

# Molecular Cloning of cDNA for CENP-B, the Major Human Centromere Autoantigen

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**Abstract.** We have isolated a series of overlapping cDNA clones for ~95% of the mRNA that encodes CENP-B, the 80-kD human centromere autoantigen recognized by patients with anticentromere antibodies. The cloned sequences encode a polypeptide with an apparent molecular mass appropriate for CENP-B. This polypeptide and CENP-B share three non-overlapping epitopes. The first two are defined by monoclonal antibodies elicited by injection of cloned fusion protein. Epitope 1 corresponds to a major antigenic site recognized by the anticentromere autoantibody used to obtain the original clone. Epitope 2 is a novel one not recognized by the autoantibody. These epitopes were shown to be distinct both by competitive binding experiments and by their presence or absence on different subcloned portions of the fusion protein. The third independent epitope, recognized by a subset of anticentromere-positive patient sera, maps to a

region substantially closer to the amino terminus of the fusion protein. DNA and RNA blot analyses indicate that CENP-B is unrelated to CENP-C, a 140-kD centromere antigen also recognized by these antisera. CENP-B is the product of a 2.9-kb mRNA that is encoded by a single genetic locus. This mRNA is far too short to encode a polypeptide the size of CENP-C. The carboxy terminus of CENP-B contains two long domains comprised almost entirely of glutamic and aspartic acid residues. These domains may be responsible for anomalous migration of CENP-B on SDS-polyacrylamide gels, since the true molecular mass of CENP-B is ~65 kD, 15 kD less than the apparent molecular mass deduced from gel electrophoresis. Quite unexpectedly, immunofluorescence analysis using antibodies specific for CENP-B reveals that the levels of antigen vary widely between chromosomes.

**T**HE structure and function of the centromere (CEN)<sup>1</sup> regions of mitotic chromosomes have recently become the focal point of investigations by workers in three diverse disciplines. First, cell biologists have known for a number of years that the centromere is both the site of sister chromatid pairing (the mechanism of which is currently unknown) and the site of mitotic spindle attachment. The latter site, the kinetochore, has been well-characterized at the ultrastructural level (5, 40–42). The kinetochore is a trilaminar plaque structure embedded in the chromatin at the surface of the chromosome. It is solidly anchored in the chromosomal infrastructure and is found in the chromosome scaffold fraction (13).

Second, geneticists have long been interested in the role of *cis*-acting DNA sequences in the control of chromosomal

segregation. Substantial advances have been made with lower eukaryotes such as in *Saccharomyces*, where DNA from 12 of the 16 CENs has been cloned (6, 15, 20, 21, 28, 36, 45). Each CEN sequence consists of ~120 base pairs (bp) of DNA organized into three subdomains and assembled into a characteristic chromatin structure (2). In the fission yeast *Schizosaccharomyces*, the three centromere regions have also been cloned by chromosome walking (33). These centromere regions are much larger and more complex (roughly 50 kb with a conserved subdomain of 4,000 base pairs), and as yet have not been amenable to functional analysis. We therefore know much about the DNA organization of centromeres in these two yeasts; however, the proteins that interact with the CEN sequences remain unknown.

Finally, rheumatologists became interested in centromere structure when it was observed that centromere components are the target of a highly selective autoimmune response in certain patients with rheumatic diseases (see references 16, 32, 46). Anticentromere antibodies (ACAs) occur in patients with Raynaud's phenomenon (hypersensitivity of the peripheral vasculature to cold), many of whom also have

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1. *Abbreviations used in this paper:* ACA, anticentromere antibody; CEN, centromere.

scleroderma. This observation has proven seminal in recent studies of centromere structure in higher eukaryotes, since the antibodies are sensitive probes for both structural and biochemical analyses. We previously showed that ACA(+) patient sera recognize three immunologically related centromere antigens, CENP-A (17 kD), CENP-B (80 kD), and CENP-C (140 kD) (10). Both CENP-A and CENP-B have been recognized independently by several other research groups (CENP-A: 8, 17, 34, 49, 50; CENP-B; 49). CENP-B is referred to as the major centromere antigen, since antibodies to it are present at high titer in all ACA(+) patient sera, while the titer of antibodies to CENP-A and CENP-C is often lower (11).

Very little is known about the functions of CENP-A, CENP-B, and CENP-C. Although an early study by Brenner et al. (4) suggested that some or all of the autoantigens recognized by one ACA(+) serum might be localized to the trilaminar kinetochore structure, it is not known which of the CENP antigens was thus localized. In addition, we have shown that all three of the antigens are absent from the latent (inactive) centromere of a stable dicentric X chromosome (9). Others have also found that the antigens are either absent, or present in much reduced amounts, at the latent centromeres of other stable dicentric chromosomes (31, 37). These results suggest that the antigens might be involved in microtubule binding. However, the detailed function and distribution of individual CENP antigens in vivo is currently unknown.

To begin a more thorough analysis of centromere antigens, we have now used immunological screening of a human  $\lambda$ gt11 cDNA expression library to isolate cloned cDNA sequences encoding polypeptides recognized by anticentromere antibodies. A series of overlapping clones corresponding to >90% of the mRNA encoding CENP-B have been obtained. Five independent lines of biochemical and immunological evidence authenticate the identity of the clones. CENP-B is encoded by a single human gene whose unusual protein product contains two long stretches of residues comprised almost entirely of acidic residues.

## Materials and Methods

### Patient Sera

All sera come from the collection at the University of Connecticut, and are derived from individuals examined by the staff of the Division of Rheumatic Diseases. This collection currently includes sera from 54 patients with anticentromere antibodies, 32 age- and sex-matched normal controls, and 230 individuals with similar clinical manifestations but lacking anticentromere antibodies. All patient sera are characterized both by indirect immunofluorescence and by immunoblotting versus the proteins of isolated chromosomes.

### Isolation of cDNA Clones

An initial cDNA clone was isolated using the  $\lambda$ gt11 expression method of Young and Davis (54, 55; see also reference 43) from a cDNA library constructed from human endothelial cell mRNA. (This library was provided by M. Chow, D. Littman, and R. Axel.) Approximately  $9 \times 10^5$  recombinant phage were plated on lawns of *Escherichia coli* Y1090, production of fusion protein induced by overlaying nitrocellulose filters (BA83; Schleicher & Schuell, Inc., Keene, NH) impregnated with isopropylthiogalactoside (Sigma Chemical Co., St. Louis, MO), and immunopositive colonies detected by processing the filter as though for immunoblotting (see below).

Filters were screened with patient serum GS (containing ACA) at a dilu-

tion of 1:1,000. Preadsorption of this serum with bacterial lysates (43) was found to be unnecessary. By this method, one clone was detected and eventually plaque purified. This clone,  $\lambda$ CNPB1, contained a cDNA insert of 1,282 bp.

Phage clones containing longer cDNA inserts were obtained by screening a cDNA library constructed from human placental mRNA (Clonetech Laboratories, Palo Alto, CA) by DNA plaque hybridization. (Further screening of  $2.7 \times 10^5$  phage from the original library yielded no phage with cDNA inserts longer than  $\lambda$ CNPB1.) The probes used, number of phage screened, and longest clone obtained are summarized in Table I. In all cases the plaque lifts and were processed by standard procedures (29). Probes were labeled with  $\alpha$ - $^{32}$ P-dATP or  $\alpha$ - $^{32}$ -dCTP using random priming with either reverse transcriptase (Life Sciences, St. Petersburg, FL) (42a) or DNA polymerase I, Klenow fragment (New England Biolabs, Beverly, MA) (14).

## Immunological Procedures

**Affinity Purification.** Antibodies were affinity purified from antigens immobilized on nitrocellulose blots by exposure to 3 M  $\text{NH}_4$ -K-thiocyanate as previously described in detail (10).

**Immunoblotting.** Antigen on nitrocellulose strips was preincubated with PTX buffer plus BSA (10 mM  $\text{NaPO}_4$ , pH 7.5, 0.2% Triton X-100, 0.15 M NaCl, 1 mM EGTA, 1 mM  $\text{NaN}_3$  containing 4% BSA; Miles Laboratories, Naperville, IN) for 20 min. Antibody was added to this and incubated for the desired time. Nonbound antibody was removed by washing five times for 3 min with buffer GB (50 mM triethanolamine-HCl, pH 7.5, 0.5% Triton X-100, 0.1 M NaCl, 2 mM K-EDTA, 0.1% SDS), the strips were then rinsed briefly with one change of PTX buffer, and incubated with PTX-BSA for 5 min.  $^{125}$ I-Protein A (or rabbit anti-mouse IgG for the mAbs) was then added ( $1 \times 10^6$  cpm/ml PTX-BSA) for 1 h, after which the strips were again washed five times for 3 min each with buffer GB. Subsequently, the strips were dried, mounted on cardboard, and bound probe was detected by autoradiography.

**Indirect Immunofluorescence.** The protocol for indirect immunofluorescence using colcemid-arrested HeLa cells spread by centrifugation on glow-discharged coverslips has been described in detail previously (9). Briefly, bound antibody was detected with biotinylated second antibody (anti-human, anti-rabbit, or anti-mouse: all from Vector Laboratories, Inc., Burlingame, CA) and streptavidin linked to Texas Red (Bethesda Research Laboratories, Bethesda, MD). Slides were examined with an Olympus BH-2 microscope and images were obtained on Kodak Tri-X film, ASA-1000.

## Preparation of Polyclonal Anticentromere Antibodies

Fusion protein fp- $\beta$ gal-CNPB1 was isolated from Y1089 lysogens harboring phage  $\lambda$ CNPB1 by a simple differential sedimentation procedure (to be described in detail elsewhere). Briefly, frozen induced lysogens were disrupted with lysozyme, EDTA, sonication, and detergent, releasing a granule of fusion protein. This granule was separated from soluble and membrane proteins by three cycles of sedimentation at 10,000 g (15 min) in the presence of 1% Triton X-100 in TEN buffer (10 mM Tris, pH 7.4, 1 mM Na EDTA, 50 mM NaCl). The granule was then dissolved in 8 M urea in TEN buffer at 65°C, insoluble material removed by centrifugation at 12,500 g for 10 min, and then dialyzed overnight versus TEN buffer.

Two male rabbits (6 mo; Harlan Sprague Dawley, Inc., Indianapolis, IN) were injected at days 0, 29, 80, 122, and 152 with 70  $\mu$ g of solubilized fp- $\beta$ gal-CNPB1. For the first injection we used Freund's complete adjuvant; for subsequent injections we used Freund's incomplete adjuvant. Injections were given at multiple sites: subcutaneous, intramuscular, and intraperitoneal. Animals were bled from the ear on days 0, 38, 92, 135, and 169 by venipuncture. One rabbit responded by producing antibodies that recognize CENP-B in immunoblots of isolated chromosomes. This polyclonal anticentromere antiserum is designated ra-ACA1.

## Preparation of Monoclonal Anticentromere Antibodies

Eight BALB/c mice were injected intraperitoneally with 200  $\mu$ g of crude fp- $\beta$ gal-CNPB1 on days 0 and 30. The mice were bled on day 40 and tested for production of antibodies binding to CENP-B. Two mice responded and were given subsequent booster injections on days 52, 53, 54, and 55. On day 55, spleens from these two mice were removed; spleenocytes were mixed together and subsequently fused to the P3-X63-Ag8.653 nonsecreting myeloma cell line (23, 24). The fusion mixture was plated in four 96-well plates (Nunc, Roskilde, Denmark). As colonies appeared, culture superna-

**Table I. Identification of CENP-B cDNA Clones**

Round	Probe (base pairs)	cDNA library	Number of phage screened	Longest cDNA clone obtained
1	Serum GS	Endothelial cell	9 × 10 <sup>5</sup>	λCNPB1 (1282)
2	λCNPB1 Eco RI-Kpn I (320)	Endothelial cell	2.7 × 10 <sup>5</sup>	1282
3	λCNPB1 Eco RI-Kpn I (320)	Placental	8 × 10 <sup>5</sup>	λCNPB2 (2133)
4	λCNPB2 Eco RI-Sac II (138)	Placental	8 × 10 <sup>5</sup>	λCNPB3 (2578)
5	λCNPB2 Eco RI-Sac II (138)	Placental	1.2 × 10 <sup>6</sup>	λCNPB4 (2617)
6	λCNPB4 Eco RI-Xho I (223)	Placental	4 × 10 <sup>6</sup>	2617

tants were screened by ELISA against fp-βgal-CNPB1 and β-galactosidase (Sigma Chemical Co.). Wells positive for the former and negative for the latter were designated possible anti-human candidates and were cloned in soft agar (24, 27). Wells positive on both ELISAs were assumed to be producing anti-β-galactosidase, and several of these were cloned as well.

Primary clones were screened by immunoblotting against fp-trpE-CNPB1. Positive wells were immediately re-cloned in soft agar. All clones that showed binding to fp-trpE-CNPB1 also bound to chromosomal CENP-B. Four independent clones (m-ACA1, m-ACA2, m-ACA3, and m-ACA4) were obtained. Competitive binding experiments (24) showed that m-ACA1 and m-ACA4 bind to overlapping determinants on fp-trpE-CNPB1. The same is true of m-ACA2 and m-ACA3.

### Competitive Binding Experiments

Competitive binding experiments were performed to examine the ability of the autoantibody and ra-ACA1 to block binding of m-ACA1 and m-ACA2 to fp-trpE-CNPB2 and to chromosomal CENP-B. Bacterial lysates containing fp-trpE-CNPB2, or alternatively, isolated HeLa chromosomes prepared as previously described (12, 26), were subjected to SDS PAGE. The separated proteins were subsequently transferred to nitrocellulose by electroblotting (48). The nitrocellulose filters were then cut into strips and treated as follows.

**Autoantibody.** Nitrocellulose strips were incubated overnight with a 1:100 dilution of human serum (either autoantibody GS or normal serum WE). In the morning, the antibody solutions were removed and replaced by an equal volume of hybridoma culture supernatant from either m-ACA1 or m-ACA2. This incubation proceeded for 3 h, following which the unbound antibodies were removed (five 3-min washes with GB followed by one 2-min wash with PTX). PTX-BSA was then added, and after 5 min <sup>125</sup>I-goat anti-mouse IgG was added to 1 × 10<sup>6</sup> cpm/ml. 2 h later, unbound probe was removed by five 3-min washes with GB. The filter strips were then dried, mounted, and autoradiographed.

**ra-ACA1.** Competitive binding experiments were also performed with ra-ACA1 as described above with the following exceptions: a 1:10 dilution of the ra-ACA1 rabbit serum was used for blocking, and incubation with the hybridoma culture supernatants was for 2 h.

### Expression of CENP-B as Fusion Proteins in Bacteria

For expression of cDNA from CNPB1-4 as fusion proteins with the bacterial protein trpE, appropriate regions of cDNA were cloned into one of the pATH series of bacterial expression plasmids (provided by T. J. Koerner, Duke University, Durham, NC). This series of plasmids was specifically engineered to contain a polylinker of unique restriction endonuclease sites within the carboxy-terminal coding sequences of the trpE protein. Because the polylinker is inserted in each of the three different reading frames in the various pATH plasmids, any exogenous cDNA fragment can conveniently be cloned such that its encoded polypeptide is in frame with the trpE protein in one of the plasmids (provided that compatible restriction sites are available).

For expression of CNPB1, CNPB2, CNPB3, and CNPB4, each cDNA was excised with Eco RI (from the original λ clones or from pUC9 sub-clones) and the fragment was isolated after electrophoresis on low melting temperature agarose gels. The gel slices were melted and added the the appropriate pATH plasmid that had been linearized with Eco RI and treated with alkaline phosphatase. The mixtures were then diluted into DNA ligase buffer (New England Biolabs), brought to room temperature, and DNA ligase added for 2 h to overnight. Ligation reactions were transformed by standard methods into *Escherichia coli* strains DH1 (18) or CAG-456 (a temperature-sensitive strain of *E. coli* that contains a mutation in the lon protease gene [43]). Transformants were selected by ampicillin resistance

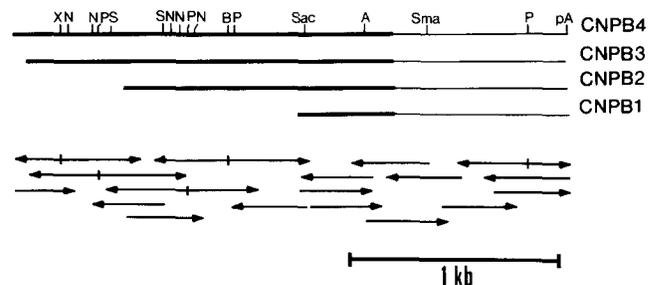
and plasmids carrying the recombinant trpE-CNPB fusion gene were identified by colony hybridization. Restriction mapping of isolated plasmid DNA identified those plasmids into which the cDNA had been cloned in the proper orientation.

TrpE-CNPB fusion proteins were induced from overnight cultures (grown at 30°C in M9 [29] supplemented with 100 μg/ml ampicillin, and 20 μg/ml tryptophane) of CAG-456 transformants harboring the desired plasmids. The overnight culture was diluted 1:10 into the same medium without tryptophane. The bacteria were incubated at 30°C for 2 h and the tryptophane operon induced for an additional 2 h by addition of indoleacrylic acid (Sigma Chemical Co.) to 5 μg/ml. Bacteria were lysed by boiling in SDS gel sample buffer and proteins resolved by PAGE (25).

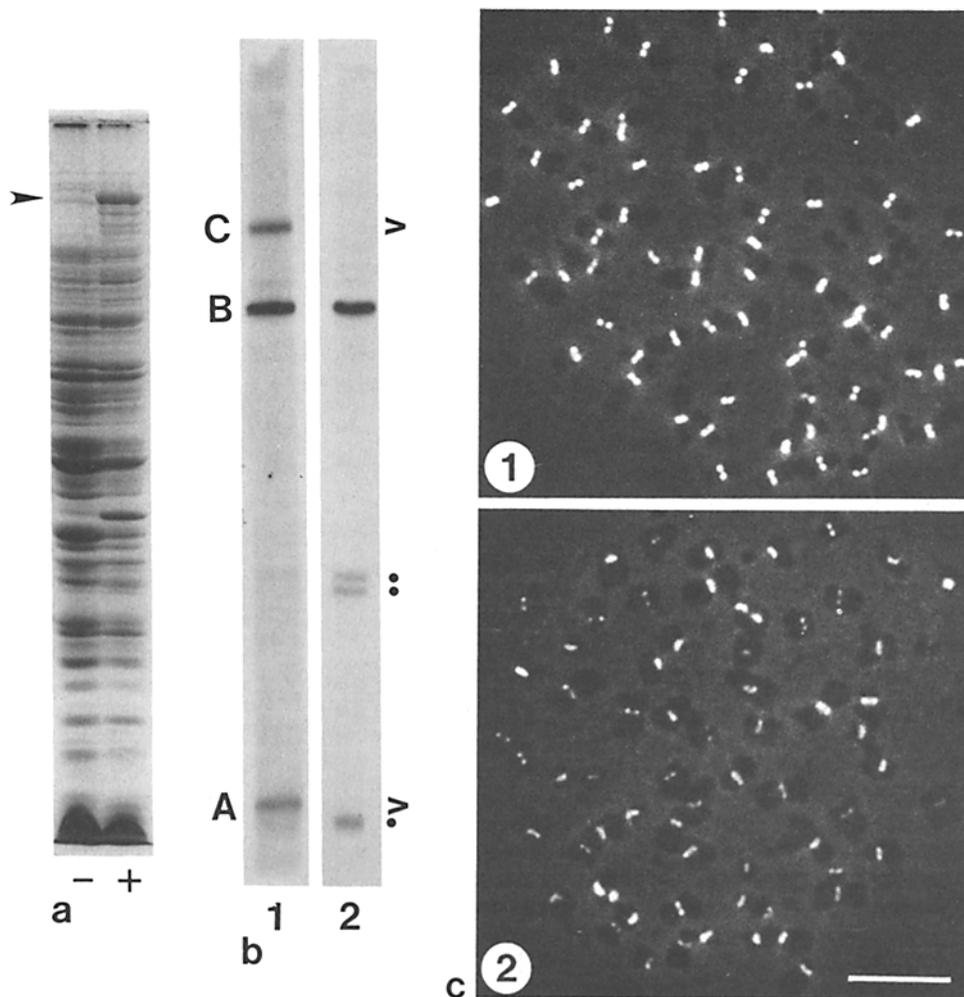
For expression of amino- and carboxyl-terminal sequences of CNPB1, a subclone of CNPB1 in pBR322 was linearized at the unique Asp 718 site within the coding portions of the cDNA (see Fig. 1). The 5' overhang produced was filled in by Klenow DNA polymerase and 1 μM dTTP, dGTP, dCTP, and dATP. A portion of the reaction was digested with Eco RI and the 318-base fragment corresponding to the more NH<sub>2</sub>-terminal sequences of CNPB1 was cloned into pATH 11 that had been digested with Eco RI and Sma I. For expression of the carboxy-terminal sequences of CNPB1, the remaining aliquot was digested with Hind III (at the pBR322 Hind III site, 29 bp 3' to the terminus of CNPB1 sequences), the appropriate fragment purified by gel electrophoresis, and cloned into pATH 3 (digested at the Bam HI site in the polylinker [which was then filled in with Klenow] and then digested with Hind III).

For expression of the amino-terminal portion of CNPB4, CNPB4 cDNA was double digested with Bam HI and Eco RI and the 1,050-base fragment corresponding to the 5' portion of CNPB4 (see Fig. 1) was cloned into Eco RI-Bam HI-digested pATH 11.

For expression of CNPB2 as a fusion protein with a smaller bacterial moiety (consisting of 16 amino-terminal residues: four from β-galactosidase and 12 from polylinker sequences), the entire CNPB2 cDNA was subcloned into the Eco RI site of pUC9. This construction placed CNPB2 expression in frame and under control of the lac operon. Fusion protein was induced in a fashion analogous to that for trpE proteins with the exception that the inducer was 10 μM isopropylthiogalactoside.



**Figure 1.** Schematic drawing and sequencing strategy for four overlapping cDNA clones for the mRNA encoding CENP-B. Schematic representations of the cDNA portions of four independent cDNA clones for CENP-B are shown along with a partial restriction endonuclease map. Bold lines indicate the protein coding sequences, whereas thin lines represent the 3' untranslated region of the mRNA. Beneath the schematic is the sequencing strategy used for determining the complete cDNA sequence. X, Xho I; N, Nae I; P, Pst I; S, Sac II; B, Bam HI; Sac, Sac I; A, Asp 718; Sma, Sma I; pA, site of polyadenylation.



**Figure 2.** Identification of the epitope encoded by  $\lambda$ CNPB1. Lysogens were prepared from  $\lambda$ CNPB1, production of fusion protein was induced, and autoantibodies binding to this fusion protein were affinity purified and characterized by immunoblotting and immunofluorescence. (a) Induction of fusion protein fp- $\beta$ gal-CNPB1 in a lysogen. Coomassie Blue staining of SDS PAGE is shown. (Lane 1) The basal polypeptides in an uninduced culture; (lane 2) polypeptides after the induction of lytic growth and exposure of the cells to IPTG. The fusion protein is indicated with an arrowhead. (b) Immunoblots of chromosomal proteins. Whole autoimmune serum GS and antibodies affinity purified from it by binding to fp- $\beta$ gal-CNPB1 were used to probe immunoblots of proteins present in isolated HeLa chromosomes. (Lane 1) Binding of whole serum GS (dilution 1:1,000); (lane 2) antibody affinity purified from fp- $\beta$ gal-CNPB1 (dilution 1:20). (As discussed in the text, the binding to histones [solid circles] is due to the presence of denatured antibody resulting from the harsh conditions used to elute the antibodies.) (c) Indirect immunofluorescence of

human chromosomes. Binding of (panel 1) whole serum GS (diluted 1:5,000) and (panel 2) antibodies (diluted 1:2) affinity purified from fp- $\beta$ gal-CNPB1 to centromeres of human chromosomes. This and all subsequent immunofluorescence figures are simultaneous double exposures of the indirect immunofluorescence (Texas Red label) and phase-contrast images. Bar, 10  $\mu$ m.

### Blot Analysis of RNA and DNA

Gels for separation of RNA were poured from 1% agarose containing 2.2 M formaldehyde (3). RNA was transferred to nitrocellulose according to Thomas (47). Electrophoresis of DNA was performed in 0.8% agarose gels containing 40 mM Tris-Cl, pH 8.1, 20 mM Na acetate, and 2 mM EDTA. Gels were stained with 1  $\mu$ g/ml ethidium bromide and transferred according to Southern (44). For both RNA and DNA blots, specific sequences were localized by hybridization with DNA probes labeled with  $^{32}$ P (3,000 Ci/mmol) by the random priming method (42a). Hybridization conditions were 50% formamide, 750 mM NaCl, 75 mM sodium citrate, 0.02% albumin, 0.02% polyvinylpyrrolidone, 0.02% ficoll, 500  $\mu$ g/ml yeast RNA, and 50  $\mu$ g/ml sonicated herring sperm DNA. Blots were washed at 53°C in 0.1% SDS, 15 mM NaCl, before visualization by exposure at  $-80^{\circ}$ C to X-ray film (XAR-2, Kodak) with Lightning Plus intensifying screens (DuPont Co., Wilmington, DE).

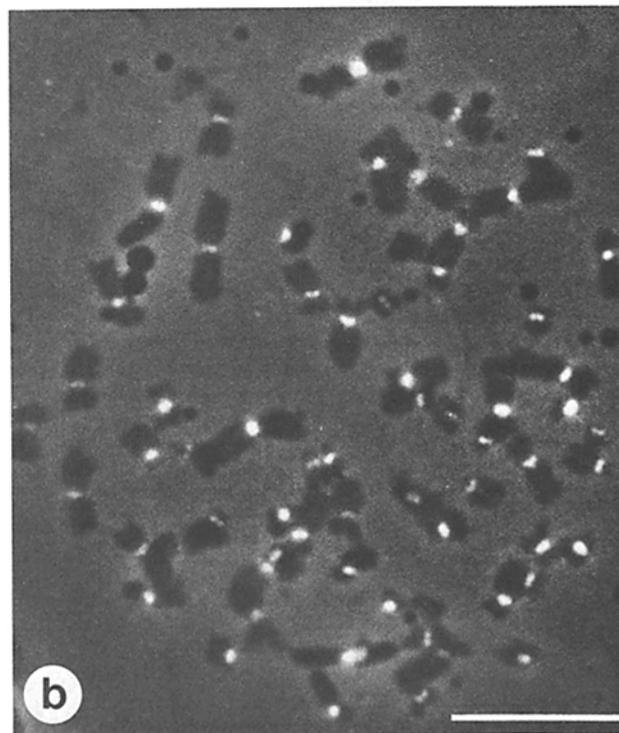
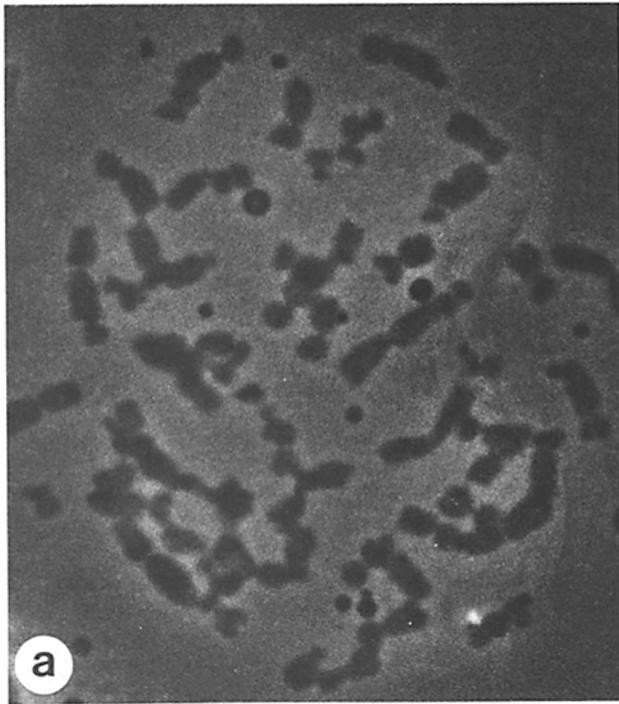
### Results

#### Isolation of a cDNA Clone Encoding an Epitope Present on Human CENP-B

To identify cDNA clones for human centromere autoantigens, we screened a  $\lambda$ gt11 expression library (54, 55) using serum from a patient with anticentromere antibodies (ACAs).

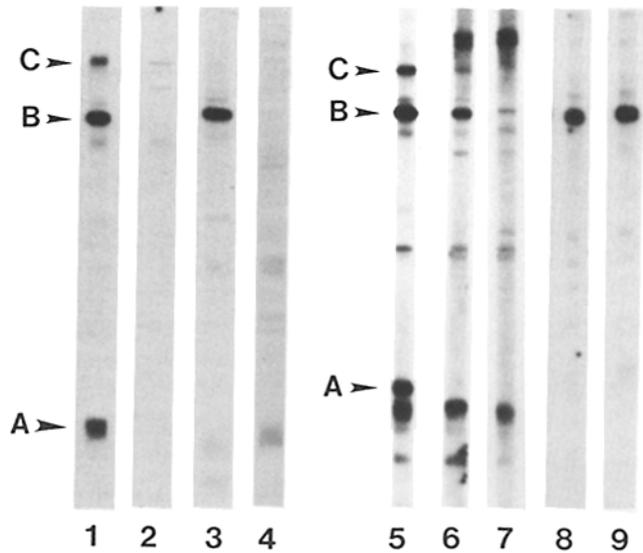
Selection of the antiserum was critical. Many antisera from these patients have high titers of ACAs, but most also have other autoantibodies that recognize a variety of cellular components. Serum GS (from a patient with Raynaud's phenomenon and scleroderma) was chosen as the most suitable of the 38 ACA(+) antisera then available to us. This serum contains 375  $\mu$ g/ml (or 3% of the total) of IgG specific for the 80-kD centromere autoantigen CENP-B (based on a quantitative ELISA using cloned CENP-B (Rothfield, N. F., C. Marino, D. W. Cleveland, and W. C. Earnshaw, manuscript in preparation), and is negative for other common autoantibodies, including Ro, La, Sm, DNA (ss and ds), RNP, Scl-70 (topoisomerase I), actin, and intermediate filaments. Serum GS binds detectibly to CENP-B in immunoblots of isolated chromosomal proteins at dilutions of up to 1:600,000.

When serum GS was used to screen a  $\lambda$ gt11 library constructed from human endothelial cell mRNA (the generous gift of Drs. D. Littman, M. Chow, and R. Axel), immunopositive plaques were detected at a frequency of about 1/50,000. After plaque purification, Eco RI digestion of purified phage DNA from an initial isolate,  $\lambda$ -CNPB1, liberated a single cDNA fragment of 1,282 bp.



**Figure 3.** Indirect immunofluorescence of human chromosomes using rabbit polyclonal antibody ra-ACA1, raised against fp- $\beta$ gal-CNPB1. (a) Preimmune serum (dilution 1:1,000). (b) Immune serum, ra-ACA1 (dilution 1:1,000).

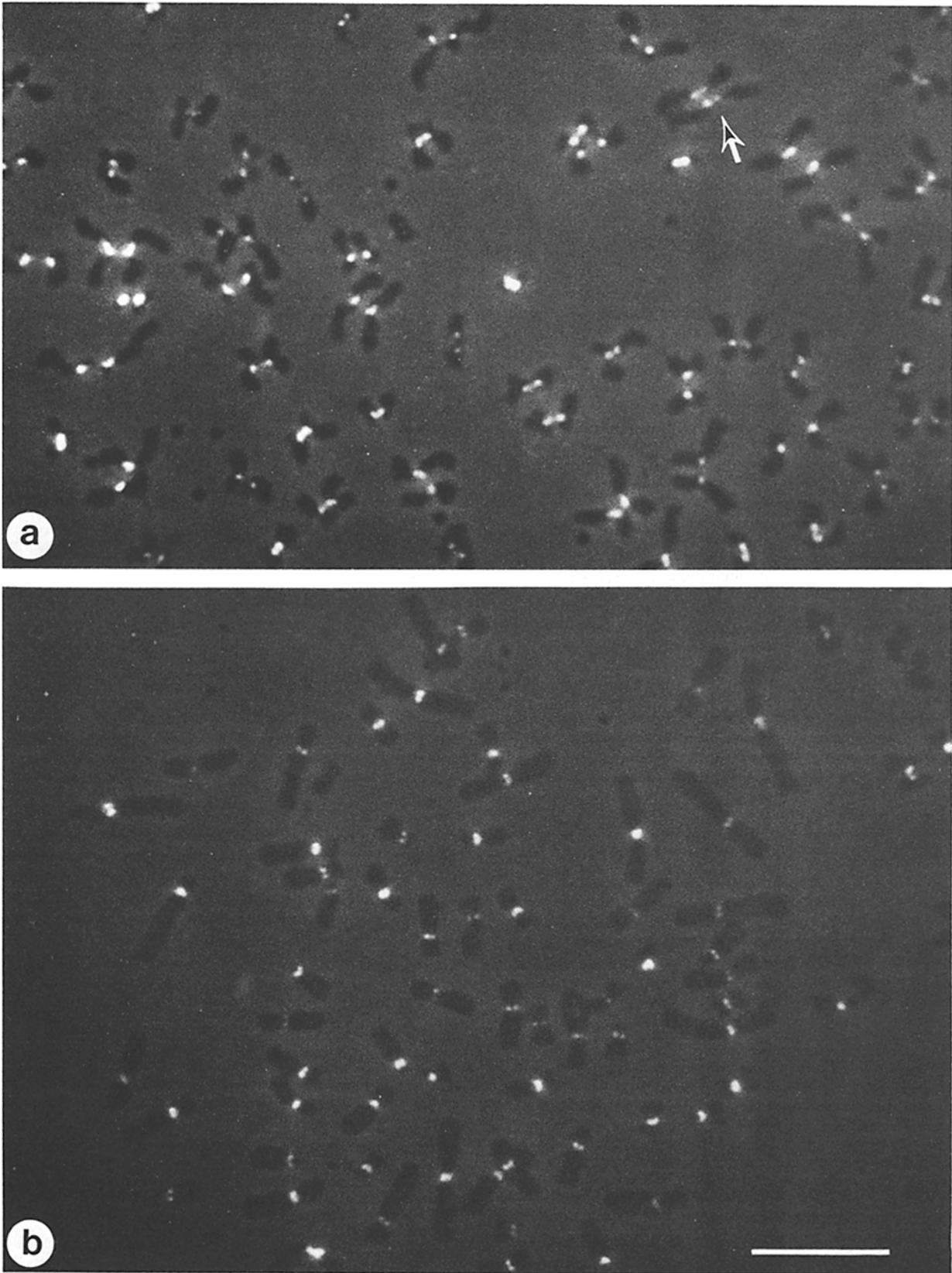
Using nucleic acid hybridization with this insert, we found no longer cDNA inserts in the original library, but a second  $\lambda$ gt11 cDNA library (from human placenta) yielded three longer clones ( $\lambda$ -CNPB2 [2,133 bp],  $\lambda$ -CNPB3 [2,578 bp], and  $\lambda$ -CNPB4 [2,617 bp]—see Fig. 1 and Table I for details).



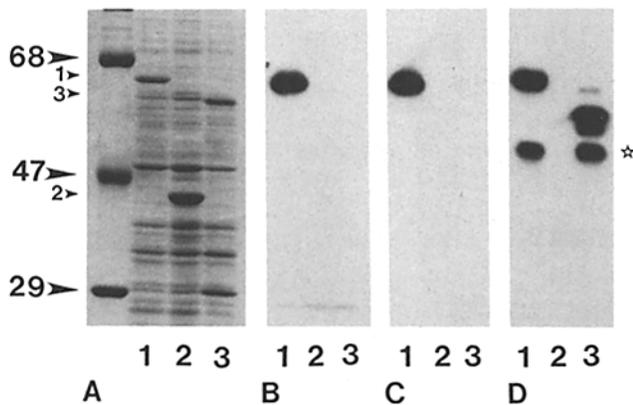
**Figure 4.** fp- $\beta$ gal-CNPB1 used as an immunogen elicits polyclonal and monoclonal antibodies that bind to chromosomal CENP-B in immunoblots. Proteins of isolated HeLa chromosomes were separated by SDS PAGE and electrophoretically transferred to nitrocellulose as previously described (48). Immunoblots of chromosomal proteins were probed with (lane 1) autoantiserum GS (dilution 1:10,000); (lane 2) rabbit preimmune serum (dilution 1:2,000); (lane 3) ra-ACA1 (from the same rabbit) (dilution 1:2,000); (lane 4) serum from a rabbit immunized with  $\beta$ -galactosidase (dilution 1:2,000); (lane 5) auto-antiserum GS (dilution 1:1,000); (lanes 6 and 7) sera from two mice immunized with fp- $\beta$ gal-CNPB1 (dilution 1:100); (lanes 8 and 9) ascites fluid from monoclonal anti-CENP-B antibodies m-ACA1, and m-ACA2, respectively (dilution 1:2,000).

#### **Determination That the Isolated cDNA Clones Contain Sequences Corresponding to an mRNA Encoding an Epitope on CENP-B**

When bacteria lysogenic for  $\lambda$ -CNPB1 were induced to express the lac operon (43), they produced a 150-kD polypeptide consisting of the cloned protein fused to  $\beta$ -galactosidase (Fig. 2 a). Autoantibodies from GS antiserum purified by binding to this fusion protein (immobilized on nitrocellulose) bound to centromeres (Fig. 2 c) and to CENP-B on blots of human chromosomal proteins (Fig. 2 b), but not to CENP-A or CENP-C. (The affinity-purified antibodies also bound weakly to histones H1 and H3 [identified by Ponceau S staining of the nitrocellulose strips—see solid circles in Fig. 2 b]). Several observations suggest that this binding is due to nonspecific sticking of denatured antibody to the histones. [a] No antibody from whole serum bound to histones provided that blots were washed stringently [Fig. 2 a, lane 1]. [b] Antibody affinity purified from histone H1 did not rebind to H1 on blots of chromosomal proteins [10]. [c] Prolonged exposure of the autoantibody to 3 M  $\text{NH}_4\text{-K-thiocyanate}$  during elution increases binding to histones [Saunders, W., and W. C. Earnshaw, unpublished results], suggesting that histone binding may involve antibody species denatured by exposure to elevated pH. [d] Sequence analysis of clone  $\lambda$ -CNPB1 reveals no homology between the cloned protein and histones. There is thus no evidence that ACAs



**Figure 5.** Indirect immunofluorescence analysis of human chromosomes using monoclonal anti-CENP-B. HeLa chromosomes were centrifuged onto glow-discharged coverslips (12) and then prepared for indirect immunofluorescence using monoclonal antibodies (a) m-ACA1 and (b) m-ACA2 (both monoclonals were ascites fluid used at a dilution of 1:2,000). The arrow in *a* points out a large metacentric chromosome whose centromere has apparently split into two halves during spreading. (This effect has previously been observed using affinity-purified anti-CENP-B [10].) Bar, 10  $\mu$ m.



**Figure 6.** Demonstration that m-ACA1 and m-ACA2 bind to separate epitopes on fp-CNPBI. The cDNA from CNPBI was digested at the unique Asp-718 site and the two subfragments subcloned into the appropriate pATH vectors where they were expressed as fusion proteins in frame with the bacterial trpE polypeptide (see Materials and Methods for details). (A) Expression of the various fusion proteins: (lanes 1 in all panels) fp-trpE-CNPBI; (lanes 2) fp-trpE-3'CNPBI, the 3' Asp 718-Hind III fragment of  $\lambda$ CNPBI; and (lanes 3) fp-trpE-5'CNPBI, the 5' Eco RI-Asp 718 fragment of  $\lambda$ CNPBI. (A) Coomassie staining of SDS PAGE of the various lysates. The positions of each induced fusion protein are denoted by arrows at the left. Also noted at the left are molecular mass markers (68 kD [bovine serum albumin], 47 kD [ovalbumin], and 29 kD [carbonic anhydrase]). B-D show the results of probing blots of these fusion polypeptides with (B) autoantiserum GS; (C) m-ACA1, and (D) m-ACA2.

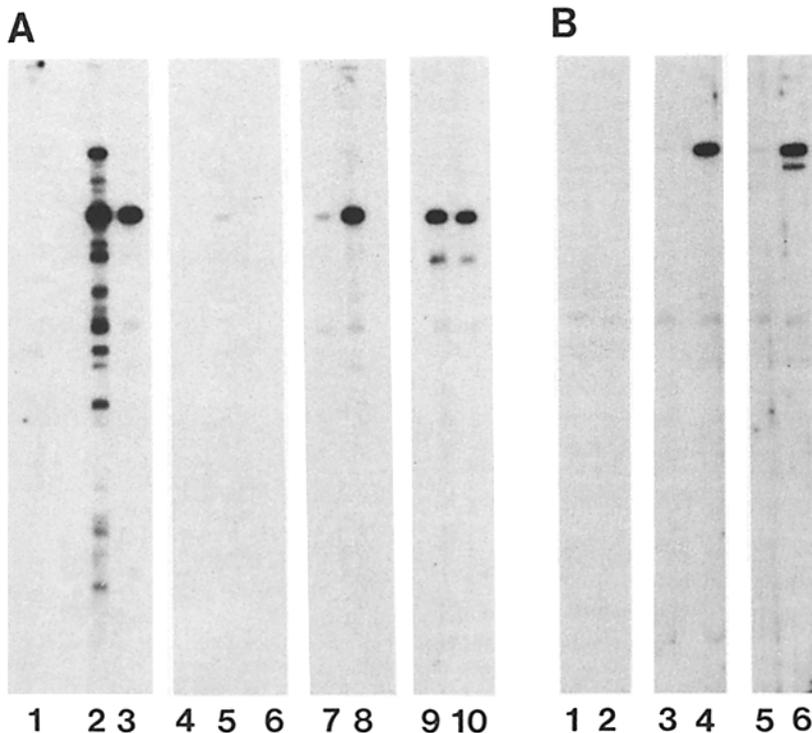
are related to antihistone antibodies often observed in patients with systemic lupus erythematosus [19, 38, 39].)

**Demonstration That the Polypeptide Encoded by  $\lambda$ -CNPBI Carries At Least Two Antigenic Sites in Common with CENP-B: Isolation of Polyclonal and Monoclonal Antibodies to fp- $\beta$ gal-CNPBI**

To verify that the protein encoded by  $\lambda$ -CNPBI is authentic CENP-B, we prepared polyclonal antibodies in a rabbit and murine monoclonal antibodies to the CNPBI protein fused to  $\beta$ -galactosidase (fp- $\beta$ gal-CNPBI). The isolated protein used for immunization was  $\sim$ 40% pure (see Materials and Methods).

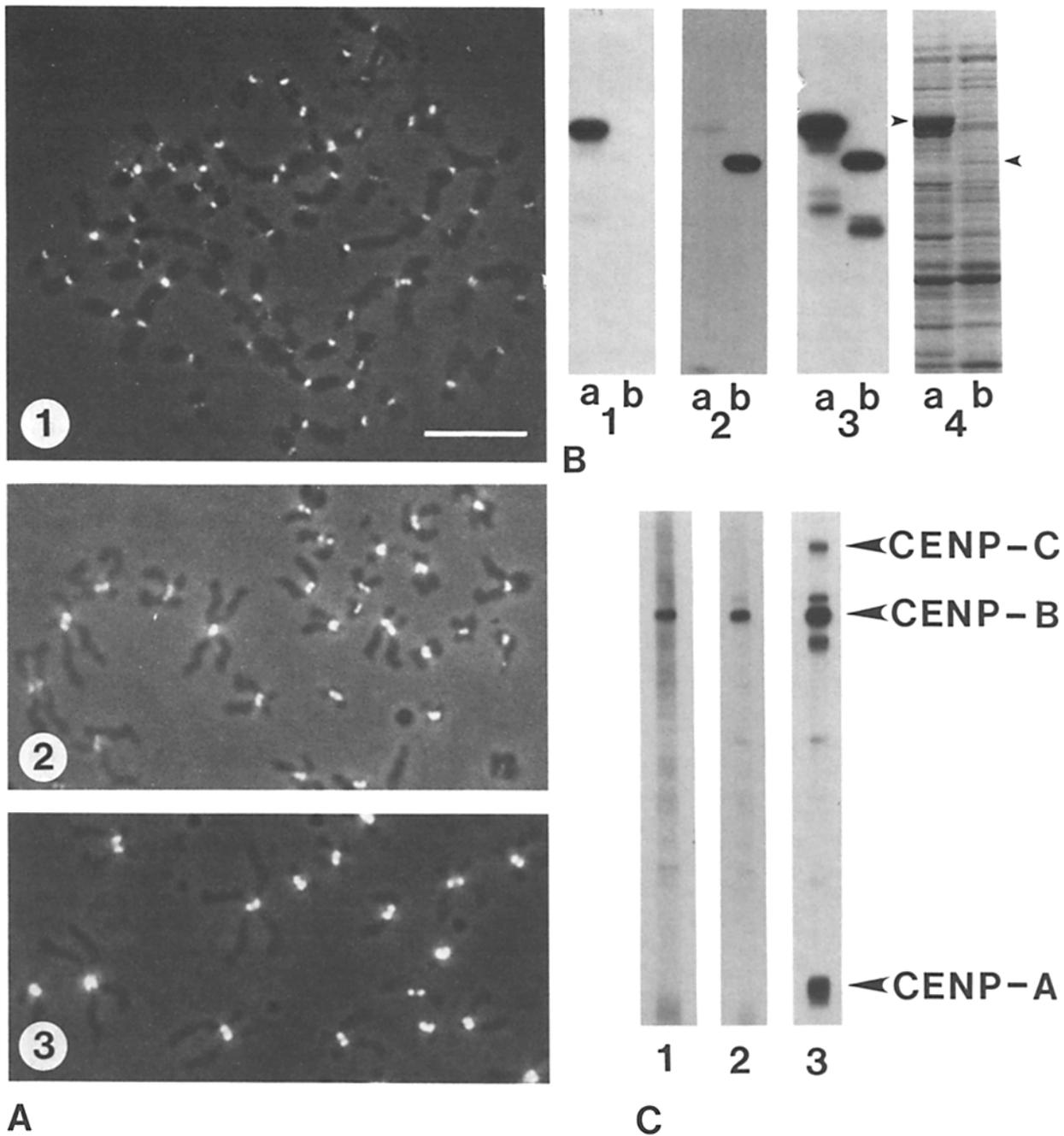
One rabbit produced antibodies (designated ra-ACA1) that not only recognized centromeres by indirect immunofluorescence (Fig. 3 b) but also bound to CENP-B on immunoblots of total chromosomal proteins (Fig. 4, lane 3). Preimmune sera did not bind in these assays (Fig. 3 a) nor did the immune serum bind to CENPs A or C (Fig. 4, lane 3).

Mice were immunized with fp- $\beta$ gal-CNPBI and hybridomas were screened with a trpE-CNPBI fusion protein to obtain two cloned cell lines secreting monoclonal antibodies to CNPBI (m-ACA1 and m-ACA2). (Use of the trpE fusion protein enabled us to readily eliminate hybridomas recognizing  $\beta$ -galactosidase.) m-ACA1 and m-ACA2 bind to centromeres (Fig. 5, a and b) and CENP-B on blots of chromosomal proteins (Fig. 4, lanes 8 and 9). When tested for binding to trpE fusion proteins containing 104 residues from the NH<sub>2</sub> terminus (Fig. 6, lanes 3) or 43 residues from the



**Figure 7.** Autoantibody GS and m-ACA1 bind to a common epitope on fp-trpE-CNPBI; m-ACA2 binds to a separate determinant. A bacterial lysate containing fp-trpE-CNPBI was separated by SDS PAGE and electrophoretically transferred to nitrocellulose. Individual strips of the resultant blot were then probed for immunoreactivity with the following sera. (A) lanes 1-3: positive controls. (Lane 1) Normal human serum WE (dilution 1:1,000); (lane 2) rabbit anti-CENP-B (dilution 1:1,000); (lane 3) autoantibody GS (dilution 1:5,000). Antibody binding was detected with <sup>125</sup>I-protein A. Lanes 4-6: negative controls. (Lane 4) PTX-BSA buffer; (lane 5) autoantibody GS (dilution 1:100); (lane 6) normal serum WE (dilution 1:100). Bound human antibody was detected with <sup>125</sup>I-labeled goat anti-mouse IgG. Note that the anti-mouse secondary antibody does cross-react weakly with the human autoantibody. (Lanes 7-10) Epitope blocking experiment. Each nitrocellulose strip was incubated overnight with autoantiserum GS (dilution 1:100; lanes 7 and 9) or normal serum WE (dilution 1:100; lanes 8 and 10). The strips were then washed and probed with culture supernatant of m-ACA1 (lanes 7 and 8) or m-ACA2 (lanes 9 and 10). Bound mouse antibody was detected with <sup>125</sup>I-goat anti-mouse IgG. (B)

Binding of both mAbs m-ACA1 and m-ACA2 is blocked by preadsorption with rabbit anti-CENP-B (ra-ACA1). (Lanes 1 and 2) Binding of immune (lane 1) or preimmune (lane 2) rabbit serum ra-ACA1 (both diluted 1:10), detected with <sup>125</sup>I-goat anti-mouse IgG. There is no visible cross-reaction. (Lanes 3-6) Epitope blocking experiment. Nitrocellulose strips were incubated overnight with immune serum ra-ACA1 (lanes 3 and 5) or preimmune serum (lanes 4 and 6), both at a dilution of 1:10. After washing, the strips were probed with (lanes 3 and 4) m-ACA1 or (lanes 5 and 6) m-ACA2. In both cases pretreatment with the rabbit polyclonal serum abolished subsequent binding of the monoclonal anti-CENP-B.



**Figure 8.** A determinant from the amino-terminal portion of CNPB4 represents a third epitope shared by fp-trpE-CNPB4 and chromosomal CENP-B. (A) Indirect immunofluorescence of chromosomes using (panel 1) antibodies affinity purified against fp-trpE-5'CNPB4, (panel 2) antibodies affinity purified against fp-trpE-CNPB1, and (panel 3) whole KG serum. Affinity-purified antibodies were used at a dilution of 1:40 for immunoblots and 1:2 for immunofluorescence. Whole serum KG was used at a dilution of 1:4,000 throughout. Lysates from induced bacteria were prepared from clones expressing either fp-trpE-5'CNPB4 (B, lanes a) or fp-trpE-CNPB1 (B, lanes b). Parallel samples were subjected to SDS PAGE and three were electroblotted to nitrocellulose, while a fourth (B 4) was stained with Coomassie Blue. (Note that the 5' fusion accumulates to a much greater level than does fp-trpE-CNPB1.) Each blot was probed as follows: (B 1) Antibodies affinity purified from serum KG by binding to fp-trpE-5'CNPB4; (B 2) antibodies affinity purified from serum KG by binding to fp-trpE-CNPB1; and (B 3) whole serum KG. (C) Immunoblotting of chromosomal proteins probed with antibodies affinity purified against fp-trpE-5'CNPB4 (lane 1), affinity purified against fp-trpE-CNPB1 (lane 2), and whole KG serum (lane 3).

COOH terminus of CNPB1 (Fig. 6, lanes 2), m-ACA2 bound to the NH<sub>2</sub>-terminal region (Fig. 6 D) but neither autoimmune serum GS nor m-ACA1 bound to either subfragment of CNPB1 (Fig. 6, B and C). Therefore, m-ACA1 and 2 bind to distinct epitopes on fp-trpE-CNPB1, a conclusion that is further supported by the observation that m-ACA2, but not

m-ACA1, binds to a proteolytic fragment derived from the fusion protein (see the star in Fig. 6). The data also suggest that the autoepitope spans the junction between the two sub-cloned portions of CNPB1 or alternatively, that the epitope may be located close to this site and may not adopt a native conformation in the trpE fusion protein.

In competitive binding experiments on blots of fp-trpE-CNPB2, antiserum GS inhibits binding of m-ACA1 but not m-ACA2 (Fig. 7 A). Similar results were obtained using chromosomal CENP-B rather than fp-trpE-CNPB2 as antigen (not shown). In a control experiment, ra-ACA1 completely inhibited the binding to fp-trpE-CNPB2 of both m-ACA1 (Fig. 7 B, lanes 3 and 4) and m-ACA2 (Fig. 7 B, lanes 5 and 6). Therefore, m-ACA 1 binds at, or adjacent to, an epitope recognized by the human antiserum.

Overall, these data demonstrate that m-ACA 1 and 2 bind to distinct sites on fp-trpE-CNPB1. Since both antibodies also bind to CENP-B (Fig. 4), the cloned fusion protein and CENP-B contain at least two epitopes in common.

### ***A Third Epitope Shared by CNPB4 and Chromosomal CENP-B***

To test whether any epitopes recognized by autoimmune sera are present in the NH<sub>2</sub>-terminal region of CNPB4, we constructed a plasmid that produces a fusion protein (fp-trpE-5'CNPB4) consisting of the 347 NH<sub>2</sub>-terminal amino acids of CNPB4. None of these residues is present on the original antibody-positive fusion protein CNPB1. When 22 ACA(+ ) patient sera were screened for binding to fp-trpE-5'CNPB4, five sera showed significant binding (Fig. 8 B, lane *a* in gel 3; and Earnshaw, W. C., manuscript in preparation). Since the residues in 5'CNPB4 and CNPB1 do not overlap, these five sera must recognize a second, independent autoepitope on CNPB4.

Affinity purification demonstrates that this epitope is also present on authentic CENP-B. ACA(+ ) serum KG bound to the newly identified epitope as well as it did to the original epitope on CNPB1, thereby permitting us to prepare KG-anti-5' (affinity purified from fp-trpE-5'CNPB4) and KG-anti-3' (purified from fp-trpE-CNPB1). These affinity-purified antibodies are highly specific (Fig. 8 B). For example, KG-anti-5' recognizes only 5'CNPB4 and not CNPB1 in immunoblots (Fig. 8 B, lanes *a* and *b* in gel 1). Most importantly, this new 5' epitope is also present on authentic CENP-B (Fig. 8 C) and at centromeres (Fig. 8 A, panel 1). In control experiments, whole serum KG and KG-anti-3' also bound to CENP-B and to centromeres (Fig. 8 A and C). (KG-anti-3' also bound weakly to fp-trpE-5'CNPB4 [Fig. 8 B, lanes *a* and *b* in gel 2].) We attribute this slight binding to the fact that CNPB1 is a very "sticky" protein (Earnshaw, W. C., unpublished observations) that may adsorb low levels of anti-5' antibodies. These would readily be detected, since fp-trpE-5'CNPB4 is the most abundant polypeptide present in the induced lysate [Fig. 8 B, lane *a* in gel 4].)

We have thus demonstrated that fp-CNPB4 and chromosomal CENP-B share at least three independent epitopes (two defined as epitopes and one defined by m-ACA2). We conclude that clone CNPB4 is derived from an mRNA that encodes CENP-B.

### ***Sequence Analysis of CENP-B Shows It to Be an Acidic Protein Containing Two Long Clusters Comprised Almost Entirely of Acidic Amino Acids***

The nucleotide sequence (determined according to the strategy in Fig. 1) of the 2,617 bases of cDNA in CNPB4 is shown in Fig. 9. The sequence contains a single open reading frame encoding 594 amino acid residues, followed by a 3' untranslated region of 832 bases that terminates at a short poly

A sequence. The correct reading frame of CENP-B was specified both by this single long open translation unit and by the production of an immunoreactive fusion protein when the cDNA was translated in this reading frame. Since the cDNA sequence does not contain an appropriate methionine translation initiation codon, it is unlikely that CNPB4 carries the entire CENP-B coding sequence.

Judging from a blot of HeLa cell RNA, a single mRNA species 2,900 bases long encodes CENP-B (Fig. 10 A). Assuming a poly A tail of 100–200 residues, this suggests that CNPB4 is 50–150 bases (or 16–50 amino acids) short of full length.

At first glance these results seem inconsistent with the apparent size of 80 kD for authentic CENP-B, since the single open reading frame encodes a polypeptide of only 64,400 D. However, as tabulated in Table II, all fusion proteins containing CENP-B sequences migrate anomalously in SDS PAGE. For example, electrophoresis of the 47,400-D human CNPB2 polypeptide fused to a 16-amino acid leader (including four amino acids from  $\beta$ -galactosidase and 12 encoded by the polylinker sequence of pUC9) yielded an immunoreactive hybrid protein with an apparent molecular mass of 71,000.

This anomalous behavior may result from a region of primary sequence that contains two extended clusters exceedingly rich in acidic residues. In one stretch of 62 amino acids (in boxed areas of Fig. 9) 79% of the residues are glutamic or aspartic acid (45 glu, 4 asp). A second 31-amino acid region occurring 45 residues closer to the carboxy terminus is comprised of 85% acidic residues (12 glu, 15 asp).

The overall sequence predicts that the cloned portion of CENP-B is an acidic polypeptide with a pI of  $\sim$ 5.2. Two-dimensional immunoblots of chromosomal proteins confirm that authentic CENP-B has a pI of  $\sim$ 5.7 (not shown).

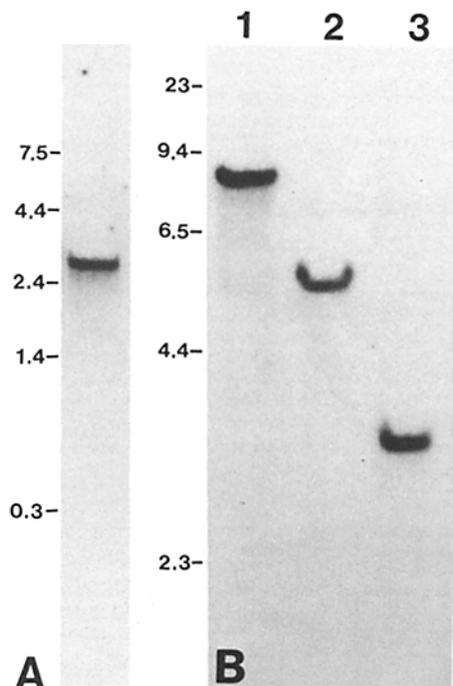
### ***CENP-B Is Encoded by a Single Copy Human Gene***

To determine the complexity of human DNA sequences with high homology to CENP-B, genomic DNA blot analysis was performed with cDNA from cloned  $\lambda$ -CNPB1. The resultant autoradiogram is shown in Fig. 10 B, lanes 1–3. Since only a single hybridizing fragment is present in any of three digests of human DNA, we conclude that CENP-B is encoded by a single copy gene.

### ***Discussion***

We have isolated and characterized cloned cDNAs for CENP-B, the major human centromere autoantigen. Five independent lines of evidence demonstrate that this clone encodes authentic CENP-B. First, expression of the cDNA in a bacterial vector yielded an immunoreactive fusion polypeptide that bound (and could be used to affinity purify) antibodies against CENP-B. Second, the apparent size on SDS PAGE and acidic pI of the nearly full-length cloned protein are appropriate for CENP-B. Third, polyclonal antibodies raised against the cloned fusion protein also recognize chromosomal CENP-B. Fourth, two mAbs that recognize distinct, non-overlapping epitopes on the cloned fusion protein also recognize the corresponding epitopes on chromosomal CENP-B. Fifth, autoimmune sera recognize at least two distinct sites present both on authentic CENP-B and on the polypeptide encoded by the cloned CNPB4 cDNA. On the





**Figure 10.** CENP-B is encoded by a single-copy human gene that is transcribed into a 2,900-base mature mRNA. (A) Poly A(+) RNA (1  $\mu$ g) from HeLa cells was analyzed by RNA blotting after electrophoresis on a denaturing formaldehyde agarose gel. CENP-B RNAs were detected by autoradiography of a blot hybridized to a  $^{32}$ P-labeled probe from CNPB1. For accurate determination of CENP-B mRNA size, a collection of RNAs of known sizes (from Bethesda Research Laboratories) were run in an adjacent lane and their migration positions (marked in kilobases at the left) visualized by staining with ethidium bromide. (B) Genomic human DNA was digested with each of three restriction endonucleases and blotted to nitrocellulose after electrophoresis and denaturation. Fragments homologous to CENP-B were identified by autoradiography after hybridization to a  $^{32}$ P-labeled probe constructed from CNPB1. (Lane 1) Eco RI; (lane 2) Hind III; (lane 3) Bam HI.

protein migrates extremely anomalously in SDS PAGE. Therefore, CENP-B is highly unlikely to be derived from CENP-C by proteolysis. Nor are CENP-B and CENP-C the products of closely related genes, since only a single gene for CENP-B was detected in genomic DNA.

Evidence previously obtained with affinity-purified antibodies further highlights the complexity of the relationship between CENP-B and CENP-C. When anti-CENP-C antibodies were affinity purified from serum GS, they did not rebind to CENP-B (10). However, the anti-CENP-B antibodies

from this serum did recognize epitopes common to CENP-B and CENP-C (10).

The relationship between CENP-A and CENP-B is less clear. CENP-A could be derived from CENP-B by proteolytic cleavage, since antibodies affinity purified from it also recognize CENP-B in immunoblots (10). However, if CENP-A were in fact derived from CENP-B, the shared epitope must reside in the extreme NH<sub>2</sub>-terminal region, since autoantibodies affinity purified from our longest CENP-B fusion protein (fp-CNPB4) do not recognize CENP-A.

Although we have screened our cDNA libraries extensively for clones that correspond to CENP-A and CENP-C, none have yet been found. Possible explanations for this are as follows.

(a) The epitopes on CENP-C may be localized near the NH<sub>2</sub> terminus, and cDNAs of sufficient length (>3,000 bp) may be rare in our libraries.

(b) Similarly, the epitope(s) on CENP-A might originate from the very NH<sub>2</sub> terminus of CENP-B, and these might also be present only rarely in our libraries.

(c) The epitopes common to CENPs A and B and CENPs B and C might arise from posttranslational modification of the proteins. Such epitopes cannot be cloned by the  $\lambda$ gt11 method, since *E. coli* does not perform such modifications. However, if this is the case, then two distinct modifications on CENP-A and CENP-C must be involved since affinity-purified anti-CENPs A and C do not cross-react (10).

(d) CENPs A and C might be the products of independent genes and might be transcribed from mRNAs present at very low abundance in the cells from which our cDNA libraries were made.

(e) CENP-A could be a centromere-specific histone subtype, as suggested by the experiments of Palmer and Margolis (35). In this case, CENP-A could be encoded by a poly A(-) mRNA that would be absent from our cDNA libraries.

We cannot at present distinguish among these possibilities.

### Significance of the Poly-acidic Domain of CENP-B

Initially we were surprised that a chromatin protein like CENP-B would have highly acidic subdomains. However, other chromatin proteins (the most notable example being HMG-1 [51]) also have acidic subdomains. It seems unlikely that such acidic regions would engage in direct interactions with the DNA. Rather, they must interact with basic regions of other chromosomal proteins.

The most striking examples of basic protein domains are the NH<sub>2</sub>-terminal regions of the core histones (22). These domains are, in general, ~20–30 residues in length, and are highly lysine and arginine rich. It was initially assumed that these regions would anchor the histones to the DNA and be

**Table II. Anomalous Migration of Fusion Proteins Containing CENP-B**

Fusion protein	Vector	True molecular mass	Apparent molecular mass	Discrepancy
		kD	kD	
fp- $\beta$ gal-CNPB1	$\lambda$ gt11	133	150	17
fp-trpE-CNPB1	pATH 11	50	68	18
fp-trpE-CNPB2	pATH 3	79	102	23
fp- $\beta$ gal-CNPB2	pUC9	48.5	71	22.5
fp-trpE-CNPB4	pATH 11	97	123	26

important for nucleosome structure, but subsequent analyses suggest that their function is more subtle (see review by McGhee and Felsenfeld [30]). If the NH<sub>2</sub>-terminal domains are removed by proteolysis (52), the truncated histones retain the capability of assembly into nucleosomes (53; reviewed in reference 30). Therefore, these domains do not appear to be essential for intra-nucleosomal contacts.

Nucleosomes constructed from truncated histones do, however, differ in one significant respect from normal nucleosomes—they are not readily induced to form 30-nm higher-order structures (1, 53). Therefore the basic domain appears to participate in nucleosome packing.

We suggest that the acidic regions of certain chromatin proteins (such as CENP-B) may serve to “capture” the NH<sub>2</sub>-terminal histone domains. This capture might then remove the domains from participating in stabilization of the higher-order chromatin fiber, causing the fiber to open into the 10-nm “beads on a string” conformation. The open conformation would then permit other proteins (or other domains of the original protein) to more readily scan the DNA for specific recognition sequences such as have been shown to specify centromere location and function in *Saccharomyces* (6, 15, 20, 21, 28, 36, 45).

### *The Amount of CENP-B Varies between Chromosomes*

The micrographs of Figs. 2, 4, and 5 reveal a very unexpected result concerning the distribution of CENP-B in the various chromosomes: the level of CENP-B antigen varies widely. This variation is seen with affinity-purified antibodies from the autoantiserum (Fig. 2 c, panel 2), with rabbit polyclonal anti-CENP-B (Fig. 4 b), and, most convincingly, with monoclonal anti-CENP-B (Fig. 5).

This finding has important implications for the possible structural distribution and physiological role of CENP-B in vivo. To our knowledge, the size of the kinetochores on various chromosomes has not been rigorously compared; however, our expectation is that kinetochore size is relatively constant. In contrast, the amount of C-banding material (thought to be at least in part centromeric heterochromatin—reference 7) varies widely from chromosome to chromosome. The human Y chromosome is C-band negative and also lacks detectible CENP-B (Earnshaw, W. C., and G. Stettin, manuscript in preparation). The obvious conclusion to draw from these results is that CENP-B is somehow associated with centromeric heterochromatin.

Earlier immunolabeling (4) suggested that centromeric autoantigens are restricted to the kinetochore, where they occur throughout all layers. Based on our results, we predict that CENP-B occurs in the basal layer of the kinetochore, in association with centromeric heterochromatin. We therefore predict that CENP-B is unlikely to be involved in microtubule binding.

Examination of chromosomes stained with whole autoantiserum (Fig. 2 c, panel 1) shows all centromeres stained more or less equally. Thus, we infer that other components recognized by such autoantisera (i.e., CENP-A and CENP-C) may show a more uniform distribution and are better candidates for components of the kinetochore trilaminar plaque structure and/or microtubule binding.

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