

# A Human Centromere Protein, CENP-B, Has a DNA Binding Domain Containing Four Potential $\alpha$ Helices at the NH<sub>2</sub> Terminus, Which Is Separable from Dimerizing Activity

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**Abstract.** The alphoid DNA-CENP-B (centromere protein B) complex is the first sequence-specific DNA/protein complex detected in the centromeric region of human chromosomes. In the reaction, CENP-B recognizes a 17-bp sequence (CENP-B box) and assembles two alphoid DNA molecules into a complex, which is designated complex A (Muro, Y., H. Masumoto, K. Yoda, N. Nozaki, M. Ohashi, and T. Okazaki. 1992. *J. Cell Biol.* 116:585-596). Since CENP-B gene is conserved in mammalian species and CENP-B boxes are found also in mouse centromere satellite DNA (minor satellite), this sequence-specific DNA-protein interaction may be important for some kind of common centromere function. In this study we have characterized the structure of CENP-B and CENP-B-alphoid DNA complex. We have shown by chemical

cross-linking that CENP-B formed a dimer, and have estimated by molecular weight determination the composition of complex A to be a CENP-B dimer and two molecules of alphoid DNA. The DNA binding domain has been delimited within the NH<sub>2</sub>-terminal 125-amino acid region containing four potential  $\alpha$ -helices using truncated CENP-B made in *Escherichia coli* cells. We have shown that CENP-B had sites highly sensitive to proteases and that the DNA binding domain was separable from the dimerizing activity by the proteolytic cleavage at 20 kD from the COOH terminus of the molecule. Thus, CENP-B may organize a higher order structure in the centromere by juxtaposing two CENP-B boxes in the alphoid DNA repeat through both the DNA-protein and protein-protein interactions.

**T**HE centromere of higher eukaryotes is defined as a primary constriction locus in mitotic chromosomes visible by optical microscopy. On the surface of the centromere of mammalian chromosomes, a specific trilaminar disk-shaped structure, a kinetochore, is formed during mitosis, and serves as the site of attachment for spindle microtubules (Brinkley et al., 1989; Pluta et al., 1990; Willard, 1990). Chromosomal movement during mitosis has been precisely examined in terms of the interaction between the kinetochore and spindle microtubules at morphological and mechanochemical levels (Mitchison and Kirschner, 1985a,b; Rieder and Alexander, 1990; Hyman and Mitchison, 1991). The centromere is also believed to play a central role in the pairing and disjunction of daughter chromosomes in mitosis and meiosis, but the fundamental molecular mechanisms of these events have not yet been elucidated.

In *Saccharomyces cerevisiae*, a DNA sequence of  $\sim$ 125 bp (centromere; CEN)<sup>1</sup> has been genetically defined as a *cis*-acting centromeric functional unit (Clarke and Carbon, 1980; Hieter et al., 1985; Clarke, 1990), and proteins that

specifically interact with CEN have been detected and characterized (Cai and Davis, 1990; Lechner and Carbon, 1991; Kingsbury and Koshland, 1991). In contrast, in *Schizosaccharomyces pombe*, centromere DNA is composed of much longer and more complexed sequences (Chikashige et al., 1989; Hahnenberger et al., 1989). In human chromosomes, alpha satellite DNA, or alphoid DNA, has been shown to be localized in the centromeric region (Willard, 1990). Alphoid DNA is a highly repetitive sequence composed of the fundamental unit of  $\sim$ 170 bp, and it clusters in the centromeric region over a few megabases (Willard and Wayne, 1987; Willard, 1990). Using autoimmune sera from scleroderma patients, specific antigens have been detected in the centromeric region (Moroi et al., 1980; Moroi et al., 1981; Brenner et al., 1981). Three human centromere-associated proteins were detected as the centromere antigens; centromere protein A (CENP-A) (17 kD), CENP-B (80 kD), and CENP-C (140 kD) (Earnshaw and Rothfield, 1985; Valdivia and Brinkley, 1985). CENP-A has been shown to be a histone H3-like protein (Palmer et al., 1991). A cDNA-encoding CENP-B has been cloned and characterized from human and mouse cells (Earnshaw et al., 1987; Sullivan and Glass, 1991). A fourth (50 kD) centromere-associated pro-

1. *Abbreviations used in this paper:* ACA, anticentromere antibody; CENP-B, centromere protein B; HLH, helix-loop-helix.

tein, designated CENP-D, has been identified also using the autoimmune sera (Kingwell and Rattner, 1987). Recently, it has been suggested that a motor protein, cytoplasmic dynein, takes part in centromere function (Pfarr et al., 1990; Steuer et al., 1990). Several monoclonal antibodies that recognize the centromeric region of human chromosomes have been isolated using scaffold proteins as antigens (Cooke et al., 1987; Compton et al., 1991); one of the antigens, a 250–300-kD protein, was found to be localized to centromere only at early stages of mitosis and was named CENP-E (Yen et al., 1991). Thus, several centromeric proteins have been found to date, but in most cases their roles at the molecular level remain to be elucidated. It is important to study the interaction between DNA and proteins at the centromeric region because the DNA sequence itself must be the primary determinant of the structure and function of the centromere. We have previously reported that CENP-B binds to subtypes of alphoid DNA that contain a 17-bp sequence, the “CENP-B box” (Masumoto et al., 1989). We have shown that CENP-B highly purified from HeLa cells binds to alphoid DNA at the CENP-B box (Muro et al., 1992). The stable complex formed in vitro, which we referred to as complex A, contained two DNA molecules, suggesting that this DNA-protein interaction may be important in organizing a higher-order structure of long alphoid DNA repeats (Muro et al., 1992). Because CENP-B is a highly conserved protein in mammalian cells (Sullivan and Glass, 1991) and CENP-B boxes are found also in a mouse minor satellite DNA repeat, this protein–DNA interaction may be of general importance in organizing centromeric DNA in mammalian cells.

In this work, we have examined the molecular structure of CENP-B in native state, and of the alphoid DNA·CENP-B complex made in vitro using the short segment of alphoid DNA containing a CENP-B box and either the 0.6 M NaCl extract of HeLa nuclei or purified CENP-B. We show by chemical cross-linking with glutaraldehyde that CENP-B forms a dimeric structure, and estimate that the stable DNA/protein complex, complex A, is composed of a CENP-B dimer and two molecules of alphoid DNA. Using truncated CENP-B expressed in *Escherichia coli* cells, we delimit the DNA binding domain within the NH<sub>2</sub>-terminal 125-amino acid polypeptide. Using partial digestion of native form of CENP-B with proteases, we detect sites highly sensitive to Glu-C protease, chymotrypsin, and thrombin, and show that the specific DNA binding activity dissociates from dimerizing activity by the cleavage of the polypeptide. By the analyses of change in molecular weight of CENP-B before and after protease digestion and the analyses of relative mobility of the complexes formed between alphoid DNA and the proteolytic products of CENP-B, we suggest that the dimerizing activity of CENP-B locates within the COOH-terminal region. We propose on the basis of these results that CENP-B has a new DNA binding motif and juxtaposes two CENP-B boxes located intra- and/or intermolecularly in alphoid DNA repeats in the centromere through both DNA-protein and protein-protein interactions.

## Materials and Methods

### Cell Culture

HeLa S3 cells were grown in suspension culture with RPMI 1640 medium

(Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 5% calf serum (Flow Laboratories, North Ryde, Australia) at 37°C.

### Antisera

Anticentromere antibody (ACA)-positive serum from scleroderma patient M. I. contained antibodies against the three major centromere autoantigens: CENP-A (17 kD), CENP-B (80 kD), and CENP-C (140 kD). Normal serum contained no antibodies against the centromere proteins. Monoclonal antibody mACA-1 was a gift from Dr. W. C. Earnshaw.

### Preparation of Extracts from HeLa Nuclei and Purification of CENP-B

Nuclear proteins were extracted from the isolated nuclei of HeLa cells ( $\sim 2 \times 10^{10}$ ) with extraction buffer (1 × EB: 20 mM Hepes, pH 8.0, 0.5 mM EDTA, 0.5 mM DTT, 15% (vol/vol) glycerol, 1 mM NaHSO<sub>3</sub>, 0.5 mM PMSF, 0.5 μg/ml pepstatin, and 2 μg/ml leupeptin) containing NaCl as described (Muro et al., 1992) except that final extraction was carried out with 200 ml of 1 × EB containing 0.6 M NaCl (0.6 M NaCl nuclear extract,  $\sim 5$  mg protein/ml). CENP-B was purified from 200 ml of the 0.6 M NaCl nuclear extract by Q-Sepharose column chromatography (the CENP-B fraction I;  $\sim 200$  mg protein/ml) and then DNA-Sepharose column chromatography (the CENP-B fraction II;  $\sim 8$  mg protein/ml, purity of CENP-B  $\sim 20\%$ ) as described (Muro et al., 1992). Part of the CENP-B fraction II was further purified by SDS-PAGE. CENP-B in the gel was transferred to polyvinylidene difluoride membrane (Millipore Corp., Bedford, MA) and the amino acid composition of the purified CENP-B was determined. The result confirmed that the purified protein was identical to the CENP-B encoded by the cDNA cloned by Earnshaw et al. (1987) (data not shown).

### Synthetic Nucleotides

The chemically synthesized DNA probes were described (Muro et al., 1992). The 56- and 23-mer DNAs with CENP-B boxes were designated CB56 and CB23, respectively, in this paper. <sup>32</sup>P-CB59 and <sup>32</sup>P-CB25 were obtained from CB56 and CB23, respectively, by endlabeling using [ $\alpha$ -<sup>32</sup>P] dTTP pr [ $\alpha$ -<sup>32</sup>P] dCTP (110 TBq/mmol; Amersham International, Amersham, UK), other three nonradioactive dXTPs (Pharmacia LKB Biotechnology AB, Uppsala, Sweden) and DNA polymerase Klenow fragment (Takara Shuzo Co., Ltd., Japan). CB<sup>d</sup>56 is 56-mer DNA containing defective CENP-B box owing to base replacement (Muro et al., 1992).

### DNA Binding Reaction and Gel Mobility Shift Analysis

Unless otherwise indicated, the following conditions were used. The DNA binding reaction was carried out at 0°C for 2–12 h in a 10-μl reaction mixture containing 10 mM Tris-HCl, pH 8.0, 10% (vol/vol) glycerol, 1 mM EDTA, 1 mM DTT, 150 mM NaCl, 0.1–6 μg sonicated salmon sperm DNA, 0.2–0.5 ng <sup>32</sup>P-labeled DNA, and the proteins from the 0.6-M NaCl nuclear extract or the purified CENP-B fraction I or II (1–3 μl). A 5-μl aliquot was then electrophoresed through a 5% polyacrylamide slab gel (15 × 15 × 0.1 cm) for 2.5 h at 200 V at 4°C in 0.25 × TBE (1 × TBE: 90 mM Tris, 90 mM boric acid, 7.5 mM EDTA-Na<sub>2</sub>) after pre-run for 3 h. When the affinity of a shifted band for the antibody was checked, 1 μl of ACA serum (1:10 dilution) or mACA-1 (1:10 dilution) was added after the DNA binding reaction and the mixture was incubated at 0°C for 1 h before gel electrophoresis. The gel was subjected to autoradiography after drying as described previously (Muro et al., 1992).

### Glycerol Density Gradient Sedimentation

For measurement of the sedimentation rate of alphoid DNA·CENP-B complex, an aliquot of the 0.6-M NaCl nuclear extract was dialyzed against 1 × EB–0.5 M NaCl without glycerol for 5 h at 4°C and 30 μl of the dialysate was used for DNA binding reaction in a 100-μl cocktail described in the preceding section, except for omission of glycerol. After the binding reaction the cocktail was layered over a 3.6-ml 5–20% glycerol linear gradient containing 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM DTT, and 0.05% NP-40, with 0.2 ml of 50% glycerol at the bottom as a cushion and centrifuged at 46,000 rpm for 12 h at 4°C in an SW 56 Ti rotor (Beckman Instrs. Inc., Fullerton, CA). A mixture of 10 μg each of BSA, catalase, and thyroglobulin dissolved in 100 μl DNA binding cocktail without nuclear extract was cocentrifuged as external size markers. After centrifugation the gradient was collected in 150-μl aliquots from the bottom. For measurement

of the sedimentation rate of native CENP-B, 100  $\mu$ l of the nuclear extract after the dialysis was layered over a 3.6-ml 5–20% glycerol linear gradient containing 20 mM Hepes, pH 8.0, 0.5 mM DTT, 1 mM NaHSO<sub>3</sub>, 0.5 mM PMSF, 0.5  $\mu$ g/ml pepstatin, 2  $\mu$ g/ml leupeptin, 0.05% NP-40, and 0.5 M NaCl with 0.2 ml of 50% glycerol at the bottom and centrifuged at 48,000 rpm for 24 h at 4°C. To determine the sedimentation rate of CENP-B in the presence of sarcosyl (sodium N-sarcosinate), we added sarcosyl to the sample after the dialysis to the final concentration of 0.1% and the sample was centrifuged through the glycerol gradient supplemented with 0.1% sarcosyl with the same condition as mentioned above. A mixture of horse myoglobin, BSA, and mouse IgG dissolved in 100  $\mu$ l EB–0.5 M NaCl was centrifuged as external size markers.

### Reactivation of DNA Binding Activity of CENP-B

After the HeLa extract was subjected to glycerol density sedimentation in the presence of 0.1% sarcosyl, the proteins in each fractionated sample were recovered by acetone precipitation. The recovered proteins were completely denatured and then renatured as described by Hager and Burgess (1980) with a slight modification. Shortly, the acetone pellet of each fraction was dissolved in 3  $\mu$ l of 6 M guanidine-HCl and held at room temperature for 20 min. The sample was diluted with 150  $\mu$ l of 1  $\times$  EB–0.5 M NaCl, kept at room temperature for 1 h and dialyzed against the same buffer for 5 h at 4°C. Approximately 20% of the DNA binding activity of CENP-B was reactivated.

### Chemical Cross-Linking of CENP-B

1  $\mu$ l of 5 mM glutaraldehyde (Nacalai Tesque, Inc., Kyoto, Japan) was added to 10  $\mu$ l of the CENP-B fraction II or 10 pmol of BSA dissolved in 10  $\mu$ l of 1  $\times$  EB–0.5 M NaCl, and the mixtures were kept at 25°C for 0–20 min. Equal volume of 2 $\times$  Laemmli reducing SDS-loading buffer was then added, and the samples were boiled for 10 min (Kato et al., 1992). The samples were electrophoresed through 5% SDS-PAGE in Weber-Osborn buffer system (Weber and Osborn, 1969).

### Construction of Truncated CENP-B Molecules

A 2.7-kb DNA fragment containing the CENP-B gene was cloned from a SacI–BamHI double digest of TIG-7 genomic DNA into the pUC119 vector (pUCCBS-B). This fragment contained 1,057 bases of the gene encoding the NH<sub>2</sub>-terminal part of CENP-B protein and 1.5 kb of its upstream region. The T7 expression system was used to express CENP-B in *Escherichia coli* (Studier et al., 1990). We first chemically synthesized the nucleotide sequence covering from the start codon to the first AccIII site of CENP-B gene downstream of a EcoRI and NcoI restriction sequence, and ligated it to the 4.1-kbp EcoRI–AccIII fragment of pUCCBS-B. A 1,058-bp NcoI–BamHI fragment from this construct was recovered and cloned in the pET-3d vector (pETCBN-B; encoding NH<sub>2</sub>-terminal 1–1,057 nucleotides of CENP-B). By the combination of partial digestion with SacI and complete digestion with BamHI of the clone containing part of CENP-B cDNA in the EcoRI–NotI site of bluescript II (a gift from Dr. K. Sugimoto), the 1.7-kbp BamHI–SacI fragment was obtained, which contained the COOH-terminal part of CENP-B gene, the poly(A) tail (1,058–2,700), and the short stretch of vector sequence. To construct a full size CENP-B gene, the 1.7-kbp BamHI–SacI fragment was ligated to the BamHI–SacI site of pETCBN-B (pETCENP-B). Thus, the pETCENP-B encodes the complete CENP-B polypeptide, and was propagated by transfection into *Escherichia coli* BL 21 (DE3) pLys. The deletion mutants of CENP-B were constructed by step-wise digestion from the COOH-terminus using exonucleaseIII, mung bean nuclease, and Klenow fragment (Takara Shuzo Co., Ltd., Kyoto, Japan) (Henikoff, 1984; Yanisch-Perron, 1985), and the clones thus obtained were propagated in *Escherichia coli* BL21 (DE3), pETCB $\Delta$ 10–25 was a deletion mutant of pETCENP-B from which DNA sequence encoding 16 amino acids from 10th to 25th was spontaneously deleted from CENP-B cDNA. The COOH-terminal deletion clones were referred to by the encoding amino acid numbers from the NH<sub>2</sub> termini of the CENP-B and the truncated polypeptides produced in *Escherichia coli* contained short foreign peptides derived from the vector sequence at the COOH termini (Studier et al., 1990) as indicated in the parentheses: pETCBN-411 (23 amino acids [aa]), pETCBN-333 (4 aa), pETCBN-234 (5 aa), pETCBN-160 (4 aa), pETCBN-143 (5 aa), pETCBN-137 (21 aa), pETCBN-125 (5 aa), pETCBN-104 (5 aa), and pETCBN-63 (5 aa).

### Protease Digestion of CENP-B

The CENP-B fraction I or II was digested with endoprotease Glu-C (Glu-C protease) from *Staphylococcus aureus* V8 (Boehringer Mannheim GmbH),  $\alpha$ -chymotrypsin from bovine pancreas (Sigma Chem. Co., St. Louis, MO), and thrombin from human plasma from human placenta (Boehringer Mannheim GmbH). Glu-C protease hydrolyzes specifically peptide bonds at the carboxylic side of glutamic acid. Chymotrypsin preferentially catalyzes the hydrolysis of peptide bonds involving aromatic L-amino acids (Tyr, Phe, and Trp). Thrombin hydrolyzes peptide bonds specifically at the carboxylic side of arginine residues. This protease also recognizes some specific tertiary structure of proteins and therefore hydrolyzes only limited number of arginine residues in proteins. To ensure reproducibility of the protease digestion, we divided original solutions of these proteases into small fractions and stored at –20°C, and used in each experiment immediately after thawing. 0.1 vol of the diluted protease solution was added to the CENP-B fraction and incubated at 0°C for 60 min. The reaction was stopped by the addition of diisopropyl fluorophosphate (Sigma Chem. Co.) to the final concentration of 1%. For preparation of the samples for SDS-PAGE, the digests were precipitated by acetone and resuspended in SDS sample buffer containing 1% diisopropyl fluorophosphate. The suspensions were left for 30 min at room temperature instead of boiling before electrophoresis.

### Western and Southwestern Blottings

The proteins, separated by SDS-PAGE as described (Laemmli, 1970; Weber and Osborn, 1969), were transferred to a polyvinylidene difluoride membrane according to the procedure of Towbin et al. (1979). Unless otherwise indicated, SDS-PAGE was performed in Laemmli buffer system. The membrane was preincubated with 10% skim milk (Difco Laboratories, Inc., Detroit, MI) and then incubated with ACA serum (1:1,000 dilution), and goat anti-human IgG HRP conjugate (Bio-Rad Laboratories, Richmond, CA) (1:3,000 dilution). Color development was carried out with Konica Immunostain (Konica Co., Ltd., Tokyo, Japan). DNA binding activity specific to CENP-B box of the proteins on the membrane was determined after the renaturation treatment by the reaction with <sup>32</sup>P-labeled CB59 DNA in the presence of 0.1 mg/ml sonicated salmon sperm DNA and detected by autoradiography as described (Muro et al., 1992; Masumoto et al., 1989; Celenza and Carlson, 1986).

### Recovery of CENP-B Fragments from SDS-PAGE

30  $\mu$ l each of the CENP-B fraction II was digested with 0.02 mg/ml of Glu-C protease, 3  $\mu$ g/ml of chymotrypsin and 3 mg/ml of thrombin for 1 h at 0°C. The reaction was stopped by 1% diisopropyl fluorophosphate and the digests were subjected to 14% SDS-PAGE. Referring to the data of Western blot analysis (not shown), the regions of 60 kD for Glu-C protease digestion, 21 kD for chymotrypsin digestion, and 16–16.5 kD for thrombin digestion were cut out and proteins were electroeluted from the gel blocks. The eluates were dialyzed against 10 mM Tris-HCl (pH 8.0) containing 1 mM EDTA, 0.1% SDS, 0.5  $\mu$ g/ml pepstatin, 1  $\mu$ g/ml leupeptin, and 0.5 mM PMSF for 5 h at 4°C. These samples and 15  $\mu$ l of the CENP-B fraction II without protease digestion were precipitated with acetone and the reactivation treatment was performed as described previously. The volume of each sample after dialysis was 50–100  $\mu$ l.

### Results

#### Detection of CENP-B·Alphoid DNA Complexes by Gel Mobility Shift Analysis

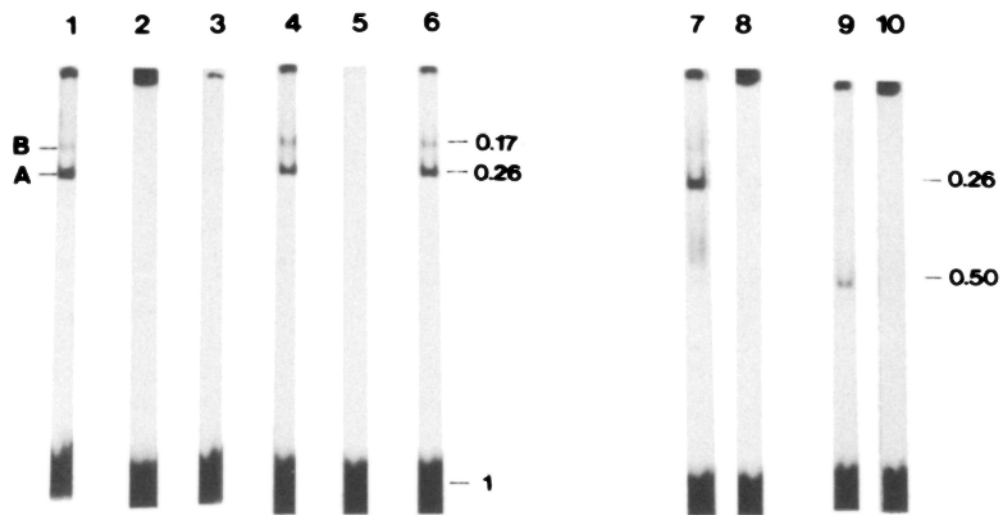
We have examined the properties of the protein/DNA complexes formed between CENP-B and alphoid DNA containing the “CENP-B box” (CTTCGTTGGAAACGGGA) by gel mobility shift analysis (Fig. 1). A <sup>32</sup>P-labeled 59-mer DNA probe (CB59) was incubated with the nuclear extract of HeLa cells and then electrophoresed at 4°C. Two shifted bands of relative mobilities 0.26 and 0.17 (expressed as the mobilities relative to that of CB59 DNA) were detected (complexes A and B) (Fig. 1, lane 1). When ACA-positive serum (M.I.) was added after the binding reaction, both complexes disappeared and were apparently trapped at the origin

region (Fig. 1, lane 2). Formation of the complexes was inhibited by the addition of ACA serum to the crude nuclear extract before the binding reaction (Fig. 1, lane 3). These results suggested that both complexes A and B contained protein(s) antigenic to ACA serum. The  $^{32}\text{P}$ -CB59 DNA molecules in complexes A and B were competed out by a 50-fold excess of unlabeled CB56 DNA (Fig. 1, lane 5) but not by the same amount of CB $^{56}$  DNA which contains a defective CENP-B box (Fig. 1, lane 6), suggesting that both complexes A and B were formed by a CENP-B box-specific interaction of CENP-B. We have shown previously that complex A was formed between alphoid DNA and a highly purified preparation of CENP-B (Fig. 7 A, lane 1) (Muro et al., 1992). When the reaction mixture was electrophoresed at 22°C, in addition to complexes A and B, a smear appeared in the region between the relative mobility 0.26 (complex A) and 0.50 (Fig. 1, lane 7). Complex A and the smear apparently converged to the band of relative mobility 0.50 when electrophoresed at 37°C (Fig. 1, lane 9). Formation of the complex of relative mobility 0.50 was not due to degradation of the proteins, since complex A appeared when the reaction mixture was electrophoresed at 4°C after incubation of 37°C for >2 h (data not shown). Taking into account of the fact that complex A contains two molecules of alphoid DNA (Muro et al., 1992), these results suggest that the protein component of complex A forms a dimeric structure that can be dissociated at higher temperature.

#### **CENP-B Forms a Dimer in Native Conditions, and a CENP-B Dimer May Bind Two Molecules of Alphoid DNA**

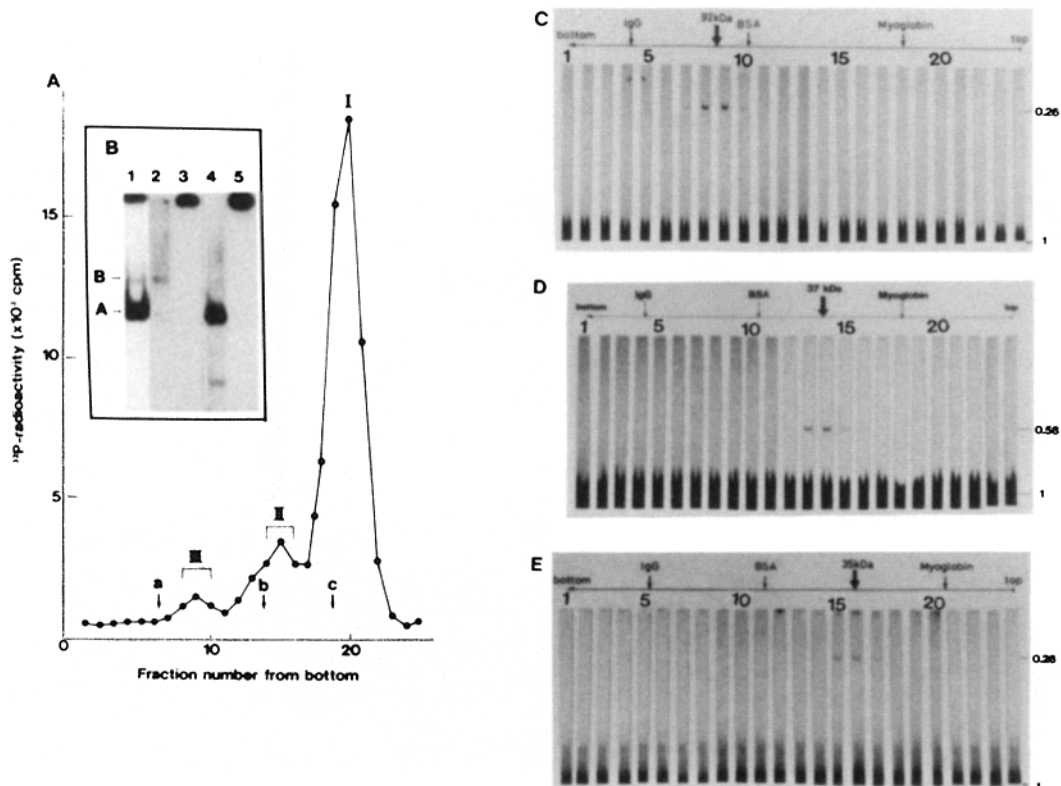
To determine the molecular weight of complex A, we fractionated the nuclear extract after incubation with  $^{32}\text{P}$ -CB 59 DNA by glycerol density gradient centrifugation (Fig. 2 A). Three radioactive peaks were detected. Peak I was free  $^{32}\text{P}$ -CB59 DNA, and its relative molecular mass was calculated as 60 kD from its sedimentation rate relative to marker proteins at positions a, b, and c. Fig. 2 B shows the results of

gel mobility shift analysis of peak II and peak III in Fig. 2 A, indicating that the peaks II and III represented complexes A and B, respectively. Complex A was calculated as 190 kD and complex B as 520 kD. For relative molecular mass determination of CENP-B under native conditions, the nuclear extract of HeLa cells was also fractionated by glycerol density gradient centrifugation before and after Glu-C protease digestion, and the DNA binding activity of CENP-B in each fraction was monitored by gel mobility shift assay after incubation with  $^{32}\text{P}$ -CB59 DNA (Fig. 2, C and D). From the results shown in Fig. 2, C and D, CENP-B was calculated as 92 kD, whereas after Glu-C protease digestion its relative molecular mass was largely reduced to 37 kD. As shown in the later section, digestion of CENP-B with Glu-C protease produced an ~60-kD fragment as measured by SDS-PAGE (Fig. 6 A, lanes 2 and 3), and this fragment formed the complex with  $^{32}\text{P}$ -CB59 DNA of relative mobility 0.58 (Fig. 7 A, lane 2), the complex with the same mobility as detected in Fig. 2 D. CENP-B measured by SDS-PAGE was ~80 kD (Muro et al., 1992; Earnshaw et al., 1987). If we assume that the proportionality between the mobility and the molecular mass of the full size and cleaved CENP-B molecules is kept same in the glycerol gradient as it is in the SDS gel, then the molecular mass of full size CENP-B could be estimated as  $37\text{ kD} \times 80/60 = 49\text{ kD}$ . The value is approximately half of 92 kD, which is the molecular mass of full size CENP-B in native conditions (Fig. 2 C). When an ionic detergent, sodium N-lauroyl sarcosinate, was present in the glycerol density gradient, CENP-B was reduced to 35 kD (Fig. 2 E), as judged by the location of the activity of the DNA/protein complex formation after renaturation. This value would represent the molecular mass of the denatured but full size CENP-B, because the 35-kD polypeptide after renaturation was capable of forming the DNA/protein complex with the relative mobility of 0.26, the mobility of complex A (Fig. 2 E). These results suggest that CENP-B forms a dimer in native conditions. To confirm dimer formation of CENP-B molecules, we have purified CENP-B from the nuclear ex-



**Figure 1.** Detection of CENP-B-alphoid DNA complex by gel mobility shift analyses. The nuclear extract from HeLa cells was subjected to gel mobility analysis after the binding reaction with 0.2 ng  $^{32}\text{P}$ -CB59 DNA ( $10^3$  Bq/ng). Lane 1 and other lanes contained 1  $\mu\text{l}$  of the nuclear extract. In lane 3, the nuclear extract was preincubated with a 0.01 vol of ACA serum for 1 h at 0°C. After binding at 0°C for 12 h, 1  $\mu\text{l}$  of ACA serum (1:10 dilution) was added in lanes 2, 8, and 10 and normal serum (1:10 dilution) in lane 4, and the mixtures were incubated at 0°C for 1 h.

Lanes 5 and 6 contained 10 ng of CB56 and CB $^{56}$  DNAs, respectively. Samples were electrophoresed at 4°C (lanes 1–6), 22°C (lanes 7 and 8), or 37°C (lanes 9 and 10). The mobility of each band relative to the mobility of CB59 DNA was indicated to the left side of lane 6 or 10. Lanes 1–6, lanes 7 and 8, and lanes 9 and 10 were the same exposures, respectively.

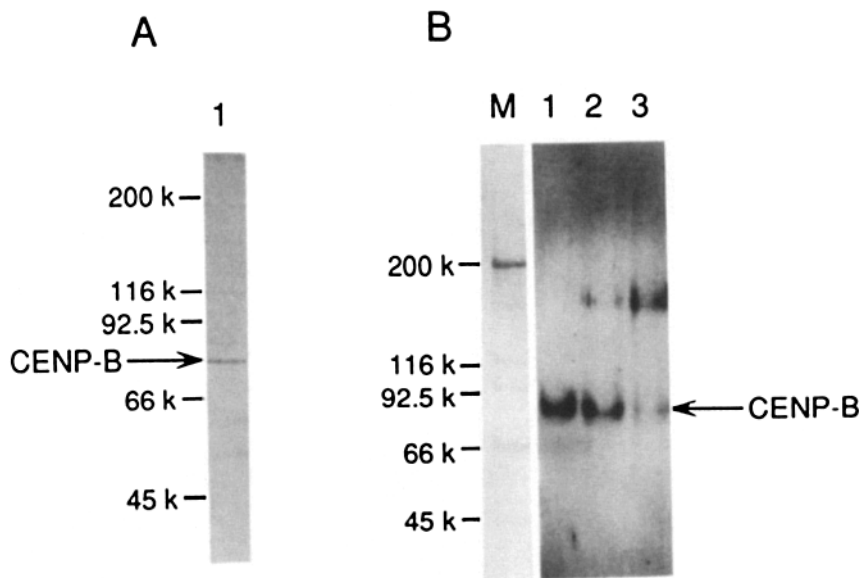


**Figure 2.** Relative molecular mass measurement of complex A and CENP-B by glycerol density gradient sedimentations. (A) Glycerol density gradient sedimentation was performed after the binding reaction between CENP-B in the extract and <sup>32</sup>P-CB59 DNA. The Cerenkov radiation of each fractionated sample was counted. The positions of size markers were indicated by arrows (*a*, thyroglobulin, 670 kD; *b*, catalase, 340 kD; *c*, BSA, 66 kD). (B) Regions bracketed in A were pooled, and 5  $\mu$ l of each fraction was electrophoresed on a 5% polyacrylamide gel; lane 1, the unfractionated sample; lanes 2 and 3, region III; lanes 4 and 5, region II. 0.1 vol of ACA serum (1:10 dilution) was added to lanes 3 and 5 before gel electrophoresis. (C and D) Glycerol density gradient sedimentation of the nuclear extract before (C) and after (D) Glu-C protease digestion (0.1 mg/ml). After fractionation, 3  $\mu$ l of each fraction was incubated with <sup>32</sup>P-CB59 DNA and then subjected to gel mobility shift analysis. (E) Glycerol density gradient sedimentation of the extract in the presence of 0.1% sarcosyl. After fractionation, proteins in each fraction were recovered by acetone precipitation, and DNA binding activity of CENP-B was reactivated by the renaturation process. 3  $\mu$ l of each fraction was subjected to gel mobility shift analysis. (C–E) The positions of size markers were indicated by arrows (mouse IgG, 167 kD; BSA, 66 kD; horse myoglobin, 14.3 kD), and the relative mobility of each complex was indicated on the right. The following sets of lanes were the same exposures: lanes 1–19 and 20–24.

tract of HeLa cells as described in Materials and Methods, and molecular mass of the purified CENP-B before and after cross-linking with glutaraldehyde was examined by Western blot analysis after SDS-PAGE using ACA serum (Fig. 3 B). After cross-linking reaction, the intensity of the ~80-kD CENP-B band decreased concomitantly with the appearance of a new band of 160 kD, whereas the mobility of BSA was unaffected by the same treatment (not shown). As shown in Fig. 3 A, the purified CENP-B fraction contained 80-kD molecules as a major component and no other major protein bands were detected around the 80-kD region. From these results we conclude that CENP-B forms a dimer in native conditions. If a CENP-B dimer of 92 kD (Fig. 2 C) binds two molecules of <sup>32</sup>P-CB59 DNA (60 kD  $\times$  2) (Fig. 2 A), the molecular mass of complex A is calculated as 212 kD, which is in good agreement with 190 kD, the value deduced from the sedimentation rate of complex A in the glycerol gradient (Fig. 2 A). All these results are in line with the idea that complex A is formed between a CENP-B dimer and two molecules of alphoid DNA.

#### Localization of the DNA Binding Domain Using Truncated CENP-B Polypeptides

To characterize DNA binding property of CENP-B, we cloned the CENP-B cDNA (pETCENP-B) and expressed in *Escherichia coli* cells as described in Materials and Methods. Various deletions from the COOH terminus and an internal deletion near the NH<sub>2</sub> terminus of the CENP-B gene were constructed and the truncated polypeptides expressed in *Escherichia coli* cells were detected with Western blot analysis (Fig. 4 A). The DNA binding activities specific to the CENP-B box of these polypeptides were determined by Southwestern blot analysis (Fig. 4 B). The expressed CENP-B protein, which showed the same mobility as that of full-size CENP-B from HeLa cells (data not shown), was reactive for ACA serum (Fig. 4 A, lane 2), and had the DNA binding activity specific to the CENP-B box (Fig. 4 B, lane 2). The polypeptide consisting of amino acid residues 1–160 (numbering for the NH<sub>2</sub> terminus) (Fig. 4 B, lane 6) and longer (lanes 2–5) showed full DNA binding activity, but the



**Figure 3.** (A) SDS-PAGE of the purified CENP-B. 10  $\mu$ l of the purified CENP-B fraction II was electrophoresed through a 7.5% SDS-polyacrylamide gel in Laemmli buffer system. The gel after electrophoresis were subjected to silver staining (lane 1). Molecular weight of marker proteins were indicated on the left. (B) Chemical cross-linking of CENP-B with glutaraldehyde. The purified CENP-B fraction II (10  $\mu$ l) was incubated at 25°C for 0 (lane 1), 5 (lane 2), and 20 min (lane 3) with 0.5 mM glutaraldehyde. After the reaction, the samples were electrophoresed through a 5% SDS-polyacrylamide gel in Weber-Osborn buffer system. The proteins in the gel after electrophoresis were transferred to a polyvinylidene difluoride membrane. The membrane containing marker proteins (lane M) was cut apart and was stained with Coomassie brilliant blue R250. The rest of the membrane containing samples were subjected to Western blot analysis using ACA serum. Myosin (200 kD), *Escherichia coli*  $\beta$ -galactosidase (116 kD), phosphorylase B (92.5 kD), BSA (66.2 kD), and ovalbumin (45 kD) used as marker proteins were indicated on the left.

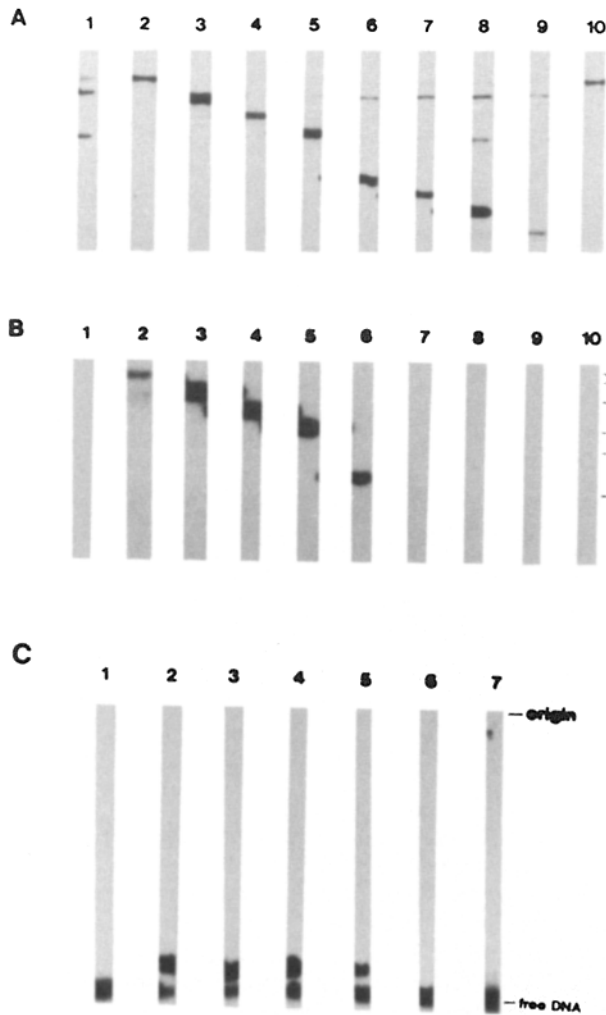
activity was weak with the polypeptide spanning from amino acid residues 1 to 143 (Fig. 4 B, lane 7) and was absent in the polypeptide from 1 to 104 and the shorter (Fig. 4 B, lanes 8 and 9). The polypeptide with a deletion spanning from 10 to 25 had no DNA binding activity (Fig. 4 B, lane 10). These results show that the DNA binding domain exclusively locates at the NH<sub>2</sub>-terminal region. It is difficult to detect the DNA binding activity of a short polypeptide by Southwestern blot analysis since an essential part of the polypeptide might be used to immobilize itself on a membrane. Therefore, DNA binding activities of the short NH<sub>2</sub>-terminal polypeptides were reexamined by the gel mobility shift analysis (Fig. 4 C) and by the immunoprecipitation (not shown). Full DNA binding activity was detected with the NH<sub>2</sub>-terminal 125-amino acid polypeptide (Fig. 4 C, lane 5), but it was completely abolished when size of the NH<sub>2</sub>-terminal polypeptide was shortened to 104 amino acids (lane 6). Therefore, DNA binding domain resides within the NH<sub>2</sub>-terminal 125 amino acids.

#### ***CENP-B Has Sites Highly Sensitive to Glu-C Protease, Chymotrypsin, and Thrombin***

To examine molecular structure of CENP-B·alphoid DNA complex, we have limitedly digested the purified CENP-B molecules from HeLa cells with various proteases at 0°C in native conditions and subjected the digests to gel mobility shift analyses after incubation with <sup>32</sup>P-CB59 DNA. Digestions with Glu-C protease (Fig. 5, lanes 2-4), chymotrypsin (lanes 6-8), and thrombin (lanes 9-12) produced complexes with different relative electrophoretic mobilities characteristic to each protease. The aliquots of the reaction products used in Fig. 5 were subjected to Western (Fig. 6 A) and Southwestern (Fig. 6 B) blot analyses. A diffuse band of ~60 kD that was produced by the limited digestion with Glu-C protease (Fig. 6 A, lanes 2-4) did not react with mACA-1

(Fig. 6 C, lane 4), whose epitope resides in the COOH-terminal portion of CENP-B (amino acid numbers from the NH<sub>2</sub> terminus 452-599; see Fig. 8), suggesting that this fragment lacked the COOH-terminal portion of the CENP-B molecule. As shown in Fig. 6 B, lanes 2 and 3, the 60-kD fragment had CENP-B box-specific DNA binding activity. With increasing amounts of Glu-C protease, fragments of ~52 and 39 kD with DNA binding activities became detectable (Fig. 6 B, lanes 3 and 4). The 60-kD fragment, recovered from SDS-PAGE and renatured, formed a complex with <sup>32</sup>P-CB59 DNA with relative mobility 0.58 in gel mobility shift analysis (Fig. 7 A, lane 2). The complex with relative mobility 0.67 shown in Fig. 5, lane 3, may contain the 39-kD fragment detected in Fig. 6 B, lane 3. When the purified CENP-B fraction was digested with chymotrypsin (Fig. 6 A, lanes 6-8) and thrombin (Fig. 6 A, lanes 9-11), sharp bands of ~60 and 66 kD appeared, respectively. The 60-kD chymotrypsin fragment bound mACA-1 (Fig. 6 C, lane 6) but lacked DNA binding activity (Fig. 6 B, lanes 6-8), indicating that this fragment contains the COOH-terminal portion of CENP-B but lacks the NH<sub>2</sub>-terminal portion. The 66-kD thrombin fragment also apparently lacks the NH<sub>2</sub>-terminal portion because it had no DNA binding activity (Fig. 6 B, lanes 10 and 11). To detect the fragments with DNA binding activity after digestion with chymotrypsin or thrombin, we digested a large amount of purified CENP-B and analyzed the Western and Southwestern blots. As shown in Fig. 6 D, lane 3, an antigenic 21-kD band was detected after digestion with chymotrypsin as the sole fragment with the CENP-B box-specific DNA binding activity (Fig. 6 D, lane 4). This 21-kD fragment formed the complex of relative mobility 0.83 in gel mobility shift analysis (Fig. 7 A, lane 3). As shown in Fig. 6 D, lane 5, two bands corresponding to fragments of 16 and 16.5 kD were detected after digestion with thrombin. Although we failed to detect DNA binding activity





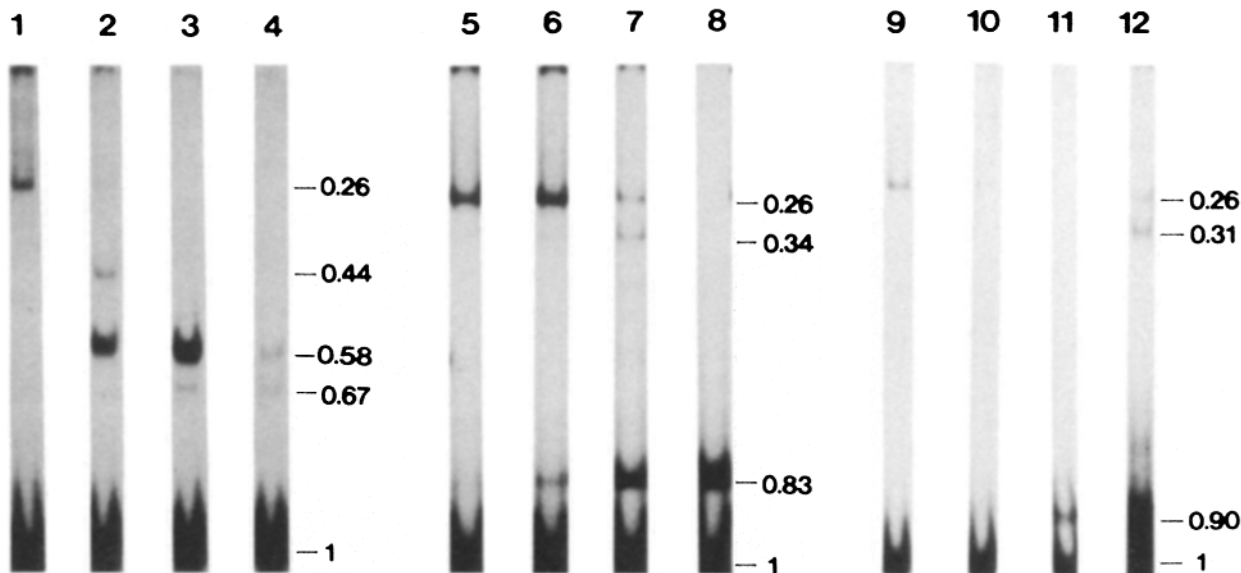
**Figure 4.** Western (A) and Southwestern blot analysis (B) of the truncated CENP-B polypeptides expressed in *Escherichia coli* cells. The crude lysate of each transformant with a plasmid carrying CENP-B cDNA or its deletion derivative was electrophoresed through duplicate 15% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes. One membrane was subjected to immunoblot analysis using ACA serum (A) and the other to Southwestern blot analysis using  $^{32}\text{P}$ -CB 59 DNA as a probe (B). Lanes 1, pET vector; lanes 2, pETCENP-B; lanes 3, pET-CBN-411; lanes 4, pETCBN-333; lanes 5, pETCBN-234; lanes 6, pETCBN-160; lanes 7, pETCBN-143; lanes 8, pETCBN-104; lanes 9, pETCBN-63; and lanes 10, pETCB $\Delta$ 10-25. Horizontal bars on the right side indicate the positions of prestained marker proteins (Bio-Rad Laboratories); approximate molecular masses are 130, 75, 50, 27, and 17 kD from the top, respectively. Each lane of A and B was derived from the same membranes, respectively. (C) Gel mobility shift analysis of NH<sub>2</sub>-terminal CENP-B polypeptides. Roughly equal amounts of expressed CENP-B polypeptides were subjected to gel mobility shift analysis after incubation with  $^{32}\text{P}$ -CB 59 DNA. Lane 1, pET vector; lane 2, pETCBN-160; lane 3, pETCBN-143; lane 4, pETCBN-137; lane 5, pETCBN-125; lane 6, pETCBN-104; and lane 7, pETCBN-63. Lanes 1-7 are the same exposure.

specific to the CENP-B box by Southwestern blot analysis (Fig. 6 D, lane 6), either or both of these fragments formed the complex of relative mobility 0.90 in gel mobility shift analysis (Fig. 7 A, lane 4). Based on these results, the ap-

proximate cleavage sites for these proteases were mapped on CENP-B molecule as shown in Fig. 8. The properties of the protease fragments were summarized in Table I.

#### **Properties of DNA/Protein Complexes Formed with Proteolytic Products of CENP-B in Native States: The DNA Binding Domain Is Separable from Dimerizing Activity**

As shown in Fig. 3, CENP-B forms a dimer in native conditions and complex 0.26 (complex A) is most probably composed of CENP-B dimer and two molecules of DNA. Complex A changed to a complex with relative mobility 0.50 when electrophoresed at 37°C (Fig. 1, lane 9), which may probably reflect the dissociation of a CENP-B dimer to monomers under the elevated temperature. As shown in Fig. 5, weak digestion of CENP-B with the proteases produced the paired complexes, one with relative mobility >0.5 and the other <0.5; 0.58 and 0.44 by Glu-C protease (lane 2), 0.83 and 0.34 by chymotrypsin (lanes 6 and 7), and 0.90 and 0.31 by thrombin (lane 12). The complexes 0.58, 0.83, and 0.90 were previously shown to be formed with the 60-, 21-, and 16 (16.5)-kD fragments (Fig. 7 A), respectively. Although the 60-kD Glu-C protease fragment lacked only 20-kD COOH-terminal region of the 80-kD full-size CENP-B molecule (Fig. 8), the relative mobility of the complex drastically changed to 0.58 (Fig. 5, lanes 2-4) from 0.26 (Fig. 5, lane 1). Results of the glycerol gradient centrifugation of the 60-kD Glu-C protease fragment also indicated the drastic decrease in the molecular mass of the native form from that of the full-size molecule (Fig. 2 D and Table I). An appealing interpretation of these results is that CENP-B dimer molecules would be cut into two parts by the proteolytic cleavage, the 60-kD Glu-C protease fragment and the rest (a 20-kD fragment plus a full-size CENP-B molecule); and the 60-kD Glu-C protease fragment would have lost dimerizing activity as a result of truncation of the COOH-terminal region although it retains the full DNA binding activity. If this is the case, both of the complexes 0.58 and 0.44 would contain only one molecule of DNA and the complex 0.44 would contain the COOH-terminal portion. As shown in Fig. 7 B, the complexes 0.44 and 0.58 both contained only one DNA molecule since no additional band appeared by the addition of non-radioactive CB23 DNA to the binding mixture containing  $^{32}\text{P}$ -CB59, whereas complex A (relative mobility 0.26) contained two DNA molecules as shown by the appearance of a new band (arrowhead in Fig. 7 B, lane 3) between the complex formed with  $^{32}\text{P}$ -CB 59 DNA (lane 1) and that formed with  $^{32}\text{P}$ -CB 25 DNA (lane 6) as reported by Muro et al. (1992). The same analysis with complexes 0.34, 0.83, 0.31, and 0.90 indicated that they also contained only one DNA molecule (data not shown). As shown in Fig. 7 C, lanes 1 and 3, complex 0.44 had epitope for mACA-1, whereas complex 0.58 did not, indicating that complex 0.44 contained the COOH-terminal portion of CENP-B as well as a single DNA binding domain. Thus, the lack of activity of the 60-kD Glu-C protease fragment in juxtaposing two DNA molecules into a complex is parallel with loss of the 20-kD COOH-terminal region, which may be necessary for dimerization. On the other hand, the bound protein of complex 0.44 seemed to be larger than the intact CENP-B monomer, because its relative mobility was <0.50. These results suggest that com-



**Figure 5.** Gel mobility shift analysis of the purified CENP-B after protease digestion. 40  $\mu$ l of the CENP-B fraction I was digested with proteases at 0°C for 1 h. A 3- $\mu$ l aliquot of each protease digest was subjected to gel mobility shift analysis. The rest of the digests were used in Fig. 6: lanes 1 and 5, no protease; lanes 2-4, Glu-C protease 0.01, 0.1, and 1 mg/ml; lanes 6-8, chymotrypsin 0.01, 0.1, and 1  $\mu$ g/ml; lanes 9-11, thrombin 0.01, 0.1, and 1 mg/ml; lane 12, overexposure of lane 11. Lanes 1-4, 5-8, and 9-11 were the same exposures, respectively.

plex 0.44 may contain a heterodimer of a full-size CENP-B and the COOH-terminal 20-kD fragment of CENP-B, which may be produced by excision of a 60-kD Glu-C fragment from a CENP-B dimer. The molecular structure of the complex with relative mobility of <0.5 will be discussed in the Discussion under the light of the independent location of the DNA binding and dimerization domains.

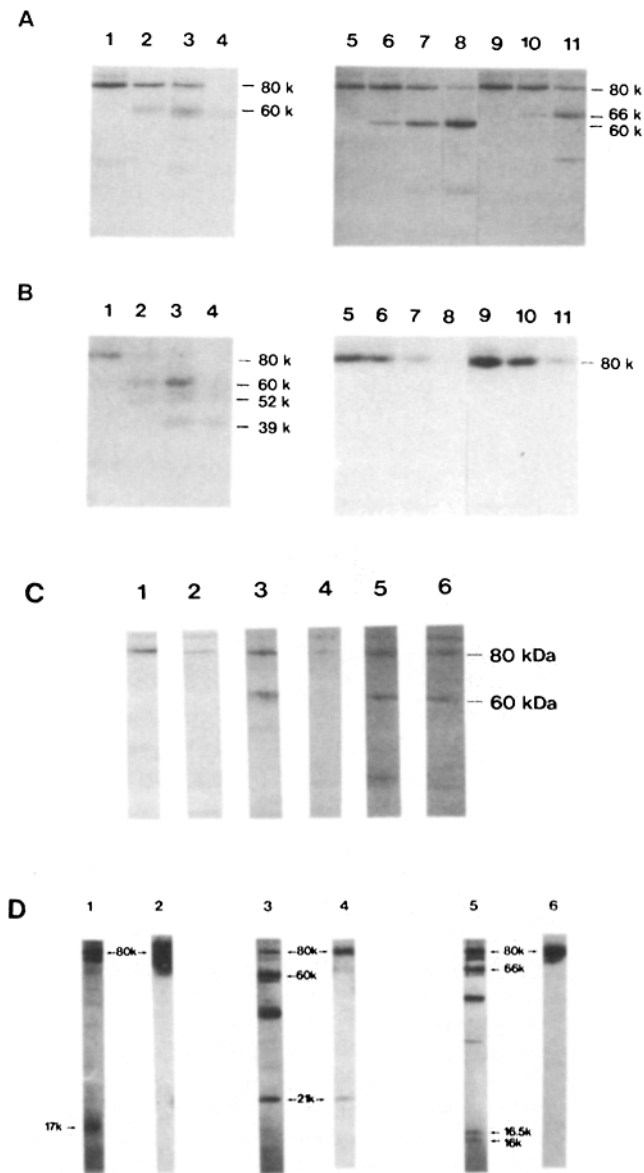
## Discussion

### Molecular Structure of the CENP-B·Alphoid DNA Complex

In this report, we have examined the structure of the CENP-B·alphoid DNA complex made *in vitro*. We have shown that CENP-B forms a dimer in native conditions which, together with molecular weight analyses, suggested that a stable CENP-B·alphoid DNA complex, complex A, was formed between a CENP-B dimer and two molecules of DNA. The DNA binding domain was delimited to the NH<sub>2</sub>-terminal 125 amino acids by the analyses of truncated CENP-B molecules expressed in *Escherichia coli* cells. Using CENP-B molecules purified from HeLa cells, we then showed that CENP-B had sites highly sensitive to Glu-C protease, chymotrypsin, and thrombin. The approximate cleavage sites of the proteases are summarized in Fig. 8. The gel mobility shift analysis of the complexes formed between <sup>32</sup>P-CB59 DNA and the CENP-B fragments produced by the protease digestions in native conditions showed that newly appeared complexes contained only one molecule of DNA. As far as the major complexes with the relative mobilities more than 0.5 are concerned, the loss of activity of CENP-B in juxtaposing two DNA molecules into a complex was paralleled with the removal of the COOH-terminal region. The longest

fragment thus far obtained that lacked juxtaposing activity was the 60-kD fragment produced by Glu-C protease digestion. Therefore, the removed 20-kD region may be necessary for dimerization of CENP-B. To deduce further the molecular structure of various complexes formed with protease-digested CENP-B, we have analyzed for each of the complexes the relationship between its relative mobility and the molecular weight of the bound protein. For this purpose we made use of the findings that the relative mobility of a protein/DNA complex in the gel mobility shift assay diminishes in proportion to the logarithm of the molecular weight of the bound protein (Schreiber et al., 1988; Tan and Richmond, 1990). When the logarithm of the molecular weight of each CENP-B fragment with DNA binding activity was plotted against the relative mobility of the complex formed between <sup>32</sup>P-CB59 DNA and the fragment (Fig. 9, closed circle), a good linear correlation was observed. From this linear correlation, the bound protein of the complex 0.50 (Fig. 1, lane 9) was deduced to be ~80 kD (Fig. 9, open circle), which supports the previous estimation that the DNA·CENP-B complex detected after electrophoresis at 37°C may contain a CENP-B monomer molecule. We examined whether the same relationship may be applicable to the complexes with relative mobility of <0.50 shown in Fig. 5, lanes 2, 7, and 12, all of which seemed to contain the proteins >80 kD. These slower mobility complexes were produced only transiently by weak protease digestion and always appeared concomitantly with the major complexes with the relative mobility more than 0.50. In contrast with the complexes with relative mobility of >0.5 that contained one DNA molecule but lacked the COOH-terminal portion of CENP-B, the complexes of relative mobility <0.5 contained both one DNA molecule and the COOH-terminal portion of CENP-B (Fig. 7 C, lanes 1 and 3; data not shown). Based on these results,





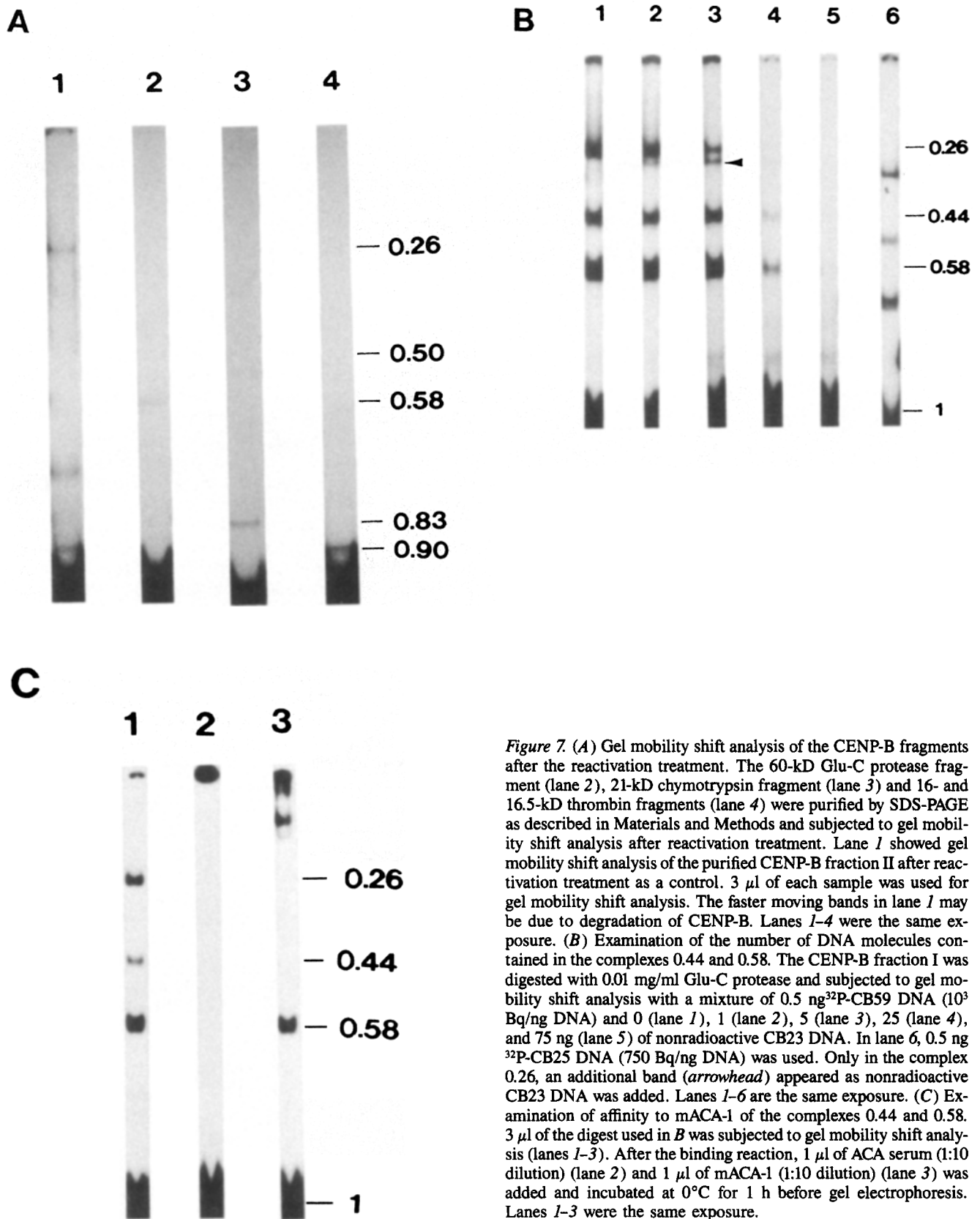
**Figure 6.** Western (A) and Southwestern (B) blot analyses of the purified CENP-B after limited digestion with proteases. The rest of each digested sample used in Fig. 5 was divided into two parts, electrophoresed through duplicate 14% SDS-polyacrylamide gels, and transferred to polyvinylidene difluoride membranes. One membrane was subjected to immunoblot analysis with ACA serum (A) and the other to Southwestern blot analysis using <sup>32</sup>P-labeled CB59 DNA as a probe (B). Lanes 1 and 5, no protease; lanes 2-4, Glu-C protease; lanes 6-8, chymotrypsin; lanes 9-11, thrombin. Protease concentration of each lane was same as in Fig. 5. (C) Immunoblot analysis of CENP-B fragments with mACA-1. Each 20  $\mu$ l of protease digest (lanes 1 and 2, no protease; lanes 3 and 4, Glu-C protease 0.01 mg/ml; and lanes 5 and 6, chymotrypsin 0.1  $\mu$ g/ml) was electrophoresed through a 14% SDS-polyacrylamide gel, and transferred to a polyvinylidene difluoride membrane. The membrane was cut into strips. Each strip was incubated with either ACA serum (1:1,000 dilution) (lanes 1, 3, and 5) and mACA-1 (1:500 dilution) (lanes 2, 4, and 6). (D) Western and Southwestern blot analysis after protease digestion of a large amount of CENP-B. 30  $\mu$ l of the CENP-B fraction II was digested with no protease (lanes 1 and 2), 1  $\mu$ g/ml of chymotrypsin (lanes 3 and 4), or 1 mg/ml of thrombin (lanes 5 and 6). The molecular masses of the fragments and their DNA binding activity specific to CENP-B box were examined

we postulate that the DNA binding domain and the dimerization domain are located independently in the NH<sub>2</sub>- and COOH-terminal regions, respectively, as depicted in Fig. 10 A. In early protease reaction, only one cut would be introduced into a CENP-B dimer by random collision of a protease to a CENP-B dimer, resulting in the cleavage of complex A into two parts (Fig. 10 B); one with relative mobility >0.5 and the other <0.5. Then, the bound protein in each complex with relative mobility of <0.5 would be calculated as CENP-B dimer (160 kD) minus bound protein in the counterpart complex with a mobility >0.5. Thus, the bound proteins of the complexes, 0.44, 0.34, and 0.31 are predicted as 160 kD - 60 kD = 100 kD, 160 kD - 21 kD = 139 kD, and 160 kD - 16 (16.5) kD = 144 (143.5) kD, respectively. When logarithm of these values were plotted against the relative mobilities of the corresponding complexes, they fell on the straight line as shown in Fig. 9 (open triangles). These results support the idea that the complex of relative mobility of <0.50 would be composed of a heterodimer consisting of a full-size CENP-B and a fragmented CENP-B that lacks the DNA binding domain (Fig. 10 B). The 60-kD chymotrypsin fragment and the 66-kD thrombin fragment shown in Fig. 6 A, lanes 8 and 11 are candidates for the components of the heterodimer complexes with relative mobility of 0.34 and 0.31, respectively. Thus, the results of molecular mass estimation of the bound protein of the complexes were also in line with the hypothesis that the dimerization domain of the CENP-B molecule would locate within the COOH-terminal 20-kD region, the smallest fragment predicted to be in the heterodimers (Fig. 8).

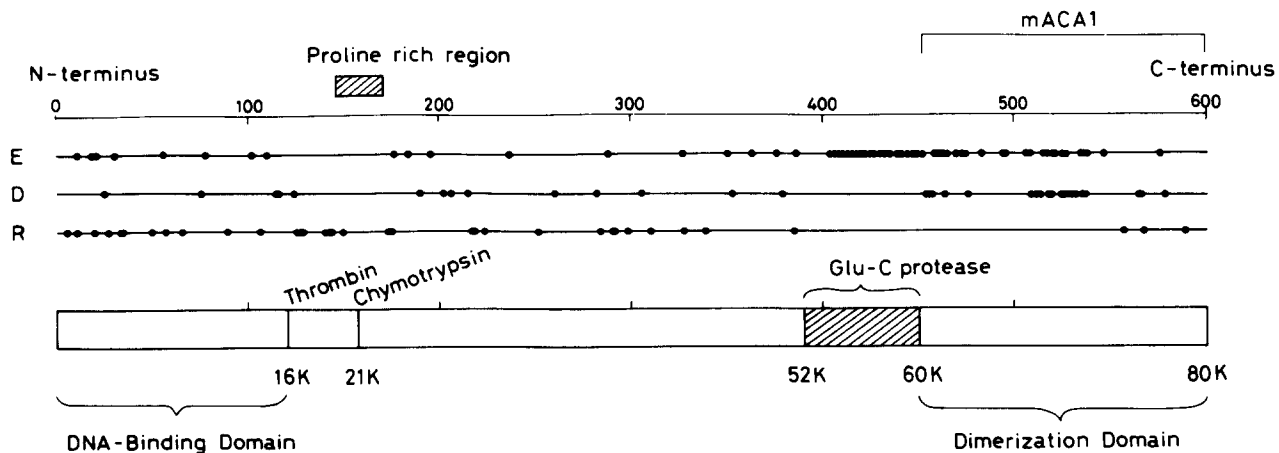
### Domain Structure of CENP-B

Three hinge regions have been predicted from the amino acid sequence of CENP-B (Pluta et al., 1990). One is a proline-rich sequence similar to those found in tau and MAP-2. Tau and MAP-2 contain in the hinge region sites highly sensitive to chymotrypsin and thrombin, respectively (Aizawa et al., 1988; Joly et al., 1989). The present results have demonstrated that sites sensitive to chymotrypsin and thrombin indeed exist near the proline-rich sequence of CENP-B (Fig. 8). The second predicted hinge region is a glutamic acid cluster located in the COOH-terminal region. The sites most sensitive to Glu-C protease were detected around the COOH-proximal glutamic acid cluster (Fig. 8). The region was also highly sensitive to proteinase K or thermolysin (data not shown). Therefore, a second hinge sequence may exist in the region around the COOH-proximal glutamic acid cluster. Thus, the results shown in this study suggest that CENP-B consists of at least three structural domains. The DNA binding domain and dimerization domain may reside as the NH<sub>2</sub>- and COOH-terminal regions of CENP-B, respectively, separated by a central domain of unknown

by Western (lanes 1, 3, and 5) and Southwestern (lanes 2, 4, and 6) blot analyses. Purified CENP-A (17 kD), added to lanes 1 and 2, was also used as a protein size marker. The bands appearing beneath the 80-kD CENP-B, and 60-kD and 66-kD CENP-B fragments were artifacts due to overloading CENP-B protein. The following proteins were used as molecular mass markers in each experiment: rabbit muscle phosphorylase b (97.4 kD), BSA (66.2 kD), hen egg white ovalbumin (42.7 kD), and bovine cardiac anhydrase (31.0 kD) not shown).



**Figure 7.** (A) Gel mobility shift analysis of the CENP-B fragments after the reactivation treatment. The 60-kD Glu-C protease fragment (lane 2), 21-kD chymotrypsin fragment (lane 3) and 16- and 16.5-kD thrombin fragments (lane 4) were purified by SDS-PAGE as described in Materials and Methods and subjected to gel mobility shift analysis after reactivation treatment. Lane 1 showed gel mobility shift analysis of the purified CENP-B fraction II after reactivation treatment as a control. 3  $\mu$ l of each sample was used for gel mobility shift analysis. The faster moving bands in lane 1 may be due to degradation of CENP-B. Lanes 1–4 were the same exposure. (B) Examination of the number of DNA molecules contained in the complexes 0.44 and 0.58. The CENP-B fraction I was digested with 0.01 mg/ml Glu-C protease and subjected to gel mobility shift analysis with a mixture of 0.5 ng<sup>32</sup>P-CB59 DNA (10<sup>3</sup> Bq/ng DNA) and 0 (lane 1), 1 (lane 2), 5 (lane 3), 25 (lane 4), and 75 ng (lane 5) of nonradioactive CB23 DNA. In lane 6, 0.5 ng <sup>32</sup>P-CB25 DNA (750 Bq/ng DNA) was used. Only in the complex 0.26, an additional band (*arrowhead*) appeared as nonradioactive CB23 DNA was added. Lanes 1–6 are the same exposure. (C) Examination of affinity to mACA-1 of the complexes 0.44 and 0.58. 3  $\mu$ l of the digest used in B was subjected to gel mobility shift analysis (lanes 1–3). After the binding reaction, 1  $\mu$ l of ACA serum (1:10 dilution) (lane 2) and 1  $\mu$ l of mACA-1 (1:10 dilution) (lane 3) was added and incubated at 0°C for 1 h before gel electrophoresis. Lanes 1–3 were the same exposure.



**Figure 8.** Mapping of the sites highly sensitive to the proteases. Amino acid numbers from the NH<sub>2</sub> terminus were shown above the horizontal lines. The approximate sites to each protease were shown below. These sites were located proportional to the molecular mass of each fragment assuming that intact CENP-B is 80 kD. See text for details of the determination of these sites. Sites of glutamic acid (E), aspartic acid (D), and arginine (R) in CENP-B were indicated with dots on each line. A proline-rich region was indicated by a shaded box. The region where the epitope of mACA-1 resides was bracketed (Earnshaw et al., 1987).

function. The central domain may contain most of the glutamic acid cluster with which other proteins might interact. It has been reported that tubulin interacts with MAP-2 or cytoplasmic dynein at an EGEE sequence located at the COOH-terminus of tubulin (Paschal et al., 1989; Cleveland et al., 1990). CENP-B has many EGEE sequences in the Glu-C cluster region.

#### DNA Binding Motif in CENP-B

As Sullivan and Glass (1991) have suggested, the DNA binding domain of CENP-B seems to be composed of the helix-loop-helix (HLH) motif. Four  $\alpha$ -helices (amino acids 10–22, 46–56, 77–93, and 100–111) were predicted in the NH<sub>2</sub>-terminal 125 amino acids by the Chou-Fasman algorithm (Fig. 11 A). It seems that a tertiary structure composed of the four  $\alpha$ -helices is necessary for DNA binding activity, since the specific DNA binding activity was completely abolished by a deletion of helix 1 (10–22) or helix 4 (100–111)

(Fig. 4). Relatively long loops intervene between helix 1 and helix 2 and between helix 2 and helix 3. When helix 3 (77–93) was used to search the NBRF/PIR protein data base using the program of Lipman and Pearson (1985), we detected 41% similarity over a 17-residue overlap to the third  $\alpha$ -helix of homeodomain proteins (Fig. 11 A). Although the region of similarity was very short, the most conserved amino acid sequence among every homeodomain protein, Trp48 Phe49 Gln50 Asn51, corresponded with Trp84 Phe85 Gln86 Gln87 of CENP-B, and one of the core residues Ile45 corresponded with Leu81 (Fig. 11 A). Helix 3 is linked to helix 4 (100–111) with a short stretch (six amino acids) of  $\beta$  structure, and helix 4 is indispensable for DNA binding activity (Fig. 4 C). Helix 4 forms a typical amphipathic helix as shown in Fig. 11 B (Segrest et al., 1990) and might be important for formation of tertiary structure in the DNA binding domain of CENP-B. Sullivan and Glass (1991) have suggested that the DNA binding domain of CENP-B is homologous to the helix-loop-helix (HLH) fam-

**Table 1.** Summary of the Properties of CENP-B Fragments Produced by the Limited Digestion with Proteases

Protease used	Apparent molecular weight (kD) measured by		Affinity to mACA-1 <sup>d</sup> (epitope: COOH terminus 452–599)	DNA-binding activity <sup>e</sup> (DNA binding domain: NH <sub>2</sub> terminus 1–125)	Alphoid DNA-protein complex	
	SDS-PAGE <sup>a</sup>	Glycerol gradient <sup>b</sup> sedimentation			Relative mobility <sup>f</sup>	Number of bound <sup>g</sup> DNA
No	80	92 <sup>c</sup>	+ (W & G)	+ (S & G)	0.26	2
Glu-C protease	60	37	– (W & G)	+ (S & G)	0.58	1
Chymotrypsin	21		– (G)*	+ (S & G)	0.83	1*
	60		+ (W)	+ (S)		
Thrombin	16 (16.5)		– (G)*	+ (G)	0.90	1*
	66		n.t.**	– (S)		

\* Data not shown.

\*\* Not tested.

<sup>a</sup> These values were taken from Fig. 6 A and D.

<sup>b</sup> These values were from Fig. 2 C and D.

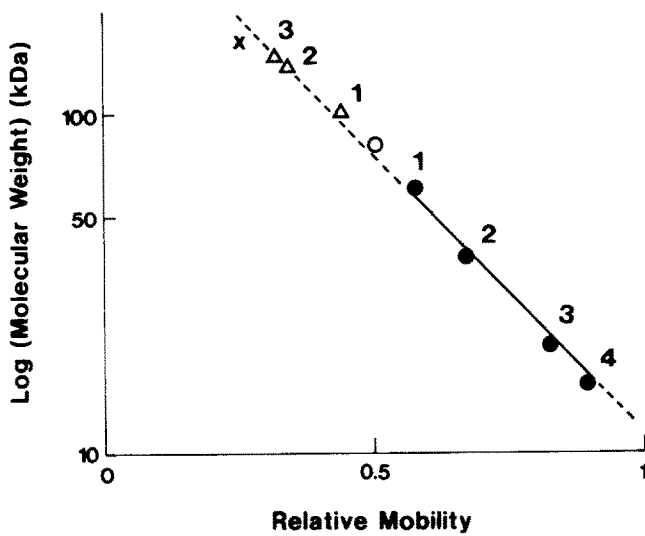
<sup>c</sup> This value represented a dimeric form of CENP-B.

<sup>d</sup> Affinity to mACA-1 was tested by Western blot analysis (W) (Fig. 6 C) or gel mobility shift analysis (G) (Fig. 7 C).

<sup>e</sup> These were examined by Southwestern blot analysis (S) (Fig. 6 B and D) or gel mobility shift analysis (G) (Fig. 5 and Fig. 7 A).

<sup>f</sup> These values were from Figs. 5 and 7 A.

<sup>g</sup> These values were from Muro et al. (1992) and Fig. 7 B.



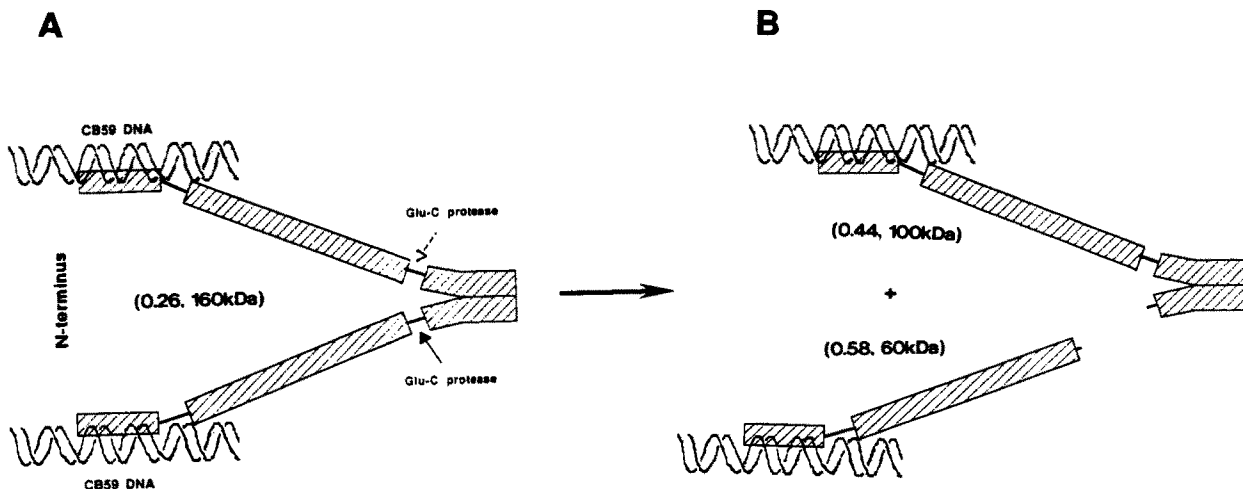
**Figure 9.** Logarithm of the molecular mass of bound protein was plotted against the relative mobility of the DNA-protein complex. The points were taken from the following results: (● 1 and ● 2) Glu-C protease digestion, Figs. 5 and 6 B, lane 3 and Fig. 7 A, lane 2; (● 3) chymotrypsin digestion, Fig. 7 A lane 3; (● 4) thrombin digestion, Fig. 7 A, lane 4. (○) Fig. 1, lane 9. (Δ1) (0.44, 100 kD) (Glu-C protease digestion), Fig. 5, lane 2 (100 kD = 160 kD - 60 kD); (Δ2) (0.34, 139 kD) (chymotrypsin digestion), Fig. 5, lane 7 (139 kD = 160 kD - 21 kD); (Δ3) (0.31, 144 (143.5) kD) (thrombin digestion), Fig. 5, lane 12 (144 (143.5) kD = 160 kD - 16 (16.5) kD). See text and Fig. 10 for details. (X) Complex A (0.26, 126 kD), Fig. 1, lane 1.

ily of proteins, although they also discussed the possibility that CENP-B represents a distant member of the HLH family with altered protein-protein interactions. The NH<sub>2</sub>-terminal region containing basic amino acids (residues 1-14) partially overlaps with the HLH motif as proposed by them. However, the successive  $\alpha$ -helix region of our model is different from

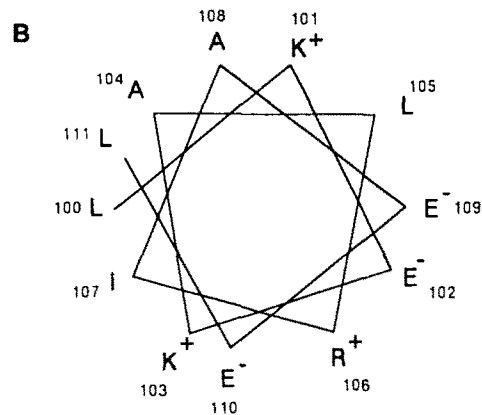
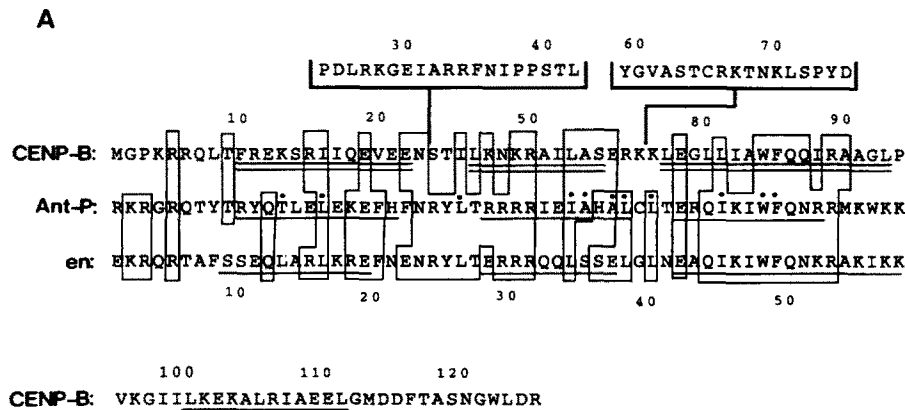
what they proposed. The presence of a proline residue at position 24 may not be compatible with the formation of an  $\alpha$ -helix in the position they suggested for helix 1. More importantly, in HLH proteins the HLH motif mediates dimerization whereas the upstream basic region is involved in DNA binding (Davis et al., 1990). In CENP-B, however, we could separate the DNA binding activity from dimerizing activity by protease digestion, and our results show that the DNA binding activity of CENP-B is independent of dimerization. Therefore, the DNA binding motif of CENP-B is likely to be different from that of the typical HLH family of proteins. CENP-B is unlikely to be a transcriptional regulatory protein but is likely to be a structural protein interacting specifically with the CENP-B box sequences of highly repetitive alphoid DNA in the centromeric region. CENP-B might represent a new type of DNA binding protein with a unique functional role in centromeric region, and the observed partial similarity with the homeodomain proteins or HLH proteins might simply reflect similar structural constraints imposed by a common function.

### Localization and Hypothetical Function of CENP-B In Vivo

The structure of the CENP-B-alphoid DNA complex shown in Fig. 10 A implies a physiological role for CENP-B in juxtaposing two CENP-B boxes located intra- and/or intermolecularly, and to form a higher order structure through DNA-protein and protein-protein interactions at the centromeric region. In addition to complex A, we have detected complex B (Fig. 1, lane 1) which seems to be much larger than complex A (Fig. 2 A). This suggests that complex A is not the sole complex formed between CENP-B and alphoid DNA, but a higher order complex, such as a multimer of complex A or a complex containing other protein(s), might be formed between CENP-B and alphoid DNA. Pluta et al. (1992) have recently shown that the NH<sub>2</sub>-terminal 158



**Figure 10.** Hypothetical structure of complex A (A) and its digestion with Glu-C protease (B). This model is based on the hypothesis that the DNA binding domain and the dimerization domain are independently located in the NH<sub>2</sub>- and COOH-terminal regions, respectively. Glu-C protease digestion is shown as an example. At early digestion, only one cut is introduced into a CENP-B dimer, following random collision of CENP-B dimer and a protease molecule. Then complex A is separated into two parts (B): one is the major complex of relative mobility 0.58 that contains the bound protein 60 kD, and the other is the minor complex of relative mobility 0.44 that contains the bound protein 160 kD - 60 kD = 100 kD.



*Figure 11. (A) Comparison of the DNA binding domain of CENP-B and homeo-domain sequences. The amino acid sequence of CENP-B was taken from Earnshaw et al. (1987) and Sullivan and Glass (1991).  $\alpha$  Helices of CENP-B predicted by the Chou-Fasman algorithm are double-underlined, and those of Ant-P (Qian et al., 1989) and en. (Kissinger et al., 1990) were underlined. The core amino acid residues of Ant-P were indicated with dots over each residue. (B) Helical wheel analysis of the helix 4 (100-111).*

amino acid residues of CENP-B is necessary and sufficient for CENP-B to localize to the centromeric region by the method expressing epitope-tagged deletion derivatives of CENP-B in HeLa cells. They have also shown that the same NH<sub>2</sub>-terminal fragment of CENP-B has the DNA binding activity specific to CENP-B box in vitro using the whole cell extract from African green monkey (COS) cells transfected with an SV-40 origin-containing plasmid carrying the CENP-B truncated gene. Sugimoto et al. (1992) have shown that the DNA binding activity of CENP-B locates within the NH<sub>2</sub>-terminal 134 amino acid residues using the truncated CENP-B expressed in *Escherichia coli* cells. These results together with the present results strongly suggest that CENP-B actually localizes to the alphoid DNA repeats at centromere regions through specific interaction of the NH<sub>2</sub>-terminal region of CENP-B with the "CENP-B box." If the dimerization domain of CENP-B exists in the COOH-terminal region as suggested by the present results, the internally expressed CENP-B truncates which have been deleted with the NH<sub>2</sub>-terminal region but contained the COOH-terminal region might have been expected to be localized to the centromeric region through protein-protein interaction. The results of Pluta et al. (1992) did not fulfill this expectation. By immunoelectron microscopy observation, CENP-B has been shown to be localized mainly in the central region of the centromere beneath the kinetochore in HeLa cell chromosomes where alphoid DNA is located (Cooke et al., 1990). It has also been reported that antigens to ACA serum

were localized at the kinetochore in PtK<sub>2</sub> or CHO cell chromosomes (Brenner et al., 1981; Moroi et al., 1981). Minor satellite DNA from *Mus musculus* has been reported to be confined to the region of the kinetochore domain (Wong and Rattner, 1988). Microinjection of anticentromere antibodies purified from ACA serum has been reported to prevent the chromosomes from undergoing prometaphase movements in the subsequent mitosis (Bernat et al., 1990; 1991; Simerly et al., 1990), suggesting that anticentromeric antigens including CENP-B may play a role in some stage of chromosome movement. Interestingly, it has been shown that microinjection of anticentromere antibodies before the end of S phase inhibited formation of a kinetochore structure (Bernat et al., 1991). Zinkowski et al. (1991) have proposed a repeat subunit model, in which the centromere/kinetochore complex is a collection of functional kinetochore units. It is attractive to assume that repetitive appearance of the CENP-B box in alphoid DNA might in some way be related to such a repeating unit. The CENP-B gene is highly conserved between human and mouse (Sullivan and Glass, 1991). In particular, the DNA binding domains are identical between the two species. We have also detected the CENP-B genes in genomic DNA of African green monkey, calf, Indian muntjac, and hamster cells by polymerase chain reaction (data not shown). On the other hand, the DNA sequences of the centromeric regions of these species are diverged. To date, the presence of CENP-B boxes has been confirmed in the  $\alpha$ -satellite DNA repeats from human, chimpanzee, and

gorilla chromosomes and in the minor satellite DNA from *Mus musculus* mouse. In other mammalian species, however, the predicted target sequence for CENP-B has yet to be found.

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