Centromere Protein B Assembles Human Centromeric α-satellite DNA at the 17-bp Sequence, CENP-B Box

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Abstract. We purified 15,000-fold from HeLa cell nuclear extract the centromere antigen that reacts specifically with the 17-bp sequence, designated previously as CENP-B box, in human centromeric α-satellite (alphoid) DNA by a two-step procedure including an oligonucleotide affinity column. The purified protein was identified as the centromere protein B (CENP-B) by its mobility on SDS-PAGE (80 kD), and reactivities to a monoclonal antibody raised to CENP-B (bacterial fusion protein) and to anticentromere sera from patients with autoimmune diseases. Direct binding by CENP-B of the CENP-B box sequence in the alphoid DNA has been proved using the purified CENP-B by DNA mobil-

ity-shift assay, Southwestern blotting, and DNase I protection analysis. The binding constant of the antigen to the CENP-B box sequence is 6×10^8 M^{-1}. DNA mobility-shift assays indicated that the major complex formed between the CENP-B and the DNA contains two DNA molecules, suggesting the importance of the CENP-B/CENP-B box interaction in organization of higher ordered chromatin structures in the centromere and/or kinetochore. Location of DNA binding and dimerization domains in CENP-B was discussed based on the DNA mobility-shift assays performed with a protein fraction containing intact and partial cleavage products of CENP-B.

THE centromere is the essential domain in eukaryotic chromosomes for proper segregation of chromosomes to daughter cells at mitosis and meiosis (7). Sister chromatids are held together at the centromere after DNA replication until the coordinate disjunction takes places at the metaphase to anaphase transition. The centromere provides attachment sites for spindle microtubules on a chromosome by organizing kinetochore (36), a unique proteinaceous matrix having contact with centromeric chromatin on inner side and dynamic association with spindle microtubules on its outer layer (8, 28) and is seen by electron microscopy as a tri-layered disc-like structure (35, 37). Motor molecules required for positioning of chromosomes during the metaphase and anaphase stages may also reside within or near the centromere/kinetochore domain (16, 17, 22, 34, 39). These multiple centromere functions must in some way be directed by a *cis*-acting DNA sequence located in the centromere region. Indeed, specific centromere DNA sequences (CEN-DNA) in yeast *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* were identified both by physical mapping of centromere region DNA and by activity assay to select DNA segment responsible for imparting mitotic and meiotic stability to plasmids containing autonomously replicating sequences (9, 12, 18, 21). Although the minimal essential sequence of CEN-DNA for *S. pombe* is not completely delimited yet, the CEN-DNAs of two yeast species are quite different in chain length and lack obvious sequence conservation (11). The

mammalian centromere is cytologically defined as the primary constriction in metaphase chromosome. However, identification of the DNA segment executing mammalian chromosome segregation has not been straightforward because of the high complexity of the DNA in centromere domain and the difficulty encountered in constructing an activity assay.

The primary constriction of mammalian chromosomes is made up of heterochromatin composed predominantly of highly repetitive DNA sequence (satellite DNA) (44). However, satellite DNA families located in the primary constriction of chromosomes in different mammalian orders (e.g., rodents and primates) appear largely unrelated in their sequences and unit length. Thus, all human chromosomes contain at the primary constriction the α-satellite (alphoid) DNA composed of tandem repeats of 170-bp diverged monomers organized into groups of polymeric units that are arranged tandemly in a larger unit repeated further in 300–5,000-kb area (25, 41, 45, 47). Human alphoid DNA thus varies in amount and sequence from chromosome to chromosome (44, 45). A kinetochore-related DNA sequence has not been identified with human cells. As for the mouse, *Mus musculus*, it has been shown by in situ hybridization that a minor-satellite DNA composed of tandem repeats of 120-bp monomer units localized on the outer surface of the centromere corresponding to the kinetochore domain (46).

With the use of centromere-specific autoantibodies (an-

ticentromere antibodies; ACA[1]) in serum of patients with autoimmune diseases, the localization of specific antigenic proteins has been determined at the centromere-kinetochore region of chromosomes from a number of mammalian species (6, 13, 29). With human cells, three major centromere-specific proteins (centromere protein [CENP]-A, B, and C) have been identified using ACA sera (14, 31). CENP-A (17-kD) is a histone-like protein component of a centromere-specific nucleosome (32, 33). The gene for CENP-B (80-kD) has been cloned and sequenced, and the property of the antigen is the most extensively studied among the three centromere antigens (7, 15). It was reported recently that CENP-B is a highly conserved protein in mammalian cells (40). The property of CENP-C (140-kD) is least known.

Previously, we have shown with HeLa cells that alphoid DNA and centromere antigens colocalize in both mitotic chromosomes and interphase nuclei by double-label in situ hybridization and immunofluorescence (27). We have shown subsequently that proteins from HeLa cell nuclear extract form a complex containing CENP-B with alphoid DNA containing the 17-bp motif (PyTTCGTTGGAAPuCGGGA). The 17-bp motif has been designated as "CENP-B box", since Southwestern blot analysis on a protein fraction, obtained from HeLa cell nuclear extract by immunoprecipitation with an ACA serum, had suggested strongly that CENP-B itself binds the 17-bp motif (26). The CENP-B box is found in only a subset of alphoid monomers but the CENP-B box–containing alphoid monomers are detected in all centromeres of human chromosomes except Y chromosome (26). The possibility of presence of a small amount of CENP-B box in Y chromosome alphoid DNA is still under investigation. Interestingly, the CENP-B box is also present in mouse minor satellite (26, 46). The conserved CENP-B and CENP-B box interaction in the mammalian centromere might offer a key to solve the common function carried by the centromeric heterochromatin without rigid sequence conservation.

To investigate the alphoid DNA and centromere antigen interaction further in molecular level, we purify in this study the CENP-B box binding protein from HeLa crude nuclear extract and show that CENP-B itself binds CENP-B box sequence in alphoid DNA. We then show that a DNA–protein complex thus formed contains two DNA molecules. This suggests the possible commitment of this DNA–protein interaction to the higher ordered structure formation in centromere domain.

Materials and Methods

Antisera and Monoclonal Antibody

ACA-positive sera were obtained from a lupus erythematosus patient, I.H., and a scleroderma patient, K.G. By immunoblotting analysis, these sera recognize CENP-A, B, and C (31). Normal human serum (donor: N.N.) does not recognize any centromere antigens. Anti–CENP-B monoclonal antibody (m-ACA1), which recognizes a site present within carboxy-terminal 147 amino acid residues in CENP-B (15), was the gift from Dr. W. Earnshaw (Johns Hopkins University, Baltimore, MD).

Buffers

Washing buffer was 20 mM Hepes, pH 7.6, 20 mM KCl, 0.5 mM EDTA, 0.5 mM DTT, 0.1 mM PMSF. Extraction buffer was 20 mM Hepes, pH 7.6,

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

0.5 mM EDTA, 0.5 mM PMSF, 0.5 μg/ml Pepstatin A, 4 μM Leupeptin (Sigma Chemical Co., St. Louis, MO). Buffer A was 20 mM Hepes, pH 7.6, 0.5 mM EDTA, 10% glycerol, 1 mM DTT, 0.2 mM PMSF. Buffer B was 20 mM Hepes, pH 7.6, 1 mM EDTA, 10% glycerol, 0.05% NP-40, 3 mM DTT, 0.1 mM PMSF. Binding buffer was 20 mM Hepes, pH 7.6, 10% glycerol, 1 mM EDTA, 3 mM DTT, 0.05% NP-40, and 150 mM NaCl (final concentration). Binding buffer II was as described by Masumoto et al. (26). TBE buffer was 12.5 mM Tris, pH 8.0, 12.5 mM boric acid, 0.5 mM EDTA.

Nuclear Extracts

HeLa cell culture and preparation of HeLa nuclei were performed as described by Masumoto et al. (26). The isolated HeLa nuclei were suspended in washing buffer (see above for buffer components) at the concentration of 2×10^8 nuclei/ml, mixed with equal volumes of extraction buffer supplemented with 0.4 M NaCl and 30% (vol/vol) glycerol, incubated 1 h at 0°C with gentle agitation, and then centrifuged for 5 min at 1,500 rpm in a rotor (model TS-9; Tomy Seiko, Tokyo, Japan). The pellet was extracted again with extraction buffer supplemented with 0.5 M NaCl and centrifuged at 25,000 rpm for 1 h in a rotor (type 40; Beckman Instruments Inc., Palo Alto, CA). The supernatant fraction was recovered and stored at −80°C (0.2–0.5 M NaCl nuclear extract).

Probes

The following complementary oligonucleotides were synthesized chemically and used for the probes or competitors containing a CENP-B box sequence.

The 56-mer DNA:

5'-TCAGAGGC [CENP-B box] TTTCTTCATATTATGCT AGACAGAATAATTC-3'
    CTCCG   AAAGAAGTATAATACGA TCTGTCTTATTAAGAGT

The 23-mer DNA:

5'-GGC [CENP-B box] TTT-3'
   G   AAACC

The 29-mer DNA:

5'-TCAGAGGC [CENP-B box] TTTC-3'
    CTCCG   AAAGAGT

The Py and Pu in the box was C and A, respectively. The defective 23-mer DNA had pyrimidine to purine base alterations at the underlined positions in the CENP-B box: 5'-TATCGTTGGAAAAGGGA-3'.

The end-labeled probe DNA molecules were prepared from the 23-mer, 29-mer, or 56-mer DNA by repairing the single-stranded ends with DNA polymerase I Klenow fragment and radioactive nucleotides. Resulting molecules were called the end-labeled 25-bp DNA, 32-bp DNA, or 59-bp DNA.

Preparation of Oligonucleotide-Sepharose

The oligonucleotide-sepharose was prepared according to the procedure described by Kadonaga and Tjian (23) with slight modification. The 56-mer DNA was ligated to form concatemers up to ~1,500 bp. The concatemeric oligonucleotides were then coupled to Sepharose-4B (Pharmacia Fine Chemicals, Uppsala, Sweden) with CNBr. About 50 μg of the oligonucleotides was covalently linked to 1 ml of Sepharose-4B with 40% coupling efficiency.

Purification of CENP-B

Nuclear extract (50 ml) was diluted with 50 ml of buffer A and loaded onto a 20-ml Q-sepharose column equilibrated with buffer A plus 250 mM NaCl. The column was washed with 100 ml of buffer A containing 250 mM NaCl and bound proteins were eluted with linear NaCl gradients, from 250 to 500 mM in 100 ml buffer A and then from 0.5 to 1.0 M in 100 ml of the same buffer. The Q-sepharose fractions with the specific binding activity to the end-labeled 59-bp DNA (fractions 39–54 in Fig. 1) were pooled together and diluted 3.1-fold with buffer B. After the addition of sonicated salmon sperm DNA at a final concentration of 25 μg/ml, the pooled material (94 ml) was mixed with 500 μl of the oligonucleotide-Sepharose resin and incubated for 2 h at 0°C with gentle agitation. The resin was packed into a column, washed with 5 ml of buffer B plus 150 mM NaCl, and bound pro-

teins were eluted with a 30-ml linear gradient of NaCl concentration from 150 mM to 1 M in buffer B. Fractions with specific binding activity to the 59-bp DNA were stored at −80°C.

DNA Mobility Shift Assays

End-labeled 59-bp DNA (10 fmole or indicated amount) was incubated in 20 μl of binding buffer containing 1 μl or indicated amounts of a protein fraction and poly(dI·dC)·poly(dI·dC) for 2 h at 0°C. Where specified, end-labeled 25-bp or 32-bp DNA containing CENP-B box was used as the probe instead of the 59-bp DNA. The reaction mixtures (10 μl) were electrophoresed on polyacrylamide gels in TBE buffer at 20 V/cm at 4°C, and gels were dried and exposed to film (XAR-5; Eastman Kodak Co., Rochester, NY). Where specified, 1 μl of ACA-positive or healthy control serum (dilution 1:30 with PBS) was added to the mixtures after the binding reaction and incubated for 30 min at 0°C.

Renaturation of Proteins from SDS-PAGE

2.5 ml of the affinity column-purified material (fractions 11–15, 300 μl each; fractions 16–20, 200 μl each) was precipitated with 10 ml of acetone and the precipitates were fractionated on a 7.5% SDS-PAGE. 80-kD and 65-kD regions of the gel were excised according to molecular weight markers (Bio-Rad Laboratories, Cambridge, MA), and the proteins were electroeluted from gels in a half strength of Laemmli electrode buffer (24) containing 5 mM DTT. After the extract was precipitated with 4 vol of acetone, the precipitates were then dissolved in 4 μl of 6 M guanidine-HCl in buffer B and allowed to stand for 30 min at 4°C. The samples were then diluted 50-fold with buffer B containing 0.5 M NaCl and dialyzed against the same buffer at 4°C for 10 h.

Immunoprecipitation of DNA Complexed with Centromere Antigen

The binding activity of the centromere antigen to CENP-B box-containing DNA was determined by immunoprecipitation as described by Masumoto et al. (26) with the following modifications. The end-labeled 59-bp DNA (0.4 ng) was incubated with 1 μl of a nuclear protein fraction in 100 μl of binding buffer containing 0.4 μg poly(dI·dC)·poly(dI·dC) for 2 h at 0°C. 1 μl of an ACA-positive serum was added to the mixture and incubated for 30 min on ice. Then 20 μl of protein G–Sepharose (Pharmacia Fine Chemicals) was added to the mixture followed by a 30-min incubation on ice with gentle agitation. The mixture was centrifuged, the precipitate was washed three times with 0.5 ml of binding buffer containing 0.5% NP-40, and its radioactivity was determined by a liquid scintillation counter (Beckman Instruments Inc., Fullerton, CA). One unit of the binding activity of centromere antigens is defined as the activity that makes 1 fmole of the end-labeled 59-bp DNA immunoprecipitable with an ACA-positive serum.

Immunoblotting and Southwestern Blotting

Immunoblotting was done by the methods of Masumoto et al. (26) with the following modifications. ACA was used at 1:600 dilution. HRP-conjugated anti-human IgG or anti-mouse IgG (Bio-Rad Laboratories, Richmond, CA) was used at 1:3,000 dilution as the second antibody.

DNA binding activity of purified proteins immobilized to a polyvinyldene difluoride membrane (Millipore Continental Water Systems, Bedford, MA) (Southwestern blotting) was analyzed by the method described by Masumoto et al. (26) using the binding buffer II containing 100 mM NaCl instead of 50 mM. No competitor DNA was added to the reaction.

DNase I Protection Analysis

DNA fragments containing α169 sequence were restricted from plasmid pUCα169 (26) and end labeled with DNA polymerase I Klenow fragment, [α-^{32}P]dNTP (3,000 Ci/mmol; Amersham International, Amersham, UK) and the other three nonradioactive dNTPs as follows. For the labeling of L-strand, pUCα169 was cleaved by DdeI and end labeled with [α-^{32}P]-dCTP. For the labeling of R-strand, pUCα169 was cleaved by EcoRI and HincII and end labeled with [α-^{32}P]dTTP. DNA fragments (1.2 ng), end labeled at one of the complementary strands as above, was mixed with the specified amounts of purified CENP-B (oligonucleotide-sepharose fraction 17) in a 50-μl binding buffer containing 40 ng of poly(dI·dC)·poly(dI·dC) and incubated for 2 h at 0°C. To the mixture, 50 μl of 6 mM MgCl₂ and 1 mM CaCl₂, and 2 μl of 50 μg/ml solution of DNase I (Takara Shuzo, Kyoto, Japan) were added and DNA was digested for 1 min at 20°C. 50 μl of a DNase stop solution (95 mM EDTA, 0.8% SDS, 0.3 mg/ml salmon

sperm DNA) and 50 μl of 10 M ammonium acetate were added to the reaction mixture and DNA was precipitated with ethanol, collected by centrifugation, dried, resuspended in 8 μl of formamide loading buffer, and then electrophoresed on a 6% polyacrylamide sequencing gel. The gel was dried and exposed to film (XAR-5; Eastman Kodak Co.).

Determination of Apparent Equilibrium Constant (Kapp)

A series of standard binding reactions were performed using the fixed concentration of the purified CENP-B (oligonucleotide-sepharose fraction 17) and varying concentrations of the end-labeled 59-bp probe DNA (Ds°) in 10 μl reaction mixtures for 2 h at 0°C. 90 μl binding buffer containing 150 mM NaCl was then added to each reaction mixture and the amount of the probe DNA complexed with CENP-B (B·Ds) was determined by the DNA immunoprecipitation as described above. The concentration of unbound probe DNA (Ds) was determined by the difference; Ds = Ds° − BDs. The apparent equilibrium constant (Kapp) was determined by fitting the data according to the statistical method of Wilkinson (43). The apparent equilibrium constant of the binding reaction (Kapp) can be calculated according to the equation:

$$[BDs]/[Ds] = -Kapp[BDs] + Kapp[B°].$$

B° is the total amount of CENP-B. Ds and BDs are the free and bound species of the probe DNA, respectively. The nonspecific binding constant is determined by titrating a constant amount of CENP-B and the end-labeled 59-bp DNA with increasing amounts of unlabeled sonicated pUC119 DNA in average chain length of 400 bp. Dñ, which is nonspecific DNA (pUC119) present, is expressed as moles of the 17-bp equivalent in pUC119 DNA (moles of pUC119 × 3162 ÷ 17). The relationship among Kapp, Ks, and Kn is given by the equation:

$$1/Kapp = 1/Ks + Kn/Ks × [Dñ].$$

Ks and Kn are the equilibrium constants for specific and nonspecific binding reactions of CENP-B, respectively.

Other Procedures

Protein concentrations were determined by the method of Bradford (5) or assayed with the Bio-Rad Laboratories kit (20) with BSA as the protein standard. Protein was visualized after the gel was silver-stained (30).

Results

Purification of CENP-B Box Binding Protein

To purify the CENP-B box binding protein, DNA binding activity of the centromere antigen was routinely monitored by DNA immunoprecipitation (26) and DNA mobility shift assay using the end-labeled 59-bp DNA that contains the CENP-B box as described in Materials and Methods. The centromere antigen was detected by immunoblotting using ACA. HeLa 0.2–0.5 M NaCl nuclear extract was first chromatographed on a cationic exchange resin, Q-sepharose. This step separated the CENP-B box specific DNA binding activity and 80-kD centromere antigen (CENP-B) from 97% of the loaded proteins with good recovery of both activities (Table I, Fig. 1). The active Q-sepharose fractions were pooled and further purified with DNA recognition site affinity column (oligonucleotide-sepharose) as described in Materials and Methods (Fig. 2). This step brought 630-fold purification and 25% recovery of the specific DNA binding activity. On both columns, the distribution of the CENP-B box specific DNA binding activity and the 80-kD antigen (CENP-B) were correlated. Thus, 15,000-fold overall purification was achieved with a 15% recovery for the DNA binding activity as estimated by the DNA immunoprecipitation assay. The recovery of CENP-B was also ~15% as estimated by the method of immunoblotting (data not shown).

As shown in Fig. 2 A, the DNA binding activity of centro-

Table I. Purification of CENP-B

Fraction	Volume	Total protein	DNA binding activity*		Yield		Purification	
			Total	Specific	Per step	Overall	Per step	Overall
	ml	*μg*	*U*	*U/μg*	*%*		*-fold*	
Nuclear extract	100	200,000	75,000	0.38	–	–	1	1
Q-sepharose	32	5,200	45,000	8.7	60	60	23	23
Oligonucleotide-sepharose	7	2	11,000	5,500.0	24	15	630	15,000

* One unit corresponds to specific DNA binding activity to precipitate 1 fmole alphoid DNA by immunoprecipitation.

mere antigens was eluted from the oligonucleotide-sepharose column in two peaks. In the DNA mobility shift assay with the end-labeled 59-bp DNA, fractions from the minor peak produced four weak bands, whereas fractions from the major peak gave a strong band with the slowest mobility of the four (Fig. 2 *B*). In accordance with this result, an extra antigenic band in 65 kD was detected in the fractions from the minor peak (Fig. 2 *C*). A representative fraction from each peak was characterized further by immunoblotting and Southwestern blotting. With fraction 17 from the major peak, a major polypeptide band was detected at 80-kD mobility by the silver staining and a single reactive band was detected at

the same position with monoclonal antibody for CENP-B, m-ACA1 (Fig. 3, *A* and *B*). The end-labeled 59-bp DNA also bound to the 80-kD polypeptide (Fig. 3 *B*). These results show that the affinity-purified 80-kD protein is indeed CENP-B. Fraction 13 from the minor peak was concentrated to 10-fold and used for immunoblotting. An 80-kD antigen accompanied with similar amounts of 65-kD antigen was detected with both ACA serum and m-ACA1 (Fig. 3 *C*). An extra-faint band of intermediate mobility was also detected by m-ACA1. The Southwestern blotting showed that both the 80- and 65-kD bands bound specifically to the CENP-B box containing probe DNA, but the binding activity of the 65-kD
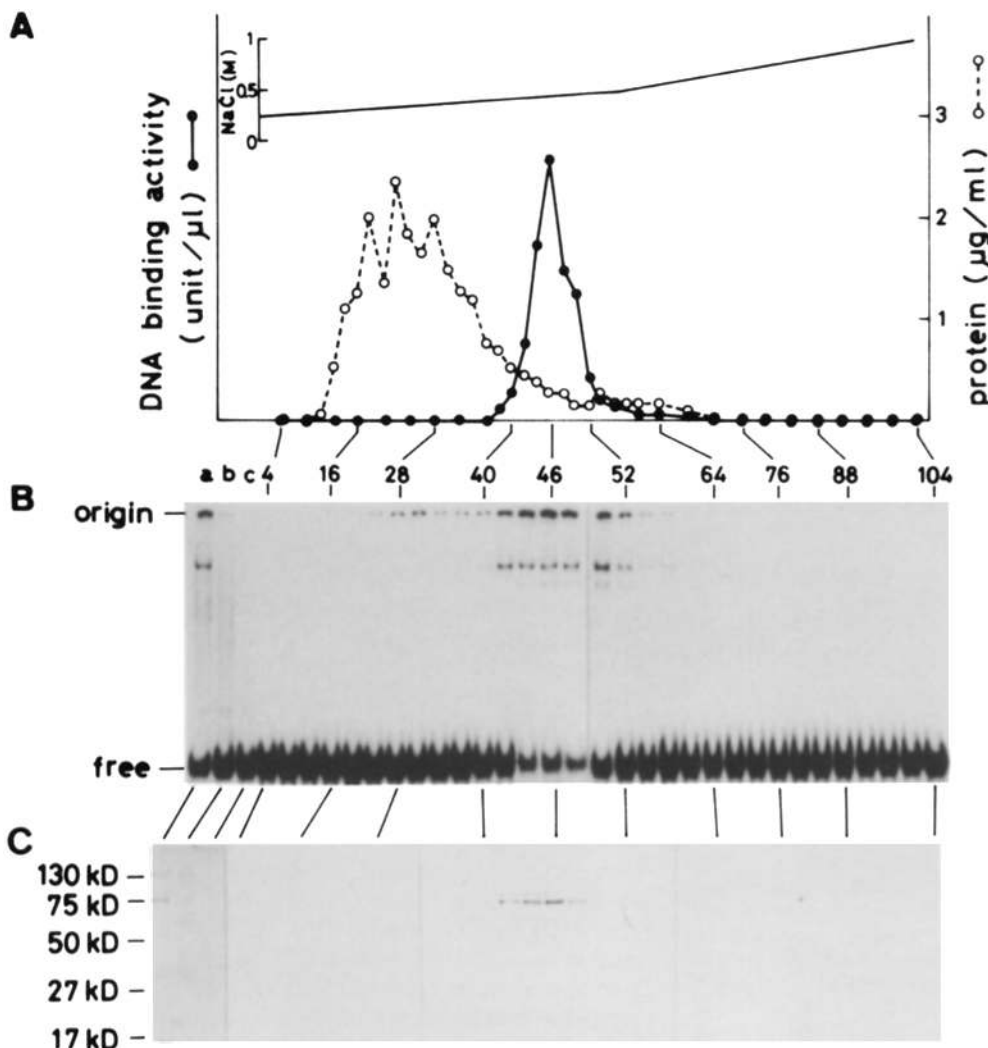

Figure 1. Fractionation of HeLa nuclear extract by Q-sepharose chromatography. HeLa nuclear extract was prepared as described in Materials and Methods and loaded onto a Q-sepharose column at 250 mM NaCl. The column was developed successively with two linear gradients of NaCl, 250–500 mM and 0.5–1 M. (*A*) The DNA binding activity (●) was determined by DNA immunoprecipitation assay using 1 μl of each fraction and ³²P-labeled 59-bp DNA containing CENP-B box. Protein concentrations (○) were determined by the Bradford dye assay. (*B*) 1 μl of each fraction was incubated with the end-labeled 59-bp DNA, and the resulting DNA–protein complex was analyzed by gel retardation assay. (*C*) Centromere antigen was detected by immunoblotting. 10 μl of each fraction was electrophoresed in 10% gel, transferred to the membrane, and probed with ACA (human IH serum). Lane *a*, HeLa nuclear extract; lane *b*, flow-through fraction; lane *c*, wash fraction; lanes *4–104*, fractions eluted from the Q-sepharose column.
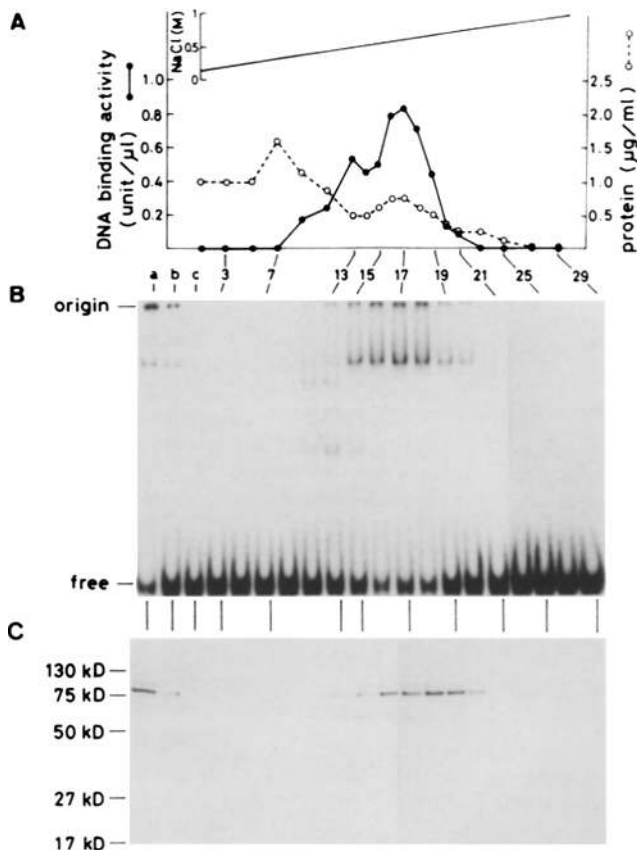
Figure 2. Purification of CENP-B box binding protein by DNA recognition site affinity chromatography. Q-sepharose fractions with specific DNA binding activity were pooled and affinity purified on an oligonucleotide-sepharose column as described in Materials and Methods. (*A*) Protein concentrations (○) were determined by a Bio-Rad Laboratories kit and specific DNA binding activity (●), which was determined as described in Fig. 1 *A*. (*B*) 1 μl of each fraction was incubated with the end-labeled 59-bp DNA and the DNA–protein complex was analyzed by gel retardation assay. (*C*) Centromere antigen was analyzed by immunoblotting as described in Fig. 1 *C*. Lane *a*, Q-sepharose pooled fraction; lane *b*, flow-through fraction; lane *c*, wash fraction; lanes *1–29*, fractions eluted with NaCl gradient buffer (0.15–1.0 M NaCl, 30 ml) from the oligonucleotide-sepharose column.

antigen was considerably weaker than that of the 80-kD antigen. A faint radioactive band was also detected at the intermediate position. In addition, a weak radioactive band was detected above the 80-kD band. It might be the same band detected in Figs. 1 and 2 above the 80-kD antigen.

Interaction Sites of CENP-B on Alphoid DNA

The binding sites of CENP-B on a 169-bp alphoid monomer (α169) have been analyzed by DNase I protection analysis using the affinity-purified CENP-B preparation and the α169 containing DNA fragments which have been labeled at the 3' end of the L- or R-strand (Fig. 4). In both L- and R-strands, affinity-purified CENP-B (fraction 17) protected a 25-nucleotide sequence centered over the CENP-B box. Protection of other sites on the probe DNA was not detected. An enhanced DNase I cleavage site (*arrowheads* in Fig. 4) was observed at nucleotides 248 and 246 on the R- and L-strand of α 169 DNA, respectively. Similar results were obtained

using the oligonucleotide-sepharose fraction 13 (data not shown).

Equilibrium Constants for CENP-B Binding to Alphoid DNA

The binding reaction was further characterized by kinetic studies. The analysis was performed on the assumption that the interaction between CENP-B and DNA was a simple bimolecular action. The equilibrium constant for CENP-B binding was determined by titrating a constant amount of purified CENP-B with increasing amounts of the end-labeled 59-bp DNA and analyzing the binding reaction using the DNA immunoprecipitation assay. A typical titration curve is shown in Fig. 5 *A*. The data in section *A* can be used to determine the concentration of active CENP-B (B°). The curve drawn corresponds to a B° of 1.1×10^{-9} M. The concentration of active CENP-B in fraction 17 can be determined using B° and the molecular mass of CENP-B, which is calculated as 65 kD from the amino acid composition (40). The concentration, 0.72 ng/μl, is about the same as the protein concentration of fraction 17, 0.75 ng/μl. Kapp, which must correspond to Ks in this assay without competitor DNA, is calculated as 6.4×10^{8} M^{-1}, according to section *B*, to which the data in section *A* are replotted. From the experimental data shown in Fig. 5 *C*, Kn/Ks and 1/Ks can be obtained as the slope of 5.5×10^{-5} and as the y intercept of 1.6×10^{-9} M, respectively. From these results, Kn is estimated as 3.4×10^{4} M^{-1}. Using this value, we can calculate the equilibrium constant for binding of CENP-B to "CENP-B box" (Ks) to be 6.3×10^{8} M^{-1}. This data well corresponds to above Kapp, which was determined in the assay without competitor DNA.

Properties of DNA–Protein Complexes Detected by Gel Electrophoresis

Properties of CENP-B fractions with the different affinity to the oligonucleotide-sepharose column have been analyzed further by DNA band shift in gel electrophoresis using the end-labeled 59-bp DNA. With fraction 17, one major DNA–protein complex showing the slow mobility (referred to as complex A) was formed. With fraction 13, complex A and three additional complexes (complexes α, β, and γ) with faster mobilities were formed (Figs. 2 *B* and 6). Formations of all these bands were competed out with the excess amount (100-fold) of nonradioactive 23-mer DNA carrying the CENP-B box, while no competition was observed with the excess amount of the 23-mer DNA having sequence alterations in the CENP-B box (the defective 23-mer [see Materials and Methods]), indicating the dependence of all complex formation on CENP-B box sequence. The addition of an ACA serum after the binding reaction caused trapping of these complexes at the origin, but the addition of normal serum did not, indicating the presence of antigenic polypeptide(s) in all complexes (Fig. 6). These results, taken together with the results in Fig. 3 *C*, strongly suggest that all the complexes with different electrophoretic mobilities were formed by the specific interaction between the probe DNA and CENP-B and/or partial degradation products of CENP-B.

To determine the number of DNA molecules in complex A, the DNA band shift assay was performed using fraction
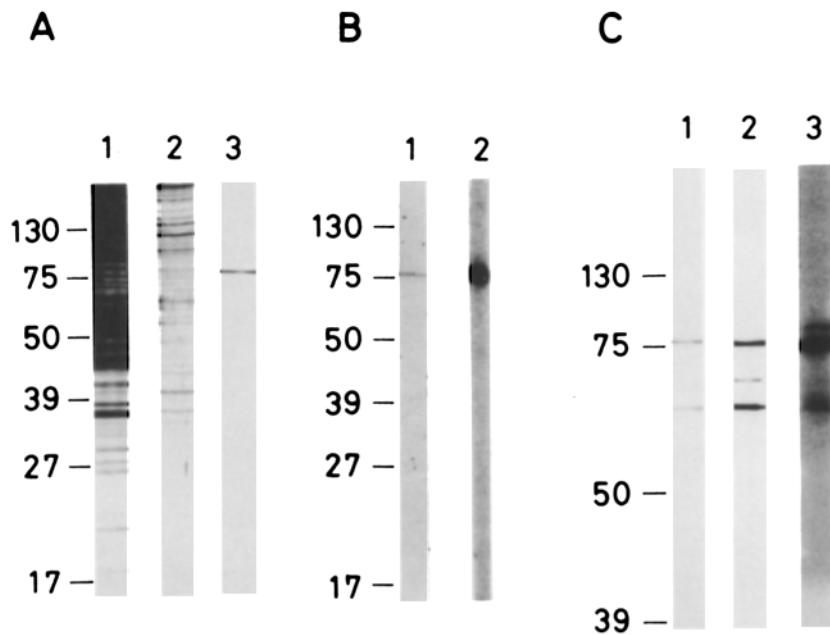
A   **B**   **C**

Figure 3. Characterization of antigens in fraction 13 and 17 from oligonucleotide-sepharose column by immunoblotting and Southwestern blotting. (*A*) Silver staining of proteins in different purification stages after SDS-PAGE. Gel concentration was 10.0%. Lane *1*, 1 μl of nuclear extracts; lane *2*, 10 μl of Q-sepharose-pooled fraction; lane *3*, 100 μl of fraction 17. (*B*) Immunoblotting analysis and DNA binding activity of fraction 17. Gel concentration was 10.0%. Lane *1*, 10 μl of sample was electrophoresed on SDS-gel, transferred to polyvinyldene difluoride membrane, and probed with m-ACA1 (1:500 dilution with PBS). Lane *2*, 10 μl of sample was electrophoresed and transferred to polyvinyldene difluoride membrane in parallel with the sample in lane *1*. The membrane was incubated with ³²P-labeled 59-bp DNA, washed, and analyzed by autoradiography. (*C*) Immunoblotting analysis and DNA binding activity of fraction 13. Gel concentration was 7.5%. Lane *1*, 100 μl of sample was precipitated with 80% acetone and treated as described in *B* for immunoblotting. Human KG serum was used as ACA. Lane *2*, immunoblotting performed in parallel with sample in lane *1* using m-ACA1 (1:500 dilution with PBS). Lane *3*, 200 μl of fraction 13 was precipitated with 80% acetone and Southwestern blotting was performed as described in *B*. Positions for mol wt (kD) marker proteins are indicated on the left of lane *1* in all panels.

17 and two probe DNA molecules in different chain lengths. When the end-labeled 59-bp or the 25-bp DNA with the CENP-B box was used as a probe in a binding reaction, a radioactive band was formed at A^{59} or A^{25} position, respectively (Fig. 7 *A*, lanes *1* and *8*). When an increasing amount of the nonradioactive 23-mer and 56-mer molecules were added to the reaction mixtures for lanes *1* and *8*, respectively, an extra band with the same intermediate mobility and an increasing amount appeared (Fig. 7 *A*, lanes *2–4* and *5–7*). Even with various ratios of competitors, no additional bands were detected. These results indicate that the complex A contains two molecules of DNA (see Fig. 9 for the possible structure of complex A).

The numbers of the DNA molecules in the other complexes (α, β, γ), were determined similarly by the band shift assay using fraction 14, which also contains 80- and 65-kD components. Fraction 14 formed the four complexes (complexes A, α, β, and γ) using the end-labeled 59-bp or 32-bp DNA (Fig. 7 *B*). When an increasing amount of the nonradioactive 29-mer and 56-mer molecules were added to the reaction mixtures for lanes *1* and *8*, respectively, no extra bands appeared for complex α, β, or γ, while an extra band appeared for complex A (Fig. 8 *B*, lanes *3–7*). These results indicate that the complex α, β, and γ contain one molecule of DNA.

To assess the constituting polypeptide in each complex, DNA band shift assays were performed using polypeptides recovered from 80- and 65-kD regions of SDS gels (Fig. 8
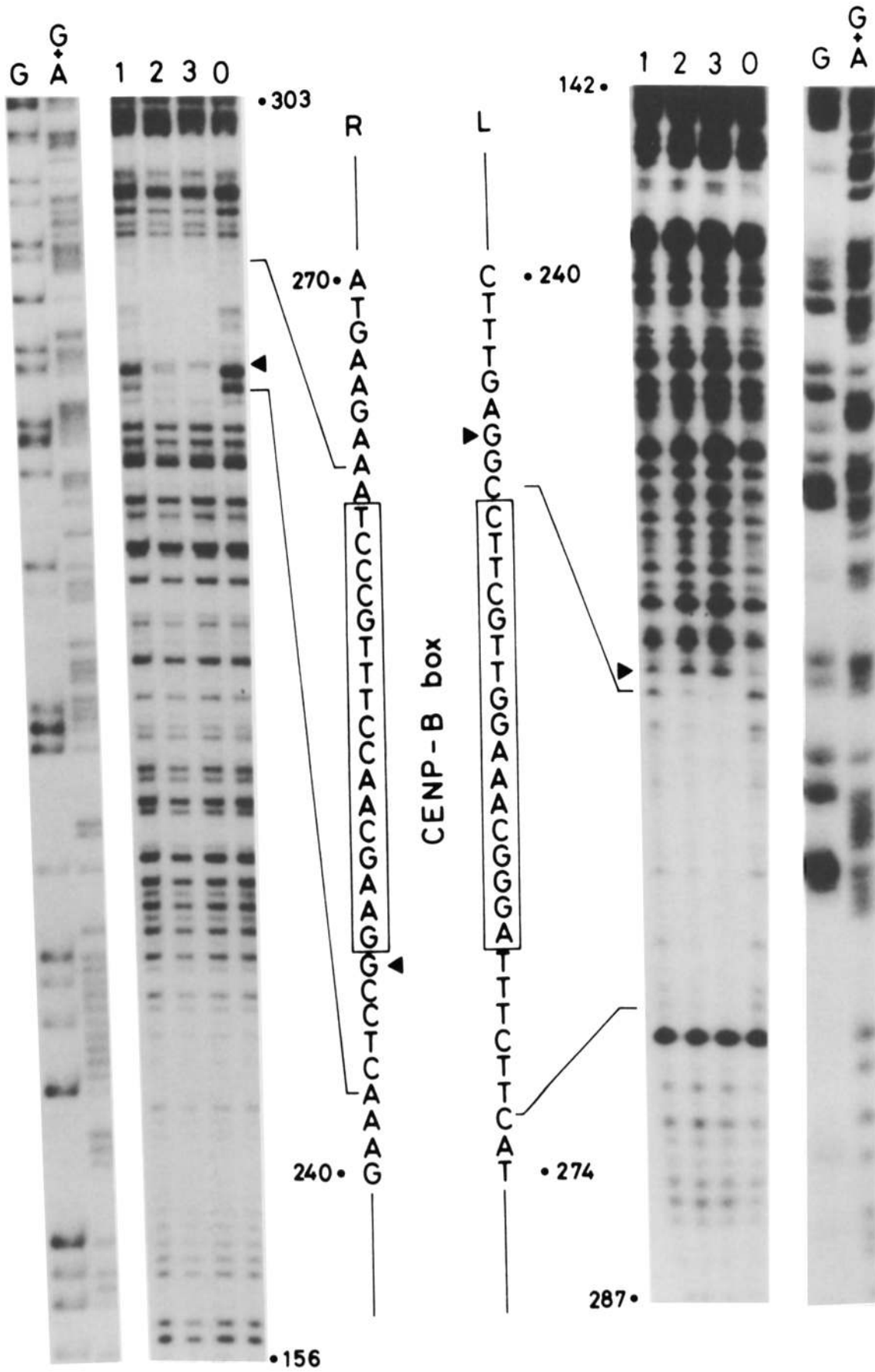
A). Using renatured 80-kD polypeptide, two bands were formed at the positions of complex A and β. Complex A and β may contain dimer and monomer of CENP-B, respectively, as discussed below. In the case of renatured 65-kD polypeptide, one major band was formed at the position of complex γ. The band at the position of complex α was not formed with either polypeptide. The susceptibility of the complexes formed with fraction 14 to the anti–CENP-B monoclonal antibody was examined (Fig. 8 *B*). The addition of m-ACA1 after the binding reaction caused trapping of complexes A, α, and β at or near the origin, but formation of complex γ was not influenced. Since complex γ was trapped at the origin by an ACA serum (Fig. 6), the polypeptide in the complex γ seems to be a fragment of CENP-B which lacks the epitope for m-ACA1, the COOH-terminal 147 amino acid residues in CENP-B. The result of complex γ is puzzling, since the immunoblotting analysis of fraction 13 has shown that the 65-kD band was reactive to both an ACA serum and m-ACA1 (Fig. 3 *C*). The 65-kD band may contain two species of antigens both derived from CENP-B but lost the different domains in the polypeptide (see Discussion for details).

Discussion

The Specific Interaction of CENP-B with the CENP-B Box Sequence

We have purified the CENP-B box binding protein 15,000-

Figure 4. DNase I footprinting of purified CENP-B on α169 DNA. DNase I protection analysis of α169 DNA was analyzed as described in Materials and Methods. A- and G-specific (lane *A+G*) and G-specific (lane *G*) sequencing reactions and DNase I digestion sites on the L-strand and R-strand, are shown. 1.2 ng of DNA end labeled at one of the complementary strands was incubated with 1 (lane *1*), 3 (lane *2*), 9 (lane *3*) or 0 (lane *0*) μl of fraction 17 from the oligonucleotide-sepharose column. Nucleotide sequence numbering corresponds to that in Fig. 3 of Masumoto et al. (27). Arrows (▲) indicate enhanced DNase I cleavage site on R strand at nucleotide 249 and L-strand at nucleotide 245.

R

•303

270• A
T
G
A
A
G
A
A
A
A
T
C
C
G
T
T
T
C
C
A
A
C
G
A
A
G
G
C
C
T
C
A
A
A
240• A
G

•156

CENP-B box

L

142•

C
T
T
T
G
A
G
G
C
C
T
T
C
G
T
T
G
G
A
A
A
C
G
G
G
A
T
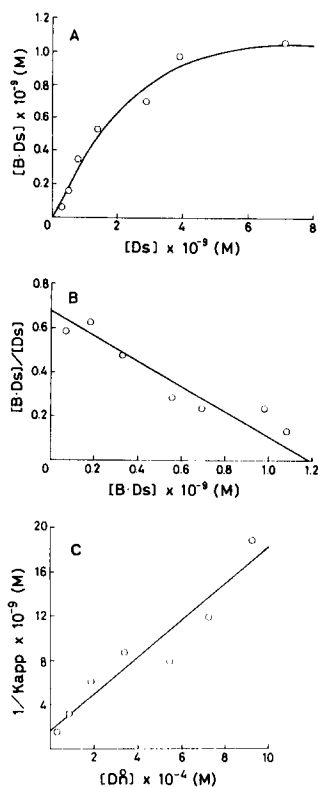T
T
C
T
T
C
A
T

•240

•274

287•

Figure 5. Titration of CENP-B with alphoid DNA. Binding reactions were carried out with constant amount of CENP-B (fraction 17 from oligonucleotide-sepharose column) and increasing amounts of ^{32}P-labeled 59-bp DNA and analyzed as described in Materials and Methods. Reaction mixtures contained no competitor DNA. (*A*) CENP-B was titrated with increasing amounts of ^{32}P-labeled 59-bp DNA. (*B*) The data of Ds and B·Ds were plotted to calculate the apparent equilibrium constant. Ds and B·Ds are the free and bound species of the 59-bp DNA, respectively. The solid lines were drawn by fitting the data under the method of Wilkinson (43). (*C*) Determination of Kn. Binding reactions were carried out as described in *A*, except that the concentration of ^{32}P-labeled 59-bp DNA was kept constant (1 nM), whereas the amount of nonspecific DNA (sonicated pUC119) was varied. Kn was determined by linear least squares fit of the 1/Kapp vs. Dñ data (*solid line*). Dñ, nonspecific DNA (pUC119).

fold from HeLa nuclear extract by a two-step procedure (Table I). Judging from the protein mobility on SDS-PAGE (80 kD), the reactivity to both ACA sera and m-ACA1 (a monoclonal antibody recognizing COOH-terminal residues of CENP-B), and copurification of CENP-B box specific DNA binding activity and 80-kD centromere antigen throughout two-step column chromatography, we concluded that CENP-B

itself binds the CENP-B box. Indeed, DNase I protection analysis of alphoid DNA showed that a 25-bp region centered over CENP-B box was protected with the purified preparation of CENP-B (Fig. 4). We delimited previously the 17-bp recognition sequence by the immunoprecipitation of the two deletion series of alphoid DNA starting from each end of the α 169 DNA (26). Although the consensus recognition sequence is yet to be determined, the fact that the CENP-B box was commonly found in various immunoprecipitable alphoid monomers (although flanking sequences were variable), reinforces the notion that the recognition sequence resides within the CENP-B box sequence (26). However, the 17-bp DNA alone was inactive as a probe for the immunoprecipitation assay or a competitor for the DNA-mobility shift assay (data not shown). Also, the efficiency of the complex formation of CENP-B was greater with the 59-bp probe than with the 25-bp probe (Fig. 7 *A*). Taking together all these results, one may easily argue that the binding areas outside the CENP-B box are also important for the stable complex formation.

Judging from the amount of purified CENP-B (50 pmol from 1×10^{10} of HeLa cells) and the recovery of the specific DNA binding activity as well as that of 80-kD antigen ($\sim$15% for both), the number of CENP-B molecules per HeLa cell is $\sim$20,000. This value is consistent with the results of Cooke et al. (13), who estimated an average of 375 copies of CENP-B per chromatid by immunoelectron microscopy.

The equilibrium constant for binding reaction of CENP-B (fraction 17) to CENP-B box (59-bp DNA) was determined by assuming that each binding site independently interacts with DNA. The value was 6×10^8 M^{-1}, which is relatively low compared with that of transcription factor tau of yeast (5×10^{10} M^{-1}) (1), the adenovirus major late transcription factor (1×10^{10} M^{-1}) (10), or the 5S rRNA transcription factor TFIIIA of xenopus (1×10^9 M^{-1}) (19). However, it should be pointed out that the equilibrium constant for a yeast centromere binding protein CPI to CEN3 sequence is 3×10^8 M^{-1}, close to the above value (2). Under our ex-
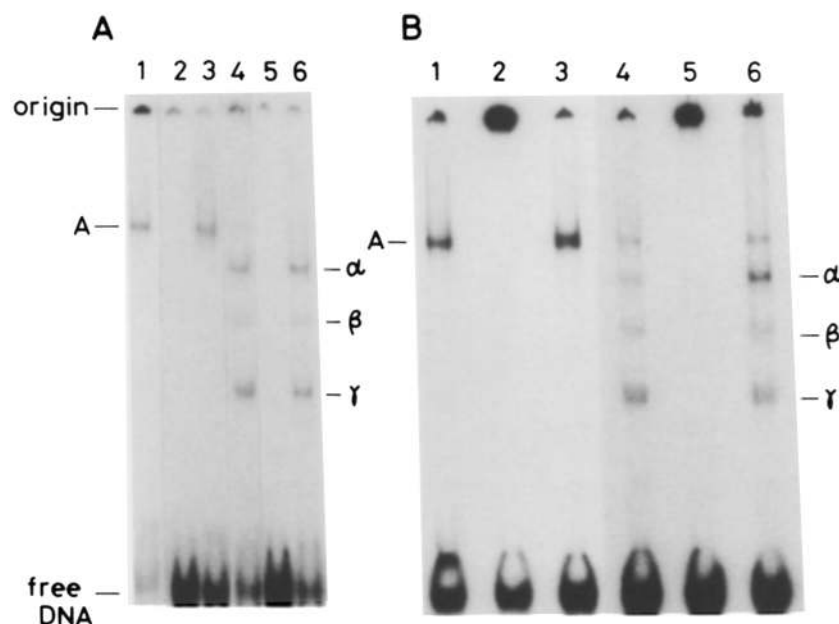

Figure 6. DNA mobility shift assays using fraction 13 or 17 from oligonucleotide-sepharose column. Binding reactions for *A* and *B* were performed as described in Materials and Methods using 1 μl of fraction 17 (lanes *1–3*) or fraction 13 (lanes *4–6*) and 10 fmole of the ^{32}P-labeled 59-bp DNA. (*A*) Competition with the nonradioactive 23-mer DNAs: lanes *1* and *4*, binding reaction without competitors; lanes *2* and *5*, addition of 1 pmole of the 23-mer DNA with a CENP-B box; lanes *3* and *6*, addition of 1 pmole of the defective 23-mer DNA. (*B*) Effects of serum addition after the DNA binding reactions: lanes *1* and *4*, no serum added; lanes *2* and *5*, addition of 1 μl ACA serum (KG) (1:30 PBS); lanes *3* and *6*, addition of 1 μl normal healthy serum (1:30 PBS). *A*, α, β, and γ indicate complexes with different mobilities.

A

1 2 3 4 5 6 7 8

origin –

A^{59} –

– A^{25}

free
59bp –

B

1 2 3 4 5 6 7 8

origin –

A^{59} –

α^{59} –

β^{59} –

γ^{59} –

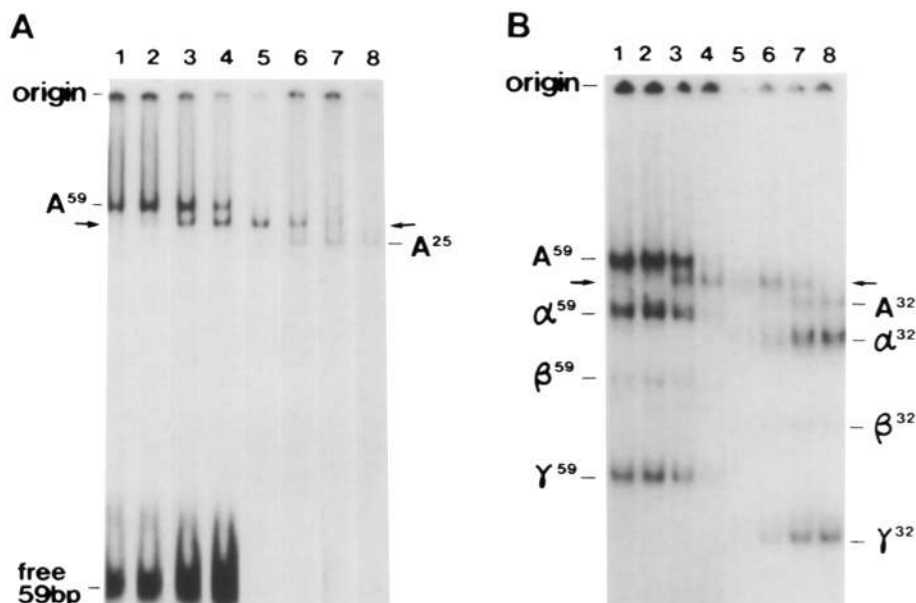– A^{32}

– α^{32}

– β^{32}

– γ^{32}

Figure 7. Determination of number of DNA molecules in complexes by gel shift assays. (*A*) Binding reactions were performed with 1 μl of fraction 17, 10 fmole of the end-labeled 59-bp DNA (10^6 cpm/18 ng) (lanes *1–4*) or 25-bp DNA (10^6 cpm/7 ng) (lanes *5–8*) and 10, 40, and 160 fmole of the nonradioactive 23-mer DNA (lanes *2–4*, respectively), or 80, 20, and 5 fmole of the nonradioactive 56-mer DNA (lanes *5–7*, respectively). Mobility shift of protein-bound DNA was determined as described in Materials and Methods. A^{59} and A^{25} show the positions of complex A formed with 59-bp and 25-bp DNA, respectively. Arrowhead indicates that the complex in the intermediate mobility appeared in the presence of two-chain length DNAs. (*B*) Binding reactions were performed using 1 μl of fraction 14, 10 fmole of the end-labeled 59-bp DNA (10^6 cpm/18 ng) (lanes *1–4*) or 32-bp DNA (10^6 cpm/10 ng) (lanes *5–8*) and 5, 20, and 80 fmole of the nonradioactive 29-mer DNA (lanes *2–7*, respectively), or 40, 10, and 2.5 fmole of the nonradioactive 56-mer DNA (lanes *5–7*, respectively). An arrow indicates the complex A formation at the intermediate mobility.

perimental conditions, no cooperation was found. Since a major complex made between CENP-B and alphoid DNA contains two DNA molecules as described below, further analysis under different conditions is required to clarify this point.
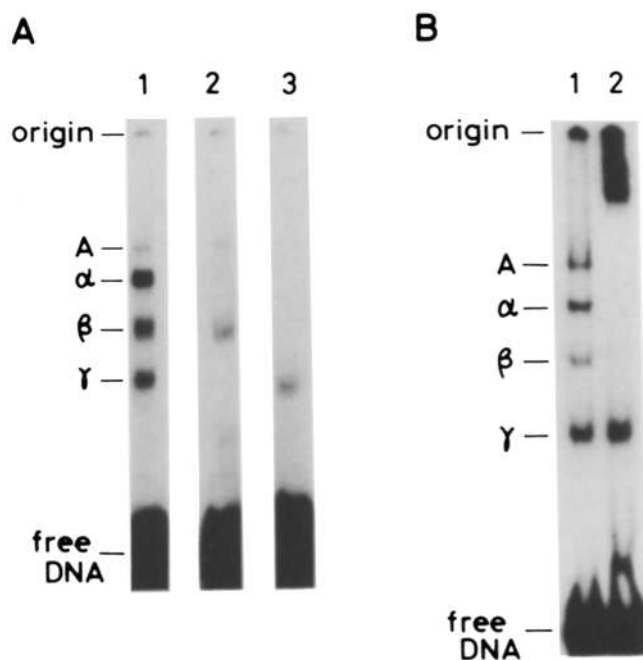

A

1 2 3

origin –

A –
α –
β –
γ –

free
DNA –

B

1 2

origin –

A –
α –
β –
γ –

free
DNA –

Figure 8. Analyses of polypeptides in DNA–protein complexes formed with fractions from the minor peak. (*A*) 80-kD or 65-kD polypeptide in fraction 11–20 was obtained as described in Materials and Methods, and complex formation with the end-labeled 59-bp DNA (10 fmole) was examined by DNA mobility shift assay. Lane *1*, 1 μl of fraction 13; lane *2*, 6 μl of 80-kD polypeptide; lane *3*, 6 μl of 65-kD polypeptide. (*B*) Susceptibility to the monoclonal antibody was analyzed using 1 μl of fraction 14 and 10 fmole of the end-labeled 59-bp DNA. Lane *1*, no antibody added; lane *2*, addition of 1 μl of m-ACA1.

Although both the antigenicity and DNA binding activity were detected only at the 80-kD position using the peak fraction of the oligonucleotide-sepharose column (fraction 17), an extra 65-kD band with the same antigenicity and specific DNA binding activity was detected as a minor component using the fraction 13 (Fig. 3). Since repeated freezing–thawing of the crude nuclear extract, the digestion of partially purified CENP-B fractions with various proteases, or purified CENP-B (fraction 17) kept on ice for one month produced m-ACA1 reactive antigenic polypeptides in the same size and/or with specific DNA binding activity (data not shown), it is very likely that the 65-kD antigens are degradation products of CENP-B. The same possibility was suggested earlier by Balczon and Brinkley (3) on the ACA-reactive 65-kD antigen that had been purified with an antitubulin affinity column. The DNA mobility shift assay showed that CENP-B and its putative degradation products form four complexes in different mobilities (Fig. 6). All four complexes have shown binding specificity to the CENP-B box sequence and reactivity to an ACA serum (Fig. 6), but the reactivity to m-ACA1 or the number of DNA molecules involved in the complex formation proved to be different (Figs. 7 and 8). Complex A, for example, contained two molecules of DNA while the faster moving three complexes (complexes α, β, and γ) contained only one molecule of DNA (Fig. 7). Complexes A, α, and β have reacted to the m-ACA1, whereas complex γ did not.

Our current view for the molecular constitution of the four complexes is shown below. The protein components of complex A and complex β may be dimer and monomer of 80-kD CENP-B, respectively, from the results as follows. (*a*) The DNA-mobility shift experiment using the 80-kD antigen recovered from SDS-gel (Fig. 8). (*b*) The number of DNA molecules in the complex A and β (Fig. 7). (*c*) Molecular weight of complex A determined by glycerol gradient sedimentation, which corresponds approximately to the sum of the molecular weight of two molecules each of CENP-B and probe DNA (our unpublished results).

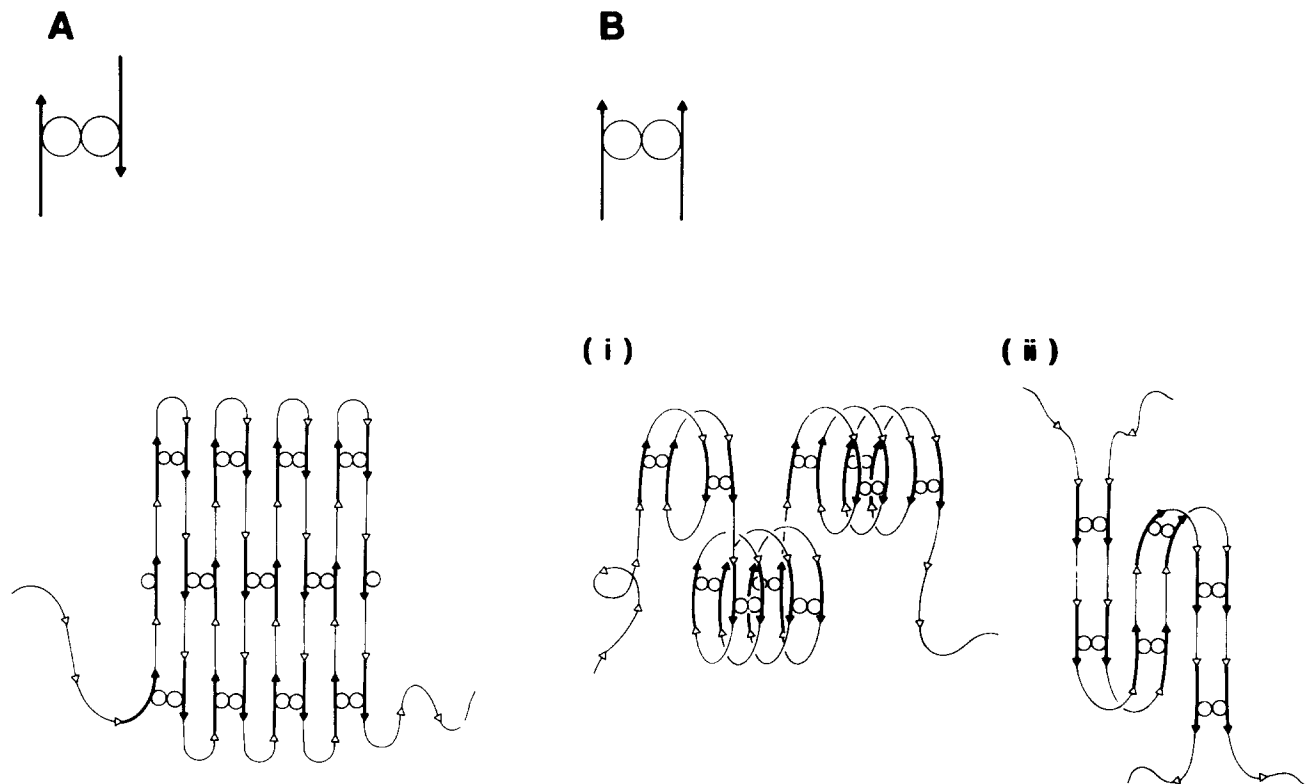Most 80-kD polypeptides eluted from the SDS-gel seemed

Figure 9. Models of higher ordered structures made with long alphoid DNA and CENP-B. In *A* and *B*, two DNA molecules in complex A orient the opposite direction and the same direction, respectively. In (*i*) and (*ii*), intrastrand and interstrand assembly, respectively, of alphoid DNA molecules by CENP-B molecules are shown. Tandemly repeating alphoid DNA monomers with and without a CENP-B box are depicted as heavy and thin arrows, respectively. CENP-B molecules binding to CENP-B boxes are shown as open circles. The complex A is depicted tentatively as dimer of the CENP-B molecules and two DNA molecules assembled together.

unable to recover dimerization activity even after renaturation treatment, since complex β was the major reaction product (Fig. 8 *A*).

A band corresponding to the mobility of complex γ was formed between the probe DNA and the 65-kD polypeptides eluted from the SDS-gel (Fig. 8). The complex γ formation was not disturbed by the addition of m-ACA1 (Fig. 8), although it was disturbed by an ACA serum (Fig. 6). Since the epitope for m-ACA1 resides within COOH-terminal 147 amino acid residues in CENP-B (15), the 65-kD polypeptide in complex γ probably lacks the corresponding COOH-terminal domain. The resistance of complex γ to m-ACA1 was unexpected, since immunoblotting analysis of fraction 13 has shown that 65-kD polypeptide reacted with both m-ACA1 and an ACA serum. These results suggest a possibility that another polypeptide with the same 65-kD electrophoretic mobility would exist in fraction 13 and this polypeptide would carry the COOH-terminal region (therefore reactive to m-ACA1) but would lack the NH$_2$-terminal region instead. The NH$_2$-terminal location of DNA-binding domain was also indicated by our current analyses: the polypeptide encoded by a cDNA clone of CENP-B lacking more than half of the COOH-terminal region showed DNA binding activity, while the polypeptide with a deletion of 16-amino acid residues at NH$_2$-terminal region lacked binding activity (our unpublished results). Given by its electrophoretic mobility, complex α may contain a heterodimer of one 80 kD and a degradation product of CENP-B. The 65-kD polypeptide with the reactivity to m-ACA1 is a candidate of the degradation product with the putative dimerization domain.

Functional Role of CENP-B and CENP-B Box Interaction

The CENP-B box sequence is present in various subclasses of alphoid repeats covering almost all chromosomes (26). The present results revealed that CENP-B molecules assemble alphoid DNA with CENP-B box. This CENP-B and CENP-B box interaction may play a role in gathering up the long alphoid DNA repeats in the centromere domain to make up regular higher structure or holding of sister chromatids after replication (Fig. 9). The CENP-B we have used in this analysis was obtained from interphase cells. It is important to know how stages in the cell cycle would affect the property of the CENP-B.

In support of our present and previous results of specific interaction between CENP-B and alphoid DNA (26), an in vivo association between alphoid DNA and CENP-B was suggested by an immunocytogenetic analysis of a rearranged centromere of human chromosome 17 (42). Cooke et al. (13) showed by immunoelectron microscopy that CENP-B is largely confined to the chromatin of the central domain beneath the kinetochore in HeLa cells. The DNA sequence distribution within centromere is well documented in the genus *Mus*. A CENP-B box was also found in the 120-bp unit sequence of the mouse minor satellite, the satellite DNA present in the discrete region of primary constriction at or adjacent to the site of the centromere antigens in most mouse species tested (46). Recently, CENP-B gene was isolated from mouse and high conservation of the CENP-B genes in the human and mouse cells was shown (40). The in vivo and

in vitro association of alphoid DNA with CENP-B, and the conservation of CENP-B and CENP-B box sequence in human and mouse cells, suggest that CENP-B plays a fundamental role in structure or function of the centromere chromatin.

The functional role of centromere antigens has been addressed recently in two microinjection studies using ACA. Bernat et al. (4) reported that injection of anticentromere antibodies into HeLa cells during interphase disrupted chromosome movements at mitosis and that all sera that inhibited mitosis had high levels of anti–CENP-B antibodies, but variable levels of anti–CENP-A or CENP-C by immunoblotting analysis. They suggest that CENP-B may be the target antigen for the observed mitotic disruption. Simerly et al. (38) also found that an autoantibody, which detected the centromere/kinetochore complex in permeabilized mouse cells or mouse oocytes and crossreacted with an 80-kD centromere antigen, interfered with the prometaphase chromosome alignment for mouse cells. These results suggest the possible link of CENP-B activity in executing centromere function. Recently, Zinkowsky et al. (48) proposed a repeat subunit model of kinetochore in which the centromere/kinetochore complex is assembled from repetitive subunits tandemly arranged on a continuous DNA/protein fiber. The CENP-B and alphoid DNA complex might be one of the components of such a repetitive unit. Thus, the reaction of CENP-B and CENP-B box may offer a key to elicit a common function performed by centromeric satellite DNA with highly variable sequence among mammalian species.

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