. Tension, microtubule rearrangements, and the proper distribution of chromosomes in mitosis. *Chromosoma.* 98:33–39.

Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl. 1990. Current Protocols in Molecular Biology. John Wiley and Sons, New York.

Bernat, R. L., G. G. Borisy, N. F. Rothfield, and W. C. Earnshaw. 1990. Injection of anticentromere antibodies at interphase disrupts events required for chromosome movement at mitosis. *J. Cell Biol.* 111:1519–1533.

Brenner, S., D. Pepper, M. W. Berns, E. Tan, and B. R. Brinkley. 1981. Kinetochore structure, duplication and distribution in mammalian cells: analysis by human autoantibodies from scleroderma patients. *J. Cell Biol.* 91:95–102.

Brinkley, B. R., and E. Stubblefield. 1966. The fine structure of the kinetochore of a mammalian cell in vitro. *Chromosoma.* 19:28–43.

Bloom, K., and J. Carbon. 1982. Yeast centromere DNA is in a unique and highly ordered structure in chromosomes and small circular minichromosomes. *Cell.* 29:305–317.

Camerini-Otero, R. D., and G. Felsenfeld. 1977. Histone H3 disulfide dimers and nucleosome structure. *Proc. Natl. Acad. Sci. USA.* 74:5519–5523.

Clarke, L., and J. Carbon. 1980. Isolation of a yeast centromere and construction of functional small circular chromosomes. *Nature (Lond.).* 287: 504–509.

Constanzo, G., E. Dimauro, G. Salina, and R. Negri. 1990. Attraction, phasing and neighbor effects of histone octamers on curved DNA. *J. Mol. Biol.* 216:363–374.

Cooke, C. A., R. L. Bernat, and W. C. Earnshaw. 1990. CENP-B: a major human centromere protein located beneath the kinetochore. *J. Cell Biol.* 110:1475–1488.

Devereaux, J., P. Haberli, and O. Smithies. 1984. A comprehensive set of sequence analysis programs for the VAX. *Nucl. Acids Res.* 12:387–395.

Earnshaw, W. C., and N. Rothfield. 1985. Identification of a family of human centromere proteins using autoimmune sera from patients with scleroderma. *Chromosoma.* 91:313–321.

Earnshaw, W. C., K. F. Sullivan, P. S. Machlin, C. A. Cooke, D. A. Kaiser, T. D. Pollard, N. F. Rothfield, and D. W. Cleveland. 1987. Molecular cloning of cDNA for CENP-B, the major human centromere autoantigen. *J. Cell Biol.* 104:817–829.

Feinberg, A. P., and B. Vogelstein. 1983. A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* 132:6–13.

Funk, M., J. H. Hegemann, and P. Philippsen. 1989. Chromatin digestion with restriction endonucleases reveals 150-160 bp of protected DNA in the centromere region of chromosome XIV in *Saccharomyces cerevisiae*. *Mol. & Gen. Genet.* 219:153–160.

Felgner, P. L., T. R. Gadek, M. Holm, R. Roman, H. W. Chan, M. Wenz, J. P. Northrop, G. M. Ringold, and M. Danielsen. 1987. Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. *Proc. Natl. Acad. Sci. USA.* 84:7413–7417.

Hill, A., and K. Bloom. 1989. Acquisition and processing of a conditional dicentric chromosome in Saccharomyces cerevisiae. *Mol. Cell. Biol.* 9: 1368–1370.

Hyman, A. A., K. Middleton, M. Centola, T. J. Mitchison, and J. Carbon. 1992. Microtubule-motor activity of a yeast centromere-binding protein complex. *Nature (Lond.).* 359:533–535.

Kurth, P. D., E. N. Moudrianakis, and M. Bustin. 1978. Histone localization in polytene chromosomes by immunofluorescence. *J. Cell Biol.* 78:910–918.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680–685.

Lechner, J., and J. Carbon. 1991. A 240 kd multisubunit protein complex, CBF3, is a major component of the budding yeast centromere. *Cell.* 64: 717–725.

Mann, R. K., and M. Grunstein. 1992. Histone H3 N-terminal mutations allow hyperactivation of the yeast GAL1 gene in vivo. *EMBO (Eur. Mol. Biol. Or-*

gan.) J. 11:3297–3306.

Masumoto, H., H. Masukata, Y. Muro, N. Nozaki, and T. Okazaki. 1989. A human centromere antigen (CENP-B) interacts with a short specific sequence in alphoid DNA, a human centromeric satellite. *J. Cell Biol.* 109:1963–1973.

Middleton, K., and J. Carbon. 1994. *KAR3*-encoded kinesin is a minus-end-directed motor that functions with centromere binding proteins (CBF3) on an *in vitro* yeast kinetochore. *Proc. Natl. Acad. Sci. USA.* 91:7212–7216.

Mirzabekov, A. D., V. V. Shik, A. V. Belyavsky, and S. G. Bavykin. 1978. Primary organization of the nucleosome core particle of chromatin: sequences of histone DNA arrangement along the DNA. *Proc. Natl. Acad. Sci. USA.* 75:4184–4188.

Morgan, B. A., B. A. Mittman, and M. M. Smith. 1991. The highly conserved N-terminal domains of histones H3 and H4 are required for normal cell cycle progression. *Mol. Cell. Biol.* 11:4111–4120.

Moroi, Y., C. Peebles, M. J. Fritzler, J. Steigerwald, and E. M. Tan. 1980. Autoantibody to centromere (kinetochore) in scleroderma sera. *Proc. Natl. Acad. Sci. USA.* 77:1627–1631.

Nicklas, R. B. 1988. The forces that move chromosomes in mitosis. *Annu. Rev. Biophys. Chem.* 12:431–449.

Nigg, E. A. 1993. Cellular substrates of $p34^{cdc2}$ and its companion cyclin-dependent kinases. *Trends Cell Biol.* 3:296–301.

Niman, H. L., R. A. Houghten, L. E. Walker, R. A. Reisfeld, I. A. Wilson, J. M. Hogle, and R. A. Lerner. 1983. Generation of protein-reactive antibodies by short peptides is an event of high frequency: implications for the structural basis of immune recognition. *Proc. Natl. Acad. Sci. USA.* 80:4949–4953.

Palmer, D. K., and R. L. Margolis. 1985. Kinetochore components recognized by human autoantibodies are present on mononucleosomes. *Mol. Cell. Biol.* 5:173–186.

Palmer, D. K., K. O'Day, M. H. Wener, B. S. Andrews, and R. L. Margolis. 1987. A 17 kD centromere protein (CENP-A) copurifies with nucleosome core particles and with histones. *J. Cell Biol.* 104:805–815.

Palmer, D. K., K. O'Day, H. L. Trong, H. Charbonneau, and R. L. Margolis. 1991. Purification of the centromere-specific protein CENP-A and demonstration that it is a distinctive histone. *Proc. Natl. Acad. Sci. USA.* 88:3734–3738.

Pehrson, J. R., and V. A. Fried. 1992. MacroH2A, a core histone containing a large nonhistone region. *Science (Wash. DC).* 257:1398–1400.

Pluta, A. F., N. Saitoh, I. Goldberg, and W. C. Earnshaw. 1992. Identification of a subdomain of CENP-B that is necessary and sufficient for localization to the human centromere. *J. Cell Biol.* 116:1081–1093.

Polizzi, C., and L. Clarke. 1991. The chromatin structure of centromeres from fission yeast: differentiation of the central core that correlates with function. *J. Cell Biol.* 112:191–201.

Richmond, T. J., J. T. Finch, B. Rushton, D. Rhodes, and A. Klug. 1984. Structure of the nucleosome core particle at 7Å resolution. *Nature (Lond.).* 311:532–537.

Ris, H., and P. L. Witt. 1981. Structure of the mammalian kinetochore. *Chromosoma.* 82:153–170.

Saitoh, H., J. Tomkiel, C. A. Cooke, H. Ratrie, 3d., M. Maurer, N. F. Rothfield, and W. C. Earnshaw. 1992. CENP-C, an autoantigen in scleroderma, is a component of the human inner kinetochore plate. *Cell.* 70:115–125.

Schulman, I., and K. Bloom. 1991. Centromeres: an integrated protein/DNA complex required for chromosome movement. *Annu. Rev. Cell Biol.* 7:311–336.

Shrader, T. E., and D. M. Crothers. 1990. Effects of DNA sequence and histone-histone interactions on nucleosome placement. *J. Mol. Biol.* 216: 69–84.

Simmerly, C., R. Balczon, B. R. Brinkley, and G. Schatten. 1990. Microinjected kinetochore antibodies interfere with chromosome movement in meiotic and mitotic mouse oocytes. *J. Cell Biol.* 111:1491–1504.

Skibbens, R. V., V. P. Skeen, and E. D. Salmon. 1993. Directional instability of kinetochore motility during chromosome congression and segregation in mitotic newt lung cells: a push-pull mechanism. *J. Cell Biol.* 122:859–875.

Sullivan, K. F., and C. A. Glass. 1991. CENP-B is a highly conserved mammalian centromere protein with homology to the helix-loop-helix family of proteins. *Chromosoma.* 100:360–370.

Takebe, Y., M. Seiki, J. I. Fujisawa, P. Hoy, K. Yokota, K. I. Arai, M. Yoshida, and N. Arai. 1988. SR alpha promoter: an efficient and versatile mammalian cDNA expression system composed of the simian virus 40 early promoter and the R-U5 segment of human T-cell leukemia virus type 1 long terminal repeat. *Mol. Cell. Biol.* 8:466–472.

Taylor, J. D., S. E. Wellman, and W. F. Marzluff. 1986. Sequence of 4 mouse histone H3 genes: implications for evolution of mouse histone genes. *J. Mol. Evol.* 23:242–249.

Tomkiel, J., C. A. Cooke, H. Saitoh, R. L. Bernat, and W. C. Earnshaw. 1994. CENP-C is required for maintaining proper kinetochore size and for a timely transition to anaphase. *J. Cell Biol.* 125:531–545.

Wells, D., and C. McBride. 1989. A comprehensive compilation and alignment of histones and histone genes. *Nucleic Acids Res.* 17(Suppl):r311–r346.

Wilson, R., R. Anscough, K. Anderson, C. Baynes, M. Berks, J. Bonfield, J. Burton, M. Connell, T. Copsey, J. Cooper, A. Coulson, M. Craxton et al. 1994. 2.2 Mb of contiguous nucleotide sequence from chromosome III of *C. elegans. Nature (Lond.).* 368:32–38.

Yoda, K., K. Kitagawa, H. Masumoto, Y. Muro, and T. Okazaki. 1992. A human centromere protein, CENP-B, has a DNA binding domain containing four potential alpha helices at the NH2 terminus, which is separable from dimerizing activity. *J. Cell Biol.* 119:1413–1427.