MOLECULAR AND CELLULAR BIOLOGY, Sept. 1996, p. 5169–5177 Vol. 16, No. 9
0270-7306/96/$04.00+0

Centromere Protein B of African Green Monkey Cells: Gene Structure, Cellular Expression, and Centromeric Localization

KINYA YODA,[1] TAKAO NAKAMURA,[2]† HIROSHI MASUMOTO,[2] NOBUTAKA SUZUKI,[2] KATSUMI KITAGAWA,[2] MEGUMI NAKANO,[2] AKIHISA SHINJO,[3] AND TUNEKO OKAZAKI[2]*

Bioscience Center,[1] and Department of Molecular Biology, School of Science,[2] Nagoya University, Chikusa-ku, Nagoya 464-01, and Ryukyu University, Faculty of Agriculture, Okinawa,[3] Japan

Received 26 February 1996/Returned for modification 17 April 1996/Accepted 20 May 1996

Centromere protein B (CENP-B) is a centromeric DNA-binding protein which recognizes a 17-bp sequence (CENP-B box) in human and mouse centromeric satellite DNA. The African green monkey (AGM) is phylogenetically closer to humans than mice and is known to contain large amounts of α-satellite DNA, but there has been no report of CENP-B boxes or CENP-B in the centromere domains of its chromosomes. To elucidate the AGM CENP-B–CENP-B box interaction, we have analyzed the gene structure, expression, biochemical properties, and centromeric localization of its CENP-B. The amino acid sequence deduced from the cloned AGM CENP-B gene was established to be highly homologous to that of human and mouse CENP-B. In particular, the DNA binding and homodimer formation domains demonstrated 100% identity to their human and mouse counterparts. Immunoblotting and DNA mobility shift analyses revealed CENP-B to be expressed in AGM cell lines. As predicted from the gene structure, the AGM CENP-B in the cell extracts exhibited the same DNA binding specificity and homodimer forming activity as human CENP-B. By indirect immunofluorescent staining of AGM mitotic cells with anti-CENP-B antibodies, a centromere-specific localization of AGM CENP-B could be demonstrated. We also isolated AGM α-satellite DNA with a CENP-B box-like sequence with CENP-B affinity. These results not only prove that CENP-B functionally persists in AGM cells but also suggest that the AGM genome contains the recognition sequences for CENP-B (CENP-B boxes with the core recognition sequence or CENP-B box variants) in centromeric satellite DNA.

The centromere of eukaryotic chromosomes plays an essential role in the pairing and partitioning of replicated chromosomes in mitosis and meiosis. Functional centromeres have been isolated and characterized from monocellular eukaryotes such as the budding (*Saccharomyces cerevisiae*) and fission (*Schizosaccharomyces pombe*) yeasts. The functional centromere (CEN) of *S. cerevisiae* centromeres features ~125 bp of DNA composed of three elements (CDEI, CDEII, and CDEIII) but free of repeated sequences (6, 7, 15). Also in *S. cerevisiae*, *trans*-acting elements specifically interacting with the CEN sequence have been detected and characterized (2, 8, 12, 17, 18, 23). In contrast, the centromere regions of *S. pombe* and higher eukaryotes contain much longer and more complex sequences. Fully functional centromere sequences in *S. pombe* are 40- to 100-kb segments containing a chromosome-specific central core sequence (4.1 to 6.8 kb) flanked on both sides by large inverted repeats (5, 14). Recently, *Drosophila* centromeric DNA in a stable minichromosome has been defined within a 200-kb heterochromatin region (30). The essential core of the centromere consists predominantly of a block of middle-repetitive and/or single-copy DNA. The centromere of mammalian chromosomes is a huge area cytologically defined as the primary constriction of the metaphase chromosomes (34, 35). The attachment sites of microtubules, the kinetochores, have been found in this area by electron microscopy as trilaminar structures (3, 32, 46). The centromere regions of mammalian chromosomes contain large, complex arrays of highly repetitive DNA sequences (satellite DNA) (45, 47), whose characteristics differ from species to species. Although DNA is believed to be the primary determinant for formation of the functional structure of mammalian centromeres or kinetochores, identification of the responsible DNA segment(s) is hindered by difficulties in developing an activity assay with mammalian cells. α-Satellite DNA is one of the most extensively studied mammalian centromeric satellite DNAs (1, 11, 19, 20, 40, 41, 43, 44). It is a primate-specific, centromeric satellite family based on a monomer repeat length of 171 bp. It was originally found in the African green monkey (AGM) genome, where it comprises more than 10% of the total AGM genomic DNA (36). In the human genome, α-satellite is known to be organized in a chromosome-specific manner with diverged α-satellite subsets encompassing up to a few megabases within the centromere region (46, 47). We have shown previously by in situ hybridization experiments that α-satellite DNA is colocalized with centromere proteins (CENPs) detected with anticentromere autoantibodies (ACAs) throughout the cell cycle (16, 25). ACAs detect paired antigenic spots within the region of the primary constriction of metaphase chromosomes from a number of mammalian species (27), and three antigenic proteins (CENP-A, -B, and -C) have been demonstrated in nuclear extracts of human cells (9, 10). Using HeLa cell nuclear extracts, we have shown that CENP-B binds to a 17-bp sequence (CENP-B box) which is found in many subtypes of α-satellite DNA in the human genome (alphoid DNA) (24). In vitro studies of CENP-B proved that it forms a homodimer by a reaction requiring the carboxy terminus of the polypeptide, while each amino terminus binds to a CENP-B box sequence (22, 51). This results in formation of a complex that contains

* Corresponding author. Mailing address: Department of Molecular Biology, School of Science, Nagoya University, Chikusa-ku, Nagoya 464-01, Japan. Phone: 052-789-2984. Fax: 052-789-3001.

Present address: Tsukuba Research Institute, Banyu Pharmaceutical Co., Ltd., Okubo, Tsukuba 300-33, Japan.

two CENP-B polypeptides and two DNA molecules (29, 51), suggesting that the function of CENP-B in vivo might be to organize the higher-order structure of centromeric satellite DNA arrays by juxtaposing pairs of CENP-B box sequences. The long-range distribution of the α-satellite DNA monomers that contain CENP-B boxes has been analyzed in human chromosome 21 DNA (16), and it was revealed that one (α21-I) of the two long α-satellite arrays (α21-I and α21-II), each encompassing over 1 Mb and located side by side in the centromere region, was composed of α-satellite monomers containing CENP-B boxes predominantly at every other monomer position. The α21-I array colocalized with the antigenic sites of ACA staining in both the interphase nucleus and metaphase chromosomes. CENP-B boxes (a 16-of-17-bp match to the human consensus) have been found in mouse (*Mus musculus*) minor satellite DNA (24), which is similarly located at the primary constrictions of metaphase chromosomes and composed of 120-bp repeat units whose nucleotide sequence is unrelated to that of α-satellite DNA except for the CENP-B box (31, 49). Highly homologous cDNAs encoding CENP-B have been cloned from human and mouse (*M. musculus*) cells (10, 38). The results suggest that the CENP-B–CENP-B box interaction is conserved among mammals and may play an important role (directly or indirectly) in establishment of the specific structure of the centromere region or kinetochore. Although the AGM is phylogenetically closer to humans than to mice and is known to contain large amounts of α-satellite DNA, no one has thus far succeeded in finding CENP-B boxes in its DNA or CENP-B in the centromere domains of its chromosomes. This raises a question about the essentiality and/or extent of conservation of the CENP-B–CENP-B box interaction in mammalian centromeres.

We report in this paper CENP-B gene cloning and the presence, biochemical properties, and centromeric localization of CENP-B in AGM cells. We show that the predicted amino acid sequence, deduced from the nucleotide sequence of the cloned AGM CENP-B gene, is 98% identical to that of the human gene. We also demonstrate that CENP-B is expressed in the same order as in human or mouse cells and has the same DNA binding specificity and homodimer forming activity as human CENP-B. Using a slightly modified indirect immunofluorescent staining procedure, we further show that antigens recognized by anti-CENP-B antibodies are colocalized with the ACAs at the centromere region of the metaphase AGM chromosomes. In addition, we isolated AGM α-satellite DNA containing a CENP-B box-like sequence which has affinity for CENP-B. These results not only prove that CENP-B is present in AGM cells but also suggest that the AGM genome contains recognition sequences for CENP-B in satellite centromere DNA.

MATERIALS AND METHODS

Cell culture. HeLa (human) and CV-1 (AGM) cells were cultured in Dulbecco modified Eagle medium (Nissui) containing 10% calf serum. COS cells (AGM) were cultured in the same medium containing 10% fetal calf serum.

Antibodies. ACA-positive sera from scleroderma patients (I and G) contained antibodies against the three major centromere autoantigens: CENP-A (17 kDa), CENP-B (80 kDa), and CENP-C (140 kDa). Normal serum contained no antibodies against centromere proteins. The antibody against the NH_2-terminal region of human CENP-B (BN1) was described earlier (22). The antibody against the COOH-terminal region of human CENP-B (BC1) was raised by immunizing rabbits with polypeptide G (positions 541 to 599) (22) and affinity purified with the same polypeptide, and then antibodies against glutathione *S*-transferase (GST) were removed by using a GST-Sepharose column.

Southern hybridization. The genomic DNA of AGM cells was digested with various kinds of restriction enzymes and electrophoresed through 0.6% agarose gels. The DNA bands in the gels were transferred to nylon membranes (Hybond N+; Amersham International, Amersham, England) under vacuum and fixed by baking for 30 min at 80°C. The 1.5-kbp *Apa*I fragment (A1500) that encodes the NH_2-terminal region of human CENP-B (positions 3 to 1462) was prepared from pET-3d CENP-B and used for hybridization with AGM DNA as a probe after labeling with [α-^{32}P]dCTP (110 TBq/mmol; Amersham), using a T7 Quick Prime labeling kit (Pharmacia). Hybridization was performed as described previously (37) for 12 to 16 h at 42°C. After hybridization, the membranes were washed twice with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 sodium citrate)–0.1% sodium dodecyl sulfate (SDS) for 30 min at 42°C and once with 0.1× SSC–0.1% SDS for 30 min at 55°C. The membranes were then exposed to X-ray film (Kodak, XAR-5) for 1 to 5 days.

Cloning of the CENP-B gene from AGM cells (CV-1). Two hundred micrograms of AGM genomic DNA was completely digested with *Xba*I, and an approximately 5.1-kbp *Xba*I fragment containing the whole CENP-B coding region was concentrated by isolation from the agarose gel after being electrophoresed through a 0.6% agarose gel for 16 h at 70 V at 4°C. The concentrated *Xba*I fragment (155 ng) was ligated to pUC119 vector DNA (100 ng) (linearized by *Xba*I digestion and dephosphorylated with calf intestinal phosphatase), using T4 DNA ligase in a 10-μl reaction mixture containing 50 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 10 mM dithiothreitol (DTT), 0.5 μg of bovine serum albumin (BSA), and 1 mM ATP for 16 h at 16°C. One microliter of the ligated sample was transfected into *Escherichia coli* XL-Blue MR cells [Δ(*mrcA*)*183* Δ(*mcrCB-hsd SMR-mrr*)*173 supE44 endA1 gyrA96 thi-1 relA1*] by electroporation in a Gene Pulser (Bio-Rad Laboratories, Richmond, Calif.) (25 μF, 2,500 V, 200 W). The transformants (8×10^5) resistant to ampicillin were screened by colony hybridization as described previously (37). The NH_2-terminal 400-bp DNA of the human CENP-B coding sequence (N400) amplified by PCR was labeled with ^{32}P and used as a probe.

DNA sequencing. Subfragments of the cloned AGM CENP-B gene (pAGCB), generated by digestion with *Xba*I-*Hin*dIII, *Sma*I, *Pst*I, *Kpn*I, *Sac*I, or *Bam*HI, were recloned into the pUC119 vector and transfected into *E. coli* DH5α cells, and the DNA sequences were determined from M13 primer sites on the vector by using a Sequenase version 2.0 DNA sequencing kit (U.S. Biochemical) or a Cycle Sequencing kit (Bethesda Research Laboratories). The reaction conditions were according to the protocols of each company, using ^{32}P radioisotopes. The DNA sequences thus obtained were analyzed with DNASIS (Hitachi Software Engineering Co., Ltd.).

Preparation of the nuclear proteins. Nuclear proteins were prepared essentially as described earlier (51). Cultured cells at 50% confluence in a 10-cm-diameter plate were physically collected with a scraper instead of by using trypsin. The cells were washed once with phosphate-buffered saline (PBS) and twice with isolation buffer (3.75 mM Tris-HCl [pH 8.0], 0.05 mM spermine, 0.125 mM spermidine, 0.5 mM EDTA, 0.5 mM DTT, 20 mM KCl, 0.1 mM phenylmethylsulfonyl fluoride [PMSF], 2 μg of pepstatin per ml, 2 μg of leupeptin per ml). The cells (10^7/ml in isolation buffer containing 0.1% digitonin) were homogenized with a Dounce homogenizer, and the nuclei were collected by centrifugation. After the nuclei were washed once with washing buffer (20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 8.0], 0.5 mM EDTA, 0.5 mM DTT, 20 mM KCl, 0.1 mM PMSF, 2 μg of pepstatin per ml, 2 μg of leupeptin per ml), the nuclear proteins were extracted in 0.5 M NaCl–extraction buffer (20 mM HEPES [pH 8.0], 15% [vol/vol] glycerol, 0.5 mM EDTA, 0.5 mM DTT, 20 mM KCl, 0.5 mM PMSF, 2 μg of pepstatin per ml, 2 μg of leupeptin per ml) at a concentration of 10^8 nuclei per ml.

Western blotting (immunoblotting) and DNA mobility shift analysis. Western blotting and DNA mobility shift analysis were performed as described previously (51). The proteins were separated by SDS–7% polyacrylamide gel electrophoresis (PAGE) and in situ transferred to polyvinylidene difluoride membranes (Millipore) as described by Towbin et al. (39). The membranes were reacted with the indicated antibody and then anti-human or anti-rabbit immunoglobulin G (IgG) conjugated with horseradish peroxidase (Bio-Rad). Color development was carried out with the Amersham ECL protein detection system. DNA mobility shift analyses were performed with the indicated amounts of nuclear extract and a ^{32}P-labeled 59-mer double-stranded DNA oligonucleotide containing the CENP-B box (CB59) (51).

Metaphase chromosome preparation and indirect immunofluorescent staining. Metaphase COS-1 cells were recovered by pipetting and centrifugation after colcemid treatment (0.05 mg/ml for 4 h), kept in hypotonic solution containing 75 mM KCl, 1 mM $MgCl_2$, and 1 mM $CaCl_2$ at 37°C for 5 min, and then spread on a poly-D-lysine-coated coverslip by cytocentrifugation at 1,200 rpm for 4 min (Tomy, Tokyo, Japan). Metaphase chromosomes on the coverslip were fixed quickly with 95% acetone at −20°C for 30 min, dried in air, and fixed again with periodate-lysine-paraformaldehyde fixative (28) at 0°C for 30 min. The fixed chromosomes were washed with PBS three times, incubated with the mixture of ACA serum (G; 1:240 dilution) and BN1 (1:40 dilution) diluted with PBS supplemented with 0.1% BSA and 0.1% skim milk at 37°C for 2 h, washed with PBS for 3 min at room temperature three times, and then incubated with rhodamine-conjugated anti-human IgG (1:80 dilution; MBL, Japan) and fluorescein isothiocyanate-conjugated anti-rabbit IgG (1:160 dilution; MBL) at 37°C for 1 h. Chromosomal DNAs were stained with DAPI (4′,6-diamidino-2-phenylindole dihydrochloride; 1 mg/ml) and then mounted (25). The samples were observed and images were obtained with a fluorescence microscope (LSM10; Zeiss) equipped with a cooled charge-coupled device camera (PXL-1400; Photometrics) and IPLab Spectrum software (Signal Analytics) on a Macintosh computer. We ascertained that this microscopy system gave completely overlapped images

through the two-filter system (Zeiss), red and green, when centromere antigens on HeLa cells were observed by ACA as the first antibody and the mixture of anti-human IgG conjugated with rhodamine and that conjugated with fluorescein isothiocyanate as the second antibodies.

Isolation of DNA fragments with affinity for CENP-B. The procedures were basically as described by Wafik (42). Two micrograms of AGM (CV-1) genomic DNA, fragmented to ~300 bp with sonication after complete digestion with *Hin*dIII and *Eco*RI and then blunt ended with Klenow enzyme, was ligated by using T4 DNA ligase with an excess amount (5 μg) of the linker DNA made by annealing two primers, CL-A (5′ CTTGAGAATTCTAATACAAG) and CL-B (5′ CTTGTATTAGAATTCTCAAG). The linker DNA was previously phosphorylated with T4 polynucleotide kinase. The linker contained an *Eco*RI site in the middle region and was designed to form a *Hin*dIII site (underlined) when the linkers were ligated with each other. After ligation, tandemly ligated linkers were completely digested with 120 U of *Hin*dIII for 3.5 h at 37°C. The binding reaction and immunoprecipitation were performed as described earlier (51). Four hundred nanograms of the linked DNA was subjected to binding reaction with human CENP-B (partially purified on a Q-Sepharose column) (51) for 2 h and with ACA serum (1:100 dilution) for 1 h at 4°C. Then bound DNA was precipitated with protein A-Sepharose (Pharmacia). The precipitated DNA was then amplified by PCR using primers CL-A and CL-B as follows: 24 cycles of 90°C for 1 min, 45°C for 1.5 min, and 72°C for 2 min. This immunoprecipitation-PCR amplification procedure was repeated four times and the products were ligated to the pUC119 vector after digestion with *Eco*RI. The affinity of each clone (AIP clone) to CENP-B was determined by DNA mobility shift competition assay as follows: a 100-fold molar excess of each AIP DNA (~1 μg) was added to the reaction mixture for the DNA mobility shift system containing 0.2 ng of ^{32}P-CB59 DNA and HeLa nuclear extract (51), and AIP clones with competitor activity were selected after electrophoresis on the basis of their negative effect on the formation of the shifted radioactive band (complex A).

PCR. The following primers were used for amplification of the DNA segment coding for the DNA binding domain of CENP-B: pri-1, 5′ATGGGCCCCAAG AGGCGACAGCTGACGTTC (1 to 30; nucleotide numbers from the start site of the coding sequence of the human CENP-B gene); pri-2, 5′GCGGATCTGC TGGAAGCAGGCGATGAT (267 to 241); and pri-3, 5′TCACGGATCATCC AGGAGGTGGAGGAGAAT (40 to 69). The reaction conditions were essentially according to the Perkin-Elmer Cetus protocols: 95°C for 2.5 min, 55°C for 2 min, and 72°C for 2 min (1 cycle); 95°C for 1.5 min, 55°C for 2 min, and 72°C for 2 min (30 cycles); and 95°C for 1.5 min, 55°C for 1.5 min, and 72°C for 5 min (1 cycle). The PCR products were electrophoresed through a 1.7% agarose gel (Takara Co. Ltd., Kyoto, Japan), and the pri-3 oligonucleotides were labeled with [γ-^{32}P]ATP (110 TBq/mmol; Amersham), using T4 polynucleotide kinase (Boehringer), and used as the hybridization probe. After hybridization, the membranes were washed three times with 2× SSC–0.1% SDS for 15 min at room temperature and then used to expose X-ray films.

Source of genomic DNA. The genomic DNAs used for the amplification of the CENP-B gene by PCR were obtained as follows: human DNA from HeLa cells, AGM DNA from CV-1 cells, muntjac DNA from cultured cells, calf DNA from thymus, *M. musculus* DNA, *Mus caroli* DNA, hamster DNA, and chicken DNA from livers, and *Drosophila melanogaster* DNA from embryos. The DNAs were purified by phenol extraction after pronase K digestion. *Xenopus* DNA, *Caenorhabditis elegans* DNA, and *S. pombe* DNA were kindly provided by Hideki Kobayashi, Yuji Kohara, and Takashi Toda, respectively. Mice (*M. caroli*) were captured in the wild on Okinawa Island.

RESULTS

Cloning of the CENP-B gene from AGM (CV-1) cells. We undertook to clone the CENP-B gene from the AGM genome as the first step toward confirming the presence of CENP-B in AGM cells. The CENP-B gene in AGM genomic DNA was detected by Southern hybridization as shown in Fig. 1A. Restriction mapping of AGM and human DNA around the CENP-B gene (Fig. 1B) indicated similar cleavage patterns. The 5.1-kbp *Xba*I fragment was cloned into the pUC119 vector, and the base sequence encoding the whole AGM CENP-B gene was determined. As for human and mouse cells, the AGM CENP-B gene was found to contain no introns. The encoded 602-amino-acid polypeptide is longer than human or mouse CENP-B by 3 amino acids (Fig. 2). In Fig. 2, the predicted amino acid sequence of AGM CENP-B is compared with the human and mouse sequences; AGM CENP-B is 98% identical to the human CENP-B. The DNA binding and dimerization domains were found to be 100% identical in all three species. These results suggest that the physiological function of AGM CENP-B as well as the DNA binding specificity must be exactly the same as for human and mouse CENP-B.

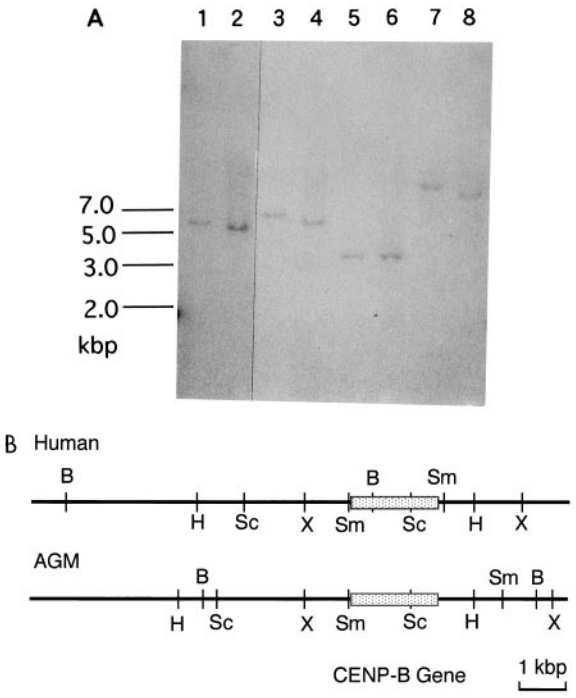


FIG. 1. Cloning of the CENP-B gene from AGM cells. (A) Detection of the CENP-B gene in AGM CV-1 cells by Southern hybridization. Five micrograms of genomic DNA of AGM cells (lanes 1, 3, 5, and 7) or HeLa cells (lanes 2, 4, 6, and 8) was digested with *Xba*I (lanes 1 and 2), *Hin*dIII (lanes 3 and 4), *Xba*I-*Hin*dIII (lanes 5 and 6), or *Eco*RI (lanes 7 and 8) and electrophoresed through a 0.6% agarose gel. The *Apa*I fragment containing the human CENP-B gene (3 to 1462) was used as a probe for the hybridization. (B) Restriction maps around the CENP-B gene region. B, *Bam*HI; H, *Hin*dIII; Sc, *Sac*I; Sm, *Sma*I; X, *Xba*I.

CENP-B expression in AGM cells. Expression of CENP-B in AGM cells (CV-1 and COS) was assessed by Western blotting with ACA serum or anti-CENP-B antibodies (Fig. 3A). In both CV-1 and COS cells, an ~80-kDa protein was detected by all three antibodies (Fig. 3A, lanes 2 to 3 [ACA serum], 5 to 6 [BN1], and 8 to 9 [BC1]). The immunoreactive species in AGM cells had the same mobility as human CENP-B (lanes 1, 4, and 7). DNA binding activity of AGM CENP-B to the CENP-B box (CTTCGTTGGAAACGGGA) was examined by DNA mobility shift analysis (Fig. 3B). A ^{32}P-labeled 59-mer DNA probe (CB59) was incubated with nuclear extracts from AGM cells and electrophoresed. DNA-protein complexes with the same mobility as those formed with human CENP-B (complex A; Fig. 3B, lane 1) appeared in lanes containing the nuclear extracts of CV-1 and COS cells (lanes 2 and 3). These complexes were reactive to ACA serum (lanes 4 to 6) or BN1 (lanes 7 to 9), indicating that the AGM CENP-B–DNA complex contains a CENP-B dimer and two DNA molecules as shown for the complex A formed with human CENP-B (29, 51). The amount of CENP-B recovered from AGM cells (which was subject to some experimental variability) could roughly be estimated from Fig. 3A and B to be ~20% of that from HeLa cells. From these results, we conclude that AGM CENP-B is expressed in AGM cells and that the AGM polypeptide has the same molecular weight, dimerizing activity, and DNA binding specificity as human CENP-B.

CENP-B localization at the centromere region of AGM metaphase chromosomes. To define the location of CENP-B expressed in AGM cells, metaphase chromosomes from COS-1 cells were prepared as described in Materials and Methods and

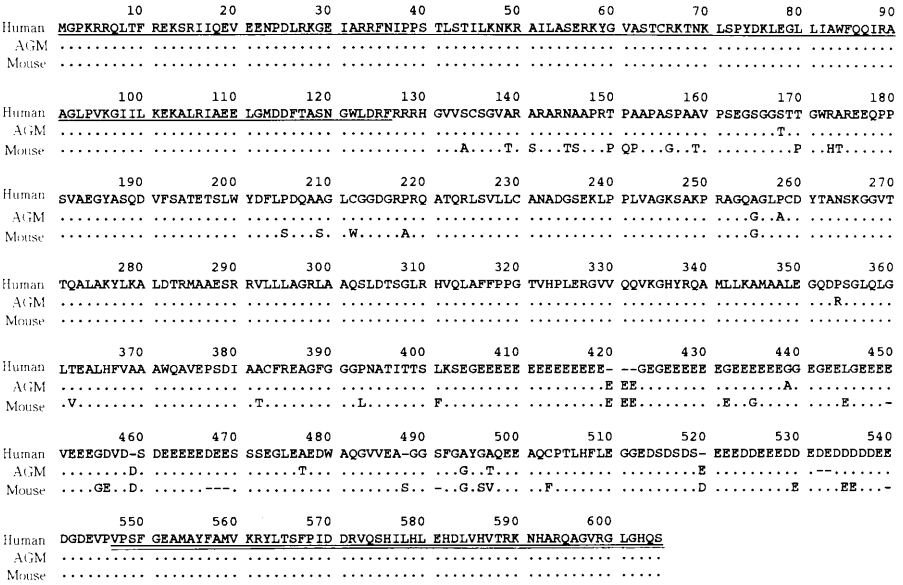


FIG. 2. Comparison of the amino acid sequences of human, mouse, and AGM CENP-B. The amino acid sequence of human CENP-B is shown in one-letter code. The same amino acids in the mouse and AGM sequence are shown as dots, and deleted amino acids are shown as dashes. Underlining and double underlining indicate the DNA binding domain and the dimerization domain, respectively, of human CENP-B.

subjected to indirect immunofluorescence staining using a polyclonal antibody against the NH_2-terminal region of human CENP-B (BN1) (Fig. 4). As shown in Fig. 4A, C, and D, AGM CENP-B was detected as pairs of dots at the outer surfaces of the primary constrictions of most COS-1 metaphase chromosomes. The results clearly indicated that AGM CENP-B is actually localized at the centromere region of AGM chromosomes. Using this antibody, we also observed human and mouse CENP-B to be located specifically at the centromere region of the chromosomes, forming clear pairs of dots overlapping the antigenic sites of ACA serum (data not shown). Pairs of dots for AGM CENP-B (Fig. 4A) mostly overlapped those for ACA antigens (Fig. 4D), but in contrast to the results seen with human or mouse CENP-B, the dots for AGM CENP-B were consistently located a little outside those for ACA antigens (Fig. 4).

Search for CENP-B recognition sequences in the AGM genome. We have shown that AGM CENP-B contains exactly the same DNA binding domain as human CENP-B and localizes specifically at the centromere region of AGM chromosomes. This clearly implies the presence of recognition sequences for CENP-B (CENP-B boxes) within AGM centromeres. To search for CENP-B boxes in the AGM genome, we first determined DNA sequences from 24 independent α-satellite monomer DNAs after cloning into the pUC119 vector. However, these monomers were highly conserved (95 to 100%) and contained neither CENP-B boxes nor the core recognition sequence defined by Masumoto et al. (23a, 26) (data not shown). Then we tried to enrich CENP-B box-containing AGM genomic DNA fragments by repeated immunoprecipitation-PCR amplification cycles using partially purified human CENP-B and an ACA serum, and the resulting fragments were cloned into the pUC119 vector as described in Materials and Methods. By further selecting the clones for affinity to CENP-B by DNA mobility shift competition assays (data not shown), we obtained two clones (AIP-1B and AIP-21) both of which contained sequences with high homology to the AGM α-satellite consensus sequence (Fig. 5A; AIP-1B, 89%; AIP-21, 94%). Binding of the AIP-1B fragment to CENP-B was confirmed by immunoprecipitation (Fig. 5B) and DNA mobility shift (Fig. 5C) assays. As shown in Fig. 5D, these selected fragments contained a CENP-B box-like sequence in which 15 of 17 nucleotides were identical to the canonical human CENP-B box sequence. It should be pointed out that the 9-bp core recognition sequence consisting of three regions, TTCG, A, and CGGG (Fig. 5D) (26), was found to be fully conserved. This CENP-B box-like sequence was different from the AGM α-satellite consensus sequence by only two nucleotides in the essential regions, TTCG versus TCCG and CGGG versus CTGG. These results suggested that this CENP-B box-like sequence might exist in α-satellite repetitive sequences in the centromere regions of AGM chromosomes.

CENP-B is conserved among mammals. To determine the extent to which CENP-B is conserved in eukaryotic cells, we amplified the NH_2-terminal region of the CENP-B gene from a number of species. This region encodes the DNA binding domain and therefore might be expected to be more conserved than other regions of CENP-B because of its functional importance. Two oligonucleotides were chemically synthesized on the basis of human CENP-B gene sequence as detailed in Materials and Methods: pri-1 (1 to 30; nucleotide numbers from the start site of the coding sequence of human CENP-B gene), encoding the NH_2-terminal 10 amino acids, and pri-2 (267 to 241), encoding 9 amino acids in the third α-helix of the DNA binding domain (51). The genomic DNAs of human (HeLa), AGM (CV-1), calf (thymus), Indian muntjac, *M. musculus* (liver), *M. caroli* (liver), hamster, chicken, *Xenopus*, *C. elegans*, *D. melanogaster*, and *S. pombe* cells were subjected to PCR as described in Materials and Methods, and the samples were electrophoresed in agarose gels (Fig. 6A). To detect the DNA fragment homologous to the CENP-B gene among various bands amplified by PCR as shown in Fig. 6A, Southern hybridization was performed with a third oligonucleotide (pri-3; 40 to 69) (see Materials and Methods). Figure 6B illustrates that the genomic segments of the same size were amplified in human (lane 1) through hamster (lane 7) samples.

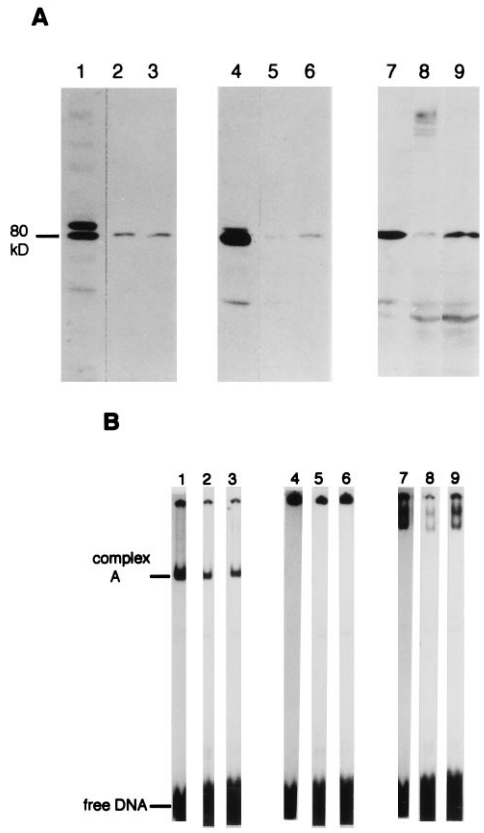


FIG. 3. Expression of CENP-B in AGM cells. (A) Western blotting. Proteins extracted from 10^6 HeLa (lanes 1, 4, and 7), CV-1 (lanes 2, 5, and 8), or COS (lanes 3, 6, and 9) nuclei with 0.5 M NaCl were subjected to SDS-PAGE and transferred to polyvinylidene difluoride membranes as described in Materials and Methods. The membranes were incubated with ACA serum (lanes 1 to 3), BN1 (lanes 4 to 6), or BC1 (lanes 7 to 9). The molecular mass of each position, calculated from the standard molecular weight markers, is indicated at the left. Bands observed around 60 kDa in lanes 8 and 9 are estimated to be degradation products of CENP-B. (B) DNA mobility shift analysis. The 0.5 M NaCl nuclear extracts (3×10^5 nuclei equivalent) of HeLa (lanes 1, 4, and 7), CV-1 (lanes 2, 5, and 8), and COS (lanes 3, 6, and 9) cells were incubated with ^{32}P-labeled CB59 DNA (0.5 ng). After the DNA binding reactions, ACA serum (lanes 4 to 6) or BN1 (lanes 7 to 9) was added, and the samples were electrophoresed in a 5% polyacrylamide gel.

Although the amount of the amplified DNA from *M. caroli* was small in lane 6, we confirmed by other PCR experiments that the same amount of CENP-B segment could be amplified in *M. caroli* as in *M. musculus* (data not shown). These results indicate that CENP-B is conserved at least among mammalian species.

DISCUSSION

Structure and biochemical activities of AGM CENP-B. In this work, we have examined both the gene structure and protein products of the AGM CENP-B gene and found the following: (i) the CENP-B gene of AGM (Fig. 1A) is highly homologous to the human CENP-B gene (98% in amino acid sequence) (Fig. 2), and the predicted amino acid sequences of the DNA binding domain and the dimerization domain are 100% identical to those of the human and mouse CENP-B genes (Fig. 2); (ii) an 80-kDa protein can be detected on SDS-PAGE of AGM nuclear extracts by immunoblotting with an ACA serum and anti-CENP-B antibodies raised against human CENP-B (BN1 and BC1) (Fig. 3A), and antigens in the AGM extract form dimers and specifically bind to the human CENP-B box (Fig. 3B); and (iii) such antigens are also specifically detectable in the centromere regions of AGM chromosomes (Fig. 4). These results indicate that CENP-B molecules are expressed in AGM cells and specifically localized to the centromere region of AGM chromosomes, very probably via specific recognition of DNA sequences in the centromere region as reported for human CENP-B. We also showed by PCR experiments that CENP-B genes are conserved, at least among mammalian cells (Fig. 6).

Expression of CENP-B in AGM cells. We detected CENP-B in AGM cells by Western blotting and by DNA mobility shift assays. The properties of the detected CENP-B molecules were found to be identical to those of human CENP-B as far as has been examined (molecular weight, dimerizing activity, and DNA binding specificity). However, the amount of CENP-B molecules extractable from AGM cells was approximately 20% of that from HeLa cells (Fig. 3). Considering that the amount of CENP-B recovered from HeLa cells was usually several times more than that from other human cultured cell lines (TIG7 or HL60) (unpublished observation), the expression level of CENP-B in the AGM cell lines appears to be in the same order as in human cell lines. It has been reported previously that CENP-B is not present in AGM cells (33), but AGM CENP-B might be sensitive to proteolytic degradation during preparation of cell extracts, since we sometimes failed to detect an ~80-kDa CENP-B band and instead detected smaller bands by Western blot analysis (data not shown and Fig. 3A).

Localization of CENP-B in the centromere regions of AGM chromosomes. Although we confirmed the expression of CENP-B in AGM cells (Fig. 3), we were at first unable to observe the CENP-B signals cytologically on AGM chromosomes with the conventional procedures used for human or mouse chromosomes. The same problems were encountered during analysis of Indian muntjac cells. Since we had experienced during the study of CENP-B signals in human or mouse chromosomes that the dot signals were somewhat unstable and sometimes became diffuse as a result of small changes in the conditions used for metaphase spreading, we tried various conditions for preparing metaphase chromosomes from Indian muntjac cells and established those best suited for detecting clear CENP-B signals (38a). When the same procedures were applied to AGM cells, CENP-B could be clearly detected as pairs of dots on the outer surfaces of the primary constrictions of mitotic chromosomes (the centromere regions) (Fig. 4), though the signals were weaker than those of human centromeres. Goldberg et al. (13) found that most of what they concluded to be AGM CENP-B was distributed diffusively throughout the interphase nucleus, and at the same time they detected faint CENP-B staining at the centromeres of mitotic AGM chromosomes. In contrast, we detected CENP-B signals in the AGM interphase nucleus in the shape of patches including the dots overlapping ACA signals (38a). The ACA signals in interphase cells of a human fetal (25) or an Indian muntjac (4) cell line were apparently decondensed and diffusive compared with those in mitotic cells. Therefore, the DNA-protein complex containing CENP-B seems to become more or less loose in interphase in some cell lines. The ACA serum used in these experiments recognizes three major centromeric antigens (CENP-A, -B, and -C) in human cells and also CENP-A (26a) and CENP-B (Fig. 3A) in AGM cells. The signals with anti-CENP-B antibody (BN1) and ACA serum detected by indirect immunofluorescent staining of human or mouse cells completely overlap one another (data not shown). Interestingly, the signals against BN1 were found to be shifted slightly but significantly toward the outside of the ACA signals (Fig.

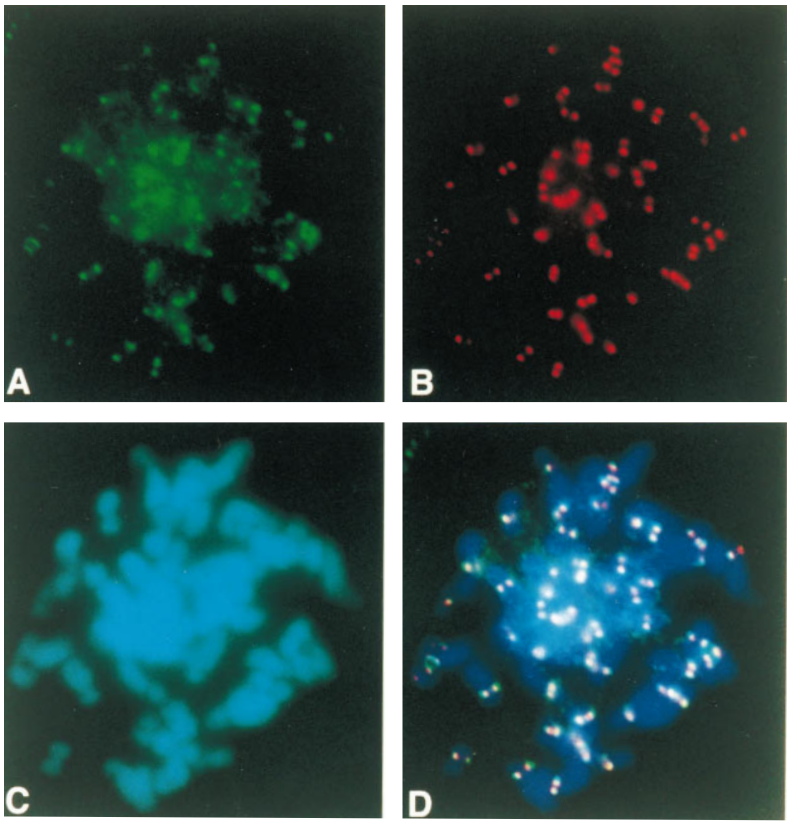
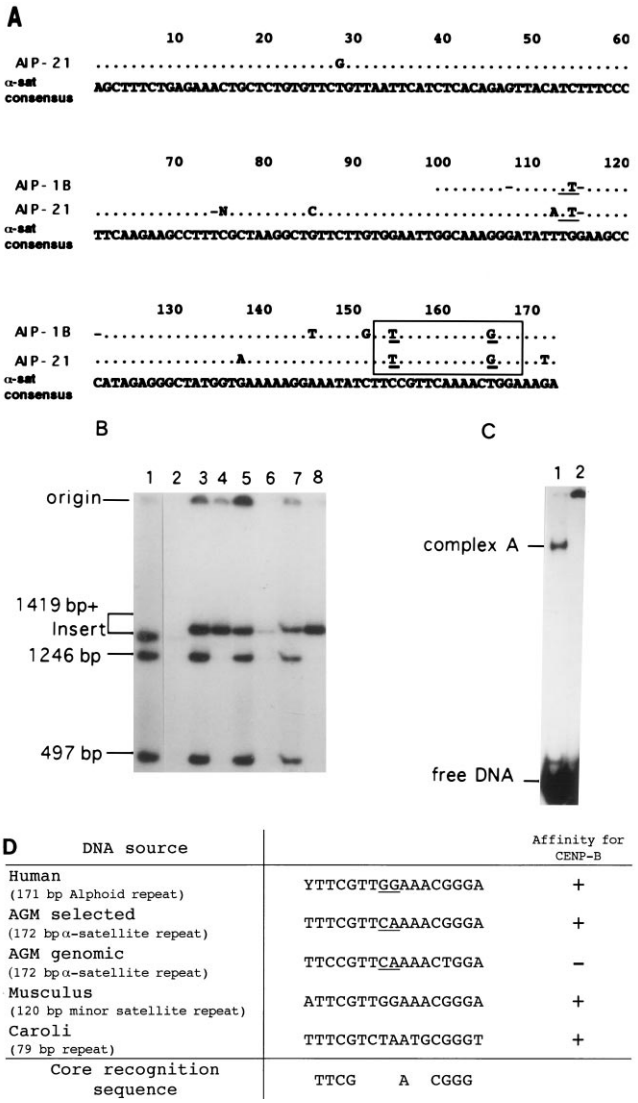


FIG. 4. Indirect immunofluorescent staining of a native metaphase spread of COS-1 chromosomes. (A) BN1; (B) CREST anticentromere serum (KG); (C) DAPI; (D) merged images of A, B, and C.

4A, B, and D), although there was major overlapping. One possible explanation for this finding is that the major epitopes in CENP-B recognized by ACA and by BN1 are different and some of the epitopes are structurally masked in AGM centromeres. Another possibility is that the titer of the antibody recognizing CENP-B in ACA is lower than those of the other antibodies (anti-CENP-A and/or -C) and the distribution of CENP-B and other CENPs in AGM centromeres is different from that in human or mouse cells. In any case, the results suggested that the higher-order structures of DNA-protein complexes at the centromere region of AGM chromosomes may be somewhat different from those in other species. Such structural differences may also be related to the observed instability and weakness of CENP-B signals during immunofluorescent staining of AGM cells.

CENP-B boxes in the centromere regions of AGM chromosomes. In human cells, it has been revealed that the NH_2-terminal DNA binding domain of CENP-B is necessary and sufficient for the localization of CENP-B to the centromere region of human chromosomes in vivo (reference 33 and our unpublished observations), which indicates that the CENP-B–CENP-B box interaction is responsible for CENP-B targeting to the centromere region. We have shown in this report that AGM CENP-B contains the same DNA binding domain as human CENP-B and localizes specifically to the centromere region of AGM chromosomes. This clearly implies the presence of the recognition sequences for CENP-B (CENP-B boxes) within AGM centromeres. Although the first α-satellite DNA was found in AGM chromosomes and its consensus sequence was determined directly by using 172-bp α-satellite monomer DNA obtained by *Hin*dIII digestion of the AGM genomic DNA (36), relatively little information is available about the subfamily organization of AGM α-satellite DNA. Furthermore, the AGM monomer consensus sequence contains no CENP-B box. To ascertain if some α-satellite monomers might carry the CENP-B box, we determined DNA sequences from 24 independent α-satellite monomer DNAs. These contained neither CENP-B boxes nor the core recognition sequence, in line with the findings in the accompanying report for 76 AGM α-satellite monomers (13). Therefore, the AGM α-satellite monomers produced by *Hin*dIII digestion may contain CENP-B boxes in only very small amounts, and to detect the CENP-B boxes expected to exist in the AGM genome, we therefore tried repeated immunoprecipitation-PCR amplification cycles using randomly fragmented AGM DNA. We thereby obtained two clones (AIP-1B and AIP-21) composed of AGM α-satellite consensus sequences containing a few base alterations (Fig. 5A). There were only three loci where the same base changes occurred in the two clones: 114 to 115, where GG is changed to T- (Fig. 5A), and the two loci (154 and 165) in the CENP-B box-like sequence. The base changes at 154 and 165 must be responsible for the affinity for CENP-B exhibited by these clones, since the altered bases were located within the 9-bp core recognition sequence of CENP-B, and no CENP-B box-like sequence was found in other regions of the clone. Since the base changes at 114 to 115 did not confer the affinity for CENP-B on the fragments, it is not likely that they were introduced and condensed in vitro during the immunoprecipitation-PCR cycles. These clones with the common base changes may represent a minor subtype



D DNA source		Affinity for CENP-B
Human (171 bp Alphoid repeat)	YTTCGTTGGAAACGGGA	+
AGM selected (172 bp α-satellite repeat)	TTTCGTTCAAAACGGGA	+
AGM genomic (172 bp α-satellite repeat)	TTCCGTTCAAAACTGGA	−
Musculus (120 bp minor satellite repeat)	ATTCGTTGGAAACGGGA	+
Caroli (79 bp repeat)	TTTCGTCTAATGCGGGT	+
Core recognition sequence	TTCG A CGGG	

FIG. 5. Analyses of DNA fragments (AIP-1B and AIP-21) isolated from AGM genomic DNA after immunoprecipitation-PCR amplification cycling. (A) AIP-1B and AIP-21 sequences were compared with the AGM monomer consensus sequence obtained from α-satellite DNA by *Hin*dIII digestion. The bases identical to the consensus sequence are marked with dots, and deleted bases are marked with dashes. Numbers are from *Hin*dIII site as published previously (36). The CENP-B like sequence is boxed, and common bases which are different from the consensus sequence are underlined. (B) Immunoprecipitation assay. The clones were digested with *Apa*LI and labeled with ^{32}P by using [^{32}P]dATP and the DNA polymerase I Klenow enzyme. Ten nanograms of each sample was subjected to immunoprecipitation as described in Materials and Methods. One-tenth of input DNAs (lanes 1, 3, 5, and 7) and the total immunoprecipitated DNAs (lanes 2, 4, 6, and 8) were electrophoresed in a 5% polyacrylamide gel. The gel was autoradiographed after drying. Lanes 1 and 2, pUC119; lanes 3 and 4, pUC119 containing a human α-satellite DNA with CENP-B box; lanes 5 and 6, pUC119 containing human α-satellite DNA with a defective CENP-B box (TATGGTGGAAAAGGAA); lanes 7 and 8, AIP-1B. (C) DNA mobility shift assay. The AIP-1B fragment isolated by digestion of the clone with *Eco*RI was labeled with ^{32}P as for panel B. One-tenth nanogram of the labeled DNA was subjected to DNA mobility shift assay as detailed in Materials and Methods (lane 1). The DNA–CENP-B complex (complex A) shown in lane 1 was supershifted by the addition of 1 μl of ACA serum (1:10 dilution) (lane 2). (D) Summary of the human, AGM, and mouse CENP-B box sequences. Nine important bases for CENP-B binding (the core recognition sequence) (23a, 26) are indicated at the top. Y = T/C. The underlined bases indicate differences between the human and AGM CENP-B boxes.

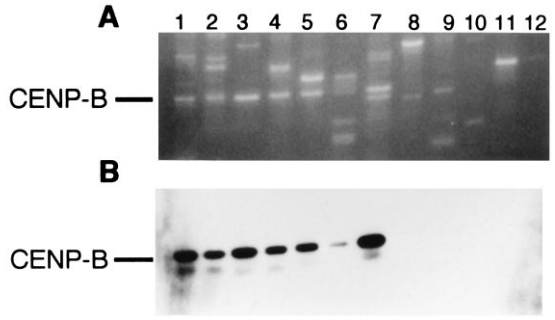


FIG. 6. Detection of CENP-B genes in genomic DNAs from various eukaryotic species. (A) Approximately 0.5-μg samples of genomic DNA of HeLa (lane 1), CV-1 (lane 2), calf (lane 3), muntjac (lane 4), *M. musculus* (lane 5), *M. caroli* (lane 6), hamster (lane 7), chicken (lane 8), *Xenopus* (lane 9), *C. elegans* (lane 10), *D. melanogaster* (lane 11), and *S. pombe* (lane 12) cells were subjected to PCR using the pri-1 (1 to 30) and pri-2 (267 to 241) primers as described in Materials and Methods. The samples were electrophoresed through a 1.7% agarose gel containing 0.5 μg of ethidium bromide per ml. The bar indicates location of the 267-bp human CENP-B fragment. (B) Southern hybridization of the PCR samples panel A. ^{32}P-labeled pri-3 (40 to 69) oligonucleotide was used as a probe.

of α-satellite sequence in the AGM cells containing the CENP-B recognition sequence. Considered together with the lack of CENP-B box-like sequences in 100 α-satellite monomer sequences (this report and reference 13), these results suggest that the amount of the selected sequences on α-satellite may be minuscule, if at all present, in *Hin*dIII monomers of AGM α-satellite DNA. The observation by indirect immunofluorescence microscopy that CENP-B localizes at the centromeres as pairs of dots (Fig. 4), however, suggests that recognition sequences for CENP-B must be clustered in AGM chromosomes at such points. We recovered many different kinds of DNA fragments as well as α-satellite-type fragments by cycling of immunoprecipitation and PCR amplification of AGM genomic DNA and found that these fragments lacked the canonical CENP-B box sequence but instead contained sequences homologous to the 9-bp CENP-B core recognition sequence. We confirmed that some of the fragments actually bind to CENP-B by immunoprecipitation and DNA mobility shift assays (data not shown). It remains to be clarified what satellite DNA families the isolated fragments are derived from and how long the repeat sizes are.

Is the CENP-B–CENP-B box interaction conserved among mammalian cells? We have shown that CENP-B is conserved in mammalian cells (Fig. 6). It should be noted that a CENP-B gene also exists in the mouse *M. caroli* (Fig. 6, lane 6). Although minor satellite DNA is localized at the centromere region of *M. musculus* and its related subspecies (*M. musculus poshivinus* and *M. musculus molossinus*), no minor satellite DNA was identified by in situ hybridization in the chromosomes of *M. caroli* (48). As the phylogenetical distance of *M. musculus* and *M. caroli* is similar to that separating humans and AGMs, the CENP-B gene of *M. caroli* would be expected to be highly homologous to that of *M. musculus*, and its CENP-B may have the same functional role. Kipling et al. recently detected a 79-bp repetitive sequence containing a CENP-B box-like sequence in the centromere region of *M. caroli* chromosomes, and binding activity of this sequence to human CENP-B was shown (21). As summarized in Fig. 5D, the sequence was found to contain the core recognition sequence of the CENP-B box, suggesting that a novel repetitive sequence that is recognized by CENP-B exists in the centromere region of *M. caroli* chromosomes in place of the minor satellite DNA.

Different kinds of repetitive sequence have been found in the centromere regions of mammalian chromosomes. The centromere DNA of *M. musculus* contains major and minor satellite DNAs, with the CENP-B boxes existing not in the major but rather in the minor satellite DNA. Human α-satellite DNA has been analyzed particularly extensively (1, 11, 16, 19, 20, 40, 41, 43, 44, 50). We have recently shown by direct sequencing using isolated nuclei of HeLa cells and by reexamination of the known α-satellite DNA sequences that human CENP-B boxes are clustered with an average distribution to every other monomer unit of the α-satellite DNA composed of 2-mer repeating units (52). Ikeno et al. (16) found that there are two megabase-sized domains of α-satellite DNA (α21-I and α21-II) in the centromere of human chromosome 21; α21-I contains many CENP-B boxes at regular intervals (mostly at every other monomer unit), and α21-II contains very few if any CENP-B boxes. They pointed out that these two clusters could be comparable to the minor and major satellite DNAs in the *M. musculus* centromere. In this respect, a repetitive DNA sequence containing recognition sequences for CENP-B (CENP-B box with the core recognition sequence or CENP-B box variants with weaker affinity to CENP-B) remains to be found in AGM centromeres. Although results of the DNA mobility shift competition assays described in the accompanying report suggested that AGM genomic DNA contains 3 orders of magnitude fewer canonical CENP-B boxes than does human genomic DNA (13), this does not rigorously exclude the presence either of a small amount of CENP-B boxes or of CENP-B box variants with weaker binding affinity. In contrast with the conservation of CENP-B genes in mammals, the DNA sequences of the centromere region are quite variable. The importance of the mammalian centromere DNA (repetitive sequence) might not be reflected in a precise DNA sequence or unit length but exist in the conservation of higher-order structures formed by the interactions between repetitive sequences and centromere proteins. Our present results together with the information on the *M. caroli* system (21) support our hypothesis that the CENP-B–CENP-B box interaction is important for centromere structure and function. It is an attractive possibility that this interaction is conserved among mammals, playing an essential role in the establishment of centromere structures.

ACKNOWLEDGMENTS

This work was supported by a Grant-in-Aid for Scientific Research on Priority Areas and a Grant-in-Aid for Scientific Research (c) from the Ministry of Education, Science, and Culture of Japan.

REFERENCES

1. **Alexandrov, I. A., T. D. Mashkova, T. A. Akopian, L. I. Medledev, L. L. Kisselev, S. P. Mitkevich, and Y. B. Yurov.** 1991. Chromosome-specific alpha satellites: two distinct families on human chromosome 18. Genomics **11:**15–23.
2. **Baker, R. E., M. Fitzgerald-Hayes, and T. C. O'Brien.** 1989. Purification of the yeast centromere binding protein CP1 and a mutational analysis of its binding site. J. Biol. Chem. **264:**10843–10850.
3. **Brinkley, B. R., M. M. Valdivia, A. Tousson, and R. D. Balczon.** 1989. The kinetochore: structure and molecular organization, p. 77–118. *In* J. S. Hyams and B. R. Brinkley (ed.), Mitosis: molecules and mechanisms. Academic Press, Orlando, Fla.
4. **Brinkley, B. R., M. M. Valdivia, A. Tousson, and S. L. Renner.** 1984. Compound kinetochores of the Indian muntjac: evolution by linear function of unit kinetochores. Chromosoma **91:**1–11.
5. **Chikashige, Y., N. Kinoshita, Y. Nakaseko, T. Matsumoto, S. Murakami, O. Niwa, and M. Yanagida.** 1989. Composite motifs and repeat symmetry in *S. pombe* centromeres: direct analysis by integration of NotI restriction sites. Cell **57:**739–751.
6. **Clarke, L.** 1990. Centromeres of budding and fission yeasts. Trends Genet. **6:**150–154.
7. **Clarke, L., and J. Carbon.** 1980. Isolation of a yeast centromere and construction of functional small circular chromosomes. Nature (London) **287:**504–509.
8. **Doheny, K. F., P. K. Sorger, A. A. Hyman, S. Tugendreich, F. Spencer, and P. Hieter.** 1993. Identification of essential components of the *S. cerevisiae* kinetochore. Cell **73:**761–774.
9. **Earnshaw, W. C., and N. Rothfield.** 1985. Identification of a family of human centromere proteins using autoimmune sera from patients with scleroderma. Chromosoma **91:**313–321.
10. **Earnshaw, W. C., K. F. Sullivan, P. S. Machlin, C. A. Cooke, D. A. Kaiser, T. D. Pollard, N. F. Rothfield, and D. W. Cleveland.** 1987. Molecular cloning of cDNA for CENP-B, the major human centromere autoantigen. J. Cell Biol. **104:**817–829.
11. **Ge, Y., M. J. Wagner, M. Siciliano, and D. E. Wells.** 1992. Sequence, higher order repeat structure, and long-range organization of alpha satellite DNA specific to chromosome 8. Genomics **13:**585–593.
12. **Goh, P., and J. V. Kilmartin.** 1993. NDC10: a gene involved in chromosome segregation in *Saccharomyces cerevisiae*. J. Cell Biol. **121:**503–512.
13. **Goldberg, I. G., H. Sawhney, A. F. Pluta, P. E. Warburton, and W. C. Earnshaw.** 1996. Surprising deficiency of CENP-B binding sites in African green monkey α-satellite DNA: implications for CENP-B function at centromeres. Mol. Cell. Biol. **16:**5156–5168.
14. **Hahnenberger, K. M., M. P. Baum, C. M. Polizzi, J. Carbon, and L. Clarke.** 1989. Construction of functional artificial minichromosomes in the fission yeast Schizosaccharomyces pombe. Proc. Natl. Acad. Sci. USA **86:**577–581.
15. **Hieter, P., D. Pridmore, J. H. Hegemann, M. Thomas, R. W. Davis, and P. Philippsen.** 1985. Functional selection and analysis of yeast centromeric DNA. Cell **42:**913–921.
16. **Ikeno, M., H. Masumoto, and T. Okazaki.** 1994. Distribution of CENP-B boxes reflected in CREST centromere antigenic sites on long-range α-satellite DNA arrays of human chromosome 21. Hum. Mol. Genet. **3:**1245–1257.
17. **Jiang, W., J. Lechner, and J. Carbon.** 1993. Isolation and characterization of a gene (CBF2) specifying a protein component of the budding yeast kinetochore. J. Cell Biol. **121:**513–519.
18. **Jiang, W., H. Middleton, H. Yoon, C. Fouquet, and J. Carbon.** 1993. An essential yeast protein, CBF5p, binds in vitro to centromeres and microtubules. Mol. Cell. Biol. **13:**4884–4893.
19. **Jorgensen, A. L., C. J. Bostock, and A. L. Bak.** 1986. Chromosome-specific subfamilies within human alphoid repetitive DNA. J. Mol. Biol. **187:**185–196.
20. **Jorgensen, A. L., C. J. Bostock, and A. L. Bak.** 1987. Homologous subfamilies of human alphoid repetitive DNA on different nucleolus organizing chromosomes. Proc. Natl. Acad. Sci. USA **84:**1075–1079.
21. **Kipling, D., A. R. Mitchell, H. Masumoto, H. E. Wilson, L. Nicol, and H. J. Cooke.** 1995. CENP-B binds a novel centromeric sequence in the Asian mouse *Mus caroli*. Mol. Cell. Biol. **15:**4009–4020.
22. **Kitagawa, K., H. Masumoto, M. Ikeda, and T. Okazaki.** 1995. 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. Mol. Cell. Biol. **15:**1602–1612.
23. **Lechner, J., and J. Carbon.** 1991. A 240 kd multisubunit protein complex, CBF3, is a major component of the budding yeast centromere. Cell **64:**717–725.

23a. **Masumoto, H.** Unpublished data.

24. **Masumoto, H., H. Masukata, Y. Muro, N. Nozaki, and T. Okazaki.** 1989. A human centromere antigen (CENP-B) interacts with a short specific sequence in alphoid DNA, a human centromeric satellite. J. Cell Biol. **109:**1963–1973.
25. **Masumoto, H., K. Sugimoto, and T. Okazaki.** 1989. Alphoid satellite DNA is tightly associated with centromere antigens in human chromosomes throughout the cell cycle. Exp. Cell Res. **181:**181–196.
26. **Masumoto, H., K. Yoda, M. Ikeno, K. Kitagawa, Y. Muro, and T. Okazaki.** 1993. Properties of CENP-B and its target sequence in a satellite DNA, p. 31–43. *In* B. K. Vig (ed.), Chromosome segregation and aneuploidy. NATO ASI series, vol. H72. Springer-Verlag, Berlin.

26a. **Morishita, S., K. Yoda, and T. Okazaki.** Unpublished data.

27. **Moroi, Y., A. L. Hartman, P. K. Nakane, and E. M. Tan.** 1981. Distribution of kinetochore (centromere) antigen in mammalian cell nuclei. J. Cell Biol. **90:**254–259.
28. **Moroi, Y., C. Peebles, M. J. Fritzler, J. Steigerwald, and E. M. Tan.** 1980. Autoantibody to centromere (kinetochore) in scleroderma sera. Proc. Natl. Acad. Sci. USA **77:**1627–1631.
29. **Muro, Y., H. Masumoto, K. Yoda, N. Nozaki, M. Ohashi, and T. Okazaki.** 1992. Centromere protein B assembles human centromeric α-satellite DNA at the 17 bp sequence, CENP-B box. J. Cell Biol. **116:**585–596.
30. **Murphy, T. D., and G. H. Karpen.** 1995. Localization of centromere function in a Drosophila minichromosome. Cell **82:**599–609.
31. **Pietras, D. F., K. L. Bennett, L. D. Siracusa, M. Woodworth-Gutai, V. M. Chapman, K. W. Gross, C. Kane-Haas, and N. D. Hastie.** 1983. Construction of a small *Mus musculus* repetitive DNA library: identification of a new satellite sequence in *Mus musculus*. Nucleic Acids Res. **11:**6965–6983.
32. **Pluta, A. F., C. A. Cooke, and W. C. Earnshaw.** 1990. Structure of the human centromere at metaphase. Trends Biochem. Sci. **15:**181–185.

33. **Pluta, A. F., N. Saitoh, I. Goldberg, and W. C. Earnshaw.** 1992. Identification of a subdomain of CENP-B that is necessary and sufficient for localization to the human centromere. J. Cell Biol. **116:**1081–1093.
34. **Rattner, J. R.** 1987. The organization of the mammalian kinetochore: a scanning electron microscope study. Chromosoma **95:**175–181.
35. **Ris, H., and P. L. Witt.** 1981. Structure of the mammalian kinetochore. Chromosoma **82:**153–170.
36. **Rosenberg, H., M. Singer, and M. Rosenberg.** 1978. Highly reiterated sequences of SIMIANSIMIANSIMIANSIMIANSIMIAN. Science **200:**394–402.
37. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
38. **Sullivan, K. F., and C. A. Glass.** 1991. CENP-B is a highly conserved mammalian centromere protein with homology to the helix-loop-helix family of proteins. Chromosoma **100:**360–370.
38a. **Suzuki, N., and H. Masumoto.** Unpublished data.
39. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76:**4350–4354.
40. **Trowell, H. E., A. Nagy, B. Vissel, and K. H. A. Choo.** 1993. Long-range analyses of the centromeric regions of human chromosomes 13, 14 and 21: identification of a narrow domain containing two key centromeric DNA elements. Hum. Mol. Genet. **2:**1639–1649.
41. **Vissel, B., and K. H. Choo.** 1991. Four distinct alpha satellite subfamilies shared by human chromosomes 13, 14 and 21. Nucleic Acids Res. **19:**271–277.
42. **Wafik, S. E.** 1992. Definition of a consensus binding site for p53. Nat. Genet. **1:**45–49.
43. **Waye, J. S., and H. F. Willard.** 1985. Chromosome-specific alpha satellite DNA: nucleotide sequence analysis of the 2.0 kilobasepair repeat from the human X chromosome. Nucleic Acids Res. **13:**2731–2743.
44. **Waye, J. S., and H. F. Willard.** 1986. Structure, organization, and sequence of alpha satellite DNA from human chromosome 17: evidence for evolution by unequal crossing-over and an ancestral pentamer repeat shared with the human X chromosome. Mol. Cell. Biol. **6:**3156–3165.
45. **Waye, J. S., and H. F. Willard.** 1987. Nucleotide sequence heterogeneity of alpha satellite repetitive DNA: a survey of alphoid sequences from different human chromosomes. Nucleic Acids Res. **15:**7550–7569.
46. **Willard, H. F.** 1990. Centromeres of mammalian chromosomes. Trends Genet. **6:**410–416.
47. **Willard, H. F., and J. S. Waye.** 1987. Hierarchical order in chromosome-specific human alpha satellite DNA. Trends Genet. **3:**192–198.
48. **Wong, A. K. C., F. G. Biddle, and J. B. Rattner.** 1990. The chromosomal distribution of the major and minor satellite is not conserved in the genus *Mus*. Chromosoma **99:**190–195.
49. **Wong, A. K. C., and J. B. Rattner.** 1988. Sequence organization and cytological localization of the minor satellite of mouse. Nucleic Acids Res. **16:**11645–11661.
50. **Wu, J. C., and L. Manuelidis.** 1980. Sequence definition and organization of a human repeated DNA. J. Mol. Biol. **142:**363–386.
51. **Yoda, K., K. Kitagawa, H. Masumoto, Y. Muro, and T. Okazaki.** 1992. A human centromere protein, CENP-B, has a DNA binding domain containing four potential α-helices at the NH_2 terminus, which is separable from dimerizing activity. J. Cell Biol. **119:**1413–1427.
52. **Yoda, K., and T. Okazaki.** Unpublished data.