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Analysis of Protein-DNA and Protein-Protein Interactions of Centromere Protein B (CENP-B) and Properties of the DNA–CENP-B Complex in the Cell Cycle

KATSUMI KITAGAWA,[1] HIROSHI MASUMOTO,[1]* MASAKO IKEDA,[2] AND TUNEKO OKAZAKI[1]

Department of Molecular Biology, School of Science, Nagoya University, Chikusa-ku, Nagoya 464-01,[1] and Medical and Biological Laboratories, Corporation Limited, Terasawa-oka, Ina, Nagano Prefecture,[2] Japan

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We previously reported that centromere protein B (CENP-B) forms a stable complex (designated complex A) containing two alphoid DNAs in vitro. Domains in the CENP-B polypeptide involved in the formation of complex A were determined in the present study with truncated derivatives expressed in *Escherichia coli* and in rabbit reticulocyte lysates. It was revealed by gel mobility shift analyses that polypeptides containing the NH_2-terminal DNA-binding domain bind a DNA molecule as a monomer, while dimerizing at a novel hydrophobic domain in the COOH-terminal region of 59 amino acid residues. This polypeptide dimerization activity at the COOH-terminal region was also confirmed with the two-hybrid system in *Saccharomyces cerevisiae* cells. The results thus proved that CENP-B polypeptides form a homodimer at the COOH-terminal hydrophobic domain, each binding a DNA strand at their NH_2-terminal domains. The dimerization and DNA-binding domains fall into two of the three completely conserved sequences found in human and mouse CENP-B, and complex A-forming activity was also detected in nuclear extracts of mouse cells. Metaphase-specific phosphorylation of CENP-B was also detected, but this had no effect on its complex A-forming activity. On the basis of the present results, we propose that CENP-B plays an important role in the assembly of specific centromere structures by forming unique DNA-protein complexes at the sites of CENP-B boxes on the centromeric repetitive DNA both in interphase nuclei and on mitotic chromosomes.

The centromere is the essential chromosomal domain required for the stable segregation of eukaryotic chromosomes at cell division. DNA and protein elements involved in centromere function have been isolated and extensively characterized in yeast species, and in *Saccharomyces cerevisiae*, the *CEN* sequence specifying *cis* activity for full centromere function has been defined (8, 17). Proteins binding to the CEN sequence, CP1, CBF3 (-A, -B, and -C), and CBF5p, have been purified and characterized (2, 20, 24). In addition, by genetic cloning approach, *NDC10* (*CBF2*, *CTF14*) and *CTF13* have been found to be responsible for chromosome segregation, turning out to be identical to the genes encoding CBF3A and CBF3C, respectively (9, 13, 19). In another yeast sp., *Schizosaccharomyces pombe*, the *CEN* sequence has been delimited to several tens of kilobases and has been shown to consist of a large inverted repeat arranged in a bilaterally symmetrical pattern about a central core sequence (7, 16).

In mammalian cells, centromeric heterochromatin is composed of satellite DNAs which are highly polymorphic families of repetitive DNA sequences. One of these satellite DNAs, alpha-satellite DNA, is composed of tandem repeats of 171-bp diverged monomer units that are arranged to form arrays of several megabases in all human chromosomes (46, 47). Introduction of alpha-satellite DNA into mammalian cells results in de novo formation of several centromere features (15, 23). The centromere region of mammalian chromosomes has also been investigated cytologically, in detail, with electron microscopic studies revealing that microtubules attach to the kinetochore, a special structure consisting of a trilaminar disk at the outer surface (35, 37). It has been suggested that the tubulin-related motor proteins, dynein and kinesin, are localized in this region (32, 41, 49). The first molecular markers for mammalian centromeres were found with centromere-specific autoantibodies from sera of autoimmune patients (28). Three major centromere-specific proteins, namely, CENP-A, -B, and -C, have been identified in this way (10). CENP-A (17 kDa) is a histone H3-like protein which seems to form a centromere-specific nucleosome (31). The CENP-B gene has been cloned and shown to be highly conserved between humans and mice (11, 44), and the properties of CENP-B (80 kDa) have been studied extensively (26, 29, 33, 43, 50). cDNA encoding CENP-C (140 kDa) has also been cloned, and the antigen has been localized to the inner kinetochore plate (38).

An important step in understanding the molecular mechanism of centromere formation is the characterization of protein components that bind specifically to DNAs at the centromere region. We have previously shown that CENP-B binds specifically to a 17-bp motif (named the CENP-B box) in centromeric alpha-satellite DNA by using CENP-B obtained from HeLa cells and have delimited the DNA-binding domain within the NH_2-terminal 125 amino acid residues which contained four potential α-helices by using truncated CENP-B polypeptides expressed in *Escherichia coli* (26, 29, 50). We have found that intact CENP-B forms a stable and unique complex in vitro, which we called complex A, containing two DNA molecules and therefore judged to exist as a dimer in its native state (29, 50). Complex A was not formed when the COOH-terminal epitope was deleted or altered through protein purification or protease digestion (29, 50). Thus, we suggested that the dimerization activity of CENP-B is located in the COOH-terminal region and is separable from the NH_2-terminal DNA-binding domain (50).

Several roles of protein-protein dimerization activity in vivo

* Corresponding author. Mailing address: Department of Molecular Biology, School of Science, Nagoya University, Chikusa-ku, Nagoya 464-01, Japan. Phone: (81)52-789-2985. Fax: (81)52-789-3001. Electronic mail address: g44478a@nucc.cc.nagoya-u.ac.jp.

have so far been reported. The dimerization activity of leucine zipper and helix-loop-helix (HLH) proteins is concerned with DNA-binding activity and regulation of gene expression in differentiation and development (6, 25, 30). On the basis of the finding that the NH_2-terminal region of CENP-B features a weak amino acid sequence homology with HLH proteins, several groups have discussed the possibility that this DNA-binding domain of CENP-B belongs to the same family (33, 43, 44). Our previous results were, however, in conflict with this view, and we suggested that CENP-B functions in bundling CENP-B boxes in repetitive DNA by its DNA-binding and dimerization activities.

In the present work, we determined that the NH_2-terminal DNA-binding domain binds DNA as a monomer and analyzed the components and structure of complex A with truncated CENP-B polypeptides expressed in *E. coli* and in rabbit reticulocyte lysates. The dimerization domain was delimited to the COOH-terminal 59 amino acid residues of CENP-B and was confirmed with the yeast two-hybrid system in vivo. Furthermore, we detected phosphorylation of CENP-B in HeLa cells specifically in metaphase, although DNA binding and dimer formation were not influenced by this and were found to be simply proportional to the total amount of CENP-B present in mitotic or interphase cells. On the basis of these results, we propose that CENP-B may play an important role in the assembly of mammalian centromere structures by formation of unique complexes repeated on the long satellite DNA, in interphase nuclei as well as on metaphase chromosomes.

MATERIALS AND METHODS

Cell culture. HeLa cells were cultured in Dulbecco's modified Eagle's medium (Nissui, Tokyo, Japan) supplemented with 10% calf serum (ICN Biomedicals) at 37°C under 5% CO_2. Mouse A9 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Bio Whittaker) under the same conditions.

Yeast strains and media. The genotype of *S. cerevisiae* L40 is *MAT***a** *trp1 leu2 his3 LYS2*::*lexA-HIS3 URA3*::*lexA-lacZ* (45). Yeast strains were grown in a rich medium (1% yeast extract, 2% Bacto Peptone, 2% glucose, and 0.1 mg of adenine per ml) or in a synthetic minimal medium with appropriate supplements (51).

Preparation of 0.5 M NaCl extract from interphase nuclei and metaphase chromosomes. Mitotic HeLa cells were obtained by selective detachment from dishes after treatment with TN-16 (0.5 μM) (Wako, Osaka, Japan) for 10 h. Metaphase chromosomes were isolated from mitotic cells with the same procedure for isolation of interphase nuclei as described by Masumoto et al. (26). Preparation of 0.5 M NaCl extracts from interphase nuclei and metaphase chromosomes was performed as described by Masumoto et al. (26). The cells blocked at mitosis were released by washing with phosphate-buffered saline (PBS) twice and incubation in fresh medium at 37°C for the described period.

Synthetic nucleotides. The chemically synthesized oligonucleotides used as probes and competitors were previously described by Muro et al. (29). The 56-, 29-, and 23-bp double-stranded DNAs containing CENP-B boxes and the 23-bp double-stranded DNA containing the defective CENP-B box were designated CB56, CB29, CB23, and defCB23, respectively. ^{32}P-end-labeled CB59 and ^{32}P-end-labeled CB32 were generated from CB56 and CB29, respectively, by end-filling reactions with Klenow fragments (Takara Shuzo, Kyoto, Japan) as previously described by Muro et al. (29). Primers P1, P2, and C1 were used in the PCR to construct glutathione-*S*-transferase (GST) fusion CENP-B polypeptide expression vectors. The primer sequences are as follows: P1, 5′CCTGGATC CCCTTGGAGGCTGAGGACT3′; P2, 5′ATGGGATCCCCGTACCCAGCTT TGGGG3′; and C1, 5′TCCGAATTCTCAGCTTTGATGTCCAA3′.

Gel mobility shift analysis. The DNA-binding reaction with HeLa or A9 nuclear extracts, as well as truncated CENP-B polypeptides expressed in rabbit reticulocyte lysates, was carried out essentially as previously described by Muro et al. (29). Indicated amounts of protein solution were mixed with 100 μl of the binding buffer (10 mM Tris-HCl [pH 8.0], 10% glycerol, 1 mM EDTA, 0.05% Nonidet P-40, 2 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 1 μg of aprotinin per ml, 1 μg of leupeptin per ml, 1 μg of pepstatin per ml, 150 mM NaCl at final concentration), supplemented with 5 μg of sonicated salmon sperm DNA and end-labeled CB59 (1 to 2 ng), and then the mixture was incubated for 1 h at 25°C. In the case of polypeptides expressed in *E. coli*, to renature the polypeptide to achieve native folding, the reaction was carried out for more than 48 h at 0°C with the binding buffer (100 μl) supplemented with 5 μg of sonicated salmon sperm DNA, 10 μg of acetylated bovine serum albumin, and end-labeled CB59 (1 to 2 ng). After the reactions, the mixture (5 μl) was electrophoresed on 5% polyacrylamide gels with TBE buffer (12.5 mM Tris-HCl [pH 8.0], 12.5 mM boric acid, 0.5 mM EDTA) at 20 V/cm at 4°C. Then, the gel was dried and exposed to Kodak XAR-5 X-ray film.

Construction of plasmids containing truncated CENP-B genes. pETCBN-125, pETCBN-160, pETCBN-411, pETCBN-516, pETCBN-578, and pETCENP-B were described previously (50). To construct pETCB368-599, a 1.0-kb fragment of the COOH-terminal coding region of CENP-B was obtained by digestion with *Dra*III and treated with T4 polymerase to produce a blunted end. This fragment was ligated into the pET-3c (42) *Bam*HI site which was blunted with the Klenow fragment. GST–CENP-B fusion polypeptide expression plasmids were constructed as follows. By complete digestion of pETCENP-B with *Dra*III (New England Biolabs), 0.7 and 1.0-kb fragments containing parts of the CENP-B gene were obtained. By partial digestion of pETCENP-B with *Dra*III, a 1.7-kb fragment containing the above two fragments (linked) was obtained. To construct pGEXCB132-599, pGEXCB368-599, and pGEXCB132-366, these 1.7-, 1.0-, and 0.7-kb fragments were blunted with T4 polymerase (Takara Shuzo) and ligated into the *Sma*I site of pGEX-3X (Pharmacia). To construct pGEXCB471-599 and pGEXCB541-599, two DNA fragments encoding COOH-terminal portions of the CENP-B gene were amplified from the pETCENP-B template using primers P1 and C1 or P2 and C1, respectively, by the high-fidelity PCR with Pfu polymerase (Stratagene). PCR products were digested with *Bam*HI and *Eco*RI (Takara Shuzo) and then ligated into the *Bam*HI-*Eco*RI site of pGEX-3X. Plasmid clones derived from PCR products were verified by sequencing analyses. To construct pGEXCB558-599, pGEXCB541-599 was digested with *Kpn*I (Takara Shuzo) blunted with T4 polymerase, digested with *Bam*HI, and blunted with the Klenow fragment, and then the obtained 5.3-kb *Bam*HI-*Kpn*I fragment was self-ligated. pGEXCB541-599 was also treated with *Kpn*I, T4 polymerase, *Eco*RI, and the Klenow fragment in sequence and then self-ligated to construct pGEXCB541-556. For the two-hybrid assay, to obtain the 1.7-kb *Bam*HI-*Eco*RI fragment containing the central and COOH-terminal regions of CENP-B, pGEXCB132-599 was digested with *Eco*RI, blunted with the Klenow fragment, and then partially digested with *Bam*HI. To obtain the 1.3-kb *Bam*HI-*Kpn*I fragment, pGEXCB132-599 was digested with *Kpn*I, blunted with T4 DNA polymerase, and then partially digested with *Bam*HI. To construct pLexACB132-599 and pLexACB132-556, these fragments were inserted into pBTM116 (45) (containing amino acids 1 to 211 of the LexA DNA-binding domain) which had been sequentially treated with *Pst*I, the Klenow fragment, and *Bam*HI. To construct pGADCB368-599, the 1.0-kb fragment was obtained by treatment of pGEXCB368-599 with *Bam*HI, the Klenow fragment, and *Eco*RI, in sequence. The product was ligated into pGAD10 (1) which had been sequentially treated with *Bam*HI, the Klenow fragment, and *Eco*RI. To construct pGADCB132-353, the 0.7-kb fragment obtained by sequential treatment of pGEXCB132-366 with *Bam*HI and the Klenow fragment was ligated into pGAD10 which had been similarly prepared. The direction of inserted fragments was certified by sequencing analyses.

Expression and purification of CENP-B polypeptides in *E. coli*. The naming of the truncated CENP-B polypeptides was done as follows. G means that the polypeptide is fused with a GST polypeptide at its NH_2-terminal side. N means that the polypeptide has the NH_2 terminus of CENP-B, C means that it has the COOH terminus of CENP-B, and NR or CR means that it comes from a reticulocyte translation. F means the full-length CENP-B polypeptide. Truncated polypeptides of CENP-B, N(1-125), N(1-129), N(1-160), N(1-411), and F(1-599), were expressed in *E. coli* with pETCBN-125, pETCBN-129, pETCBN-160, pETCBN-411, and pETCENP-B, respectively, by the T7 expression system (see Fig. 1) (42). Polypeptides of N(1-125), N(1-160), and N(1-441) were purified by the inclusion body method (39) from crude extracts, and then the bands of these polypeptides were cut out from agarose gels of the ProSieve gel system (a sodium dodecyl sulfate [SDS]-agarose gel system for electrophoretic separation of proteins) (FMC Corp.). To prepare antibodies, N(1-129) polypeptides were purified and then fractionated on a 15% polyacrylamide gel including 0.1% SDS by the Prep Cell system (Bio-Rad Laboratories). Polypeptides of F(1-599) were purified by the inclusion body method, dissolved in denaturation buffer (8 M urea, 50 mM Tris-HCl [pH 8.0], 50 mM dithiothreitol, 2 mM EDTA, 0.5 M NaCl, 2 mM phenylmethylsulfonyl fluoride), and fractionated on a Q-Sepharose column (Pharmacia) with a fast protein liquid chromatography system (Pharmacia) according to the procedure described by Muro et al. (29) for CENP-B from HeLa nuclear extract, except that 6 M urea was used in buffers A and B. Truncated CENP-B polypeptides fused with GST at their NH_2-terminal sides, G(132-599), G(368-599), G(471-599), G(541-599), G(558-599), G(541-556), and G(132-366), were expressed in *E. coli* (NM522) with pGEXCB132-599, pGEXCB368-599, pGEXCB471-599, pGEXCB541-599, pGEXCB558-599, pGEXCB541-556, and pGEXCB132-366, respectively. GST fusion proteins were expressed and purified essentially according to the method described by Guan and Dixon (14). Crude extracts were fractionated through a glutathione-Sepharose 4B column (Pharmacia). Peak fractions of the column were pooled, dialyzed with PBS (pH 7.3), and purified further by the ProSieve gel system.

In vitro transcription and translation of CENP-B polypeptides. Polypeptides of FR(1-599), NR(1-578), or NR(1-516) were generated by in vitro transcription and translation with pETCENP-B, pETCBN-578, or pETCBN-516, respectively, in a TNT-coupled transcription translation system in a rabbit reticulocyte lysate

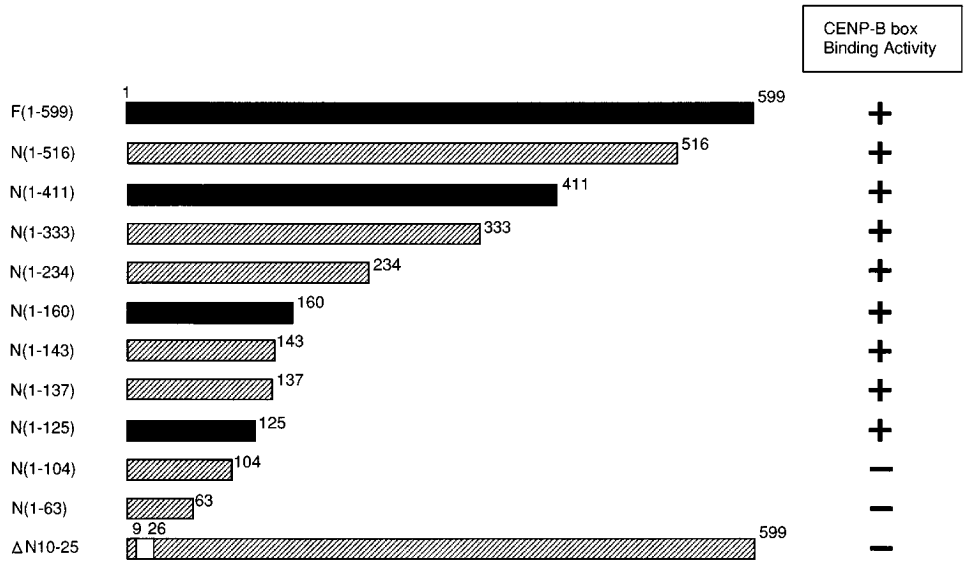

FIG. 1. DNA-binding activities of full-length and truncated CENP-B polypeptides, expressed in *E. coli*, summarized from the results previously described in the work of Yoda et al. (50). The numerals indicate the amino acid numbers from the NH_2 terminus. +, strong binding activity specific to the CENP-B box; −, no specific DNA-binding activity. The polypeptides used in the present experiments are shown as black boxes.

(Promega) according to the manufacturer's instructions. Plasmid DNAs (0.3 μg) were used in 25 μl of the reaction lysate. To confirm the self-interaction of the COOH-terminal 59-amino-acid region, polypeptides of FR(1-599) and CR(368-599), or NR(1-578) and CR(368-599), were coexpressed in the lysate with pET CENP-B and pETCB368-599, or pETCBN-578 and pETCB368-599, respectively.

Yeast transformations and β-galactosidase assays. Yeast transformations were performed according to the procedure of Vojtek et al. (45). For β-galactosidase assays, yeast colonies were picked into 3 ml of glucose minimal medium lacking leucine and tryptophan and grown until the A_{600} of the culture reached about 1.0. Assays for β-galactosidase activity were then carried out according to the procedure of Poullet and Tamanoi (34).

Preparation of antibody. Purified N(1-129) (described above) was used to elicit polyclonal antibody production in a rabbit. The serum was precipitated with 50% ammonium sulfate twice, the pellet was dissolved and dialyzed with PBS, and the immunoglobulin G fraction was obtained with protein A-Sepharose (Pharmacia). The fraction, designated BN1 antibody, was purified by affinity for a CNBr-Sepharose column (Pharmacia) coupled with N(1-129) polypeptides.

Immunoprecipitation and phosphatase treatment. Ten microliters (~20 μg of proteins) of 0.5 M NaCl extract obtained from HeLa metaphase chromosomes was incubated with 4.3 μg of BN1 antibody in 100 μl of the binding buffer supplemented with 0.5 M NaCl and 100 μg of acetylated bovine serum albumin per ml for 1 h at 0°C. Then, 10 μl of protein A-Sepharose was added. The mixture was incubated for 1 h at 4°C with gentle agitation. After precipitation, the pellet was washed twice with the binding buffer supplemented with 0.5 M NaCl and 100 μg of acetylated bovine serum albumin per ml and was washed once and resuspended (40 μl) in calf intestinal alkaline phosphatase (CIP) buffer (50 mM Tris-HCl [pH 8.5], 0.1 mM EDTA, 100 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 1 μg of aprotinin per ml, 1 μg of leupeptin per ml, 1 μg of pepstatin per ml). For dephosphorylation, the suspension was incubated with 0.5 μl of CIP (molecular biology grade, 24 U/μl, Boehringer Mannheim) in the presence or absence of phosphatase inhibitor, 10 mM sodium PP_i ($Na_4P_2O_7$) (Wako), for 1 h at 37°C. The reaction was stopped by adding the same volume of 2× SDS gel loading buffer (39). The sample was boiled for 5 min, run in SDS-polyacrylamide gel electrophoresis (PAGE), and then immunoblotted. Harvested whole cells were resuspended with 1× SDS gel loading buffer, sonicated, boiled, and subjected to SDS-PAGE.

Immunoblotting. Immunoblotting was performed as described by Masumoto et al. (26) with the following modifications. BN1 antibody was used at a 1:3,000 dilution. Horseradish peroxidase-conjugated anti-rabbit immunoglobulin G (Bio-Rad Laboratories) was used at a 1:3,000 dilution as the second antibody. After final washing of the membrane with TPBS (154 mM NaCl, 10 mM sodium phosphate buffer [pH 7.6], 0.05% Tween 20), antigen-antibody complexes were visualized with the enhanced chemiluminescence detection system (Amersham). The blots were exposed to Kodak XRP-5 X-ray films.

RESULTS

The DNA-binding domain of CENP-B polypeptide binds a CENP-B box DNA as a monomer. We showed previously that the DNA-binding domain of CENP-B is located within the NH_2-terminal 125-amino-acid region containing four potential α-helices by using truncated CENP-B polypeptides expressed in *E. coli* by gel mobility shift analysis (Fig. 1) (50). Therefore, we proposed that the DNA-binding motif of CENP-B may be a new type, different from the typical HLH motif. The HLH proteins bind DNA as a dimer (30). In order to clarify this point, we determined whether the DNA-binding domain of CENP-B bound to a DNA molecule as a monomer or as a dimer. Gel mobility shift analysis using two chain lengths of polypeptides containing the DNA-binding domain was carried out. N(1-411) and N(1-160), which, respectively, contain 411 and 160 amino acid residues from the NH_2 terminus, were mixed in the presence of 8 M urea. The mixture of the polypeptides was diluted with the binding buffer, incubated with a ^{32}P-labeled 59-bp DNA probe (CB59), and then electrophoresed in the native condition. Two shifted bands were detected at the positions corresponding to the mobilities of the complex formed with either polypeptide alone (Fig. 2). No hybrid complex with an intermediate mobility between these two bands was detected (Fig. 2a, lane 2). Under the same conditions, the truncated CENP-B polypeptides retained the specific DNA-binding activity for the CENP-B box (Fig. 2b).

These experiments demonstrated that the DNA-binding domain of the CENP-B polypeptide specifically binds to a DNA molecule as a monomer. This type of protein-DNA interaction is different from that expected for the HLH protein family, which binds DNA as a dimer, and supports our previous proposal that the DNA-binding domain of CENP-B does not belong to the HLH protein family.

The COOH-terminal region is responsible for dimerization activity. We reported previously that intact CENP-B obtained from HeLa cells forms a stable and unique complex containing two DNA molecules in vitro, complex A. We also showed that

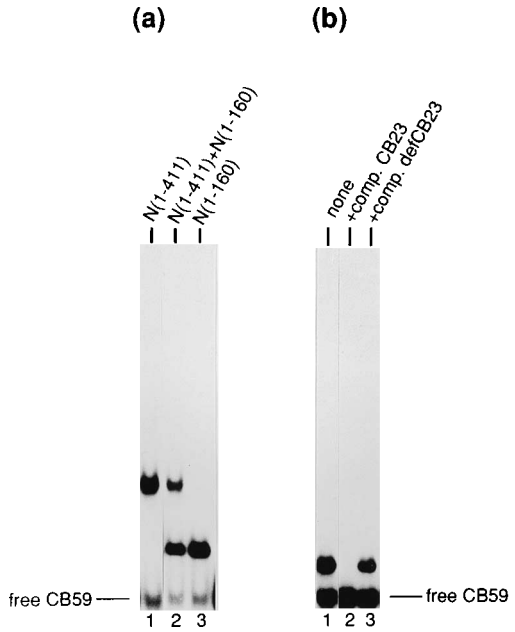

FIG. 2. Determination of the number of CENP-B polypeptides required for the specific DNA binding by gel mobility shift analysis. (a) DNA-binding reactions were carried out with end-labeled CB59 and N(1-411) (lane 1) or N(1-160) (lane 3). Equal amounts of these two polypeptides were mixed in the denaturation buffer (as described in Materials and Methods) and then renatured in a DNA-binding reaction (lane 2). (b) The DNA-binding reaction was carried out with N(1-125) (lanes 1 to 3). One-hundred-fold excess amounts of nonradioactive CB23 containing the CENP-B box (lane 2) or defCB23 containing a defective CENP-B box (lane 3) were used to supplement the mixture.

CENP-B forms a dimer under native conditions and that dimerizing activity was separable from the DNA-binding domain (50). To determine the dimerization domain of CENP-B, we first established the condition by which complex A was formed with the full-length CENP-B, F(1-599), expressed in *E. coli*, and purified with a Q-Sepharose column. When the protein solution was diluted and incubated with the end-labeled 59- or 32-bp DNA containing the CENP-B box (CB59 or CB32) in a binding reaction and then electrophoresed, a band of the complex was detected at position A^{59} or A^{32}, respectively (Fig. 3a, lane 1 or 14). When a different chain length of nonradioactive competitor CB29 or CB56 (29 or 56 bp) was added to the same reaction mixture for lane 1 or 14, respectively, with an increasing ratio, an extra band showing the same intermediate mobility appeared between A^{59} and A^{32} (Fig. 3a, lanes 2 to 7 and 8 to 13). Even with various ratios of the competitors, no additional bands were detected. Thus, the purified full-length CENP-B polypeptide expressed in *E. coli* has an ability to form a stable complex containing two DNA molecules, as does CENP-B obtained from HeLa cells (29).

However, when a similar experiment was performed with N(1-411) polypeptides, no extra band appeared, and the shifted band only faded out with the addition of the competitor (Fig. 3b). Since the NH_2-terminal DNA-binding domain of CENP-B binds to a DNA molecule as a monomer, these results indicate that full-length CENP-B forms a dimer complex and that the domain required for the self-dimerization activity of CENP-B is located within the COOH-terminal 189-amino-acid region.

Delimitation of the dimerization domain of CENP-B. To determine the dimerization domain of CENP-B, we constructed GST fusion polypeptides including various regions (see Fig. 6). GST fusion polypeptides were expressed in *E. coli*

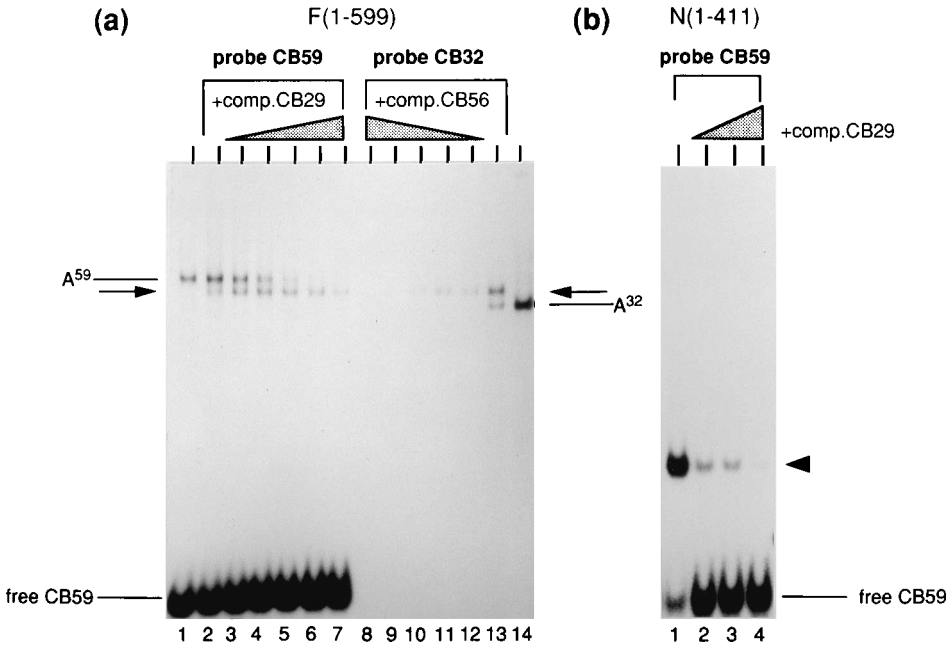

FIG. 3. Gel mobility shift analysis of complex A-forming activity of CENP-B polypeptides expressed in *E. coli*. (a) DNA-binding reactions were carried out with 0.7 ng of F(1-599) and end-labeled CB59 (lanes 1 to 7) or end-labeled CB32 (lanes 8 to 14). Excess amounts of nonradioactive CB29 (0-, 0.25-, 0.5-, 1-, 2-, 4-, and 8-fold, lanes 1 to 7, respectively) compared with the amount of end-labeled CB59 or excess amounts of nonradioactive CB56 (16-, 8-, 4-, 2-, 1-, 0.5-, and 0-fold, lanes 8 to 14, respectively) compared with the amount of end-labeled CB32 were used. A^{59} and A^{32} show the positions of complex A formed with end-labeled CB59 and end-labeled CB32, respectively. Arrows indicate the complex appearing with an intermediate mobility between A^{59} and A^{32} when two different chain lengths of DNAs exist in the reaction. (b) DNA-binding reactions were carried out with N(1-411), end-labeled CB59, and zero-, two-, four-, and eightfold excess amounts of nonradioactive CB29 (lanes 1 to 4, respectively) compared with the amount of end-labeled CB59. An arrowhead indicates disappearance of the complex with competition.

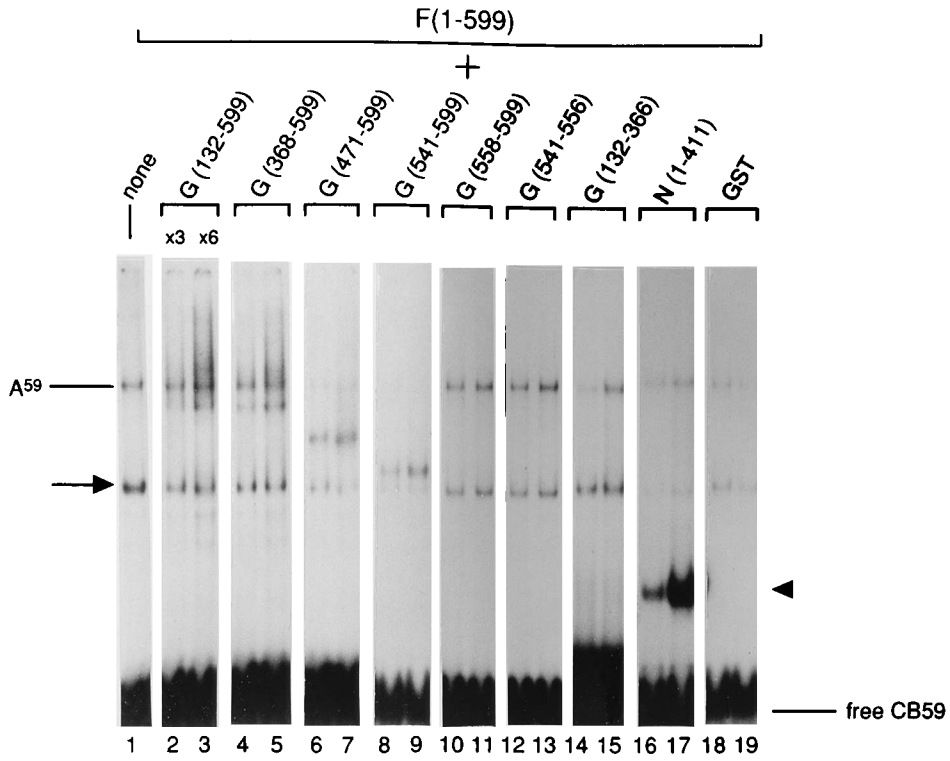

FIG. 4. Determination of the dimerization domain of CENP-B by gel mobility shift analysis. F(1-599) and one of the truncated polypeptides were mixed in denaturation buffer (as described in Materials and Methods) and then diluted 20-fold with binding buffer supplemented with 0.5 M NaCl at the final concentration. Aliquots (1 μl) of the mixed polypeptide solutions were added to 100 μl of binding mixture, and the reaction was carried out. One microliter of mixed polypeptide solutions contained 1 ng of F(1-599) and threefold (×3) or sixfold (×6) amounts of the truncated polypeptides. Polypeptides mixed with F(1-599) were as follows: none (lane 1), G(132-599) (lanes 2 and 3), G(368-599) (lanes 4 and 5), G(471-599) (lanes 6 and 7), G(541-599) (lanes 8 and 9), G(558-599) (lanes 10 and 11), G(541-556) (lanes 12 and 13), G(132-366) (lanes 14 and 15), N(1-411) (lanes 16 and 17), and GST polypeptides (lanes 18 and 19). The arrow at the left indicates the monomer complex (described in the text). The arrowhead at the right indicates the complex formed between N(1-411) and probe DNA.

and purified with glutathione-Sepharose 4B column and SDS-agarose gel approaches (ProSieve gel system) so that each polypeptide became essentially a single band on SDS-PAGE (data not shown). Each GST fusion polypeptide was mixed with full-length CENP-B, F(1-599), in the presence of denaturant (7 M urea), and diluted with the binding buffer, and then the binding reaction was carried out with end-labeled CB59 DNA (Fig. 4). In lane 1 of Fig. 4, the reaction was carried out only with purified F(1-599). The dimer complex (complex A) was formed at the A^{59} position, but the monomer complex was also formed (indicated by the arrow beside lane 1). This monomer complex contained one DNA molecule (data not shown). In each experiment using mixtures with fusion polypeptides containing the COOH-terminal 59 amino acid residues, namely, G(132-599), G(368-599), G(471-599), and G(541-599), an extra band representing the size of the heterocomplex appeared between the positions of the full-length dimer complex and the monomer complex (Fig. 4, lanes 2 to 9). However, with fusion polypeptides containing a COOH-terminal region of CENP-B shorter than that of G(541-599), namely, G(558-599) or G(541-556), no extra complex was detected (Fig. 4, lanes 10 to 13). Also, with G(132-366) containing the central region of CENP-B or with only GST polypeptide, no extra complex was detected (Fig. 4, lanes 14 and 15 and 18 and 19, respectively). Though no extra band was also detected between the dimer complex and the monomer complex with N(1-411) containing the DNA-binding domain and the central region of CENP-B, the complex formed with N(1-411) itself and probe DNA was detected (Fig. 4, lanes 16 and 17). The results of these experiments prove that the COOH-terminal 59-amino-acid region is required for the dimerization activity of CENP-B.

The freshly prepared F(1-599) polypeptides did not form the monomer complex (Fig. 3). However, storage of the polypeptides for a long period caused the formation of the monomer complex, probably owing to partial inactivation of the COOH-terminal region.

To obtain information regarding the COOH-terminal side required for the dimerization activity, we used polypeptides expressed in rabbit reticulocyte lysates because sufficient polypeptides containing the DNA-binding domain longer than N(1-411) could not be obtained by the T7 expression system in *E. coli* except for full-length CENP-B. Full-length CENP-B expressed from pETCENP-B in rabbit reticulocyte lysates, FR(1-599), was analyzed by a gel mobility shift assay similar to the one whose results are shown in Fig. 3. With increasing amounts of the shorter nonradioactive oligonucleotide (CB29) as a competitor, an extra band appeared under the A^{59} position (Fig. 5a, lanes 1 to 3). Therefore, complex A was also formed in this system. However, when NR(1-578) or NR(1-516) whose COOH-terminal 21- or 83-amino-acid region, respectively, had been deleted was used for the assay, a band was formed at a position much faster than the A^{59} position (α and β, lanes 4 to 9 in Fig. 5a), and the band only faded out with addition of the competitor CB29 (Fig. 5a, lanes 4 to 9). The bands indicated with arrowheads on the left side of lanes 1, 4, and 7 in Fig. 5a are monomer complexes composed of a single molecule of the probe DNA and a degradation product of each

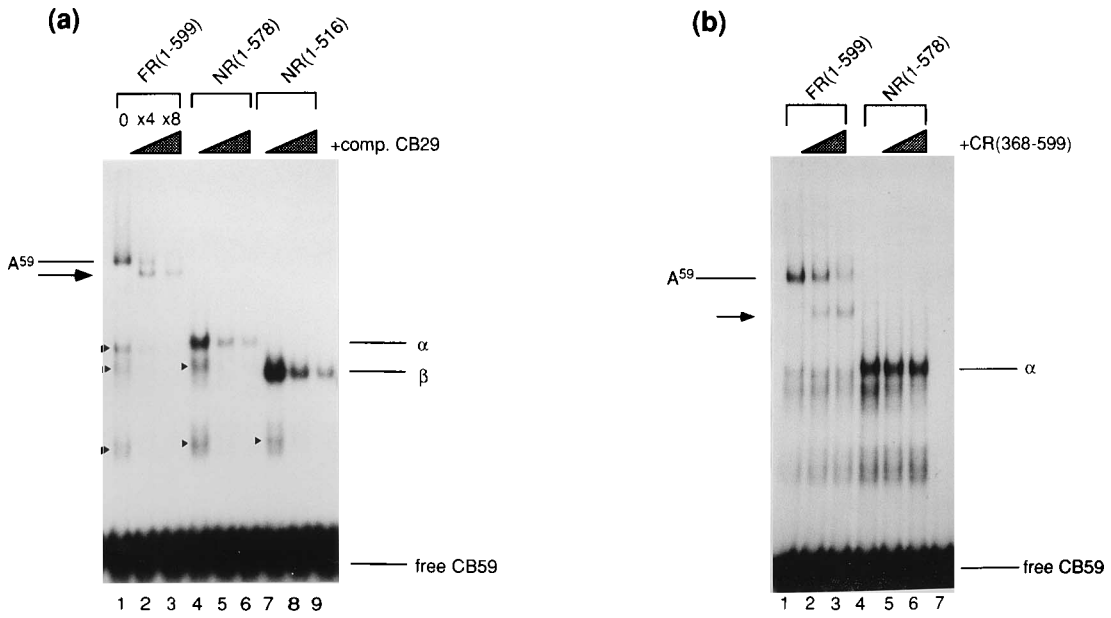

FIG. 5. Gel mobility shift analysis with polypeptides expressed in rabbit reticulocyte lysates. (a) DNA-binding reactions were carried out with 1 μl of lysate and end-labeled CB59 and zero- (0), four- (×4), or eightfold (×8) excess amounts of nonradioactive CB29. Subject polypeptides were as follows: FR(1-599) (lanes 1 to 3), NR(1-578) (lanes 4 to 6), and NR(1-516) (lanes 7 to 9). The arrow indicates the smaller complex appearing in the presence of two chain lengths of DNA. α and β indicate disappearance of complexes with competition. Bands indicated with arrowheads were formed with degraded products of each polypeptide. (b) DNA-binding reactions were carried out with lysate in which two chain lengths of polypeptides were coexpressed. Plasmids encoding FR(1-599) or NR(1-578) were coexpressed with zero-, one-, and threefold amounts of plasmid encoding CR(368-599) (lanes 1 to 3 or 4 to 6, respectively). A lysate expressing only CR(368-599) (an amount equal to that in lane 6) was used for the assay (lane 7). The small arrow indicates the hetero-complex formed with FR(1-599), CR(368-599), and probe DNA. α, complexes which were not influenced by coexpression.

CENP-B polypeptide. According to these results, the COOH-terminal 21 amino acid residues are necessary for the dimerization activity of CENP-B.

The possibility remains that the COOH-terminal 59-amino-acid region may interact with another region of the full-length CENP-B polypeptide. To examine whether this is the case, a gel mobility shift assay was carried out with lysates in which FR(1-599) and CR(368-599) containing the COOH-terminal 232 amino acid residues of CENP-B (amino acids numbered from 368 to 599) or NR(1-578) and CR(368-599) were coexpressed at different ratios. Though an extra band of the hybrid complex appeared below complex A when lysates containing FR(1-599) and CR(368-599) were used (Fig. 5b, lanes 2 and 3), no hybrid band appeared except for the monomer band formed with NR(1-578) itself and probe DNA when lysates containing NR(1-578) and CR(368-599) were used (Fig. 5b, lanes 5 and 6). These results indicate that the COOH-terminal region of CENP-B interacts with the same region of another molecule of CENP-B, clearly delimiting the dimerization domain to the COOH-terminal 59-amino-acid region (Fig. 6).

Detection of dimerization activity of CENP-B in yeast cells with the two-hybrid system. To confirm whether CENP-B forms a dimer under native conditions in the cell, we used the two-hybrid system originally described by Fields and Song (12) applying yeast cells as a mimic model of the mammalian in vivo system. In this assay, a hybrid protein containing the LexA DNA-binding domain is tested for ability to interact with another hybrid protein containing the GAL4 activation domain (45). We constructed pLexACB132-599 encoding the LexA DNA-binding domain fused with the CENP-B central and COOH-terminal domains (amino acid numbers 132 to 599). As the expression vector of a GAL4 activation domain fusion protein, pGADCB368-599, encoding the COOH-terminal domain of CENP-B (amino acid numbers 368 to 599), was constructed. These two hybrids were coexpressed in strain L40, which contains two integrated reporter constructs, the yeast *HIS3* gene and the bacterial *lacZ* gene, incorporating binding sites for the LexA protein. The presence of both hybrid proteins resulted in the expression of β-galactosidase activity significantly higher than the background level, indicating that the COOH-terminal polypeptides of CENP-B interact with each other in yeast cells (Table 1). The combination of pLex ACB132-556 encoding the fusion protein with the truncated CENP-B polypeptide (amino acid numbers 132 to 556) and pGADCB368-599 or pLexACB132-599 and pGADCB132-353 encoding the fusion protein with the central part of the CENP-B polypeptides (amino acid numbers 132 to 353) resulted in residual interacting activity similar to the background (Table 1).

These results correspond well to our in vitro results obtained from the gel mobility shift assay, suggesting strongly that CENP-B also forms a dimer at its COOH terminus in mammalian cells.

The dimerization domain is perfectly conserved in both human and mouse CENP-B. The amino acid sequence of the dimerization domain of human CENP-B (541-599) is conserved perfectly (100%) in mouse CENP-B, as is also the DNA-binding domain (1 to 125) (Fig. 7a) (44). To confirm that good conservation of two functional domains corresponds with complex A-forming activity, gel mobility shift analyses were performed with 0.5 M NaCl nuclear extracts obtained from mouse A9 cells. The complex A-forming activity was detected just as with purified CENP-B from HeLa cells (Fig. 7b) (29).

CENP-B is phosphorylated specifically at metaphase. The question of whether the complex A-forming activity changes during the cell cycle is of clear interest. We therefore analyzed the properties of CENP-B in mitotic phase. Total proteins of metaphase HeLa cells which were synchronized in TN-16 were

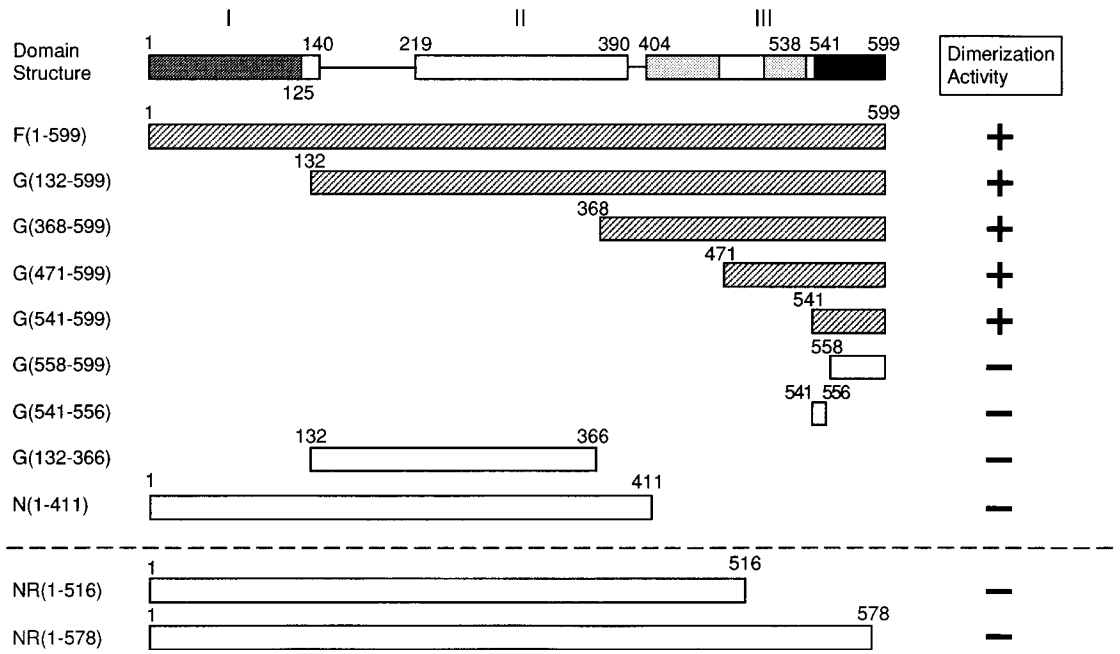

FIG. 6. Summary of dimerization activity of CENP-B polypeptides. The numerals indicate the amino acid numbers from the NH_2 terminus. +, dimerization activity of truncated polypeptides; −, no dimerization activity. The dimerization assay results above the broken line were obtained from heterodimerization activity with F(1-599). The results below the broken line depend on homodimerization activity. The domain structure of CENP-B shown is according to the work of Sullivan and Glass (44). The DNA-binding domain is deeply shaded, the acidic stretches are lightly shaded, and the dimerization domain is black.

immunoblotted with polyclonal antibody (BN1) specific to the CENP-B NH_2-terminal region. Metaphase CENP-B showed slower mobility than interphase CENP-B on SDS-PAGE analyses (Fig. 8a). When the total proteins from cells at 2 and 10 h after release from the mitotic block were blotted, the majority of CENP-B from the released cells returned to the interphase mobility within 2 h (Fig. 8a), and all of CENP-B did within 6 h (data not shown). These results indicate that CENP-B is modified specifically at metaphase. The majority of CENP-B protein was extracted from isolated HeLa metaphase chromosomes as well as from HeLa interphase nuclei under our extraction conditions. To determine whether the modification involved phosphorylation, CENP-B in the 0.5 M NaCl chromosomal extract obtained from isolated metaphase chromosome was immunoprecipitated with BN1 antibody and treated with calf intestinal alkaline phosphatase (Fig. 8b). The mobility of metaphase CENP-B after treatment with the phosphatase increased, becoming the same as that of interphase CENP-B. However, in the presence of Na-PP_i, a phosphatase inhibitor, the mobility did not change. Therefore, the metaphase-specific modification was concluded to be phosphorylation. In order to ascertain whether this modification affects the complex A-forming activity, gel mobility shift analysis was carried out with 0.5 M NaCl mitotic extracts (Fig. 8c). Since immunoblotting analysis revealed the amount of CENP-B in mitotic cells to be approximately twice that in interphase cells (lanes 1 and 2 in Fig. 8a), half the amount of mitotic CENP-B was used in the binding reaction (equivalent to the amount of CENP-B in 1×10^4 mitotic cells) in comparison with interphase CENP-B (equivalent to the amount of CENP-B in 2×10^4 interphase nuclei). The results showed that an equivalent amount of complex A was formed with mitotic CENP-B with a slight difference of the mobility of the complex (Fig. 8c). The complex A-forming activity may thus be simply correlated with the total amount of CENP-B both in interphase nuclei and on mitotic chromosomes.

TABLE 1. β-Galactosidase activity induced by dimerization of CENP-B polypeptides in a two-hybrid system

LexA DNA-binding domain fusion	GAL4 activation domain fusion[a]		
	GADCB368-599	GADCB132-353	GAD
LexACB132-599	5.4 ± 0.45	<0.1	<0.1
LexACB132-556	0.15 ± 0.04	ND	0.31 ± 0.05
LexA	<0.1	<0.1	<0.1

[a] The numbers refer to the amino acid residues of CENP-B. Values are Miller units of β-galactosidase activity, averaged from three experiments in which three independent plasmid-bearing transformants were assayed. ND, not done.

DISCUSSION

The complex formed with CENP-B and alphoid DNA. Assembly of the centromeric heterochromatin on mammalian chromosomes can be presumed to be crucial for the structure and function of the centromere. CENP-B and alphoid DNA interaction is one of the most probable candidates for achieving this assembly. The present analysis of the DNA-protein and the protein-protein interactions of the stable complex composed of CENP-B and alphoid DNA (complex A) with truncated CENP-B polypeptides expressed in *E. coli*, rabbit reticulocyte lysates, and the yeast two-hybrid system revealed two functional domains. From amino acid sequence observation of CENP-B, it was earlier reported that the NH_2-terminal region had similarity with a DNA-binding motif identified in HLH proteins (44), leading to speculation as to whether it might be a member of this family (33, 43). However, the DNA-binding domain of HLH family proteins has two α-helices, whereas we showed that the region required for specific DNA binding of CENP-B polypeptides contained four potential α-helices (50).

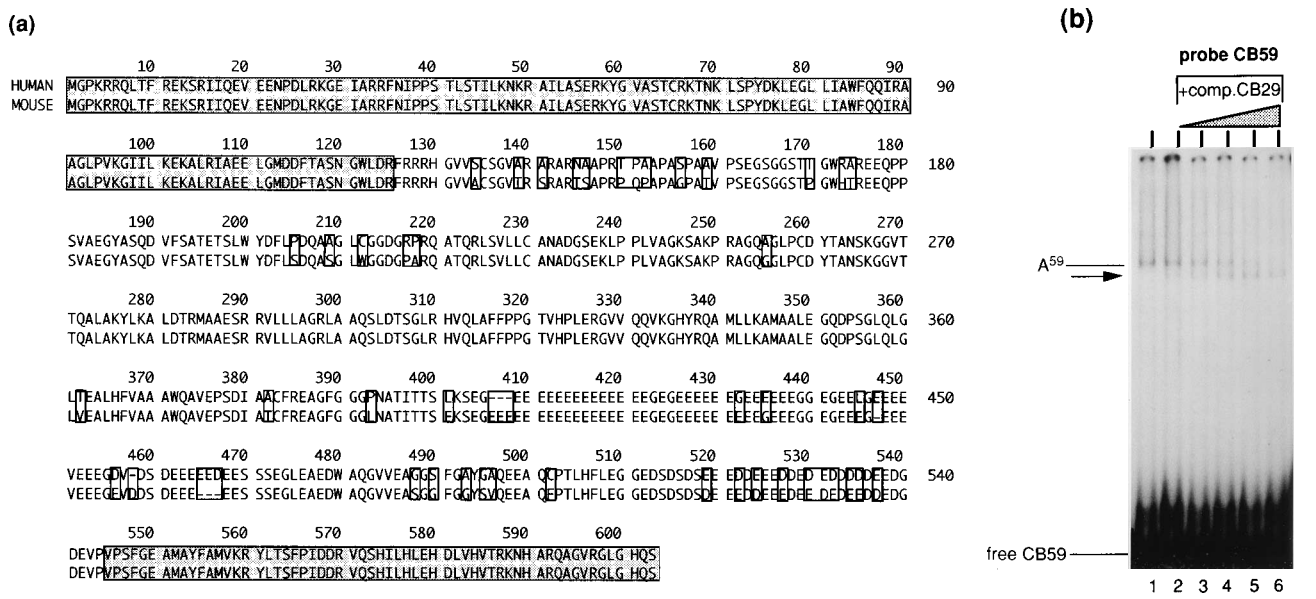

FIG. 7. Amino acid conservation of the complex A-forming domain between humans and mice. (a) Comparison of human and mouse CENP-B amino acid sequences. The shaded boxes indicate the DNA-binding and the dimerization domains, both being perfectly conserved. The blank boxes indicate nonconserved amino acids. Amino acid sequences were according to the work of Sullivan and Glass (44). –, deletion. (b) Gel mobility shift analysis using 0.5 M NaCl nuclear extract obtained from mouse cells (A9). The binding reaction was performed under the same conditions as described for lanes 1 to 6 in Fig. 3 except for use of the mouse nuclear extract. The arrow at the left indicates the smaller complex appearing in the presence of two chain lengths of DNA.

Furthermore, the DNA sequence of the CENP-B binding site, CENP-B box, is longer than that of the HLH protein binding site (26, 30). Nonpalindromic nucleotide sequences of the two major grooves in the CENP-B box were found to be responsible for the specific CENP-B binding (25a, 26), and from the available data, we therefore suggested that the DNA-CENP-B interaction differs from that of HLH proteins (27, 50). The present study shows that truncated CENP-B polypeptides containing the NH_2-terminal DNA-binding domain bind specifically to a DNA molecule as a monomer, in contrast to the HLH case in which a dimer is involved (30). This result clearly proves that CENP-B does not belong to the HLH family and that it is a new type of DNA-binding protein which recognizes and binds to a DNA molecule in a particular orientation as a single polypeptide molecule.

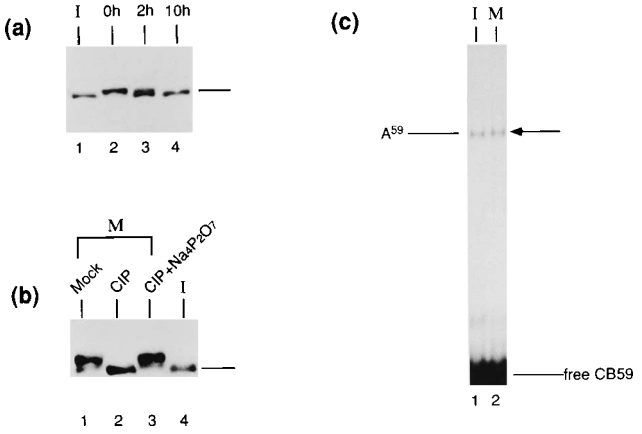

FIG. 8. Mitotic phase-specific phosphorylation of CENP-B and complex A-forming activity. (a) CENP-B in whole random cultured cells (10^5) (lane 1), whole mitotic cells (10^5) arrested by TN-16 (lane 2), and whole cells (10^5) harvested at 2 h (lane 3) and 10 h (lane 4) after release from TN-16 mitotic block was immunoblotted with BN1 antibody. Electrophoresis was carried out for 1.5 h at 2 mA constant current per cm of rod with a 7% polyacrylamide gel. The line indicates the position of interphase CENP-B. (b) Immunoblotting of immunoprecipitated and phosphatase-treated CENP-B from mitotic cell extracts (lanes 1 to 3) and immunoprecipitated interphase nuclear extract (lane 4). The extracts were treated with nothing (lane 1) and with CIP (lane 2) and with CIP plus Na-PP_i (lane 3). Electrophoresis was carried out for 4 h under the same conditions as described for panel a. (c) Gel mobility shift analysis using HeLa interphase and mitotic 0.5 M NaCl extracts. The DNA-binding reaction was carried out with an interphase extract containing an amount of CENP-B equivalent to 2×10^4 interphase cells (lane 1) and a mitotic extract containing the amount of CENP-B equivalent to 1×10^4 mitotic cells (lane 2). The complex A formed with the mitotic extract shows a slight decrease in mobility (arrow).

Glycerol density gradient sedimentation of complex A and the chemical cross-linking experiment showed that CENP-B obtained from HeLa cells forms a dimer in the native state (50). When CENP-B expressed in *E. coli* and purified to a single band in SDS-PAGE was used for the gel mobility shift analysis, it formed complex A containing two DNA molecules as in the HeLa cell case. Considering the DNA-binding properties of CENP-B documented in this paper, the results clearly indicate that CENP-B forms a dimer complex independent from its DNA-binding activity and that no other factors or specific modifications (for example, phosphorylation) are required for its functioning.

The motif required for dimerization activity. We succeeded in delimiting the dimerization domain of CENP-B within the COOH-terminal 59-amino-acid region. For the analysis of the dimerization domain, we used not only the methods described in this paper but also chemical cross-linking coupled with SDS-PAGE analysis, as reported earlier (50). With the latter method, it was very difficult to find optimal conditions in vitro especially for truncated polypeptides, with even the GST polypeptide alone forming a dimer in some cases. The dimer complex was first identified by gel mobility shift analysis in its native condition. Therefore, we used this procedure with modifications to determine the minimal domain required for the dimerization. Under conditions whereby specific DNA-binding

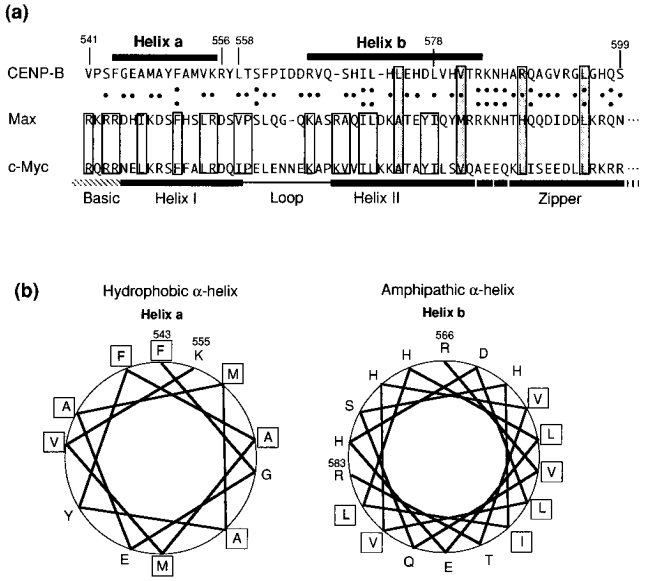

FIG. 9. (a) The dimerization domain of CENP-B shows a weak similarity with Max, one of the HLH-Zip proteins. The numerals indicate the amino acid numbers from the NH_2 terminus of CENP-B (44). Identical amino acids or similar amino acids are indicated by colons or dots, respectively. Blank boxes indicate residues that fit the consensus as derived for known HLH proteins, for example, c-Myc (4). Shaded boxes denote the heptad repeat of hydrophobic residues. The HLH-Zips have two more hydrophobic heptad repeats than the residues shown in this figure. The illustrated α-helices of the dimerization domain of CENP-B were predicted by Robson-Garnier computer analysis (helix a and helix b). (b) Helical wheel analysis of helix a and helix b of the dimerization domain. Boxes indicate hydrophobic residues.

activity at the NH_2 terminus and dimerization activity at the COOH terminus were reproduced, we could not obtain any evidence of dimer formation outside the COOH-terminal domain of CENP-B. These results obtained from in vitro analysis for the dimerization domain were in complete agreement with the results from in vivo analysis using the yeast two-hybrid system.

The dimerization domain of CENP-B shows slight homology (22%) with the dimerization domain of Max, one of the HLH zipper proteins (HLH-Zip) on FASTA and NBRF-PIR analysis (Fig. 9a) (3, 4, 21, 36). Amino acid sequence similarities and several points of difference between the dimerization domain of CENP-B and that conserved in the HLH-Zip family are presented in Fig. 9a (4). The HLH-Zip proteins have six heptad repeats of hydrophobic residues, whereas CENP-B has only four, and HLH-Zip proteins have a basic region connected to the NH_2-terminal side of helix 1 which is responsible for the DNA-binding activity, whereas CENP-B has not. However, the fundamental structure which is required for the protein-protein interacting activity may be conserved between the dimerization domain of CENP-B and the HLH-Zip domain. We can thus say that the dimerization domain of CENP-B which is separable from the DNA-binding activity is a novel motif but must bear in mind that it conserves a weak structural homology with the HLH-Zip family.

The 59-amino-acid dimerization domain is relatively rich in hydrophobic amino acids (44%). Therefore, it may be assumed that the protein-protein interaction is based on hydrophobic association (bonding). By Robson-Garnier computer analysis, two potential α-helices could be located (Fig. 9a), and when either of these was truncated, the dimerization activity vanished (Fig. 9a). It is thus likely that the structure including these two helices plays an important role in generating dimerization. A helical wheel projection of the two α-helices is shown in Fig. 9b, the hydrophobic amino acids in helix a being distributed almost symmetrically (Fig. 9b). In contrast, helix b has the potential to form an amphipathic α-helix (Fig. 9c) (40). Interestingly, the polar side of this amphipathic helix is rich in positively charged amino acids, especially histidine (Fig. 9c). Although it is not clear yet whether polypeptides of CENP-B form a dimer in parallel or antiparallel, considering the partial homology to the HLH-Zip motif, a dimer in parallel form is probable.

Possible role of complex A in assembly of centromeric heterochromatin. Amino acid sequences of both dimerization and DNA-binding domains have been shown to be perfectly conserved between human and mouse CENP-B (Fig. 7a) (44). Complex A was formed by CENP-B in 0.5 M NaCl nuclear extracts obtained from mouse cells, and the CENP-B box is also conserved in mouse minor satellite DNA (22, 26, 48). Considering this evidence, it is possible that the interaction between CENP-B and CENP-B boxes in centromeric satellite DNA might carry an important function, for example, assembling the centromeric satellite DNA. Recently, interactions of several mammalian proteins were found with the two-hybrid system (45, 52). We similarly found that the COOH-terminal domain of CENP-B polypeptides has the ability to form dimers in yeast cells, indicating that our in vitro analysis results reflect the in vivo properties of CENP-B. Ikeno et al. (18) in our group have shown recently that there are two different long arrays of alpha-satellite DNAs (α21-I and α21-II) in the centromere region on human chromosome 21. While the α21-I region in which CENP-B boxes appear with very high frequency localized as compact dots on the primary constriction, the α21-II region in which CENP-B boxes are very rare was detected as a larger, more extended area on fluorescence in situ hybridization analysis. They also revealed that the α21-I region colocalized and overlapped with the assembly sites of CREST (calcinosis, Raynaud's phenomenon, esophageal dysmotility, sclerodactyly telangiectasiae) autoimmune centromere antigens (18). It seems that the observed morphological differences between these two alphoid arrays may reflect structural differences caused by different levels of complex A formation. The difference in compaction of these two regions was observed through the interphase and the mitotic phase (18), which correlates well with our detection of complex A-forming activity in both interphase nuclear and metaphase chromosome extracts corresponding simply to the total amount of CENP-B (Fig. 8). Although the effect of other interacting proteins also requires consideration, complex A formation at the centromeric satellite DNA may thus have a primary role in assembling the specific structure required for centromere functions.

The central domain of CENP-B is also conserved perfectly between humans and mice (Fig. 7a), suggesting that it might also have an important function. Moreover, CENP-B has an acidic serine-rich region, similar to that found in the product of yeast CTF13 (CBF3C) (5, 9).

Complex A formation activity in the cell cycle. The present study revealed that CENP-B is modified at metaphase specifically and that this modification is a phosphorylation. There exists a minor possibility that this phosphorylation could have been caused by mitotic arrest with the mitotic blocker, TN-16. However, in the case of a shorter arresting period CENP-B in mitotic cells also showed the reduced mobility in SDS gels, and even with other mitotic blockers, nocodazole and colcemid, the same phenomenon was observed (data not shown). By using cells with a relatively large proportion in mitosis (~30%) ob-

tained 10 h after release from G_1/S-phase arrest by adding excess thymidine, the ratio of the CENP-B molecules showing the mobility shift corresponded to the ratio of mitotic cells (~30%) (data not shown). However, the mobility was a little higher than that of CENP-B in cells after mitotic arrest because of treatment with TN-16 for a long period. Therefore, while a minor degree of hyperphosphorylation might be caused by TN-16, we conclude that mitotic phase-specific phosphorylation of CENP-B does exist in intact cells and that hyperphosphorylated CENP-B molecules preserve the same specific DNA-binding activity and dimerization activity as in the interphase cell case.

It is probable that metaphase-specific modification is related to other activities of CENP-B, including, for example, interaction with other proteins. CENP-B has a mitogen-activated protein kinase target site (148 amino acids from the NH_2 terminus) and several casein kinase II target sites (9, 72, 181, 193, 403, 456, 466, 510, 512, 514, and 543 amino acids from the NH_2 terminus). These sites are outside the DNA-binding domain and the dimerization domain except for the casein kinase II site at the 543-amino-acid position of the latter. This site was found not to be phosphorylated by purified casein kinase II in vitro (data not shown), but clearly it is important for understanding other functions of CENP-B to test whether the mitotic phase-specific phosphorylation is performed by these or by unknown kinases.

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